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DISPOSABLE PYRUVATE BIOSENSORS FOR ROUTINE ASSESSMENT OF ONION FLAVOUR

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ABSTRACT

The UK-grown onion sector shows strong potential for growth through new product development as consumers become increasingly aware of the health and dietary benefits of fresh onion consumption. Prospects for the production of new, more palatable sweet onions will be boosted by the development of improved grower diagnostics for flavour standardisation. Growers require simple-to-use on-farm diagnostics to assure flavour quality. The introduction of pungency tests has led to increased mild onion sales, however currently tests are out-sourced and as a result costly. Through this Defra- (Department for Agriculture Food and Rural Affairs) sponsored research project (HL0164), hand-held biosensor technology, adapted from the medical sector, has been developed for improved and lower cost pungency and sweetness analysis in onions.

Following investigations on variability of pyruvate and glucose within onion bulbs, spatial and temporal profiling of these biochemical constituents revealed commonly selected equatorial sections consistently underestimated overall bulb pungency in both onion cvs. SS1 and Renate by up to +2 μmol g^{-1} FW, indicating that sampling methods should be revised with more representative quarter sections taken for pungency analysis.

Biosensors are analytical devices which generate an electronic signal which can be related to the concentration of the analyte(s) of interest. Two amperometric disposable pyruvate biosensors based on either pyruvate oxidase or pyruvate dehydrogenase have been produced, optimised and validated against pyruvate standards and more importantly through extensive field trials using real samples from a wide variety of commonly UK marketed onion cultivars. Bulbs were seed drilled and grown over two years. The field trials highlighted a number of challenges in achieving the required biosensor performances, owing to the complexity and variability of onion samples. The levels of interference were quantified in onion (cvs. Supasweet (SS1), Renate, Rose de Roscoff, Hired and Rumba, Vanetz Sherpa, Hyfort and Red Baron) with both pyruvate biosensors and was found to vary across and within cultivars. Sweet, mild onions generated higher interference responses (μC) than pungent or red types and had higher levels of ascorbic acid, a common interferent. A number of strategies to minimise the effects of interference were investigated, which included freezing onion juice, juice incubation, enzymatic removal of ascorbic acid and forced oxidation. Subsequently, issues surrounding interference and sample coverage on the biosensor surface were circumvented through the addition of 0.5% (w/w) of surfactant Tween 80 to onion samples, or a dilution 1:10 (v/v) as a simple sample preconditioning step. The detection range for the pyruvate biosensors were 12 mM and 15 mM (1 mM ≡ 1.0 μmol g^{-1} FW in onion) for the oxidase and dehydrogenase-based biosensor format's respectively. Resolution was excellent at 0.50 mM and expected values for pyruvate biosensors were generally within ±1.0 mM of those determined by the industry standard (Schwimmer and Weston, 1961) assay. Total assay time was 3 minutes, a 60-fold reduction compared to the current pyruvate method. Two successful live demonstrations were conducted in front of consortium members and Defra representatives.

A prototype glucose biosensor was developed, optimised and validated against real onion juice samples (cvs. SS1, Buffalo and a commercial sweet cultivar) using the same electrode format as used for pyruvate biosensors in order to facilitate multi-analyte pyruvate and glucose testing. Onion juices were diluted to allow determinations within the linear range of the biosensor (0 to 4 mg ml^{-1} glucose; 0 to 22.2 mM). Analysis time was reduced to as much as 40-fold as compared to conventional HPLC.

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CONTENTS

CHAPTER 1: INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

CHAPTER 3: DETAILED SPATIAL AND TEMPORAL PROFILING OF PYRUVATE AND GLUCOSE WITHIN ONION (*ALLIUM CEPA* L) BULBS

CHAPTER 4: DEVELOPMENT OF A DISPOSABLE PYRUVATE OXIDASE-BASED BIOSENSOR TO DETERMINE PUNGENCY IN ONIONS (*ALLIUM CEPA* L.)

CHAPTER 5: A PYRUVATE DEHYDROGENASE-BASED SCREEN-PRINTED BIOSENSOR FOR ASSESSING PUNGENCY IN ONIONS (*ALLIUM CEPA* L.)

CHAPTER 6: EVALUATING THE PERFORMANCE OF PYRUVATE BIOSENSORS DURING FIELD TRIALS: PART I - ASSESSING INTERFERENCE

CHAPTER 7: EVALUATING THE PERFORMANCE OF PYRUVATE BIOSENSORS DURING FIELD TRIALS: PART II - RESOLVING INTERFERENCE

CHAPTER 8: TOWARDS A MULTI-ANALYTE GLUCOSE AND PYRUVATE BIOSENSOR FOR ONIONS (*ALLIUM CEPA* L.)

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

APPENDICES

Appendix A: Investigating the stability of pyruvate oxidase

LIST OF TABLES

CHAPTER 2

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

APPENDICES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

reagents 0.6 units PDH, 0.12 mM TPP, 0.06 mM FAD, 0.16 mM Lip A, 0.12 mM CoA and 0.2 mM NAD$^+$ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Figure 5.4. Thiamine pyrophosphate (TPP) optimisation (0 to 0.2 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; 0.06 mM FAD, 1 mM LipA, 0.01 mM CoA and 0.2 mM NAD$^+$ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Figure 5.5. Flavine adenine dinucleotide (FAD) optimisation (0 to 0.1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP; 0.3 mM CoA and 0.2 mM NAD$^+$; 0.2 mM LipA (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Figure 5.6. Nicotinamide adenine dinucleotide (NAD$^+$) optimisation (0 to 1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP, 0.1 mM FAD, 0.3 mM CoA and 0.2 mM Lip A (final concentrations); 5 mM pyruvate standard ; 21°C. Standard error bars are from the mean of three experiments.

Figure 5.7. Lipoamide (Lip A) optimisation (0 to 0.2 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 80 µM TPP, 60 µM FAD, 20 µM CoA and 1mM NAD$^+$ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Figure 5.8. Coenzyme A (CoA) optimisation (0 to 0.1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP, 0.06 mM FAD; 0.2 mM LipA and 0.02 mM NAD$^+$ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Figure 5.9. Pyruvate dehydrogenase-based biosensor calibration plot with 0 – 15 mM pyruvate standards verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). Standard error bars are from the mean of three experiments. +50 mV; phosphate buffer pH 5.7; 0.1M KCl; 21°C.

CHAPTER 6

Figure 6.1. Time-course studies on background interference (µC) from onion (cv. Hyfort) juice during 3h incubation using blank-mediated pyruvate oxidase biosensors. LSD (*P* = 0.05) = 24.74 µC. +150 mV; phosphate buffer pH

CHAPTER 7

CHAPTER 8

APPENDICES

LIST OF PLATES

CHAPTER 3

CHAPTER 4

CHAPTER 9

APPENDICES

LIST OF ABBREVIATION AND SYMBOLS

$	Dollars
%	Percent
</>	Less than/Greater than
±	Plus or minus
≡	Equivalent to
°C	Degrees Celsius
μ	Micro (10^{-6})
₤	Pounds
˚Brix	Degree Brix
1-PRENCSO	1-*S*-propenyl cysteine sulphoxide
2-PRENCSO	2-*S*-propenyl-cysteine sulphoxide
ACSO	*S*-alk(en)yl-L-cysteine sulphoxides
Ag/AgCl	Silver/Silver Chloride
ANOVA	Analyis of variance
atm	Atmospheric pressure
ca	Approximately
CA	Controlled atmospheres
CoA	Coenzyme A
CRD	Completely randomised design
cvs.	Cultivars
Da	Dalton
DM	Degrees of polymerisation
E	Equilibrium potential
E°	Standard potential
E-nose	Electronic nose
EPY or P_E	Enzymatically produced pyruvate
Eq.	Equation
F	Faraday constant
FAD	Flavin adenine dinucleotide
FAO	Food and Agricultural Organisation
FIA	Flow injection analysis
Fig.	Figure
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
I or i	Current
I.U.	International units
KCl	Potassium chloride
l	Litre
LF	Lachrymatory factor
Lip A	Lipoamide

LSD	Least significant difference values
m	Mass
M	Molar
MB	Meldolas blue
MCSO	*S*-methyl cysteine sulphoxide
mg	Milligram
$MgSO_4$	Magnesium sulphate
ml	Millilitres
mM	Millimolar
MMt	Million metric tonnes
mV	Millivolts
N	Newtons
n	Number
Na_2HPO_4	Disodium hydrogen phosphate sodium
NADH	Nicotine adenine dinucleotide
NaH_2PO_4	Dihydrogen phosphate
NaOH	Sodium hydroxide
NHE	Normal Hydrogen electrode
nm	Nanometres
O_2	Oxygen
PA	Pyruvic acid
P_C	Control or background pyruvate
PCSO	*S*-propyl cysteine sulphoxide
PDH	Pyruvate dehydrogenase
pH	Percentage hydrogen ions
ppm	Parts per million
P_T	Total pyruvate
PVPP	Polyvinyl polypiridine
PyOx	Pyruvate oxidase
Q	Electrical charge
R	Gas constant
R^2; r^2	Pearson's coefficient
Redox	Reduction and oxidation
s	Second
SCE	Saturated Calomel electrodes
spp.	Species
SPR	Surface plasmon resonance
SSC	Soluble solids content
T	Absolute temperature
TCA	Trichloroacetic acid
TPP	Thiamine pyrophosphate
TSS	Total soluble solids
TTF	Tetrathiafulvalene
™	Trade Mark
V	Volts

v/v	Volume by volume
vs.	Versus
w/w	Weight by weight
β-NAD	β-nicotinamide adenine dinucleotide
γ	Gamma
µC	Micro coulombs
µl	Micro litre
µmol g^{-1} FW	Micromole per gram fresh weight

CHAPTER 1

Introduction

1.1 Background

A previous Horticultural Link (HortLink; HL0186) project “Fundamentals for Mild Onion production” resulted in the formation (based around the commercial partners) of the Sweet Onion Development Group and launch of the SupaSweet™ (cv. SS1) onion. The agreed protocol for marketing under this trademark demanded a threshold for low pungency onions with a pyruvate assay score of pungency <5.0 (≡ 5.0 µmol g^{-1} FW in onion). Pungency is synonymous with the ‘heat’ experienced when eating raw onions. The introduction of routine laboratory-based pungency assays has led to a moderate increase in sales of mild onions produced in the U.K. through better quality assurance. However, pungency analysis is irregularly undertaken; being out-sourced by growers. Pungency analysis is both slow and expensive as it is based on a colorimetric assay technique first reported in 1961 (Schwimmer and Weston, 1961). This test, validated in HL0186, is currently employed in the UK and worldwide. It currently takes approximately a week to turn around from receipt of bulb samples to results being available, hence this resulted in unanimous support by the consortium for an accurate, rapid, real-time, packhouse/field-based test. Ideally, standardisation of both strength and sweetness is required. Developing a biosensor for onion strength will facilitate regular checks from field to packhouse and underpin existing quality standards. If the sensor is further developed to include sweetness it will revolutionize quality assurance for sweet onions and be a world first for the UK industry.

1.2 Aim and objectives

1.2.1 Aim

The aim of the initiative was to develop state-of-the-art, grower-friendly, rapid quality assurance diagnostics to gain added value for the UK onion industry.

1.2.2 Objectives

The two main objectives of this research were as follows:

- To examine the feasibility of a biosensor for on-farm testing of the mildness of onions based on the pyruvate test developed in a previous Horticultural Link Project (HL0186) to produce a rapid, cheap, quality control test for onions and to establish a sampling system for onion bulb tissue for use with the biosensor and;

- To develop a multi-analyte biosensor to target the biochemical differences and produce a rapid, cheap, on-farm quality control test of the sweetness of onions.

1.3 Thesis structure

The thesis is arranged into ten chapters. Chapter two gives a necessary review of literature, initially covering the significance of onions both globally and in the UK including existing techniques for onion quality assessment. The numerous variations of the current pyruvate assay used within the industry have created problems in standardisation for the global trading of onions and is therefore discussed. Thereafter, more modern approaches such as biosensors, as well as their principles of operation are detailed. Reports on temporal biochemical changes within onion bulbs were reviewed as it was necessary to investigate variability within onion bulbs, such that the sampling technique would be representative of both strength and sweetness. Thus, Chapter three profiles the chosen analytes for biosensor research, pyruvate and glucose. Chapter four reports preliminary results from the development of the pyruvate oxidase amperometric biosensor. Although the use of amperometric sensors is now well established in the medical industry (*cf.* Abayomi *et al.,* 2006), they have since been researched for food applications such as fermentation process monitoring (Tothill *et al.,* 2001) and tropical fruit quality control (Jawaheer *et al.,* 2003). To meet the current aims, a number of indirect quantifications of onion strength may have been adopted. However, pyruvate, the standard marker for onion pungency was chosen and is generally recognised as the best biochemical marker for onion pungency. Common amperometric strategies for developing a pyruvate biosensor were researched. These include the removal of oxygen and generation of hydrogen peroxide (H_2O_2), the rate at which the electrochemical signal was generated, indicating analyte concentration and the use of mediators replacing oxygen (O_2) as an electron acceptor. The latter strategy eliminates the O_2 concentration dependency and high operating potentials required for the reduction of $O_{2,}$ or the oxidation of H_2O_2. It was subsequently decided to extend work to also use pyruvate dehydrogenase due to extended linear range and resolution. This work is described in chapter five. Chapters six and seven are concerned with the validation of both pyruvate biosensors. Whereas chapter six relates field trials and problems associated with performance with a variety of onion cultivars. Strategies to improve biosensor reliability are the focus of Chapter seven. It was determined that glucose and fructose were the most reliable markers for perceived sweetness and would readily displace “brix” in a more targeted approach to “sweetness” measurement. Glucose was consequently adopted as the most appropriate marker for sweetness assessment and hence a glucose biosensor based on the same format as the pyruvate biosensors was developed. Chapter eight relates to the glucose biosensor development and validation with real onion samples and reviews integration with the pyruvate biosensor with the view of simultaneous multi-analyte testing for onion flavour.

This is followed by a general discussion with conclusions and recommendations for further development work necessary for commercialisation. The appendices are divided into sections containing: shelf life stability trials (Appendix A); statistical tables (Appendix B); the standard operating procedure for the biosensor field trials (Appendix C); the concluding Defra project assessment (Appendix D); a list of abstracts (Appendix E), presentations and publications.

CHAPTER 2

Literature review

2.1 Introduction

Alliums belong to the family Alliaceae and are thought to have originated in central Asia. Dry bulb onions (*Allium cepa* L.) are the second most important horticultural crop after tomatoes and are consumed worldwide for their unique flavour and health-related properties (Grffiths *et al*., 2002).

2.2 Economic importance of bulb onions

Annual world production of bulb onions stood at 44 million metric tonnes (MMt) in 2003, 55 MMt in 2004 and continues to increase (FAO statistics, 2006). Onion consumption in the UK and elsewhere has increased as a result of the demand for increased choice and health promotion campaigns (Grffiths *et al*., 2002). About 100,000 tonnes of onions were imported and sold as 'mild' in the UK in 2000, as until recently no mild-flavoured varieties were suitable for growing in the UK climate. The SS1 sweet onion cultivar was launched in the UK under the “SupaSweet™ in 2002. The market for sweet onions has since seen a 50% increase year on year since its launch. Mild and sweet onions account for 25% of the US onion market (The Guardian, June 16, 2005). Over 650,000 tonnes are consumed per annum in the UK of which *ca*. 100,000 tonnes have historically been perceived as mild. These milder flavoured onions are imported into the UK from countries such as Spain and Chile. Sweet onions are a premium product, currently selling at *ca*. £2.30 per kg., which is twice the price of Spanish imported onions. Commercially, it is expected that improving onion quality assurance will reliably expand the UK market for sweet onions, substituting current levels of mild onion imports.

2.3 Flavour compounds in onion

2.3.1 Onion flavour precursors

The sulphur-containing compounds in onion are responsible for their distinctive aroma and flavour. Four individual flavour precursors, known collectively as the *S*-alk(en)yl-L-cysteine sulphoxides (ACSO's) have been identified in alliums, *S*-methyl cysteine sulphoxide (MCSO), *S*-propyl cysteine sulphoxide (PCSO), 1-*S*-propenyl cysteine sulphoxide (1-PRENCSO), and 2-*S*-propenyl-cysteine sulphoxide (2-PRENCSO) found only in garlic (Granroth, 1970; Freeman and Whenham, 1975; Hamilton *et al.*, 1998). 1-PRENCSO is a primary product upon tissue disruption, including sulphenic acids (Yagami *et al.*, 1980). Only when the bulb is cut or damaged does the familiar odour and onion flavour develop (Fig. 2.1). Allinase, *S*-alk(en)yl cysteine sulphoxide lyase (E.C. 4.4.1.4) contained within vacuoles cleaves the ACSO's leading to the release of volatiles. The precursors are contained within the cytoplasm (Lancaster and Collin, 1981). The biosynthesis of these flavour compound are still under investigation and is reviewed elsewhere (Jones *et al.,* 2004). However, the enzyme lachrymatory factor (LF) synthase (or propanthial-*S*-oxide synthase) is now believed to be responsible for the development of the tear-inducer or lachrymatory factor (Imai *et al.*, 2002; Luthra and Luthra, 2002). The pyruvic acid or pyruvate assay is now an established method for indicating onion bulb pungency or flavour intensity as perceived when eating raw onions (Yoo and Pike, 2001).

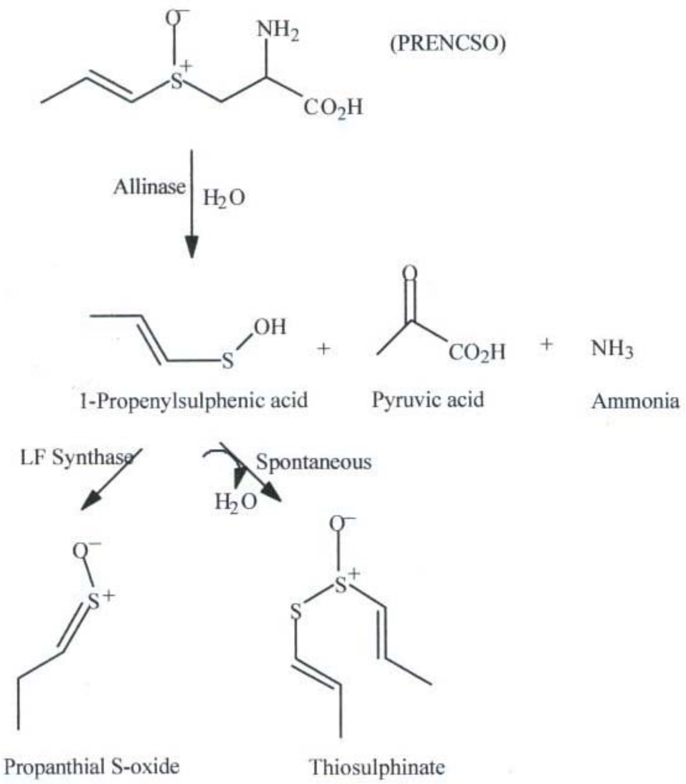

Figure 2.1. Biosynthesis of onion flavour during tissue disruption. Reproduced from Luthra and Luthra, 2002.

2.3.2 Sugars and fructans

Onions store carbohydrates as fructans (Darbyshire and Henry, 1978; Crowther *et al.*, 2005). Fructans are large chain polymers of fructose which are stored in the vacuole of onion bulbs. The role of fructans in onions is not clear, but there is evidence they are reserve carbohydrates and/or are responsible for osmotic regulation (Darbyshire and Henry, 1978; Jaime *et al*., 2001). Carbohydrate supply in the form of substrate sucrose may play a significant role, controlling the ability of the plant to synthesise polymers with higher degrees of polymerisation (DM), which in turn may be related to the concentrations of non-structural carbohydrates (Darbyshire and Henry, 1978).

The principal carbohydrates identified in onions are fructose, glucose and sucrose. The carbohydrate constituent of raw onion bulbs contribute to the perception of sweetness

(Crowther *et al.*, 2005; Terry *et al.,* 2005a). Storage carbohydrates constitute a high percentage of dry matter within onion bulbs, with significant differences between sweet cultivars and those grown for dehydration (Table 2.1). Studies by Chope *et al.* (2007) also showed the low pungency, low dry matter cultivar (cv.) SS1 was dominated by monosaccharides glucose and fructose, whereas for high pungency, high dry matter cvs. Renate and Carlos, the disaccharide sucrose dominated.

Table 2.1. The carbohydrate constituent of some onion cultivars. Adapted from Crowther *et al.*, 2005.

	White sweet Spanish	**Crossed cultivar**
% dry matter	7.5	17.2
% soluble carbohydrate as glucose and fructose	75	3
% as sucrose	18	7
% as fructans	7	90

2.4 Onion flavour classification and perception

Following a UK government sponsored project (HL0186), which validated the pungency assay over a wide range of onion cultivars marketed in the UK (Crowther *et al.*, 2005), flavour has recently been defined in the UK as sweet (less than 4 µmol pyruvate g^{-1} fresh weight), mild (4 -7 µmol pyruvate g^{-1} fresh weight) or strong (above 7 µmol pyruvate g^{-1} fresh weight). There is, however, still some confusion over these classifications. There are a wide variety of molecules known to affect sweetness with the perception of sweetness involving a sensory system with many types of sweetness receptors. Onion flavour has also been correlated with aroma and taste.

Pyruvate in onions was negatively correlated with aroma. An R^2 value of –0.96 was shown between human olfactory threshold concentration (ppm), pyruvate and perceived pungency during analysis of six cultivars (Schwimmer and Guadagni, 1961).

Each flavour precursor gives rise to different thiosulphinates that impart distinct flavour characteristics. Propenyl/propyl thiosulphinates have a green-fresh raw onion and sulphur flavour, while the methyl thiosulphinates impart the cabbage-like flavour (Randle, 1997; Uddin and MacTavish, 2003). It has been suggested the lachrymatory factor may also be involved in the development of bitterness (Lancaster and Boland, 1990). Taste studies were undertaken by a trained panel on cultivars with a wide variation in pungency, where one half of the bulb was used for pyruvate analysis and the bottom half for sensory evaluation (Wall and Corgan, 1992). A rating scale of 1 extremely mild to 5 extremely pungent was used, with assessments including inner, middle and outer scales. Three replicates of the experiment gave correlations of 0.84, 0.95 and 0.79, respectively. Hence, taste panel assessment of flavour is commonly used to assist in classifying onions. The trouble with this method is that it is time consuming, requires trained panellists and only a limited number of bulb samples can be assessed as once pungent cultivars have been assessed it becomes difficult to accurately assess milder types. According to Randle and Bussard (1993), 1 μmol pyruvate g^{-1} FW is a perceptible difference in flavour intensity. Studies examining the relationship of taste panel perception of sweetness to sugar and pyruvate levels found that pungency masked the perception of sweetness once pyruvate levels exceeded 4 μmol g^{-1} FW. There was an inverse relationship between sweetness and pyruvate concentration (Fig. 2.2).

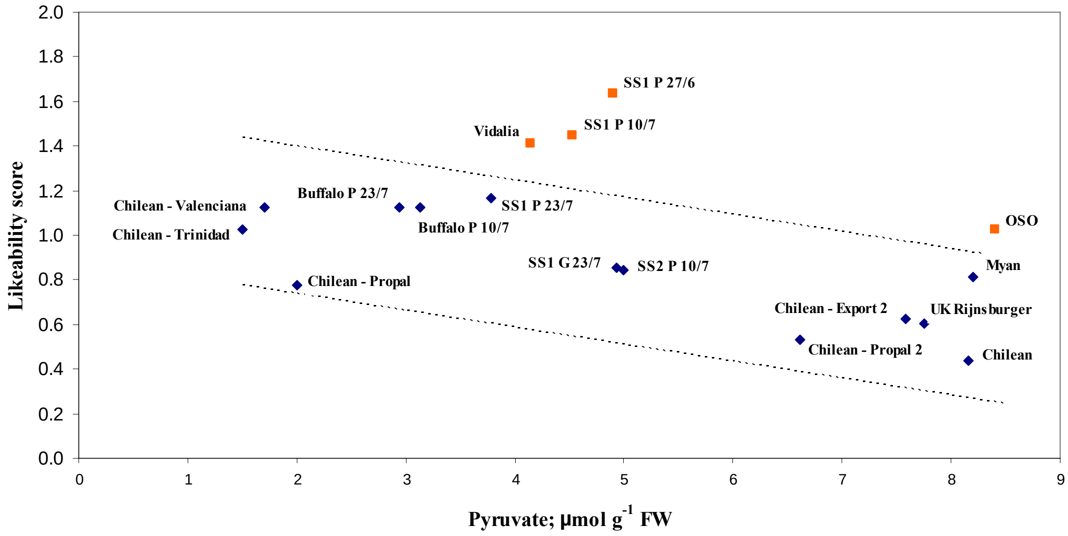

Figure 2.2. Taste panel assessment of sweetness and pungency. Source: Crowther *et al.*, 2005. Dashed lines show 95% confidence interval limits for points estimated to conform to a linear relationship between the appreciation of sweetness by taste panels and pyruvate.

2.5 Temporal and spatial biochemical changes in onion bulbs during storage

2.5.1 Pungency

Bulb onions are commonly marketed out of storage and flavour perception following long-term storage may differ from pre-storage assessments. Numerous studies have shown pyruvate changes during short and long-term storage across cultivars, including individual ACSOs (flavour precursors) (Freeman and Whenham, 1974; Kopsell *et al*., 1999). It is difficult to compare these studies however, as both preharvest and postharvest environments which were not identical have been shown to influence compositional changes across and within cultivars.

A number of enzymes have been associated with pyruvate changes in onion. For example, Uddin and McTavish (2003) studied allinase specificity during ambient and CA storage. Allinase specificity was significantly enhanced in bulbs stored in ambient conditions suggesting this change in activity explained changes in the efficiency in converting ACSOs into pyruvate and flavour volatiles following tissue disruption. In another study on onion macerates, increased concentrations of pyruvate were found with increased additions of exogenous allinase and γ-glutamyl transpeptidase compared with non-treated macerates. Hanum (1995) suggested this increase occurred as a result of the action of γ-glutamyl transpeptidase on γ-glutamyl peptides within onion, releasing flavour precursors which are then acted upon by allinase, enhancing concentrations of pyruvate.

2.5.2 Sugars

Selected conditions of storage aim to maintain bulb dormancy for as long as possible. The loss of onion dormancy can be observed by the appearance of internal sprouting which eventually extends from the neck of the bulb. Sprouting is considered a marketing defect. Onion sugar content and dormancy has been shown to be related in onions (Rutherford and Whittle, 1982; Benkeblia and Shiomi, 2004) and fructan changes observed in some studies have been shown to be dependent, in part, by storage conditions (Chope *et al*., 2007) as well as cultivar.

2.5.3 Organic acids

Organic acids in onion are malic, succinic, fumaric, citric, oxalic and ascorbic acid (vitamin C) and these have also shown to vary over storage and under different storage regimes (Gubb and McTavish, 2002; Benkeblia and Varoquaux, 2003).

2.5.4 Spatial distribution of pyruvate and bulb sampling methods

Sampling procedures for routine pungency assays including sugars commonly involve using whole, halved, quartered, cores and equatorial tissue sections in order to obtain representative samples from which to interpret assay results, thus emphasising the significance of correct sampling. Some studies have been undertaken demonstrating total changes in pyruvate within onion bulbs at various stages of maturity (Freeman, 1975; Randle *et al*., 1998) and have shown the highest concentration of flavour precursors in the stem plate and central bulb scales with lower concentrations within the outer scales. Lower concentrations are believed to be present at the neck (Lancaster *et al*., 1986; Randle *et al*., 1998) increasing progressively towards the base. Freeman (1975) and Bacon *et al.* (1999) examined the distribution of thiosulphinate and pyruvate within bulbs again showing concentration gradients across scales. None of these studies however, have detailed both horizontal and vertical flavour profiling within bulbs.

2.6 Evaluation of current methods for measuring onion pungency, flavour and aroma

2.6.1 Colorimetric methods

Confidence in the accuracy of pyruvate tests is becoming more important particularly as the popularity of low pungency onions increases (Yoo and Pike, 2001; Havey *et al*., 2002). A number of techniques and indirect methods have been used to define quality in onions. However, no one method can measure all of the compounds contributing to onion flavour. The spectrophotometric method was developed in 1961 by Schwimmer and

Weston and utilises the reaction of pyruvate with 2,4-dinitrophenylhydrazine (DNPH) to form a coloured product whose absorbance is measured at 420 nm. Enzymatically produced pyruvate (EPY or P_E) described as the difference between total pyruvate (P_T) and endogenous (control) pyruvate (P_C) is now widely used to compare cultivars because it is stable compared with some other methods still employed (Schwimmer and Weston, 1961; Thomas *et al*., 1992; Randle and Bussard, 1993), although it only measures gross flavour intensity.

Using identical samples, Havey *et al.* (2002) demonstrated significant variations existed across laboratories assaying pungency based on the Schwimmer and Weston method. There is a large potential for cumulative error due to the number of steps involved with this method, along with the absence of an adopted industry standard. These steps include tissue sampling technique, juice extraction, juice incubation period, chemical grade, reagent volumes and concentrations, and absorbance setting. These steps are also common to the other quantification methods described below. Efforts towards standardising the method of Schwimmer and Weston (S&W) commenced with the adoption of a press for tissue maceration and juice collection to reduce assessment times (Randle and Bussard, 1993). The aforementioned method was subsequently adapted to a microplate reader capable of 96 measurements in a single operation, significantly reducing measurement times (Boyhan *et al*., 1998). Yoo and Pike (1999) then further developed an automated system eliminating dilution steps and thus reducing pipetting error, whilst Gordon and Barrett (2003) devised a more sensitive method which consisted of changing the concentrations and volumes of reagents and measuring absorbance at 515 nm compared with 420 nm employed in the original method. Recently Pineda *et al.* (2004) put forward another modification using a flow injection analysis (FIA) – pyruvate method which reduced the amount of glassware, possible risk of contamination and time for analysis. Their results showed no difference between the FIA and pyruvate batch processes.

2.6.2 High performance liquid chromatography (HPLC)

Some techniques have highlighted variations in the levels of background or control pyruvate (P_C) across cultivars using some of the various analytical methods described above. Onion sprouts and garlic contained 0.84 - 0.95 µmol background pyruvate FW^{-1} (Yoo and Pike, 2001) and using the modified method of Gordon and Barrett (2003) background levels of pyruvate across a variety of sweet and strong cultivars were less than 0.38 µmol g^{-1} FW. However, most studies quantifying pungency in onion cultivars, using identical methods, reported considerably higher background pyruvate levels across cultivars, for example, up to 4 µmol gl^{-1} FW (Schwimmer and Guadagni, 1961; Randle and Bussard, 1993; Boyhan *et al*., 1999). Given that a difference of 1 µmol g^{-1} FW is perceptible on sensory evaluation, these discrepancies are significant in the marketing of sweet onions. Total and individual ACSOs have also been used to differentiate flavour (Thomas and Parkin, 1994; Randle *et al*., 1995). These methods are time consuming and vary in their ability to classify cultivars.

2.6.3 Gas chromatography/Mass spectroscopy (GC/MS)

Volatile sulphenic and thiosulphenic acids and the lachrymatory (LF) factor have been used to differentiate onion cultivars for breeding purposes. Alcala *et al*. (1998) compared gas chromatography and thiosulphinates with the S&W pyruvate assay, and their efficiencies in classifying cultivars for pungency and flavour using identical samples. There was a need for standardising as many experimental conditions as possible in order to obtain reproducible results. Whilst measuring sulphenic, thiosulphenic acids and the lachrymatory factor, detection and quantification have been achieved to varying degrees of accuracy. The important factors such as run times, incubation times for macerated onion tissue, temperature during gas chromatography (GC) injection and separation were raised (Kopsell *et al.,* 2002). Proportions of precursors in cv. Rijnsburger quantified through gas chromatography were 6:88:6 (Freeman and Whenham, 1975), 20:66:14 (Lancaster and Kelly, 1983) for MCSO, PCSO and 1 PRENCSO, respectively. In the majority of these studies allinase was not inhibited, resulting in partial degradation during the extraction leading to inaccurate and irreproducible results. Subsequently, Edwards and co-workers (1994) suggested a prolonged extraction process for quantitative removal of ACSOs and used an enzyme inhibitor to halt further allinase activity.

2.6.4 Electronic Nose technology

Electronic nose technology was evaluated against the Schwimmer and Weston (1961) method for assessing pyruvate in diced onion as an alternative method for assessing *Allium* quality. One key motivation was to save time and improve accuracy (Abbey *et al.*, 2003). Unfortunately, although showing potential, this technology still has not been sufficiently miniaturised to be portable, is expensive and also requires skilled training.

2.6.5 Refractometry

Sugars in fruit and vegetables are an important contributor to flavour. Percentage or degree Brix (˚Brix) as a measure of total soluble solids (TSS) or soluble solids content (SSC) is widely adopted by fresh produce importers and exporters as a measure of sweetness in many fruits as part of routine quality control including onion juice (Kopsell and Randle, 1997). Sweetness can also be ascertained by the levels of glucose, sucrose and fructose via chemical methods and although these methods are more accurate, in most cases the refractometer is employed owing to convenience. The use of refractometers has limitations though owing to variations in dry matter content within the onion juice. For example, taste panel evaluation of sweetness did not always correlate to TSS (Crowther *et al.*, 2003) and is more closely related to fructans in onion (Chope *et al.*, 2006).

2.7 Biosensor technology

2.7.1 Biosensors as a food quality control tool

The food industry requires pocket-sized devices capable of simplified one-shot field measurements on undiluted samples as well as on-line monitoring of one or more parameters simultaneously (Prodromidis and Karayannis, 2002; Tothill, 2003). Biosensors offer the opportunity for improved validation, including the extension of quality control tests, through the measurement of target analytes directly related to produce quality. Traditionally, developments in the biosensor market have arisen from the need for clinical monitoring. However, increasing applications are being found within the food industry (Terry *et al.,* 2005b), including the military defence and environmental diagnostics areas (Tothill, 2001).

Biosensors may be described as analytical tools incorporating biological (e.g. enzyme, whole cell or tissue) or biomimetic sensing elements either closely connected to, or integrated within a transducer (Newman *et al.*, 2004).

2.7.2 Transducer Technology

The main function of the transducer is to convert the physico-chemical change in the compound that has arisen from the reaction with the analyte, into an output signal (Fig. 2.3). The analyte selective interface is normally a bioactive substance such as enzyme, antibody, micro-organism which recognise their specific analytes and regulate the specificity and sensitivity of the device (White, 2004). Biosensors are categorised based on the type of transducer employed.

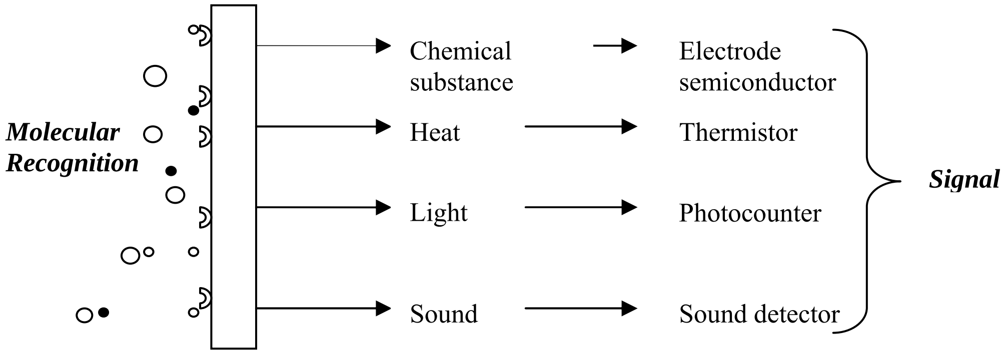

Figure 2.3. General schematic of a biosensor. Adapted from Chaubey and Malhotra (2002).

2.7.3 Types of biosensors and their potential use for food applications

Electrochemical biosensors have dominated the global market with the majority based on enzyme catalysis or whole cells. Electrochemical biosensors are based on signal production and detection arising from the electron transfer. Oxygen (O_2) and hydrogen peroxide (H_2O_2) are the co-substrate and product respectively, of many enzymatic reactions. Depending on the property to be determined, electrochemical biosensors may be subdivided into several categories, namely, potentiometry, conductometry and amperometry. Potentiometry consists of measuring voltage changes. Citings where potentiometric biosensors have been used for food analysis include assessing the concentration of isocitrate in fruit juice (Kim and Kim, 2003) and levels of urea in milk (Verma and Singh, 2003). Nevertheless potentiometric approaches are not as popular as some others as they are prone to drift including interferences from pH changes (Tothill, 2001). Conductometry involves measuring changes in conductance between a pair of metal electrodes resulting from the biological component, whilst amperometry involves measuring the changes in current on the working electrode resulting from oxidation of products from a biochemical reaction. This technique is linearly dependent on analyte concentrations and is used significantly in the development of biosensors.

Optical biosensors are based on the measurement of light absorbed or emitted. These have been used for the measurement of pH, O_2 and CO_2. According to White (2004) optical biosensors are also widely reported as they offer some advantages over electrochemical methods. They can employ a variety of techniques including chemiluminescence, surface plasmon resonance (SPR) and fluorescence. Other advantages include speed, reproducibility and no need to incorporate a biological component. BIAcore^{TM} (Uppsala, Sweden) have launched a number of commercially successful systems based on SPR transduction. They are useful for microorganism detection. The detection of microorganisms in food samples is standard practice for ensuring food safety and quality.

Colorimetric formats are based on the detection of heat evolved. Integrated circuit temperature sensitive structures have been modified with enzymes. For example, the enzyme invertase was immobilised on a thermistor system where the heat of the reaction was used to calculate the sucrose content in sugar cane (Thavarungkul *et al.,* 1999).

Piezoelectric biosensors are constructed on the principle of the generation of electric dipoles on subjecting an anisotropic natural crystal to mechanical stress. Adsorption of the analyte increases the mass of the crystal, altering its natural frequency of oscillation. Piezoelectric formats have been used for the measurement of ammonia, nitrous oxide, carbon monoxide, hydrogen and methane.

Another group of biological sensors are immunosensors, where a label such as an enzyme or fluorescent marker, is used to detect an immunological reaction. Recently, Terry *et al.* (2004) detailed some of the developments in this particular area, highlighting their potential as a food screening tool.

All biosensors suffer from a number of drawbacks. For example, optical biosensors, although highly sensitive, are restricted for use in turbid media. Thermal biosensors are of little use where there is little heat exchange and ease of handling is also poor (Chaubey and Malhotra, 2002). Amperometric enzyme biosensors have a limited linear range owing to low oxygen concentration or deactivation of the enzyme by the hydrogen peroxide produced (Luong *et al*., 1997). Notwithstanding, their simplicity favours their use as a portable, low cost means of measuring quality within the fresh produce industry.

2.7.4 *Electrodes and electrode reactions*

In order to understand and explain characteristics of the biosensors developed in forthcoming chapters, it is necessary to describe some fundamental electrochemical properties. Electrochemistry is the study of the transfer of electrons and can be used to exploit properties of chemical reactions. A typical electrode is the surface of a piece of metal in contact with a solution of salts and other chemicals dissolved in water. Reduction and oxidation (Redox) electron-transfer reactions occur at the electrode. For this reaction to occur it is necessary for the reacting molecules or ions to travel through the water and reach the metal electrode. Amperometric biosensors, the focus of the present research, are designed using either two electrodes (working and auxiliary) or three (working, auxiliary and reference). The most common type of working electrode material employed in amperometric biosensor construction are platinum, glassy carbon and gold for the oxidation of hydrogen peroxide, and graphite/carbon pastes/glassy carbon for mediator and NADH oxidation. These electrodes are relatively inert and highly conductive, resulting in low background currents. With a reference electrode included in the format, the potential of the working electrode is controlled with reference to the former whilst measuring the current between the working and auxiliary electrodes. The main challenge in the construction of amperometric biosensors is the establishment of efficient conductivity. Surface modification can also be achieved via electrodeposition or chemical modification. Other materials include semi-conductors such as metal oxides and conducting organic salts. Carbon pastes doped with cobalt, platinum, palladium and ruthenium have been reported (Prodromidis and Karayannis, 2002), including mass-producible rhodinised carbon for monitoring glucose during the fermentation process (Tothill *et al*., 2001) and assessing fruit maturity (Jawaheer *et al.,* 2003).

2.7.4.1 *Faradaic and non-faradaic reactions*

Non-faradaic reactions do not involve charge transfer but reflect changes in the structure of the electrode-solution interface. When a potential is applied, charge accumulates. This charge depends on the potential across the interface and composition of the solution.

In an electrochemical cell, the electromotive force E, the difference in potentials between the two electrodes can be measured. This potential is a measure of the energy per unit charge which is available from the oxidation/reduction reactions to drive the reaction. Oxidation is associated with a loss of electrons, whereas reduction is concerned with gaining electrons. The cell reaction is composed of two half-reactions, an oxidation half-reaction and a reduction half-reaction. Faradaic reactions occur at the electrode and involve the transfer of a charge across a metal-solution interface, resulting in oxidation or reduction. The normal hydrogen electrode (NHE) has been chosen as a reference, to which a standard potential of $E° = 0.00$ V (at 1M, 1 atmosphere and 25°C) has been assigned (Table 2.2). The values depicted indicate Zinc is the strongest reducing agent and Copper the strongest oxidizing agent. Other common reference electrodes include the silver/silver chloride (Ag/AgCl) and saturated calomel electrodes (SCE), with potentials of +0.197 and +0.242 vs. NHE, respectively (Bard and Faulkner, 2001).

Table 2.2. Standard electrode potentials in aqueous solution at 25°C.

Half- reaction	**Standard Potential E° (V)**
$Zn^{2+}(aq) + 2e^- \rightarrow Zn(s)$	-0.76
$Pb^{2+}(aq) + 2e^- \rightarrow Pb(s)$	-0.13
$Fe^{3+}(aq) + 3e^- \rightarrow Fe(s)$	-0.04
$2H^+(aq) + 2e^- \rightarrow H_2(g)$	0.00
$Cu^{2+}(aq) + e^- \rightarrow Cu^+(aq)$	+0.16
$AgCl(s) + e^- \rightarrow Ag(s) + Cl^-(aq)$	+0.22
$Cu^{2+}(aq) + 2e^- \rightarrow Cu(s)$	+0.34

Reactions involving charge transfer are governed by Faraday's law which states that the amount of reaction caused by the current flow at the electrode surface is proportional to the quantity of electricity passed. Reactions associated with charge separation and transfer may be described by:

$$O + ne^- \leftrightarrow R$$

Equation 2.3

Where n = number of electrons (e^-) transferred between oxidant (O) and reductant (R). Current results from the oxidation or reduction of a compound, following the application of a potential. At equilibrium, there is no net charge transfer and the chemical reaction is described by the Nernst equation:

$$E = E^o + \frac{RT}{F} = \ln \frac{[O]}{[R]}$$

Equation 2.4

Where E = Equilibrium potential; E^o = Standard electrode potential; R = Gas constant; T = Absolute temperature; F = Faraday's constant

The current-voltage characteristics for the system depends on the transport of reactants in the electrolyte to the electrode surface, transfer of electrons for the oxidation, reduction of electroactive chemical species and removal of resulting reactants. The resulting electric charge (Q) is governed by:

$$Q = \int_{0}^{t} + \, I dt = \frac{nFm}{M} \qquad \text{Equation 2.5}$$

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

2.7.5 Factors affecting electrode reaction rates and current

Mass transfer depends on processes such as diffusion, migration and convection. Diffusions results from the movement of ions or molecules due to a concentration gradient. Movement occurs from a high chemical potential to a region of low potential and is described by Fick's Law:

$$Q = -D\left(\frac{dc}{dx}\right) \qquad \text{Equation 2.6}$$

Where Q = charge; D = coefficient of diffusion; $\frac{dc}{dx}$ = concentration gradient

In the absence of convection and migration, diffusion only controls the flux of reactants. This diffusion is governed by Fick's second law, which relates to the change in concentration of the solute with time.

$$\frac{\partial c_s}{\partial t} = D \frac{\partial^2 c_s}{\partial x^2} \qquad \text{Equation 2.7}$$

The change in concentration with time at a location, x, is governed by the differences in flux into and out of an element of width dx.

2.7.6 Background Signals

Typically, non-Faradaic currents decay to a stable background level and are subsequently subtracted from gross signals. High background currents are undesirable. Current leakage, including small potential differences in the electronic instrumentation, dissimilar metal contacts in wire leads of the biosensor, or electrochemically active impurities may all contribute to background signals.

2.7.7 Enzyme Kinetics

Generally, biosensor signal responses are 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. Enzyme-substrate reactions may be represented by the Michaelis-Menten mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

Equation 2.8

Where E = enzyme; S = substrate; ES = enzyme-substrate complex; P = product; 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 such that:

$$V = \frac{dP}{dt} = k_2[ES]$$

Equation 2.9

A rate equation which relates the velocity of the reaction to the substrate concentration was proposed by Michaelis and Menten:

$$V = \frac{V_{MAX.}[S]}{K_M + [S]}$$

Equation 2.10

Where V_{MAX} = maximum velocity

$K_M = \frac{k_{-1} + k_2}{k_1}$ = Michaelis-Menten constant

Equation 2.10 assumes that the ES complex is in a steady state (i.e. after the initial phase, [ES] is constant) and 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.

At the working electrode, 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. At the vicinity of the electrode, the enzyme binds to their specific substrate to yield the products. Figure 2.4 depicts a typical electrocatalytic signal generated from an enzyme-substrate reaction using an amperometric biosensor.

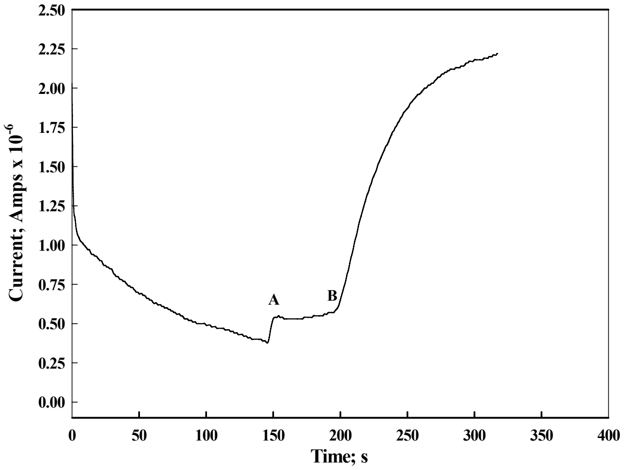

Figure 2.4. Electrocatalytic signal generated from an amperometric enzyme-substrate reaction using Cranfield carbon biosensors. 'A' depicts the addition of phosphate buffer solution and 'B' the oxidation of hydrogen peroxide (B) on the working electrode surface.

2.7.8 Sensitivity and Detection Limit

Factors determining the sensitivity of a biosensor design to a target analyte include the size of the sensor, thickness of the membranes (if included), and the mass transport of chemical species from the sample to the sensing region. The sensitivity of the device is defined as the final steady state change in the magnitude of the biosensor output signal with respect to the change in concentration of specific chemical species. This is indicated by the slope of the linear range. The lowest analyte concentration that can be detected by the sensors with an acceptable signal-to-noise ratio is defined as the detection limit. This should be small or equal to minimum analyte concentrations contained within the substrate. Ideally, the current signal generated should originate solely from the enzyme-analyte reaction.

2.8 Construction of amperometric enzyme-based biosensors

Electronic production techniques have been applied to the manufacture of electrodes and both thick and thin film technologies have been employed which broadly differ in their method of deposition of surface layers (Icaza and Bilitewski**,** 1993). Other methods adapted for the manufacture of biosensors include inkjet printing, air-brush and cavro deposition. Given the common problem of enzyme stability and other technical factors restricting the successful commercialisation of biosensors, the development of disposable biosensors must take a profitable approach. Hence, screen-printing mass production methods are often adopted (Tu *et al.,* 2001). A potential problem with the mass production of disposable biosensors however is reproducible sensitivity and linear range. Difficulty arises from variations in time, pH and temperature during storage conditions. Most commercial amperometric biosensors developed consume oxygen (oxidases), produce hydrogen peroxide, or indirectly produce the reduced form of β-nicotinamide adenine dinucleotide (e.g. dehydrogenases). The performance of a given enzyme electrode will be influenced by the specifics of the analyte, enzyme and food matrix, analyte concentration, electrochemical interferents and pH. The construction and optimisation of multi-component matrices consisting of carbon graphite powder, organic oils, enzymes, redox mediators and co-factors is still being researched and developed. Carbon is generally favoured as it can be used within a wide anodic potential range and is inexpensive compared with platinum or gold. The successful Exactech blood glucose monitor (Abott Laboratories, US., formerly MediSense, Inc., Cambridge, MA) used by diabetics utilises screen printing technology to produce the base transducer cheaply allowing one-shot use (Newman *et al,* 2004). Some examples of portable glucose biosensors are shown below (Fig. 2.5).

Figure 2.5. Commercial one-shot disposable glucose biosensors. Adapted from Cullen, 2004.

2.9 Mediated Systems

Mediators replace oxygen as an electron acceptor and allow biosensor functioning at much lower operating potentials, reducing the effects of other electrochemically active species found in many food matrices, such as onions. 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). Mediators facilitate electron transfer from an enzyme's redox centre to the electrode surface.

The 'first generation' oxygen biosensors (Clark and Lyons, 1962) utilised the ambient O_2 concentration where the rate of electrochemical reduction depended on the rate of diffusion of the oxygen from the bulk solution. This diffusion in turn was dependent on the concentration gradient, and consequently the bulk O_2 concentration. In order to overcome oxygen concentration dependency and high potentials (-0.6V versus Ag/AgCl electrode) required for the reduction of O_2, or the oxidation of H_2O_2, artificial electron acceptors evolved and gave rise to the 'second generation' of biosensors utilising mediators. The use of mediators involves a multi-step process in which the enzyme participates initially in redox reaction with the substrate, followed by reoxidation by the mediator and finally the mediator being oxidised by the electrode. 'Third generation' biosensors that directly oxidise a reduced enzyme without the use of mediators have now been constructed by coating the electronic conductors (conducting salts).

2.9.1 *Types of mediators*

A growing selection of mediators is being reported for the construction of amperometric biosensors. However, on deciding which mediator to adopt, the presence of crucial properties are sought in order to optimise biosensor performance (Table 2.3).

Table 2.3. Characteristics and advantages of using mediators. Adapted from Davis *et al.,* 1995.

Advantages	**Ideal Characteristics**
Reduced dependency on oxygen concentration	Rapid reaction with reduced enzyme
Working potential of electrode determined by oxidation potential of mediator	Exhibition of reversible heterogeneous kinetics
Interference from unwanted species is avoided	Low overpotential for regeneration of oxidised mediator and pH independent
pH insensitive where the oxidation of reduced mediator does not involve protons	Stable oxidised and reduced forms
	No reaction of reduced form with oxygen

There have been numerous reports of meldolas blue being utilised for the construction of biosensors (Table 2.4) as this mediator, through cyclic voltametry studies, has been shown to possess some of the desired properties highlighted in Table 2.3 above (Wedge *et al*., 1999). Meldolas blue was subsequently adopted for the current study and the performance characteristics in the construction of onion pyruvate biosensors are detailed in subsequent chapters.

Table 2.4. Some mediators used in the construction of biosensors

Type	**Reference**
Methylene blue	Kulys *et al*., 1994a; Munteanu *et a*l., 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

2.10 Conclusions

There is evidence that the food industry will change its traditional approach to the introduction of biosensors given the offer of improvements to quality control, safety and traceability (Terry *et al.,* 2004). New legislation and regulations may also encourage new markets for biosensors in the food industry (Luong *et al*., 1997; Tothill, 2001). In the case of disposable biosensors, profits arising from mass production must be viewed as attainable. In contrast with biosensors for clinical purposes, once a food test has been developed it must be adapted for use within a diverse range of industries and environments such as field, processing and retailing (Prodromidis and Karayannis, 2002). It has also been suggested that the food biosensor market share is being limited by the vast academic research which usually proceeds without a defined specification (Ivnitski *et al.*, 1999), also ignoring crucial parameters such as testing on real samples, stability of the biological component and cartridges that often accompany them (Wedge et al., 1999). Furthermore, it was suggested that as not all experimental parameters affecting biosensor performance are fully understood and are therefore not controlled, leading to large deviations. As a result, this prevents guaranteed applicability (Icaza and Bilitewski, 1993). Clearly, biosensors offer the potential for a quick and reliable method of assaying onion flavour. However, the onion biosensor will have to demonstrate equal performance to the established methods of analysis.

For the present study, standardisation of the preliminary steps in the process will be required in order to achieve an acceptable level of repeatability and hence confidence in the marketing of onions. Critical parameters include sampling procedure, including juice extraction and incubation. Ultimately, a multi-analyte biosensor constructed from individual sensors measuring pyruvate and sweetness would be desirable. This research focuses on the development of a laboratory prototype pyruvate biosensor and examines spatial and temporal changes within stored bulbs with the aim of recommending an onion bulb sampling procedure and establishing the design requirements for optimum biosensor performance.

CHAPTER 3

Detailed spatial and temporal profiling of pyruvate and glucose within onion (*Allium cepa* L) bulbs

3.1 Introduction

Onions are primarily consumed for their flavour, which can change during storage and marketing. Low pungency or mild onions have a pyruvate concentration of *ca*. <5 μmol g^{-1} FW and command a price premium. When onion bulb tissue is damaged, allinase, *S*-alk(en)yl cysteine sulphoxide lyase (E.C. 4.4.1.4) cleaves the alk(en)yl cysteine sulphoxides (ASCOs) flavour precursors, leading to the release of many volatiles, 1-propenylsulphenic acid, pyruvate and ammonia (Schwimmer and Weston, 1961). Pyruvate concentration has been correlated to the degree of pungency experienced using taste panel evaluation (Schwimmer and Weston, 1961; Wall and Corgan, 1992; Crowther *et al.*, 2005) but has occasionally been confused with overall onion flavour (Crowther *et al.*, 2005). Onion flavour and taste preference are complex and related to many compounds including sugars (Crowther *et al.*, 2005; Terry *et al.*, 2005). There was an inverse relationship between sweetness and pyruvate concentration (Griffiths *et al.*, 2002; Coolong and Randle, 2003). Pyruvate was positively correlated with ACSOs (Bacon *et al.*, 1999; Resemann *et al.*, 2003) and thiosulphinates (Freeman, 1975; Havey *et al.*, 2004).

Carbohydrates in onion constitute the bulk of dry matter (Henry and Darbyshire, 1978). Fructooligosaccharides, also known as fructans are the main carbohydrate reserve found in onion and accumulate during bulbing, but are generally catabolised during regrowth and sprout development. The major non-structural carbohydrates identified in onions are fructose, glucose and sucrose, but proportions vary according to cultivar and maturity. Recent work suggested that higher concentrations of both glucose and fructose in certain onion cultivars is positively correlated to taste preference (Davis *et al.*, 2007).

Bulbs with high fructan content take up or retain less water, concentrating both soluble carbohydrates and thiosulphinates responsible for pungency (Havey *et al.*, 2004). Consensus suggests that during storage, fructans are hydrolysed to lower degrees of polymerisation (DP) fructans, then disaccharides, and finally to monosaccahrides. This said, contradictory evidence suggests that fructan concentrations may increase during storage. Fructan changes during storage have been shown to be dependent, in part, by storage conditions as well as cultivar.

Fundamental to developing a robust analytical procedure is consideration of proper material sampling (Luthria *et al*., 2006). Sampling procedures for routine pungency assays, sugars, and other constituents affecting onion bulb flavour involve using either whole, halved, quartered, core sections (Randle *et al.,* 1998) or equatorial tissue sections in order to obtain representative data. In addition, processing industries are also interested in flavour distribution within onion bulbs. The highest concentration of flavour precursors related to pungency are found in the stem plate and central bulb scales and decline progressively in the outer scales (Randle *et al.,* 1998). Lower concentrations of pyruvate were present at the neck end (Lancaster *et al*., 1986) increasing progressively towards the bulb base. Longitudinal sampling with a bore just below the bulb equator was shown to be the best method for obtaining representative tissue for pyruvate analysis for Granex-type onions, which are similar to low pungent cv. SS1 (Randle *et al.,* 1998). None of these studies, however, have detailed three-dimensional flavour profiling. Furthermore, despite the aforementioned research, there has still been some discrepancy between market classification and actual pyruvate levels measured within onion bulbs (David O'Connor pers. comm., 2003). Whether this difference has been attributable to the sampling technique, assay method employed or postharvest biochemical changes within the bulb during marketing is unknown. In order to elucidate these issues, the implication of sampling was revisited in the present study. Accordingly, the effect of temporal and spatial variation in pyruvate and glucose within onion cvs. SS1 (low pungency) and Renate (high pungency) bulbs, stored under standard industry controlled atmosphere (CA) conditions was investigated, primarily to ascertain the most appropriate method required for representative sampling. Another aim of the study was to examine more closely the metabolic relationship between glucose and pyruvate changes in significantly different onion cultivars. Little research to date has followed temporal changes in glucose metabolism in onion bulbs and mobilisation within onion scales up to the break of dormancy and beyond the period of sprout suppression.

3.2 Materials and methods

3.2.1 Plant material

Commercial onion cvs. SS1 (SupaSweet) and Renate bulbs were grown in the UK using standard agronomic practices, and kindly donated by G's Marketing Ltd. (Cambs., UK) and Bedfordshire Growers Ltd. (Beds., UK), respectively. Bulbs of cvs. SS1 and Renate were obtained 2 months and 4 weeks post curing, respectively.

3.2.2 Onion storage

Onion cvs. SS1 and Renate bulbs were held at 4˚C ±1˚C under industry standard controlled atmosphere (CA) conditions (3.03 kPa CO_2 and 5.05 kPa O_2) (Smittle, 1988) for 31 days and 8 months, respectively. Controlled atmosphere was achieved using an Oxystat 2 CA system, attached to an Oxystat 2002 Controller, and Type 770 fruit store analyser (David Bishop Instruments, Sussex, UK). This system was self-calibrating every 24 h against 5% CO_2 in N_2 (British Oxygen Co., Surrey, UK). Bulbs were stored in plastic nets and held in three identical rigid polypropylene fumigation chambers (88 x 59 x 59 cm) which acted as blocks. Samples were selected randomly. The dry aerial parts and roots were removed, and any diseased or damaged bulbs discarded prior to storage. Relative humidity (%RH) was monitored throughout storage (Tinytalk loggers, Gemini Data Loggers, R.S. Components Ltd, UK). Nine onion bulbs of cvs. SS1 (every 4 days for 31 days) and Renate (every month for 8 months) were removed from store (3 from each chamber). All bulbs were allowed to acclimatise at room temperature for 24h before being sectioned.

3.2.3 Sample preparation

For accurate dissection, bulb height was measured using a digital electronic calliper (Model RS 592095; Mitutoyo, Japan) from the basal section to the neck area. The diameter was taken at the broadest section of the bulb. Bulbs were weighed and the outermost dried skins and 10 mm of neck region removed. Bulbs were cut in half with one half snap-frozen in liquid nitrogen and stored at -20ºC until use. The other portion was assayed for enzymatically-produced pyruvate. Each half of the bulb was cut into 12 regular sections: three equal horizontal sections (top, middle and base) with each divided into outer, second, third and grouped central scales as depicted in Plate 3.1.

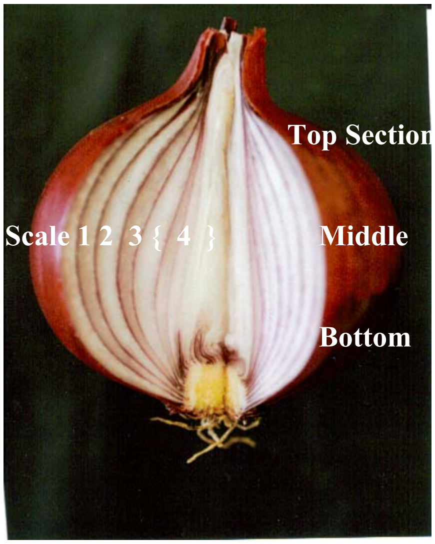

Plate 3.1: Sample onion bulb showing sections used for pyruvate and glucose profiling.

3.2.4 *Pyruvate measurement*

Pyruvate analysis was performed as previously described (Schwimmer and Weston, 1961; Crowther *et al.*, 2005) with slight modifications (Abayomi *et al.,* 2006) on all twelve sections from each bulb from each outturn from both cvs. SS1 (n = 1728) and Renate (n = 1728). Two grams from each section was homogenised for 1 minute in 3 ml distilled water. Juices were extracted using a homogenizer at 800 rpm (Polytron PT 3000, Kinematica, Switzerland). Aliquots of 1.5 ml were transferred to Eppendorf tubes and subsequently stored at -20˚C prior to analysis. Extracts were thawed at room temperature for 30 min and centrifuged at 13,000 rpm for 10 min (Biofuge pico, Kendro Laboratory Products, Germany). Samples were then diluted 10-fold in deionised water. An aliquot

(0.5 ml) of the filtrate was added to 1 ml 2, 4-dinitrophenyl hydrazine (DNPH) (0.0125%; v/v) in 2 M HCl and 1.5 ml deionised water in a boiling tube. The mixture was briefly vortexed and incubated at 37°C for 10 min. Five ml of 0.6 M NaOH was added and the absorbance at 420 nm recorded (Camspec M501, Camspec Ltd., Cambs., UK). A standard curve to allow calculation of pyruvate concentration from onion samples was produced by taking 10 ml of 5 mM pyruvic acid stock solution and diluting to 1 mM, followed by serial dilutions giving a concentration range of standards of 0.04 to 0.4 mM. Pyruvate concentration (µmol g^{-1} FW) in onions was determined from the equation of the linear standard curve.

3.2.5 *Glucose quantification*

Glucose levels were studied only where there were significant ($P < 0.001$) differences in pyruvate across and within bulbs. This occurred after 5, 9, 16, 23 and 31 days for cv. SS1 and 1, 2, 4, 6 and 8 months for cv. Renate within the same tissue sections. One bulb from each of the three blocks was assayed. For both cultivars, glucose analysis was performed on top, middle and bottom sections and towards the bulb centre, on outer, third and remaining combined central scale tissues (n = 324). Selected onion tissue sections were freeze-dried for 72h and the lyophilized tissue ground to a fine powder using a mortar and pestle before being assayed for glucose. A glucose test kit (Megazyme International Ltd., C. Wicklow, Republic of Ireland) was used according to manufacturer's instructions. Prior to performing the glucose assays, a similarly lyophilized onion sample of cv. SS1 was used to compare glucose content obtained using the test kit against that quantified using HPLC (O'Donoghue *et al.*, 2004) with slight modifications (Davis *et al.,* 2007).

3.2.6 *Bulb quality*

At the intervals previously described in section 3.2.2., three bulbs from each block were removed from store. Bulb firmness was measured on the other halves of skinless bulbs (n = 72) that had been cut longitudinally with flat side face down using an 8 mm probe mounted onto a crosshead of a conventional Instron Universal Testing Machine (Model 1122, Instron, Bucks., UK). Firmness was expressed as maximum force (N) required until tissue failure. The firmness of both halves was measured. For each cultivar, 15 bulbs were also weighed at the beginning of the study and the weight monitored separately. Sectioned tissue weights (n = 1728) were also recorded at each interval. Incidence of sprouting and rooting was monitored during the storage period and numbers recorded. Diseased bulbs were recorded and discarded periodically.

3.2.7 *Statistical analysis*

All treatments were arranged in a completely randomised design (CRD). Containers represented blocks (n = 3). Data were subjected to ANOVA using Genstat for Windows Version 7.1.0.198 (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. Pearson's Rank Coefficients were used to measure linear relationships between variables. All measurements were made in triplicate.

3.3 Results

3.3.1 *Three-dimensional changes of pyruvate and glucose in cvs. SS1 and Renate onion bulbs*

Significant ($P < 0.001$) vertical variations in pyruvate were recorded in onion cvs. SS1 and Renate bulbs (Figure 3.1) (Appendix B, Figs. B3.1.1 and B3.1.2). Bulb pyruvate concentration within cv. SS1 bulbs increased with storage time from pre-storage levels of 3.4 μmol g^{-1} FW to a maximum of 8.3 μmol g^{-1} FW after 23 days. Pyruvate content in cv. Renate declined rapidly during the first month in CA storage from 12.5 μmol g^{-1} FW to 9.6 μmol g^{-1} FW. This level was maintained for a further four months after which there was a steady increase reaching 15 μmol g^{-1} FW.

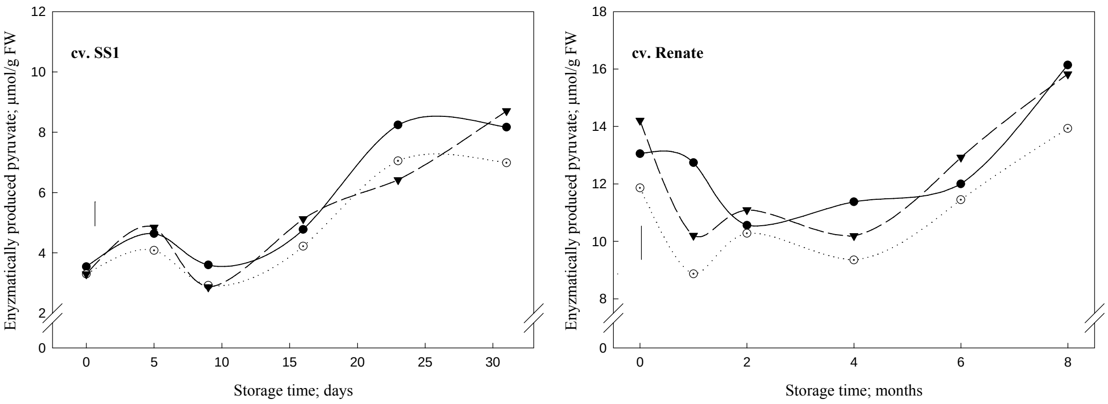

Figure 3.1. Vertical spatial variation (●, top; ○, middle, ▼, bottom sections) in pyruvate concentration measured in onion cvs. SS1 and Renate bulbs during controlled atmosphere storage. LSD ($P = 0.05$) bars shown.

Pyruvate was significantly ($P < 0.001$) lower in the middle region of both cv. SS1 ($P = 0.002$) and cv. Renate bulbs moving vertically down bulbs (Appendix B, Figs. B3.1.3 and B3.1.4), with a magnitude of +2 µmol g^{-1} FW difference between the middle and other parts for cv. Renate. Horizontally towards the centre of cv. SS1 bulbs, pyruvate within central tissues remained significantly higher ($P < 0.001$) than the outer and third scales (Figure 3.2). This, however, was not observed for cv. Renate bulbs, where conversely the outer tissues had significantly ($P < 0.001$) higher levels (+2 µmol g^{-1} FW) than central scales. There was no significant difference in pyruvate between outermost and second scales towards bulb centres for either cultivar.

Bulb glucose content for cv. SS1 increased over the first 3 days of storage to 25.9 mg g^{-1} FW, reached a minimum (17.6 mg g^{-1} FW) then climbed up again to pre-storage levels (Figure 3.3). Glucose levels in cv. Renate were almost half that of cv. SS1, with the lowest levels (*ca.* 12 mg g^{-1} FW) found at the beginning of storage. Middle sections had significantly ($P < 0.001$) higher glucose levels than top and bottom for both cultivars (Appendix B, Figs. B3.3.3 and B3.3.4).

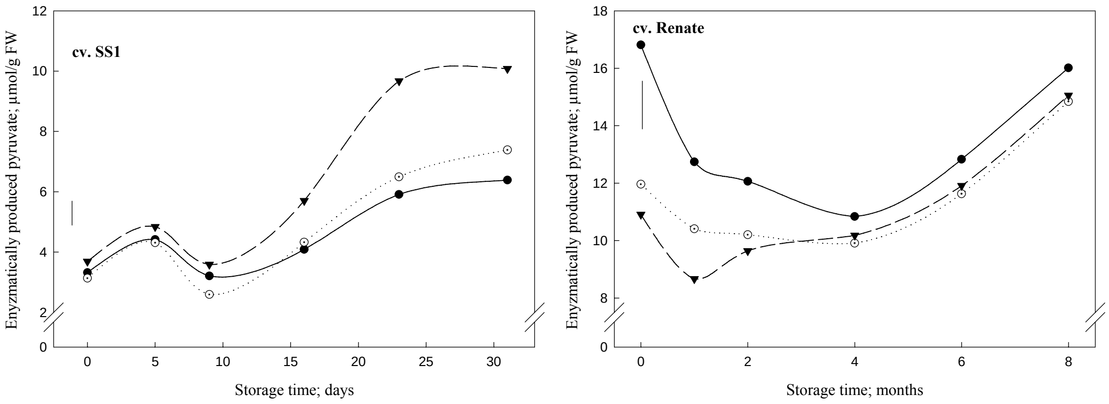

Figure 3.2. Horizontal spatial variation (●, outer; ○, middle, ▼, central) in pyruvate concentration measured in onion cvs. SS1 and Renate bulbs during controlled atmosphere storage. LSD (P = 0.05) bars shown.

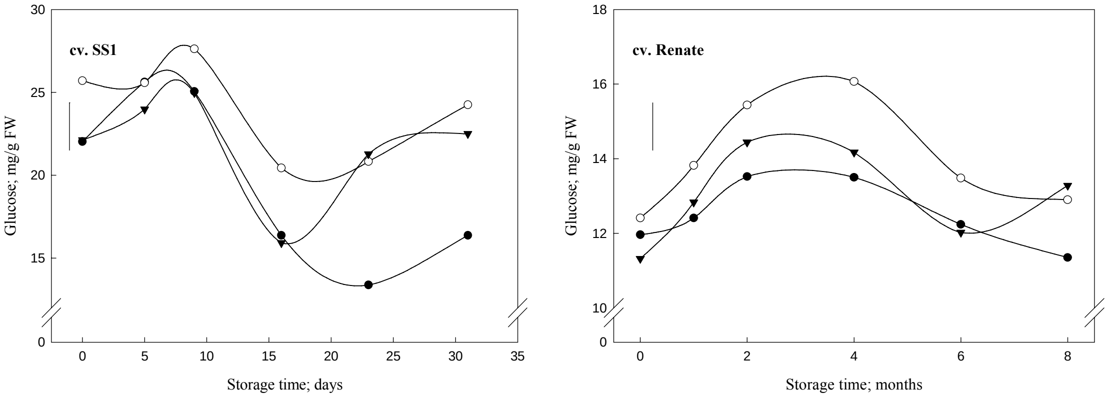

Figure 3.3. Vertical spatial variation (●, top; ○, middle, ▼, bottom sections) in glucose concentration measured in onion cvs. SS1 and Renate bulbs during controlled atmosphere storage. LSD (P = 0.05) bars shown.

In both cultivars stark differences in glucose concentration were apparent between scale sections (Figure 3.4). Glucose levels across outer, third and combined central bulb scales decreased significantly (P < 0.001) towards the centre of cv. SS1, whereas, for cv. Renate onions, the opposite occurred (Appendix B, Figs. B3.4.1 and B3.4.2). There was an inverse correlation -0.69 and -0.87 between glucose and pyruvate for cvs. SS1 and Renate, respectively.

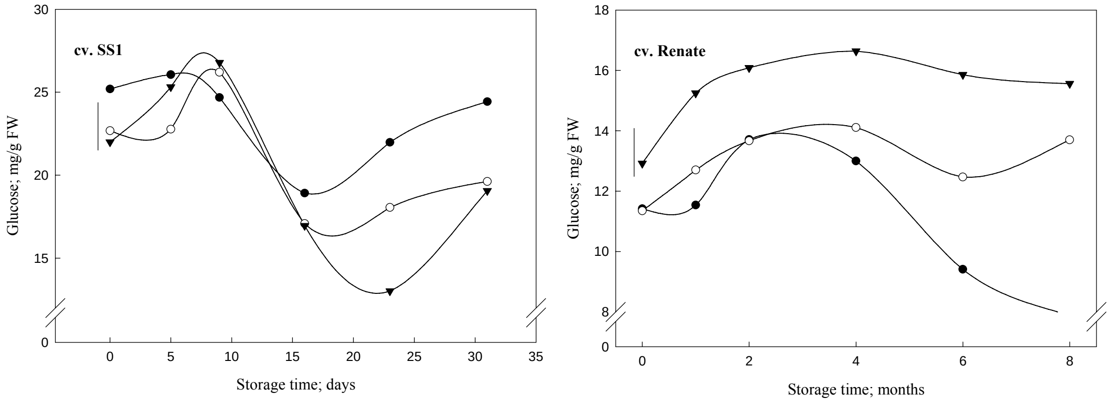

Figure 3.4. Horizontal spatial variation (●, outer; ○**,** middle, ▼**,** central) in glucose concentration measured in onion cvs. SS1 and Renate bulbs during controlled atmosphere storage. LSD ($P = 0.05$) bars shown.

3.3.2 Changes in bulb quality

There was a significant difference in firmness between cultivars with cv. SS1 exhibiting lower (*ca.* 45N) values. There was no significant change in firmness during the storage period for cv. SS1 although a significant ($P < 0.001$) reduction from 100N to 75N was observed with cv. Renate (Appendix B, Figs. B3.3.3 and B3.3.4). There were significant fluctuations in weight across scales for both cultivars during storage (results not shown). There was no net change in weight for cv. SS1. By the end of the storage period, bulbs of cv. Renate lost 7% of their prestorage weight. Tissue weight was highest towards the end of storage declining rapidly thereafter. The presence of internal rooting preceded sprouting for cv. SS1 bulbs. Only one bulb showed internal rooting at the end of the storage period for cv. Renate.

3.4 Discussion

3.4.1 *Pyruvate changes*

The present study clearly demonstrated that the concentration of pyruvate and glucose in two different onion cultivars not only changed over storage time but also that the spatial distribution of these two target analytes within the bulb differed markedly during CA storage. Although many have shown that pyruvate (Freeman, 1975; Randle *et al.*, 1998; Uddin and MacTavish, 2002) and glucose (Mikitzel and Fellman, 1994; Chope *et al.*, 2006) levels alter during storage, the present study represents the most detailed published work to date whereby both pyruvate and glucose were quantified from the same tissue sections from two radically different cultivars over prolonged storage. Consequently, the observed temporal and spatial variations in pyruvate and glucose will have implications on future sampling procedures if representative concentrations of the whole bulbs are required.

Changes in pyruvate across cultivars during various storage regimes are now well documented and have been attributed to metabolic dissimilarities in the *S*-Alk(en)yl cysteine sulphoxides associated with individual phenotypes (Bacon *et al.*, 1999; Kopsell and Randle, 1997) changes in individual precursors (Freeman and Whenham , 1974; Kopsell *et al.*, 1999) and variations in allinase activity and form (Kopsell *et al.*, 2001). In the present study, cvs. SS1 and Renate displayed very different three-dimensional profiles in the target analytes along with in-bulb fluctuations over time. Unless whole bulbs or wedges are taken, samples which are representative of the whole bulb cannot be obtained for glucose either, particularly when sampling tissue from bi or multi-centred bulbs. Onion scales have previously been shown to exhibit quite different patterns of precursor accumulation and loss (Lancaster *et al.*, 1986). Research with radio-labelled carbon (^{14}C) urea translocation studies on bulbing onions demonstrated that the younger scales had acquired most of the photosynthate transported, particularly during bulb enlargement (Mann, 1983).

3.4.2 *Sugar changes*

Low pungency, low dry matter cultivars tend to have greater concentrations of fructose including glucose as seen with cv. SS1, than more pungent, high dry matter onions (Kahane *et al.,* 2001; Terry *et al.,* 2005; Chope *et al.,* 2007; Davis *et al.,* 2007) such as cv. Renate where sucrose dominates. It should be noted, that soluble solids content is not the best method for assessing glucose. Soluble solids content is better related to fructans (Briggs and Goldman, 2002). Notwithstanding, soluble solids within inner and outer scales of cv. Walla Walla Sweet (low pungency) onion bulbs over 7 weeks at 22˚C, 35-40 % RH over two successive harvests was monitored and compared (Mikitzel and Fellman, 1994). Soluble solids of inner scales increased with time and was consistently higher than outer scales. In the second harvest, soluble solids content did not increase with time but again inner scales contained significantly ($P < 0.001$) more soluble solids (*ca.* 9.1%) than outer (*ca.* 6.0%) scales. In the present study also, cv. SS1 also had consistently higher glucose levels within the outermost scales. Contrastingly, cv. Renate, had the highest glucose levels within central scales.

3.4.3 *Pyruvate and glucose metabolism*

The relationships between enzymatically produced pyruvate and glucose assimilation, metabolism and translocation, and dormancy in onion is unclear. In the present study, increased levels of pyruvate coincided with reduced levels of glucose. The fact that cvs. SS1 and Renate have quite different levels of pyruvate and glucose contents and were at different stages of maturity did not appear significant. Other studies (Mikitzel and Fellman, 1994), even those who also worked with cv. SS1 bulbs (Chope *et al.,* 2006), showed no association between pyruvate with glucose or soluble solids. The different behaviour over successive harvests described earlier (Mikitzel and Fellman, 1994), may explain this lack of association observed by other research with cv. SS1.

3.4.4 Sugar metabolism and dormancy

Onion sugar content and dormancy has been shown to be related in onions (Rutherford and Whittle, 1982; Benkeblia and Shiomi, 2004). For example, total soluble sugar concentration in onion cv. Rouge Amposta bulbs stored under ambient peaked at 22 mg g^{-1} FW between 5 - 7 weeks, which coincided with the onset of sprouting (Benkeblia and Shiomi, 2004). In the same study, the reduction in total sugars coincided with sprouting after 5 - 8 weeks storage at both 10˚C and 20˚C. In accordance with results from Chope *et al.* (2007) as with the present study, glucose concentrations in cv. SS1 declined rapidly before increasing again when the incidence of sprouting increased. However, with cv. Renate, the rate of decrease was much slower and only more significantly occurred in outer scales. The latter trend was also observed by previous workers (Benkeblia *et al.,* 2002). For cultivars such as SS1, it is likely that glucose metabolism is controlled by other means. When onion cv. SS1 and Renate bulbs were stored under identical conditions to those used in the current study, there was no significant change in fructans for cv. SS1 but a significant (P < 0.001) decrease was observed for cv. Renate, over 2 and 7 months, respectively (Chope *et al.,* 2006). Fructan concentrations for cv Renate were 4-fold that of cv. SS1.

3.4.5 Pyruvate changes and dormancy

The increase in pyruvate observed for cv. SS1 coincided with increases in numbers of sprouted bulbs as has been previously found (Freeman and Whenham, 1976). No correlations between either sprouting or internal rooting with pyruvate were observed for cv. Renate. Again, albeit under different storage regimes, not all studies have indicated a link between pyruvate changes and sprouting even when testing similar cultivars as those used in the present study. For example, no associations were made between pyruvate changes and bulb dormancy for short-storage cvs. Pukekohe, Candy, Walla Walla Sweet, Zenith, Sweet Sandwich, Granex 33' or Dehydrator #3 (Kopsell and Randle, 1997).

3.4.6 Other physical changes

Evaluations on the role outer scales have on dormancy have indicated the properties of outer scales are likely to determine the firmness of onion bulbs (Füstös, 1997). Compared to cv. Renate, cv. SS1 bulbs have noticeably thin and fewer skins which were easily shed during handling. Consequently, firmness for cv. SS1 was expected to decrease. Firmness of cvs. SS1 and Renate was also monitored by other workers (Chope *et al.*, 2006) using similar methods and storage conditions. Cultivar Renate was 2-fold firmer than cv. SS1, in accordance with the present study. For both cvs. SS1 and Renate, the maximum weight across scales followed peak levels of pyruvate. Relative humidity averaged 98%. Hence, weight fluctuations could have been not only due to water, but also dry matter content changes (Rutherford and Whittle, 1982).

3.5 Conclusion

Significant differences in both temporal and spatial profiling of pyruvate and glucose exist, the extent of which varied across cultivars. The present study highlights the importance of appropriate sampling techniques when assaying biochemical constituents in onion and other fresh produce types. In order to measure overall bulb flavour, it is recommended that sampling procedures, particularly for routine pungency determination, should aim at obtaining bulb quarters or wedges rather than equatorial sections which consistently underestimated overall bulb pyruvate.

CHAPTER 4

Development of a disposable pyruvate oxidase-based biosensor to determine pungency in onions (*Allium cepa* L.)

4.1 Introduction

Bulb onions are the second most important horticultural crop after tomatoes (Griffiths *et al.,* 2002) and are consumed worldwide for their unique flavour. Increasingly, low pungency bulbs (often referred to colloquially as mild and/or sweet onions) are consumed raw in the USA and elsewhere. Pyruvate concentration (µmol g^{-1} FW) in macerated onion tissue is used as a quality assurance indicator of pungency (Schwimmer and Weston, 1961; Wall and Corgan, 1992; Crowther *et al.,* 2005) or flavour intensity in most onion producing countries. Typically, low pungency onions have a pyruvate concentration of *ca.* <5 µmol g^{-1} FW and command a price premium.

Despite improvements to the original Schwimmer and Weston (1961) method over the last four decades, current quality assurance assays for onion pungency (e.g. Randle and Bussard, 1993; Yoo and Pike, 2001) are still relatively time-consuming and expensive. Confidence in the accuracy of pyruvate measurements is becoming more important, particularly as the popularity of low pungency onions increases (Yoo and Pike, 2001; Havey *et al.,* 2002). Pungency tests are currently out-sourced. Decentralising the current pyruvate assay will empower growers and packers marketing low pungency onions to improve their quality assurance procedures.

The demand for reliable and inexpensive methods for the assessment of fresh produce quality is set to expand; biosensors offer a viable opportunity to fulfil this niche (Terry *et al.,* 2005b). Over the last twenty years research has been carried out to produce a pyruvate biosensor, mainly for clinical applications (Table 4.1). This study describes the development of an amperometric biosensor to detect and quantify the pyruvate concentration in juice from macerated onion tissue based on the following enzyme reaction:

$$\text{Pyruvate} + HPO_4^{-2} + O_2 \xrightarrow{\text{pyruvate oxidase (PyOx)}} \text{acetylphosphate} + H_2O_2 + CO_2 \qquad \text{Equation 4.1}$$

Mediators were used to reduce the effects of electrochemically active species, found in many food matrices (Terry *et al.,* 2005b). Meldolas blue was the preferred mediator used for this study; the reaction of which is given below. Electrons are recycled between the FAD active site of the enzyme pyruvate oxidase, the mediator and working electrode:

$$\text{Pyruvate} + HPO^{-2}{}_4 + O_2 \rightarrow \text{acetylphosphate} + \text{PyOx}(FADH_2) \qquad \text{Equation 4.2}$$

$$\text{PyOx}(FADH_2) + MB^+ \rightarrow \text{PyOx}(FAD) + MBH + 2H^+ \qquad \text{Equation 4.3}$$

$$MBH \rightarrow MB^+ + H^+ + 2e^- \qquad \text{Equation 4.4}$$

Table 4.1. A selection of biosensor formats used to detect pyruvate.

Enzyme(s)	**Detection**	**Detection Range**	**Construction Format**	**Reference**
PyOx[1]	H_2O_2 (V not stated)	1-10 mM	Chemical bonding. Polyazetidine prepolymer, nylon membrane	Mascini and Mazzei, 1987
PyOx	0.2-0.5V	0.38-1.03 mM	Modified carbon, Methylene green	Kulys *et al.,* 1992
PyOx	0.3V	1 µM-1.8 mM	Electropolymerisation, conductive redox polymer, glassy carbon	Arai *et al.,* 1999
PyOx/HRP[2]	H_2O_2 (-0.05V)	0.1-3 mM	Modified carbon , Methylene green	Bergmann *et al.,* 1999
PyOx	H_2O_2 (+0.65V)	5 µM-5 mM	Covalent attachment to polytyramine	Situmorang *et al.,* 2002

[1] Pyruvate oxidase; [2] Horseradish peroxidase

4.2 Materials and methods

4.2.1 Reagents, standards and plant material

All of the chemicals used were of analytical grade. Pyruvate oxidase (E.C. 1.2.3.3.; PyOx) derived from *Pediococcus* spp., thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), hydrochloric acid (HCl) and 2,4-dinitrophenyl hydrazine (2, 4-DNPH); pyruvic acid sodium salt ($C_3H_3O_3Na$), magnesium sulphate ($MgSO_4$), sodium hydroxide (NaOH) and trichloroacetic acid (TCA); potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma (Dorset, UK); Fisher Scientific Chemicals (Dorset, UK), and BDH. Ltd. (Leics., UK), respectively. All reagents were made up in reverse osmosis water. FAD and TPP co-factors were made up as 3 mM and 6 mM stock solutions, respectively, and stored at -20˚C until use. $MgSO_4$ was prepared as a 0.9 M stock solution and stored at 4˚C. Pyruvate oxidase solution was made up in the co-factor mix. Pyruvic acid sodium salt for deriving calibration standards was made up as a 5 mM stock solution and stored at 4˚C.

Commercially grown onion cvs. SupaSweet (SS1), Renate, Hyfort, Red Baron, UK Sturon, Crystal, Marimba and Spanish Pandero bulbs were donated by F.B. Parrish and Son Ltd. (Beds., UK), Moulton Bulb Co. Ltd. (Lincs., UK), or Bedfordshire Growers Ltd. (Beds., UK).

4.2.2 Onion pyruvate analysis

Total pyruvate was measured according to Schwimmer and Weston (1961) and Crowther *et al.* (2005) with slight modifications. Whole onion bulbs were homogenised using a domestic blender (Braun, Type 4192, Spain) (Yoo and Pike, 1999). The juice was left to incubate for 1h at room temperature. Aliquots (1.5 ml) were transferred to eppendorf tubes and centrifuged at 16060 g (rotor 3325) for 10 min (Biofuge Pico, Kendro Laboratory Products, Germany). Some samples were subsequently stored at -20˚C prior to analysis. Juices were thawed at room temperature for 30 min and diluted 15-fold in deionised water. Filtrates (0.5 ml) were added to 1 ml aliquots of 0.0125% (v/v) (2, 4-

DNPH) in 2 M HCl and 1.5 ml deionised water in boiling tubes. The mixture was briefly vortexed and incubated at 37°C for 10 min. Five ml of 0.6 M NaOH was added and the absorbance at 420 nm recorded (Camspec M501, Camspec Ltd., Cambs, UK). A standard curve to allow calculation of pyruvate concentrations from onion samples was produced by taking 10 ml of 5 mM pyruvic acid stock solution and diluting to 1 mM, followed by serial dilutions giving a concentration range of standards of 0.04 to 0.4 mM. Pyruvate concentrations (µmol g^{-1} FW) in onion were determined from the equation of the straight line on the standard curve.

4.2.3 Unmediated electrodes

Screen printed disposable plain carbon electrodes were manufactured by Cranfield University, Silsoe, UK. The electrodes comprised of a central carbon working electrode (10 mm^2), a counter electrode and a Ag/AgCl reference electrode (Plate 4.1). The electrodes were printed using a DEK 247 screen-printer (DEK Printing Machines Ltd., Dorset, UK). Sensors were connected to an Autolab workstation (Echochemi, Utrecht, The Netherlands) via custom made electrical connectors (RS Components, Northhants., UK). The Autolab was controlled by the Autolab General Purpose Electrochemical System (GPES) software.

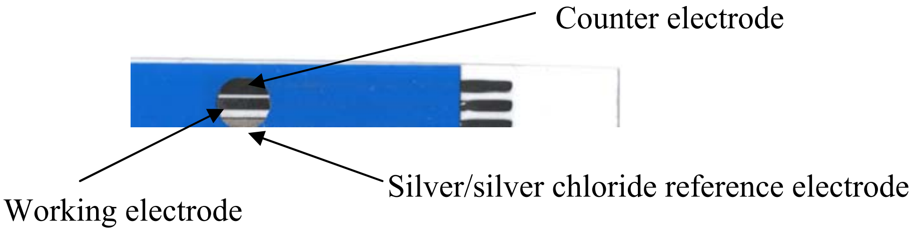

Plate 4.1. Representation of Cranfield carbon electrode (Scale 1:1).

Measurements were initially carried out at +800 mV at 21˚C in a controlled environment. All experiments were undertaken in triplicate. All electrodes were only used once before disposal.

Initially, the response of unmediated carbon electrodes without enzyme and cofactors to onion cv. Renate juice was examined. Reagents included 50 mM sodium phosphate buffer pH 6.9 and cofactor mix A: 2 units PyOx, 0.2 mM TPP, 0.01 mM FAD and 10 mM $MgSO_4$ (final concentrations). The electrochemical response to increasing pyruvate concentrations in previously frozen undiluted onion juice was also compared against a calibration curve using the modified Schwimmer and Weston (1961) assay. All measurements were made by depositing 0.1M 20 µl KCl electrolyte in phosphate buffer on the electrode surface (Plate 4.2), applying the potential then allowing a steady state current to be reached before adding 20 µl onion juice.

Plate 4.2. Experimental set-up: Portable potentiostat with three-electrode array

4.2.4 Mediated electrodes

Generic carbon, mediated with meldolas blue (C2030519D5, Gwent Electronic Materials Ltd. GEM, Gwent, UK) comprised the working electrodes (28 mm^2) and were screen printed with Ag/AgCl reference/counter electrodes onto a PVC substrate in a two-electrode configuration (Plate 4.3). Electrochemical measurements were carried out using a PalmSense potentiostat (Palm Instruments BV, The Netherlands). Electrochemical measurements were carried out at either +150 mV or +200 mV at 21°C. Resulting currents were integrated, expressed in μC.

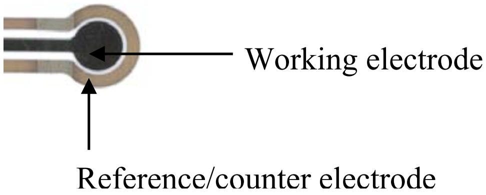

Plate 4.3. Meldolas Blue Electrodes with a two-electrode array, manufactured by Gwent Electronic Materials Ltd (GEM, UK) (Scale 1:1).

Enzyme immobilisation was achieved by depositing an enzyme cocktail containing PyOx, TPP, FAD and $MgSO_4$ made up in 0.1 M sodium phosphate buffer pH 5.7 onto the surface of the working electrode. Electrodes were left to air dry for 4h, then subsequently stored at 4°C prior to use. The optimum pH for hydrogen peroxide liberation from the reaction of pyruvate oxidase and pyruvate in the presence of phosphate and oxygen is 5.7. Irrespective of pyruvate content (2.3 – 6.6 μmol g^{-1} FW), the pH of onion juices for cvs. Red Baron, SS1, Marimba, Crystal, UK Sturon and Spanish Pandero marketed in the U.K. corresponded with the optimum for PyOx activity.

The electrochemical response to undiluted juices from individual onion cvs. SS1 and Renate bulbs of increasing pyruvate concentration were examined in two separate experiments using meldolas blue sensors.

4.2.5 Interference

The effect of operating potential on interference in juices from different onion (cvs. Red Baron, Spanish Pandero and Hyfort) cultivars were compared at +150 mV and +200 mV using cofactor mix B: 1 unit PyOx, 0.04 mM TPP, 0.1 mM FAD and 30 mM $MgSO_4$ (final concentrations).

4.2.6 Statistical analysis

All statistical analyses were carried out using Genstat for Windows Version 7.1.0.198 (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. Pearson's Rank Coefficients were used to measure linear relationships between variables.

4.3 Results and discussion

4.3.1 Response of unmediated carbon electrodes with and without PyOx and cofactors to onion juice

The electrochemical biosensor response to onion cv. Renate juice was considerably amplified with the addition of enzyme and co-factors. A significant response was also evident with the addition of juice on bare electrodes due to other electrochemically active species in onion inevitably being oxidised at +800 mV (Table 4.2). Nevertheless, these preliminary results demonstrated the feasibility of measuring pyruvate concentration in onion juice using an amperometric biosensor format, but in order to reduce the biosensor operating potential, mediated sensors were adopted for further biosensor development.

Table 4.2. Effect of addition of onion extract on standard carbon biosensor response.

Delivered solution	**Current; i (μA)**	**Standard Error**
Buffer	0.2842	0.0773
Buffer, pyruvate oxidase plus co-factors (Solution A)	0.5580	0.0659
Buffer and onion extract (100 ml l^{-1})	5.6003	0.6303
Solution A + aqueous onion extract (100 ml l^{-1})	17.467	1.7704

4.3.2 Response of mediated meldolas blue electrodes to undiluted onion juice

Meldolas blue biosensors responded positively to increasing pyruvate concentrations in onion juice at a significantly reduced potential of +200 mV compared with unmediated electrodes at +800 mV. An operating potential of +200 mV was adopted following preliminary experiments where a constant operating range between +50 mV and +250 mV was demonstrated (Fig. 4.1).

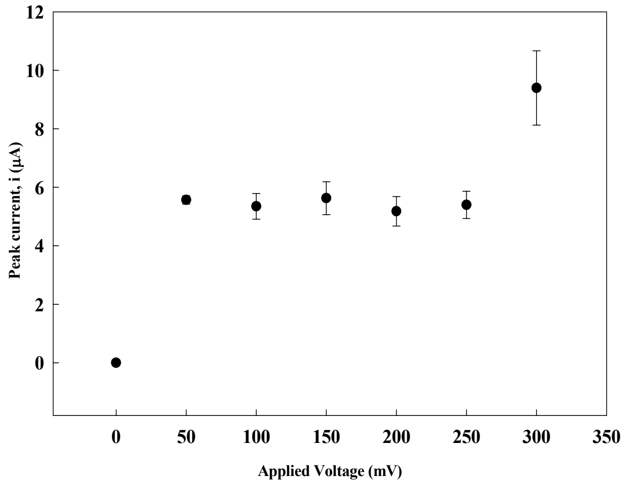

Figure 4.1. Optimising applied potential (V) on mediated meldolas blue pyruvate oxidase-based sensors with a 5mM pyruvate standard in 0.1M phosphate buffer pH 5.7; 0.1M KCl. Standard error bars are from the mean of three experiments.

Furthermore, the meldolas blue biosensor gave considerably enhanced signals compared with the standard carbon format. A good correlation between the mediated biosensor responses to known pyruvate within juices of onion cvs. SS1 (low pungency) and Renate (high pungency) was demonstrated (Figs. 4.2 and 4.3). A resolution of at least 1 mM is required by the onion industry in order to differentiate between mild and intermediate strength onions. This was clearly achieved using the developed mediated pyruvate oxidase-based biosensor. In addition, the total assay time was reduced to 3 minutes, compared with 1h employed within the industry assay.

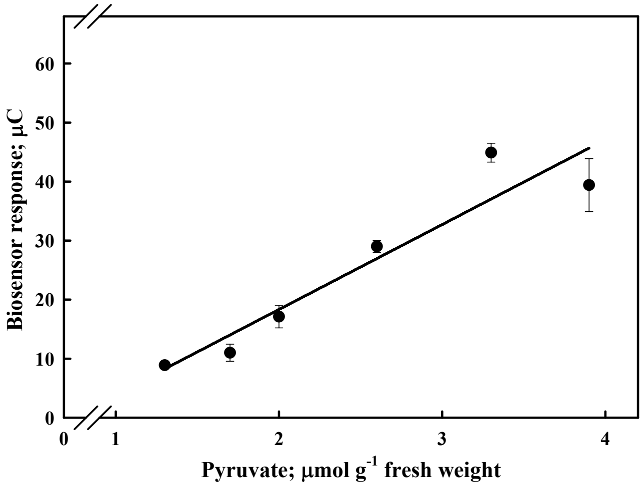

Figure 4.2. Mediated biosensor response to onion juices from six individual low pungency bulbs of increasing pyruvate concentration verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). $R^2 = 0.83$; $y = 14x - 9$; $P < 0.001$. Standard error bars are from the mean of three experiments. +200 mV; 0.1M KCl phosphate buffer pH 5.7; co-factor mix B.

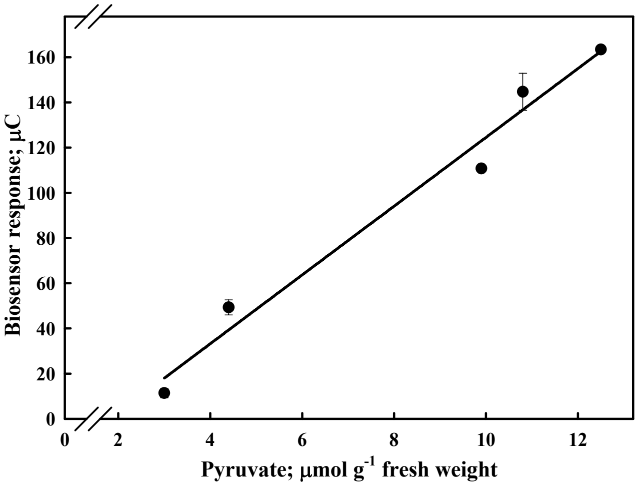

Figure 4.3. Mediated biosensor response to onion juices from two individual low pungency (cv. SS1) and three high pungency (cv. Renate) bulbs of increasing pyruvate concentration verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). $R^2 = 0.97$; Biosensor response (µC) = 15 x pyruvate concentration – 27; $P = 0.001$. Standard error bars are from the mean of three experiments. +200 mV; 0.1M KCl phosphate buffer pH 5.7; co-factor mix B.

4.3.3 Interference Experiments

There was, with some cultivars, a significant response on bare compared with enzyme immobilised mediated electrodes, indicating the presence of interference compounds. There was also a clear variation in background current across and within onion cultivars at +150 mV and +200 mV. This background response was reduced at +150 mV without any deterioration in performance. Levels of interference were 4.7% ± 1.8, 80.3% ± 5.5 and 32.0% ± 4.4 at +150 mV and 31.0% ± 3.8, 89.3% ± 1.9, 42.7% ± 8.1 at +200 mV for cvs. Red Baron, Spanish Pandero and Hyfort, respectively (Appendix B, Figs. B4.3.1 and B4.3.2). The corresponding pyruvate contents ranged between 4.3 – 6.6, 7.7 – 9.5 and 10 – 10.7 µmol g^{-1} FW for cvs. Red Baron, Spanish Pandero and Hyfort, respectively. These results suggested that major polyphenols in red-skinned onions (cv. Red Baron; Price *et al.,* 1997), were unlikely to be a major contributing factor given the low interference response.

5. Conclusion

Meldolas blue mediated electrodes were shown to be the best biosensor format tested for the amperometric detection of pyruvate in the juice from macerated onion tissue. There was a strong correlation between the biosensor response and known pyruvate concentrations (2 – 12 µmol g^{-1} FW) in onion as measured using the modified Schwimmer and Weston (1961) colorimetric assay. No sample dilution was necessary as found with some pyruvate biosensors developed for clinical applications (*cf*. Table 4.1).

This preliminary study demonstrated for the first time the possibility of replacing the standard colorimetric assay used ubiquitously by the world onion industry for determining pyruvate concentration with a more rapid method using a mediated amperometric biosensor. The introduction of a pyruvate biosensor for onions will empower growers to undertake their own quality control, rather than outsourcing pungency analysis. Subsequent research aims to further improve biosensor performance and elucidate possible variations in cultivar interference.

CHAPTER 5

A pyruvate dehydrogenase-based screen-printed biosensor for assessing pungency in onions (*Allium cepa* L.)

5.1 Introduction

The development of pyruvate biosensors over the decades has been driven by demands arising within the medical sector (Mascini and Mazzei, 1987; Kulys *et al.,* 1992; Zhang *et al.,* 1997; Bergmann *et al.,* 1999; Mizutani *et al.,* 2000). The majority of these formats have utilised oxidase-based enzyme systems such as that described in Chapter 4. In comparison, there is a lack of commercial biosensors employing dehydrogenases, supposedly owing to the need to regenerate the co-factor (Sprules *et al.,* 1996; Luong *et al,* 1997; Prodromidis, 2002), including the lack of established protocols for deydrogenase stabilisation. Nevertheless, there are an increasing number of reports on biosensor formats that use NADH cofactor-dependent dehydrogenases coupled with the mediator meldolas blue (7-dimethylamino-2,3-benzophenoxazine). These biosensors have proved successful however in detecting a number of key analytes in food including, lactate, ethanol, ammonia and malate (Wedge *et al.,* 1999, Vasilescu *et al.,* 2003; Lupu and Palleschi, 2004) and required significantly lower operating potentials, reducing the influence of oxidisable interfering compounds which may adversely affect biosensor operating performance. Consequently, the feasibility of utilising a dehydrogenase was examined. In addition, one desirable characteristic facilitating the development of a commercial biosensor is that of high output currents (pers. comm. R. Pittson) resulting from the enzyme-substrate reaction. Preliminary investigations found this to be the case with the pyruvate dehydrogenase-mediated meldolas blue format.

To date, there are no reports on the development of a disposable biosensor to determine pyruvate levels in onion bulbs utilising a pyruvate dehydrogenase/NAD^+ format, which the present study describes. A follow on study will evaluate technical performance characteristics of both oxidase and dehydrogenase pyruvate biosensors, including commercial viability.

The reaction catalysed by pyruvate dehydrogenase (PDH) is described by (Eq. 5.1). The amount of NADH generated, enzymatically, can be evaluated by monitoring its subsequent oxidation by the biosensor. At the working electrode, the current generated from the reduced mediator (Eq. 5.2) gives a measure proportional to the initial pyruvate concentration found in the sample. Biosensor performance (amperometric pyruvate) was evaluated against the standard industry colorimetric assay (Schwimmer and Weston, 1961) for assessing onion pyruvate.

$$\text{Pyruvate} + \text{NAD}^+ \xrightarrow{\text{PDH}} \text{acetyl-CoA} + \text{NADH} + \text{CO}_2 \qquad \text{Equation 5.1}$$

$$\text{NADH} + \text{Med}_{\text{OX}} \xrightarrow{\text{Electrode}} \text{Med}_{\text{RED}} + 2e^- + \text{NAD}^+ \qquad \text{Equation 5.2}$$

5.2 Materials and methods

5.2.1 Reagents and standards

Pyruvate dehydrogenase (PDH) derived from *Lactobacillus delbrueckii*, lipoamide (Lip A), coenzyme A (CoA) and β-nicotinamide adenine dinucleotide (NAD^+) were purchased from Sigma (Dorset, UK). All other reagents were as described in section 4.2.1. FAD, Lip A, NAD^+, CoA and TPP co-factors were made up as 3 mM, 1 mM, 4 mM, 1 mM and 4 mM stock solutions, respectively, and stored at -20°C until use. The required PDH concentrations were made up in the co-factor mix. Pyruvic acid sodium salt for deriving calibration standards was made up as 5 mM stock solutions and stored at 4°C.

5.2.2 Plant material

Commercially grown UK onion cvs. SupaSweet (SS1), Rumba, Musika, Rose de Roscoff, Hired and Renate bulbs were harvested in August 2005 and donated by F.B. Parrish and Son Ltd. (Beds., UK). This batch of bulbs was not cured postharvest and were tested with the biosensor after 6 weeks storage at 4°C.

5.2.3 Onion pyruvate analysis

Total pyruvate was measured according to Schwimmer and Weston (1961) and Crowther *et al.* (2005) with slight modifications as described in section 4.2.2.

5.2.4 Electrodes and electrochemical measurements

The mediated meldolas blue screen-printed (carbon-based) sensors described in the previous chapter (Plate 4.2) were used to develop the pyruvate dehydrogenase-based biosensor. Electrochemical measurements were carried out using a PalmSense potentiostat (Palm Instruments BV, The Netherlands). Electrochemical measurements were carried out at +50 mV at 21°C in a temperature controlled environment as described in section 4.2.3.

Enzyme immobilisation was achieved by depositing a known volume of enzyme cocktail containing PDH, TPP, Lip A, CoA, FAD and NAD^+ (made up in 0.1M sodium phosphate buffer pH 5.7) onto the surface of the working electrode. Electrodes were left to air dry for 4h, then subsequently stored at 4°C prior to use. The optimum pH for PDH (*Lactobacillus delbrueckii*) activity is 6.3. Previous studies showed that the pH of onion juice for a wide range of onion cultivars marketed in the UK ranged from 5.5 to 5.7 (data not shown). Hence, and for convenience, a pH in buffer solution of 5.7 was adopted. The procedure adopted for electrochemical tests was as previously described (Chapter 4). All measurements were made by depositing 0.1M 20 µl KCl electrolyte in phosphate buffer on the electrode surface prior the addition of onion juice or standards.

5.2.5 Optimisation studies

Preliminary work was conducted to determine the effect of applied potential (-100 to +200 mV). Cofactors associated with the pyruvate dehydrogenase complex where added to assess whether increased concentrations improved biosensor performance. Concentration of co-factors TPP (0 to 0.2 mM), FAD (0 to 0.1 mM) and Lip A (0 to 0.2 mM), CoA (0 to 1 mM) and NAD^+ (0 to 1 mM), enzyme loading (0 to 1 units) and temperature (5 to 40°C) on biosensor performance was evaluated. The best conditions identified for optimum PDH biosensor performance were used thereafter.

5.2.6 Biosensor calibration studies

The PDH biosensor was calibrated against pyruvate standards ranging from 0 to 7.5 mM (0 – 7.5 μmol g^{-1} FW) made up in 0.1 M sodium phosphate buffer pH 5.7. The supporting electrolyte was 0.1M KCl in the aforementioned buffer.

5.2.7 Experiments with onion juice

The electrochemical responses to freshly extracted juices from five randomly selected individual bulbs of onion cvs. SupaSweet (SS1), Rumba, Musika, Rose de Roscoff, Hired and Renate bulbs were examined using the optimised biosensor. Juices were left to incubate at 21˚C for 1h before applying to the biosensor. Pyruvate contents for individual bulbs were verified via conventional analysis (Schwimmer and Weston, 1961).

5.2.8 Statistical analysis

All statistical analyses were carried out using Genstat for Windows Version 7.1.0.198 (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. Pearson's Rank Coefficients were used to measure linear relationships between variables.

5.3 Results and discussion

5.3.1 Optimisation studies

The optimum applied potential and enzyme loading of PDH using a 5 mM pyruvate standard were +50 mV (Fig. 5.1) and 0.6 units (Fig. 5.2), respectively. The required operating potential was lower than most other mediated pyruvate biosensors reported (200-500 mV) (Kulys *et al.,* 1992; Mikki *et al.,* 1995).

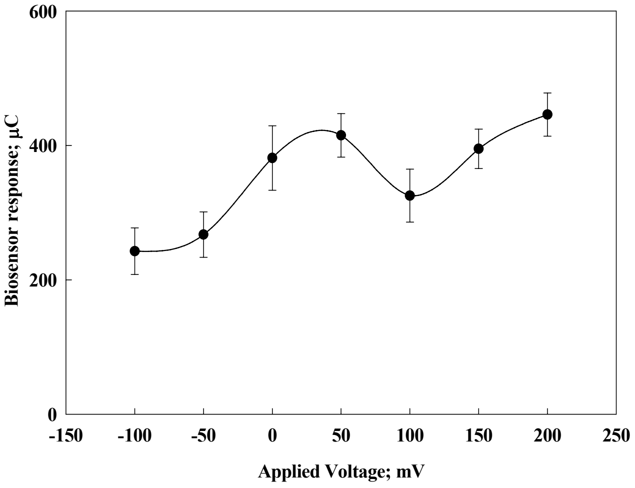

Figure 5.1. Pyruvate dehydrogenase biosensor optimisation of applied potential (V) -100 mV to +200 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; 1 unit PDH, cofactors 20 mM TPP and FAD, 1 mM Lip A, 0.29 mM CoA, 0.4 mM FAD and 1mM NAD$^+$(final concentrations). Standard error bars represent the mean of three replications.

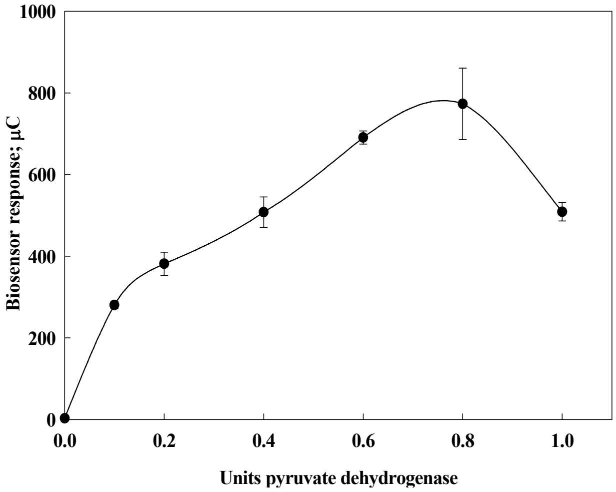

Figure 5.2. Pyruvate dehydrogenase (PDH) optimisation 0 to 1 unit; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 20 mM TPP, 0.4 mM FAD and 1 mM LipA, 0.29 mM CoA and 1mM NAD$^+$ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

The most favourable temperature for enzyme activity was between 35 and 40°C (Fig. 5.3); however experiments were conducted at room temperature for convenience. Previous studies on the pyruvate dehydrogenase complex demonstrated an optimum of 37°C (Fine and Costello, 1963).

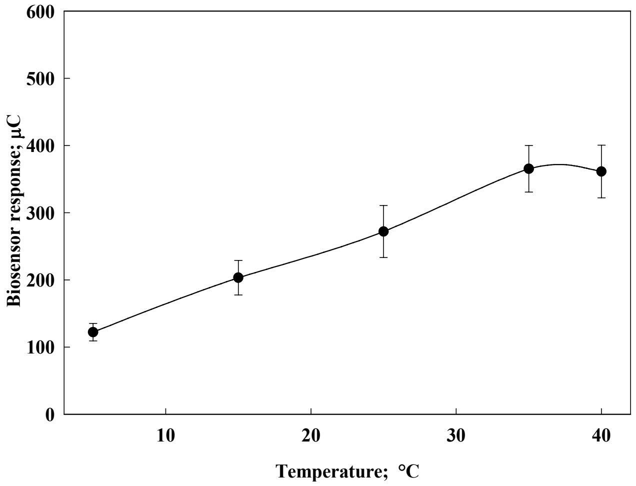

Figure 5.3. Influence of operating temperature (5 to 40 °C). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl. Optimised reagents 0.6 units PDH, 0.12 mM TPP, 0.06 mM FAD, 0.16 mM Lip A, 0.12 mM CoA and 0.2 mM NAD^+ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

The reaction within the pyruvate dehydrogenase complex is well known, involving cofactors lipoamide, coenzyme A, thiamine pyrophosphate, nicotinamide adenine dineucleotide and flavin adenine dinucleotide. The absence of thiamine pyrophosphate (TPP) within the co-factor mix had the same effect on the biosensor as when no enzyme was present (Fig. 5.4), i.e. no response. TPP is an essential co-factor for the oxidative decarboxylation of pyruvate to form acetyl-coenzyme A (Voet and Voet, 1995). Whereas NAD^+ is a coenzyme that reversibly binds to enzymes, Lip A and FAD are prosthetic groups which are permanently part of the pyruvate dehydrogenase complex. It was necessary to however examine the biosensor response to increased concentrations of these cofactors to assess whether an improved current signal would be obtained.

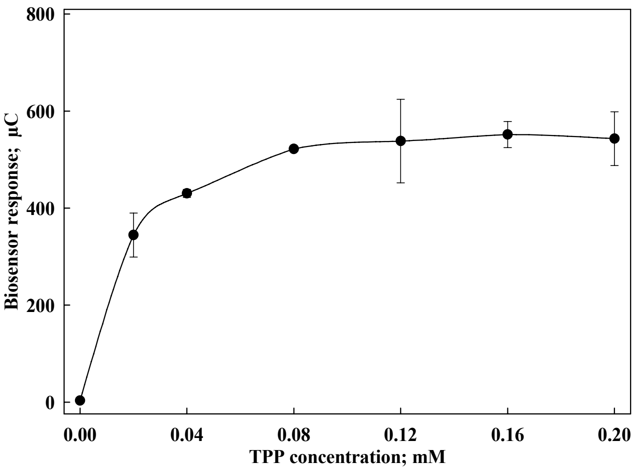

Figure 5.4. Thiamine pyrophosphate (TPP) optimisation (0 to 0.2 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; 0.06 mM FAD, 1 mM LipA, 0.01 mM CoA and 0.2 mM NAD^+ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

Additional FAD and NAD^+ contributed to an almost 0.5-fold increase in biosensor response (Figs. 5.5 and 5.6). Additional concentrations of Lip A and CoA had no effect (Figs. 5.7 and 5.8) on biosensor output and so these could be omitted from the co-factor mix. However, significant differences may not have been apparent owing to large errors and so were retained for all further experiments.

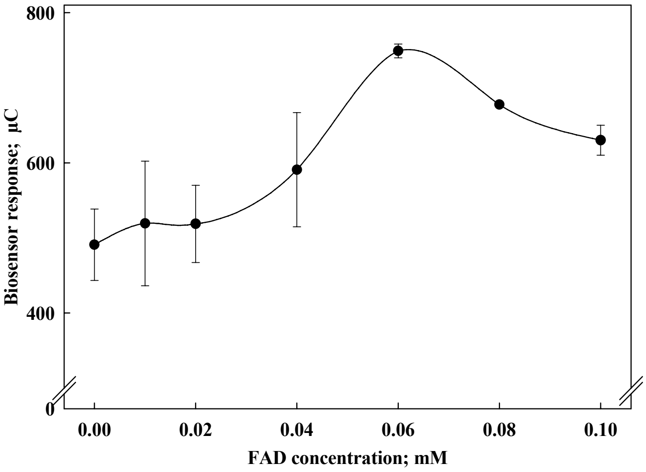

Figure 5.5. Flavine adenine dinucleotide (FAD) optimisation (0 to 0.1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP; 0.3 mM CoA and 0.2 mM NAD^+; 0.2 mM LipA (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

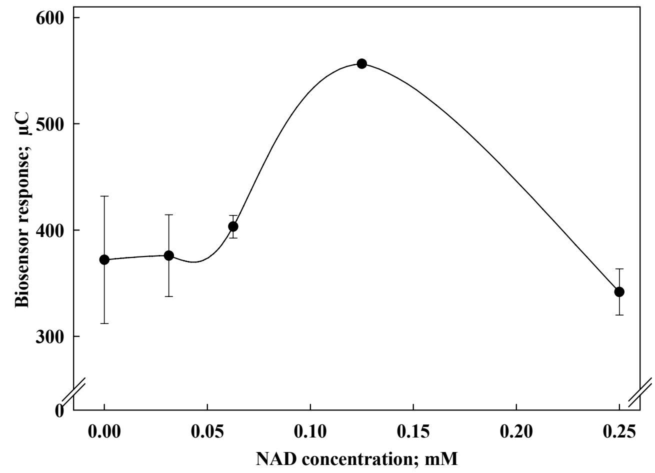

Figure 5.6. Nicotinamide adenine dinucleotide (NAD^+) optimisation (0 to 1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP, 0.1 mM FAD, 0.3 mM CoA and 0.2 mM Lip A (final concentrations); 5 mM pyruvate standard ; 21°C. Standard error bars are from the mean of three experiments.

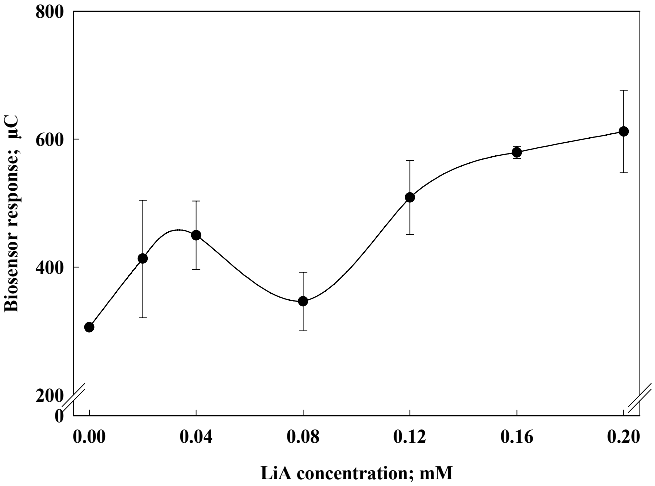

Figure 5.7. Lipoamide (Lip A) optimisation (0 to 0.2 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 80 µM TPP, 60 µM FAD, 20 µM CoA and 1mM NAD^+ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

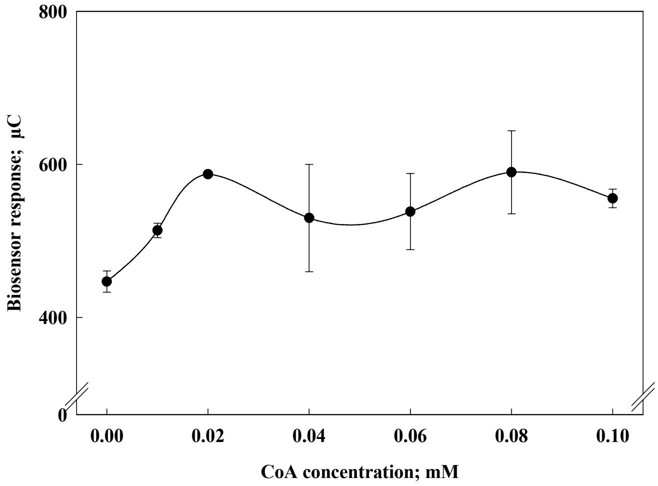

Figure 5.8. Coenzyme A (CoA) optimisation (0 to 0.1 mM). 0.6 units PDH; +50 mV; 5 mM pyruvate standard in phosphate buffer pH 5.7; 0.1M KCl; cofactors 4 mM TPP, 0.06 mM FAD; 0.2 mM LipA and 0.02 mM NAD^+ (final concentrations); 21°C. Standard error bars are from the mean of three experiments.

5.3.2 PDH biosensor calibration

An excellent linear correlation ($y = 61x$, $R^2 = 0.951$) between the pyruvate dehydrogenase biosensor response to pyruvate standards was demonstrated (Fig. 5.9).

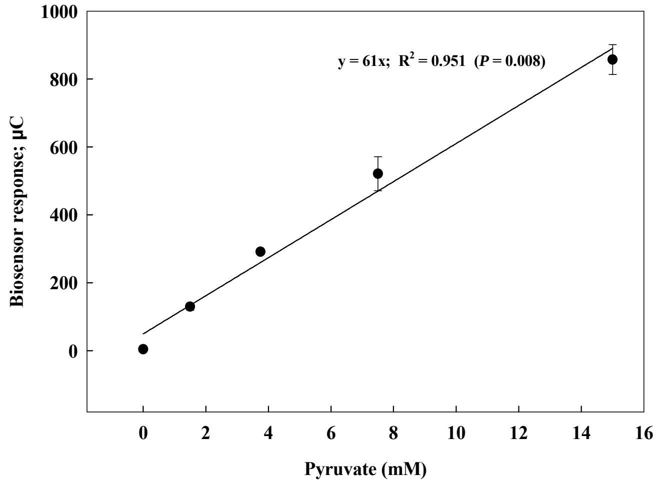

Figure 5.9. Pyruvate dehydrogenase-based biosensor calibration plot with 0 – 15 mM pyruvate standards verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). Standard error bars are from the mean of three experiments, with those for spectrophotometric pyruvate being very small; +50 mV; phosphate buffer pH 5.7; 0.1M KCl; 21°C.

5.3.3 PDH biosensor performance with real onion samples

The biosensor was able to classify onion cultivars (Table 5.1) into mild (< than 4 mM pyruvate) and pungent (>7 mM). Values highlight slight differences between pyruvate standards in buffer and pyruvate within real onion juice samples, most likely owing to other compounds present within the juice matrix. Nevertheless, the resolution was *ca.* 0.5 mM as was also the case with the pyruvate oxidase-based pyruvate biosensor (Chapter 4). The pyruvate dehydrogenase-base pyruvate biosensor was thus comparable to the current colorimetric assay employed by industry. Total time to carry out biosensor measurement was 2 minutes.

Table 5.1. Classification of six onion cvs. SS1, Rumba, Musika, Rose de Roscoff, Hired and Renate bulbs (n = 5) with the pyruvate dehydrogenase biosensor, evaluated against both the spectrophotometric assay and biosensor calibrated using standards in buffer.

	Spectrophotometric pyruvate	**PDH biosensor pyruvate calibrated from standards in buffer**	**PDH biosensor pyruvate calibrated with real onion juice samples**
Cultivar		**(µmolg^{-1} FW ≡ mM)**	
SS1	4.0	3.79	3.85
Musika	4.2	3.96	3.82
Rose de Roscoff	5.5	5.77	6.42
Hired	5.8	5.44	5.51
Rumba	6.5	6.59	6.96
Renate	7.8	7.91	7.52

Colorimetric pyruvate LSD (P = 0.05) = 0.1909, Biosensor pyruvate LSD (P = 0.05) = 0.4975

5.4 Conclusion

This is the first study demonstrating the potential for an alternative biosensor for measuring pyruvate in juice from macerated onion based on NAD$^+$-activated pyruvate dehydrogenase coupled with mediated meldolas blue electrodes. A strong correlation between the biosensor response and known pyruvate concentrations (4 - 8 mM) across six onion cultivars was found as measured using the standard industry assay. The subsequent chapter describes strategies to circumvent or eliminate possible interferences to biosensor performance across and within a wider range of commonly marketed onion cultivars.

CHAPTER 6

Evaluating the performance of pyruvate biosensors during field trials: Part I - Assessing interference

6.1 Introduction

Although biosensors are expected to play an increasing role in fresh produce quality control and food processing (Terry *et al.*, 2005), many unfortunately fail when placed in contact with food samples, generally due to the high complexity of the food matrix and non-target interference species (Luong, 1997). There are a wide variety of potential interferent compounds in onion which could detrimentally affect biosensor performance. These include non-volatile and volatile sulphur compounds, such as sulphenic and thiosulfenic acids and the lachrymator, carbohydrates, amino acids and flavonoids (Breu, 1996; Lanzotti, 2006). When onion bulb tissue is homogenised; an essential step in the Schwimmer and Weston-based assay, allinase, *S*-alk(en)yl cysteine sulphoxide lyase (E.C. 4.4.1.4) cleaves the alk(en)yl cysteine sulphoxides (ACSOs) leading to the release of volatiles, including pyruvate and ammonia in equimolar amounts (Granroth, 1970; Block, 1992).

Common interferences such as ascorbic acid, uric acid, cysteine, acetaminophen and hydrogen peroxide are oxidised on electrodes to give current responses with positive errors (Mizutani *et al.*, 2000). Many fresh produce types also include relatively high concentrations of other non-target phenylpropanoid species that are themselves electrochemically active (e.g. anthocyanins, flavonoids etc.; Terry *et al.*, 2005). Major flavonols in onion are quercetin compounds, quercetin- 3, 4’-diglucosides and quercetin-4’ glucoside (Rodrigues *et al.*, 2003).

There are a number of approaches by which interferences have been minimised. For instance, when developing a biosensor array to monitor non-structural carbohydrates in tropical fruit, Jawaheer *et al.* (2003) reduced interference caused by ascorbic acid in fruit juice by enzymatic inactivation through addition of ascorbate oxidase. Some polymers have properties based on hydrophobic and/or charge interaction between solutes and membranes and may be useful for eliminating anionic interferences. A cellulose acetate membrane was able to discriminate between interference-causing compounds and hydrogen peroxide (34Da) (Jawaheer *et al.*, 2003). For restricting the transport of neutral molecules such as L-cysteine and acetominophen, size exclusion is a technique that has been adopted. Mizutani *et al.* (2000) successfully adopted a pyruvate oxidase/polyion complex-bilayer membrane for the determination of pyruvate. The membrane excluded interferences of molecular weights >100 Da such as glucose and L-glutamic acid.

The pyruvate biosensors previously reported have operated at applied potentials of +50mV (Chapter 5) and +150 mV with a mediated meldolas blue format (Chapter 4). However, despite the apparent interference being minimal for the cultivars tested, it was deemed sensible to subsequently evaluate the mediated meldolas blue biosensor against a much wider range of commonly marketed onion cultivars with varying degrees of pungency. Most importantly, neither of the aforementioned pyruvate biosensors were evaluated on stored onion bulbs. Onion bulbs are commonly marketed out of storage for consumption or processing and biochemical constituents following long-term storage may differ from pre-storage assessments (Patil *et al.*, 1995; Benklebia, 2000). In order to fully appraise the potential of disposable mediated meldolas blue biosensors for routine pyruvate analysis, field trials were undertaken specifically focusing on possible interference issues.

6.2 Materials and methods

6.2.1 Reagents

All reagents were made up as described in sections 4.2.1. and 5.2.1.

6.2.2 Plant material

A wide selection of onion cultivars (cvs.) of varying degrees of pyruvate was selected. These included onion bulbs of cvs. SupaSweet (SS1), Renate, Musika, Red Baron, Rose de Roscoff and Rumba grown under standard UK agronomic practices and donated by F.B. Parrish and Son Ltd. (Beds., UK). Bulbs were stored in air in netted bags at 4ºC. Cultivars Vanetz Sherpa, Hyfort, Spanish Pandero and Red Baron were donated by Moulton Bulb Co. Ltd. (Lincs., UK), G's Marketing Ltd. (Cambs., UK) or Bedfordshire Growers Ltd. (Beds., UK) and were held in bulk bins as is standard industry practice until marketed. Bulbs were removed from storage at 6, 12 and 18 weeks.

6.2.3 *Conventional onion pyruvate analysis*

Total pyruvate was measured as described in section 4.2.2.

6.2.4 *Electrodes and electrochemical measurements*

Pyruvate oxidase and dehydrogenase-based biosensors were constructed as described in 4.2.4. and 5.2.4., respectively. With the exception of forced oxidation experiments, electrochemical measurements were carried out at either +50 mV or +150 mV, operating potentials for the mediated pyruvate dehydrogenase and oxidase biosensors, respectively. With the exception of incubation temperature studies, all experiments were undertaken at 21°C in a temperature controlled environment as described in section 4.2.3. Again, all electrochemical measurements were undertaken as described in sections 4.1. by depositing 0.1M 20 µl KCl electrolyte in phosphate buffer on the electrode surface allowing a steady state current to be reached before adding 20 µl undiluted onion juice or organic acid standards. All experiments were undertaken in triplicate.

6.2.5 *Evaluation of pyruvate oxidase and dehydrogenase biosensors with fresh and frozen onion juices*

The performances of the optimised PDH and PyOx biosensors were evaluated initially with freshly extracted onion (cvs. SS1, Musika, Rumba, Renate, Rose de Roscoff and Hired) juices. In a separate experiment, the biosensors were re-evaluated with the same juices which had been frozen at -20°C. Before testing, juices were thawed at room temperature for 30 minutes. Five individual bulbs from each of the six cultivars were each tested separately. Bulbs were removed from storage at 6, 12 and 18 weeks and left to acclimatise at room temperature for 24h before juice extraction (200W Moulinex, Spain). Pyruvate content in treated juices was verified against conventional analysis. Experiments were carried out in triplicate.

6.2.6 Temporal effects on background interference

To study the effect of onion juice incubation time on background current and interference, the electrochemical response to fresh onion cvs. Vanetz Sherpa and Hyfort juice were examined on electrodes with and without the PyOx enzyme at +150mV. Wedges of bulbs were homogenised for 2 minutes using a hand blender (Braun, Type 4192, Spain). The juices were passed through Whatman No. 1 filter paper. Aliquots of onion juices (20 µl) were immediately deposited on blank-mediated, then PyOx-mediated sensors at 5, 10, 20, 40, 60, 120 and 180 min intervals post homogenisation, and the current change recorded. At the above mentioned intervals, any further production of pyruvate caused by the reaction of allinase with ASCOs was arrested by adding to 80 µl of 5% (v/v) TCA to 20µl of incubated onion juices and in Eppendorf tubes. Pyruvate was assayed conventionally

The effect of onion juice incubation temperature on background current-the electrochemical response to fresh onion cv. Renate juice was also examined with the PyOx-mediated biosensor at 37°C. Small wedges (n=3) from one bulb (cv. Renate) were homogenised for 1 min using a hand blender. The juice was separated into two 50 ml Pyrex beakers then passed through Whatman No. 1 filter paper. One beaker with onion juice was left at room temperature (control) and the other immersed in a water bath at 37°C for 2 min prior to testing. The incubated sample was cooled rapidly to 21°C in dry ice before analysis. A 20 µl aliquot of either control or treated onion juice was deposited on blank electrodes at 5, 15, 25, 45, 65 and 85 minutes intervals post homogenisation. At the above mentioned intervals, any further development of pyruvate was arrested using TCA. Pyruvate was assayed conventionally

In a separate study, to examine the temporal changes and quantify biosensor background signals, these commonly marketed onion cultivars ranging from yellow to red types with low, medium and high pyruvate contents were selected. Five bulbs of each cultivar (viz. SupaSweet (SS1), Renate, Musika, Red Baron, Rose de Roscoff and Rumba) were removed from store at 6, 12 and 18 weeks postharvest. Their juices were extracted from half bulbs and left to incubate for 1h before depositing on blank-mediated meldolas blue electrodes with applied voltages of +50mV and +150mV for the pyruvate dehydrogenase (PDH) and PyOx- based biosensors respectively. Pyruvate was assayed conventionally.

6.2.7 Biosensor response to organic acids

Potentially interferent major organic acids present in onion include malic, oxalic, citric and ascorbic acid. Accordingly, standards (5 mM) of pyruvic, malic, oxalic and citric acid were made up in buffer solution and 20 µl of each acid solution presented to both blank-mediated and PyOx-mediated biosensors and the change in current recorded.

6.2.8 Quantification of ascorbic acid and hydrogen peroxide in onion juice

Uncured bulbs (n=3) of cvs. SupaSweet (SS1), Renate, Musika, Hired, Rose de Roscoff and Rumba were cut vertically down the middle and one half immediately snap frozen in liquid nitrogen and stored at -20ºC before freeze drying. Tissue was freeze dried for 72h and the lyophilized tissue ground to a fine powder using a mortar and pestle before assaying for ascorbic acid using an ascorbic acid test kit (Megazyme International Ltd., C. Wicklow, Republic of Ireland), according to manufacturer's instructions. The biosensor response with blank-mediated sensors at +50 mV (PDH biosensor) and +150 mV (PyOx biosensor), with 20 µl of freshly extracted juices also from the same bulbs, was evaluated against ascorbic acid contents. Pyruvate was assayed conventionally.

Hydrogen peroxide (H_2O_2) was determined according to Snell and Snell (1971) with slight modifications using the same bulbs assayed for ascorbic acid. Juice was extracted from bulbs (n=3) for each cultivar. Juices were incubated at room temperature for 1h. A 0.3 ml aliquot of onion juice was added to 3 ml phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 g for 25 min. Next, 3 ml of the supernatants were added to 1 ml of 0.1% titanium sulphate in 20% (v/v) H_2SO_4. The mixtures were again centrifuged at 6,000 g for 15 min and the absorbance measured at 410 nm (Camspec M501, Camspec Ltd., Cambs, UK). H_2O_2 was calculated from a standard curve prepared from serial dilutions of 30% (v/v) hydrogen peroxide.

6.2.9 Enzymatic removal of ascorbic acid

In a separate experiment enzymatic removal of ascorbic acid was examined to assess its contribution to residual background current. An onion (cv. Sprinter) bulb was homogenised for 2 minutes. The juice was allowed to stand for 1h then filtered through Whatman No. 1 paper. The biosensor response to five treatments (A-E) was compared on blank-mediated and PyOx-mediated sensors: A, untreated undiluted onion juice; B, onion juice was diluted 1:1 with sodium phosphate buffer solution (pH 5.7); C, diluted juice with addition of ascorbic acid oxidase (10 units ml^{-1}); D, diluted juice with the addition of pyruvate oxidase (10 units ml^{-1}); E, diluted juice with the addition of both pyruvate oxidase and ascorbic acid oxidase (10 units ml^{-1}). Pyruvate was assayed conventionally. Experiments were carried out in triplicate.

6.2.10 *Effect of freezing onion juice on background biosensor response*

Onion juice suspends fibre, pulp and protein. Freshly extracted or homogenised onion tissue was observed to be cloudier in appearance than juices which had been frozen then thawed and as a result their effect on background biosensor signals was compared. Quarter bulbs (n=3) of cvs. Spanish Pandero, Red Baron and Hyfort were homogenised as described above. The cultivars Spanish Pandero and Hyfort are white, whilst cv. Red Baron is a commonly UK marketed red type. Juices were left to incubate for 1h at room temperature then filtered prior to analysis with blank-mediated then PyOx-mediated biosensors (+150 mV). Some samples were transferred to Eppendorf tubes and frozen for 48h at -20°C. Thereafter, juices were thawed at room temperature for 30 min as described earlier. Juices were retested with the biosensors as described above. Juices were analysed for pyruvate before and following freezing for 48h via conventional analysis.

6.2.11 *Effect of forced oxidation*

To evaluate whether forced oxidation could reduce or eliminate the effect of potential non-target interference compounds found in onion juice, the response of the PyOx-mediated biosensor was examined prior to and following oxidation of onion cv. Vanetz Sherpa juices in four separate experiments. A 10 ml aliquot of filtered onion extract was transferred to a stirred beaker. A plain carbon electrode was immersed in the stirred beaker and either a potential of +150, +750, +1500 or +2000 mV was applied for 3 min. A 20 μl aliquot of onion juice was next removed from the beaker and deposited on blank-mediated then PyOx-mediated biosensors. Prior to and following forced oxidation, the pyruvate content of juice was assayed as described previously.

6.2.12 Statistical analysis

Data were subjected to ANOVA using Genstat for Windows Version 7.1.0.198 (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. Pearson's correlations (R^2) were used to assess relationships between variables.

6.3 Results and discussion

6.3.1 Comparative performance with juice samples across onion cultivars

The performance of PyOx-based and PDH-based biosensors was evaluated against a number of onions cvs. that were stored for 6, 12 and 18 weeks. Juice was either freshly extracted or previously frozen for 48 h when presented to the biosensors. As formerly reported (Abayomi *et al.*, 2006), the PyOx-based biosensor performed well with low pungency onions (Table 6.1). Irrespective of storage duration, results demonstrated that the dehydrogenase-based biosensor worked better with certain cvs. and depending whether fresh or frozen samples were used. Similar results were for the oxidase-based biosensor also (results not shown). Table 6.2 shows the integrated currents obtained from selected individual bulbs with similar pyruvate contents across cultivars, for both freshly extracted and frozen then thawed juices. The overall inconsistency demonstrated that these pyruvate biosensors were being differentially affected by specific interference from various cultivars.

Table 6.1. Pyruvate oxidase-based biosensor performance verified against standard industry (Schwimmer and Weston, 1961) analysis. Juices from frozen-thawed onion (cv. SS1) bulbs stored for six weeks.

Bulb Nr.	**Spectrophotometric pyruvate**	**Biosensor pyruvate**
	(μmolg^{-1} FW ≡ mM)	
1	3.47	3.79
2	3.90	3.90
3	4.23	4.61
4	4.20	4.17
5	4.80	4.29

LSD (0.05) colorimetric pyruvate= 0.276 (P < 0.001); biosensor pyruvate=0.389 (P = 0.001) (Appendix B, Tables B4.3.1 and B4.3.2).

Table 6.2. Pyruvate dehydrogenase biosensor performance during field trials. Juices are from freshly extracted and frozen-thawed onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs stored for six weeks.

		Spectrophotometric pyruvate (mM)		**PDH Biosensor pyruvate (mM)**	
Cultivar	Bulb Nr.	Freshly extracted juice	Frozen-thawed juice	Freshly extracted juice	Frozen-thawed juice
SS1	1	3.73	3.47	2.53	5.97
Musika	3	3.83	3.90	3.58	5.92
Rose de Roscoff	5	4.73	4.23	5.48	8.80
Hired	3	4.87	4.20	5.74	4.80
Rose de Roscoff	2	5.67	4.80	6.64	6.93
Rumba	1	5.80	6.07	6.33	7.10
Hired	2	6.80	6.20	6.38	5.50
Renate	5	7.03	5.67	8.29	6.94
Renate	2	9.00	6.83	6.86	6.68
Rumba	5	9.73	8.03	8.58	7.32
LSD (0.05)		0.302 ($P < 0.001$)	0.276 ($P < 0.001$)	1.86 ($P < 0.001$)	1.75 ($P < 0.001$)

6.3.2 Time-course studies

There was a significant (*P* < 0.001) decrease in background current for cv. Hyfort over the 3h incubation period, from 77.5 to 17.3 µC (Fig. 6.1). It is likely that the initial concentration of labile sulphur-based compounds produced during hydrolysis of alk(en)yl cysteine sulphoxides (ACSOs) is one of the causes of the transitory interference observed and that their rapid decline occurs within 5 minutes. Thereafter, the residual background current remained relatively stable. Background interference for cv. Vanetz Sherpa did not change (Appendix B, Tables B6.1.1 to B6.1.4) although there appeared to be a declining trend (results not shown). The variability between replicates was particularly large, thus, preventing clear differences between intervals to be realised. Upon onion tissue disruption, unstable sulphenic acids arise (Block, 1992).

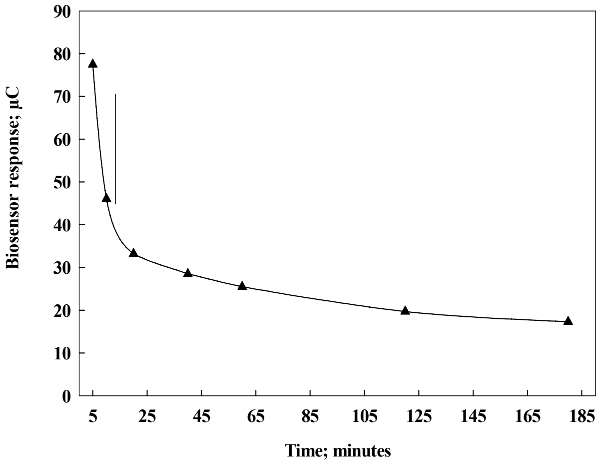

Figure 6.1. Time-course studies on background interference (µC) from onion (cv. Hyfort) juice during 3h incubation using blank-mediated pyruvate oxidase biosensors. LSD (*P* = 0.05) = 24.74 µC (n=3). +150 mV; phosphate buffer pH 5.7; 0.1M KCl;. 21°C.

The amount of lachrymator quantified between 5 and 120 seconds decreased linearly for cv. Dehydrator. This trend was less apparent for cv. Sweet Vidalia (Kopsell *et al.*, 2002). Calvey *et al.* (1997) observed a similar pattern for the same incubation period. In the present study, after 1h incubation there was no rise in pyruvate for either cultivar although it increased from 6.8 to 7.9 mM and 11.5 to 12.6 mM after 3h for cvs. Vanetz Sherpa and Hyfort, respectively. In accordance with current results, Lancaster *et al.* (1998) examined pyruvate production and found cultivars differed in their time to maximum pyruvate production following maceration. Hydrolysis of total ACSOs took place within 5 seconds. Again, Hanum *et al.* (1995) reported maximum pyruvate production after 2 hours for a yellow sweet onion cultivar. It was proposed that onion allinase decomposes the precursors at different rates (Kopsell and Randle, 1997). It has also been suggested sulphur containing compounds may also act as oxidising agents towards flavonols (Jungbluth and Waldemar, 2002). These factors, coupled with the enzymatic oxidation of flavonols via polyphenyl oxidase, were most likely responsible for the rapid loss in background current observed. Hence, increasing the homogenate standing time at room temperature for as little as 5 minutes substantially reduced level of interference and, thus, is a viable method of improving biosensor performance.

6.3.3 *Juice incubation temperature and temporal effects*

Raising the temperature of the incubating onion juice (cv. Renate) to 37°C for 2 min did not accelerate the degradation of background current when compared to control juice left at room temperature (results not shown). Temperature was demonstrated to influence ascorbic acid degradation in fruit juices, with different juices exhibiting different tolerance ranges (Ozkan *et al*, 2004) although not flavonols (Franke *et al.*, 2004) in onion. It may be that in the present study, 37°C was not sufficiently high to effect any significant current deterioration with onion juice. There was no difference in pyruvate within treated and control juices. Although commercial biosensors may incorporate a heating and temperature control device, heating above 40°C would not be a viable pre-treatment method as the optimum temperature for both pyruvate oxidase and pyruvate dehydrogenase activity is *ca.* 35°C.

There were significant currents resulting from onion juice on bare (blank-mediated) electrodes. One of the key attributes of a good mediator is to facilitate electron transfer. However, an undesirable consequence is that the mediator may also simultaneously aid the transfer of other oxidisable species within the onion juice matrix. Over 18 weeks, there were significant ($P < 0.001$) changes in background interference across cultivars (Appendix B, Tables B6.2.1 to B6.2.3) at both +150 mV (Fig. 6.2a) and +50 mV (Fig. 6.2b), decreasing for some (cvs. SS1 and Musika) whilst remaining constant or fluctuating for others. The response profile was generally the same at either applied potential for all cultivars with the exception of cv. Renate (Fig. 6.2b), suggesting an anomaly. Ascorbic acid, one potential interferent, increased linearly (0.5 mg 100g^{-1} fresh weight month^{-1}) in stored onion bulbs irrespective of temperature (Gubb and McTavish, 2002) although in another study with cvs. Red Creole, Texas Early Grano and White Majestic, ascorbic acid increased with increasing storage temperatures (0ºC, 10ºC, 20-25ºC, 22-32ºC) (Iglesias *et al*., 1987).

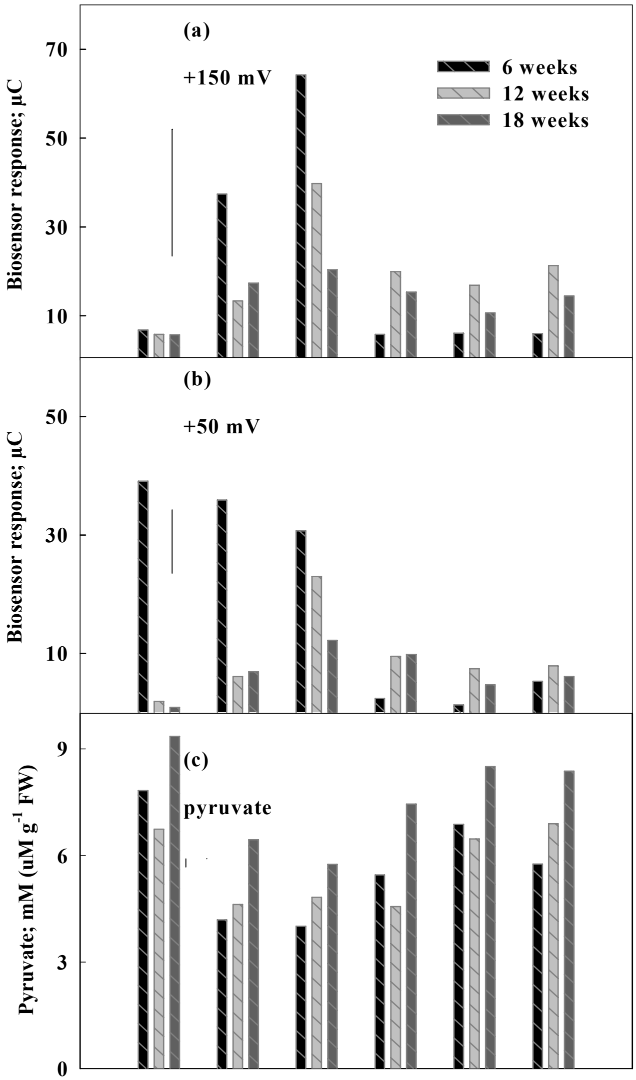

Figure 6.2. Changes in background responses (µC) for pyruvate dehydrogenase (a) and pyruvate oxidase (b) and pyruvate (c) over 18 weeks in storage at 4ºC with onion cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba juices on blank-mediated sensors. Standard error bars are from the mean of three experiments.

The reduction in background interference was most dramatic between weeks 6 to 12 for cvs. SS1 and Musika (low pungency, low dry matter content varieties). The levels of potentially oxidisable species within and across onion cultivars are highly variable (Rodrigues *et al*., 2003; Yang *et al*., 2004) and would be expected to change during storage owing to metabolic processes, so the results were not unexpected.

Background interference was greater in low pungency bulbs, probably as a result of higher intrinsic ascorbate concentrations. Once this background was reduced by freezing, more variability and error was still observed for onion cvs. with higher pyruvate levels. It was postulated that because pungent onion cvs. tend to have high dry matter (Havey *et al.,* 2004) and thus higher concentrations of fructans, extracted juice was more viscous. This viscosity may have sufficiently reduced contact between the sample and the electrode, with variations across individual bulbs.

Reducing the applied potential from +150 mV to +50 mV, the operating potential for the pyruvate dehydrogenase-mediated biosensor, again further minimised the influence of interferents by at least half. A low applied potential in a biosensor system is a commonly used strategy to minimise the level of interference caused by electrochemically active species. Decreasing the applied potential from +200mV to +150mV reduced the effect of interference compounds by as much 80% for most cultivars tested (Abayomi *et al*., 2006). Low pungency cultivars were negatively associated with higher background currents at both +50mV ($r = -0.65$) and +150mV ($r = -0.61$). There was no clear correlation between pyruvate changes and background response at either +50 mV or +150 mV over the 18 week period, where pyruvate increased significantly ($P < 0.001$) in all cultivars (Fig. 6.2c).

6.3.4 Biosensor response to major organic acids

Compared with a 5 mM pyruvate standard, the contributing signal from the major organic acids in onion were 2.3, 2.3 and 3.6 % for malic, oxalic and citric acid, respectively, confirming the specificity of pyruvate oxidase for its substrate. There was no difference in response between acids for the PyOx-mediated biosensor.

6.3.5 Hydrogen peroxide and ascorbic acid on mediated background response

Endogenous H_2O_2 is a metabolite present in plants and is also known to be produced as a result of tissue wounding (Mukherjee and Choudhuri, 1985). Both enzymatic and non-enzymatic mechanisms may give rise to the formation of H_2O_2 in onion juice. The non-enzymatic oxidation of ascorbic acid for example involves the reduction of molecular oxygen to superoxide radical by ascorbic acid in the presence of copper and iron (Serrano and Barceló, 1999). Phenolic compounds were also shown to produce H_2O_2 by autooxidation (Takahama *et al*., 2001). Moreover, its significance in the biosensor system is the possible contribution to biosensor signals via electro-oxidation on mediated electrodes according to the following reaction below:

$$H_2O_2 \xrightarrow{\text{mediated electrode}} O_2 + 2H^+ + 2e^- \qquad \text{Equation 6.1}$$

Ascorbic acid content in cvs. SS1, Musika, Hired, Renate, Rose de Roscoff and Rumba was similar to that found previously by other authors (Adam *et al*., 2000; Bahorun *et al*., 2004), ranging from 0.03 to 0.15 mg g^{-1} FW (Table 6.3).

Table 6.3. Hydrogen peroxide, ascorbic acid and pyruvate content in uncured onion (cvs. SupaSweet (SS1), Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Cultivar	**Ascorbic acid (mg g^{-1} FW)**	**Hydrogen peroxide (m/m)**	**Spectrophotometric pyruvate mM (µmol g^{-1} FW)**	**Onion type**	**Storage characteristics**
SS1	0.103	0.00020	4.40	Yellow	Short
Musika	0.145	0.00033	3.68	Yellow	Short
Renate	0.030	0.00080	7.58	Brown	Long
Rose de Roscoff	0.052	0.00089	5.08	Pink	Medium
Rumba	0.044	0.00145	7.75	Brown	Long
Hired	0.100	0.00177	6.40	Red	Long

LSD (P = 0.05) Ascorbic acid = 0.054; LSD (P = 0.05) Hydrogen Peroxide = 0.00116; LSD (P = 0.05) Pyruvate = 0.135

The levels of H_2O_2 in onion juices has not previously been cited, neither its relationship with ascorbic acid or pyruvate. There was an inverse relationship ($r = -0.79$) between ascorbic acid content and pyruvate in onion bulbs, with less pungent types having higher ascorbic acid contents. There was also a positive relationship ($r = 0.69$) between pyruvate content and H_2O_2 with more pungent cultivars containing higher H_2O_2 levels. However, there was no association between ascorbic acid and H_2O_2 in bulbs. Besides ascorbic acid, which, in studies by Yang and co-workers (2004) accounted for only 2.6% of total antioxidant activity in onion, the bulk of antioxidants are probably associated with the flavonoids quercetin, kaempferol, myricetin, anthocyanins and catechin (Nematollahi and Malakzadeh, 2003; Yang *et al.*, 2004). Anthocyanins are responsible for the coloured pigmentation in onions (Ferreres *et al.*, 1996; Price *et al.*, 1997).

Serranó and Barceló (1999) demonstrated that although phenolics in strawberry could be degraded both in the absence and presence of H_2O_2, the addition of H_2O_2 increased the decay of anthocyanins from 50% to approximately 85%. This was also the case for catechin. Other phenolics in strawberries however were not affected by H_2O_2 as was the case for quercetin-3-glucoside. According to Gennaro *et al.* (2002), flavonoids exist in complex conjugates with sugars. These are also likely to vary within and across onion cultivars. In relation to ascorbic acid, Ozkan and co-workers (2004) studied ascorbic acid degradation in orange, grape and pomegranate juice and found that not only the addition of 5 $\mu l\ l^{-1}$ H_2O_2 result in increased degradation rates, but also the presence of anthocyanins accelerated ascorbic acid degradation. Different juices displayed different susceptibilities. The presence of anthocyanins, coupled with H_2O_2 could lead to rapid anthocyanin degradation giving rise to low background interference response consistently observed for red cvs. Hired and Red Baron. Hence, it is proposed that both biochemical attributes and metabolic processes are responsible for the differences in biosensor background responses, ascorbic acid and H_2O_2 content across cultivars.

There is conflicting evidence as to the composition of phenylpropenoids across cultivars. White onions were not found to contain quercetin (Patil *et al.*, 1995; Pike *et al.*, 2004; Okamoto *et al.*, 2006), or anthocyanin (Pike *et al.*, 2004). Rodrigues *et al.*, (2003) however did find quercetin and anthocyanins in white onions albeit in lesser amounts compared with red onions. It was suggested that quercetin could also decrease the concentration of oxidants which are able to oxidise ascorbic acid by terminating radical chain reactions and scavenging O_2 radicals (Yasuhiro *et al*., 1988). According to Yang *et al*. (2004), yellow onions contained more quercetin than red, with pink and white with having the lowest content. If this is the case, it may account for the higher levels of ascorbic acid found in less pungent cultivars such as SS1 and Musika.

6.3.6 Enzymatic removal of ascorbic acid in onion

The removal of ascorbic acid from onion (cv. Sprinter) juice resulted in a significant ($P < 0.001$) reduction in background biosensor response (Table 6.4, treatment C), reaffirming conclusions that ascorbic acid was likely to have a major influence in background interference with low pungent cultivars, albeit synergistic. More importantly the biosensors performance in quantifying pyruvate in onion (treatment D) was not affected. Explaining the synergy referred to above, the presence of ascorbic acid in onion juice may provide a stabilizing effect on antioxidants. On developing a sample preparation method for determining oxidized flavonols in onion and leek, the addition of ascorbic acid was used to protect flavonols from oxidation (Jungbluth and Waldemar, 2002). Note the PyOx biosensor response for treatments A and B are the same owing to the linear range of the biosensor being reached. Surprisingly, the response obtained from the addition of pyruvate oxidase to the juice (treatment D) and the significant reduction in background current obtained was identical to the addition of ascorbic acid oxidase (treatment C). It may be that the effect of the additional H_2O_2 generated within the juice, a product of the reaction between pyruvate oxidase and pyruvate in onion, actually promotes ascorbic acid degradation via the processes described in section 6.3.5. Although the level of interference was significantly reduced by adding ascorbic acid oxidase to the homogenate, this option is not favorable as the additional cost of enzyme will substantially increase the unit cost of the disposable biosensor.

Table 6.4. Pyruvate oxidase-mediated biosensor response to the removal of ascorbic acid in onion cv. Sprinter juice (+150 mV).

Treatments	**Spectrophotometric pyruvate (mM; µmol g^{-1} FW)**	**Biosensor response (µC)**	
		Blank-mediated	**PyOx-mediated**
A	17.39	43.6	120.5
B	9.87	30.2	118.9
C	8.99	6.5	105.3
D	1.67	2.2	1.9
E	1.57	2.6	2.6

LSD (P = 0.05) Pyruvate = 0.600; LSD (P=0.05) Biosensor Response = 10.78

A = untreated undiluted onion juice; B = onion juice was diluted 1:1 with sodium phosphate buffer solution pH 5.7; C = diluted juice with the addition of ascorbic acid oxidase (10 units ml^{-1}); D = diluted juice with the addition of pyruvate oxidase (10 units ml^{-1}); E = diluted juice with the addition of both pyruvate oxidase and ascorbic acid oxidase (10 units ml^{-1}); (Appendix B, Tables B6.4.1 and B6.4.2).

6.3.7 Freezing and background interference

Pyruvate in onion juice was unchanged by freezing for 48h. Moreover, the electrochemical response with PyOx-mediated biosensors was unchanged for either control or treated onion juices and consistently correlated with pyruvate concentration validated using the modified Schwimmer and Weston (1961) colorimetric assay. Both enzymatic and non-enzymatic reactions are known to occur within freshly homogenised or damaged plant tissue, many of which are slowed down at lower temperatures. Freeman and Whenham (1974) suggested that freezing onion tissue may result in complete or partial enzyme destruction, complete or partial non-enzymic destruction of precursor combinations or both, and complete or partial enzymic hydrolysis of precursors with the loss of volatile reaction products. These processes may explain the significant reduction of background interference observed post freezing. Although using freezing as a pre-conditioning step provided a means of circumventing the problem surrounding interference for certain cultivars it may be impracticable due to time constraints.

Table 6.5. Biosensor response (µC) to onion cvs. Hyfort, Red Baron and Spanish Pandero bulb extracts using either bare-mediated or pyruvate oxidase (PyOx)-meditated sensors before and after 48h freezing at -20°C (+150 mV).

Freezing Time (h)	**Blank-mediated (µC)**			**PyOx-mediated Biosensor (µC)**		
	cv. Hyfort	cv. Red Baron	cv. Spanish Pandero	cv. Hyfort	cv. Red Baron	cv. Spanish Pandero
0	29.5	2.8	29.5	91.4	55.4	88.2
48	1.9	2.4	19.0	91.4	49.2	100.2

LSD (*P* = 0.05) Biosensor = 15.01; LSD (*P* = 0.05) Pyruvate = 0.86. Pyruvate mM (µmol g^{-1} FW) = 10.23, Hyfort; 5.80, Red Baron; 8.60, Spanish Pandero. 1 mM (µmol g^{-1} FW) = *ca*. 10 µC; (Appendix B, Tables B6.5.1 and B6.5.2).

6.3.8 Forced oxidation

There was no reduction of residual interference with the application of elevated applied voltages to the onion (cv. Vanetz Sherpa) juice (results not shown). Following the initial degradation of current, it was assumed that highly oxidisable polyphenolic compounds, abundant in onion may be further reduced by this technique. Pyruvate within onion juice was not affected by forced oxidation. Although oxidation of quercetin in solution has been successfully demonstrated using biosensors (Brett and Ghica, 2003; Nematollahi and Malakzadeh, 2003)**,** generally, free quercetin in onion has been found to be very low (Patil *et al*., 1995; Bonaccorsi *et al*, 2005) which may explain the results observed in this study.

6.4 Conclusion

Results from such extensive biosensor field trials with real biological samples are rarely available. This includes the negative results often resulting from interference with real samples compared with standards in buffer, although it is well known that this is an important factor often limiting commercialisation. This study highlighted just some of the challenges and performance requirements of commercial biosensors for food analysis owing to the complexity of food matrices. Mediated biosensor background signals were quantified across a wide variety of commonly UK marketed onion cultivars including Supasweet (SS1), Renate, Rose de Roscoff, Hired and Rumba, Vanetz Sherpa, Hyfort and Red Baron. A number of contributing compounds and mechanisms were proposed. Temporal biochemical changes were found to occur in stored bulbs which were reflected with blank-mediated biosensor responses. The influence of interference compounds on biosensor performance could be reduced by a number of pre-conditioning steps. Following the major issues of interference highlighted in this study, the subsequent chapter is concerned with resolving the interference issue and reappraising the performances of both pyruvate dehydrogenase and oxidase biosensors.

CHAPTER 7

Evaluating the performance of pyruvate biosensors during field trials: Part II - Resolving interference

7.1 Introduction

Due to the high complexity of the onion juice matrix and presence of non-target interference species, onion pyruvate biosensors were unable to yield consistent results during field trials for all cultivars tested (*cf.* section 6.1). The level of interference was shown to vary across onion cultivars and could be reduced by a number of strategies. These included reducing the applied operating potential, enzymatic removal of ascorbic acid, incubating the extracted juice for a few minutes and also freezing the onion extract. Herein the performance characteristics of the pyruvate oxidase and dehydrogenase meldolas blue pyruvate biosensors are discussed following a selection of different strategies to minimise the effects of interference.

7.2 Materials and methods

7.2.1 Reagents and plant material

In addition to chemicals described in section 6.2.1., polyvinyl polypiridine (PVPP) was purchased from Sigma (Dorset, UK). Onion cultivars used were as described in Chapter 6.

7.2.2 Onion pyruvate analysis

Total pyruvate was measured according to Schwimmer and Weston (1961) and Crowther *et al*. (2005) with slight modifications as described in section 4.2.

7.2.3 Electrodes and electrochemical measurements

Pyruvate dehydrogenase (PDH) and pyruvate oxidase (PyOx) based biosensors were constructed as described in sections 4.2.4. and 5.2.4., respectively.

Electrochemical measurements were carried out at either +50 mV or +150 mV, operating potentials for the mediated PDH and oxidase biosensors, respectively. All experiments were undertaken at 21°C in a temperature controlled environment as described in section 4.2.3.

Electrochemical measurements were undertaken as described in sections 4.1, allowing a steady state current to be reached before adding 20 µl undiluted onion juice or organic acid standards. All experiments were undertaken in triplicate. Pyruvate in onion was quantified from biosensor calibration curves constructed from pyruvate standards in 0.1 M phosphate buffer pH 5.7 and verified against conventional analysis.

7.2.4 Evaluation of PyOx and PDH biosensors with pre-treatments

7.2.4.1 Fresh onion (cv. Renate) juice dilution, PVPP, Tween 80 and filtering

Initial studies were conducted utilising both biosensors. The response to five treatments with bulbs (n=3) of onion (cv. Renate) was compared following juice extraction. Treatments included untreated undiluted onion juice (control); onion juice diluted 1:9 with buffer; passing juice through a 0.45 micron filter; the addition of PVPP (10 % w/w) to juice; and the addition of surfactant Tween 80 (0.5% w/w) to juice. Onion juice contains a matrix of protein particles and so PVPP was trialled as a potential pre-treatment owing to its precipitatory properties.

7.2.4.2 Frozen onion (cvs. Renate, Hired, Rumba and SS1) juice with dilution and Tween 80

Next, the most effective pre-treatments were repeated with frozen then thawed juices from cvs. Renate, Hired, Rumba and SS1 bulbs (n=3). These cultivars are known to vary widely in storability and hence it was assumed also in their biochemical constituents, including potential effect on biosensor performance. Initial results revealed PVPP significantly reduced pyruvate concentrations and thus could not be adopted as a viable pre-treatment.

7.2.4.3 Fresh onion (cvs. Rumba and Hired) juice dilution and Tween 80

A re-evaluation of both biosensor performances using just Tween 80 (0.5% w/w) was carried out using fresh onion (Rumba and Hired) juices (n=2).

7.2.4.4 Combining surfactant and dilution with fresh onion (cvs. Rumba and Hired) juice

The combined effect of dilution and surfactant, including optimisation studies on the dilution factor.

7.2.4.5 Optimised pre-treatments with juice from red onions (cvs. Red Globe and PX21)

Final evaluations were performed using the PDH biosensor only with fresh juices from additional red cultivars Red Globe and PX21 (n=1) and surfactant alone.

7.2.5. Statistical analysis

All statistical analyses were carried out using Genstat for Windows Version 7.1.0.198 (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.

7.3 Results and discussion

7.3.1 The influence of different pre-treatments on PyOx and PDH biosensor performance

7.3.1.1 Dilution, PVPP, Tween 80 and filtering with fresh onion (cv. Renate) juice

Preliminary experiments with freshly extracted onion (cv. Renate) juices revealed that neither filtering nor the presence of 10% (m/m) polyvinylpolypyrrolidone (PVPP) improved the performances of either biosensor (Table 7.1) (Appendix B, Tables B7.1.1 to B7.1.3). Initial results in Chapter 4, where the PyOx-based biosensor was used to quantify pyruvate in cv. Renate (12 mM; 12 μmol g^{-1} FW), were comparable to conventional analysis. However, this was not repeated in this test. The only difference with this set of experiments was the freezing pre-treatment on samples prior to testing with the biosensor.

Table 7.1. PyOx and PDH biosensor assessment of pyruvate in freshly extracted onion (cv. Renate) juices pretreated with 0.5% (w/w) Tween 80, micro filtered, PVPP (w/w) and dilution (1:9 v/v) in buffer, verified against conventional Schwimmer and Weston-based colorimetric analysis.

		Pyruvate concentration (mM; µmol g^{-1} FW)		
Treatment	**Bulb No.**	**Spectrophotometric**	**PDH Biosensor**	**PyOx Biosensor**
Control	1	13.3	9.9	6.2
	2	13.6	8.9	5.6
	3	13.5	7.2	4.9
Diluted (1:9 v/v)	1	1.1	0.9	0.7
	2	1.4	1.5	0.8
	3	1.3	0.8	0.6
Filtered	1	11.6	8.7	6.9
	2	12.3	7.3	6.0
	3	13.2	7.1	7.2
10% PVPP	1	9.2	8.7	6.1
	2	8.3	7.1	6.4
	3	12.6	10.5	7.4
0.5% (w/w) Tween 80	1	12.4	15.6	12.4
	2	13.5	15.1	9.6
	3	13.1	15.0	12.8

LSD ($P = 0.05$) pyruvate = 0.449, PDH biosensor LSD = 1.280, PyOx biosensor LSD = 1.504

7.3.1.2 *Dilution and Tween 80 with frozen onion (cvs. Renate, Hired, Rumba and SS1) juices*

A vast improvement in biosensor performance was observed from diluting juice and also the addition of 0.5% (w/w) Tween 80. From this, it could be deduced that either turbidity and/or distribution of juice on the working electrode, and hence repeatability and reliability with some cultivars was an issue but the problem could be rectified by adding a surfactant to the sample as a pre-treatment. This inhibition due to spreading was again confirmed where frozen-thawed juices from other bulbs of cv. Renate were tested (Table 7.2) (Appendix B, Table B7.2.1). Although the use of surfactants has been associated with biosensors, the usual format is where flow injection analysis (FIA) techniques are employed. A less common approach cited was where Tween 20 was incorporated into diaphorase/ferrocene-modified carbon paste electrodes for NADH measurement (Molina *et al.,* 2003). The current generated was increased 5-fold compared to the control, which was attributed to an improved interaction between enzyme, co-factor and mediator. More uniquely, Abbott Laboratories, Illinois, US (patent filed September, 2005) applied a silicone surfactant onto a mesh layer. This format facilitated transport of aqueous samples into a defined area. There are no previous publications that show that pre-treating biological samples with Tween 80 may improve the performance of amperometric disposable biosensors.

Table 7.2. Effect of various pre-conditioning treatments on pyruvate concentration of previously frozen and then thawed onion juice (cvs. Renate, Hired, Rumba and SS1) validated against the conventional Schwimmer and Weston (1961) assay or either with pyruvate dehydrogenase-based (PDH) or pyruvate oxidase-based (PyOx) mediated biosensors.

			Pyruvate concentration (mM; µmol g^{-1} FW)		
Cultivar	**Treatment**	**Bulb No.**	**Spectrophotometric**	**PDH Biosensor**	**PyOx Biosensor**
Renate	Control	1	8.20	5.04	5.05
		2	8.86	4.92	4.22
		3	9.79	4.14	5.62
	Diluted (1:9 v/v)	1	0.72	0.70	0.69
		2	0.95	0.77	0.75
		3	0.77	0.70	0.49
	0.5% (w/w) Tween 80	1	9.40	9.84	9.77
		2	12.47	9.24	9.64
		3	11.90	11.09	10.63
Hired	Control	1	5.34	4.13	3.04
		2	4.27	2.90	2.61
		3	2.75	2.81	5.15
	Diluted (1:9 v/v)	1	0.51	0.60	0.31
		2	0.48	0.92	0.26
		3	0.53	0.56	0.24
	0.5% (w/w) Tween 80	1	5.02	4.49	5.37
		2	3.66	3.12	5.26
		3	3.68	4.03	5.78

Table 7.2 continued

Rumba	Control	1	3.72	3.46	3.55
		2	5.27	3.82	3.32
		3	5.35	2.98	5.68
	Diluted (1:9 v/v)	1	0.38	0.31	0.27
		2	0.33	0.41	0.33
		3	0.32	0.32	0.29
	0.5% (w/w) Tween 80	1	5.77	5.89	6.78
		2	5.73	5.52	6.56
		3	5.47	5.31	6.35
SS1	Control	1	3.16	3.35	2.66
		2	3.46	3.25	2.68
		3	1.96	2.00	2.78
	Diluted (1:9 v/v)	1	0.28	0.13	0.22
		2	0.38	0.27	0.22
		3	0.38	0.33	0.27
	0.5% (w/w) Tween 80	1	4.61	4.38	4.65
		2	3.91	4.46	3.47
		3	4.18	4.34	4.28

LSD (*P* = 0.05) = 1.465

7.3.1.3 Dilution and Tween 80 with fresh onion (cvs. Rumba and Hired) juices

The PDH-based biosensor performance using freshly extracted onion (cv. Rumba) juice was comparable to colorimetric analysis of pyruvate (Table 7.3). However, for the PyOx-based biosensor, the measurable range appeared to have been reached. With cv. Hired, these experiments clearly demonstrated that even although surfactants resolved the issue of sample distribution on electrode surfaces by reducing surface tension, other challenges were posed.

7.3.1.4 Optimising surfactant concentration and dilution factor

Testing the PDH biosensor with a reduced surfactant concentration in juice revealed that the 0.2% (w/w) Tween-dilution combination gave an inferior performance than for 0.5% (w/w) Tween alone. In addition, the revised dilution of 1:2 (v/v) worked very well with cv. Rumba, but again less so with the red (cv. Hired) cultivar (Table 7.4). Nevertheless, results were within ± 1 mM of the conventional assay. Reliable biosensor performances with some red onion cultivars which have a particularly high level of phenylpropanoids were expected to create a particular challenge and further evaluation was thus required.

Table 7.3. PyOx and PDH biosensor assessment of pyruvate in freshly extracted onion (cv. Rumba and Hired) juices pretreated with 0.5% (w/w) Tween 80 and verified against conventional Schwimmer and Weston-based colorimetric analysis.

			Pyruvate concentration (mM; µmol g^{-1} FW)		
Cultivar	**Treatment**	**Bulb No.**	**Spectrophotometric pyruvate**	**PDH Biosensor**	**PyOx Biosensor**
Rumba	Control	1	12.60	7.99	4.58
		2	13.17	7.64	4.67
	0.5% (w/w) Tween 80	1	12.93	11.12	8.09
	Diluted (1:9 v/v)	2	12.97	12.68	7.58
Hired	Control	1	11.87	5.48	3.41
		2	12.30	5.54	3.64
	0.5% (w/w) Tween 80	1	11.53	8.31	7.25
	Diluted (1:9 v/v)	2	12.37	6.38	6.54

LSD (*P* = 0.05) = 1.570

Table 7.4. PDH biosensor assessment of pyruvate in freshly extracted onion (cvs. Rumba and Hired) juices undiluted, diluted (1:2 v/v) and combined with Tween 80 (0.2%; v/v) and diluted 1:2 (v/v).

			Pyruvate concentration (mM; µmol g^{-1} FW)	
Cultivar	**Treatment**	**Bulb No.**	**Spectrophotometric pyruvate**	**PDH Biosensor**
Rumba	Control	1	12.02	2.31
		2	11.85	1.85
	0.2 % (w/w) Tween 80 + 1:2 (v/v) dilution	1	3.01	2.21
		2	3.08	2.74
	1:2 (v/v) dilution	1	2.93	2.37
		2	3.08	2.74
Hired	Control	1	15.61	1.79
		2	11.57	2.59
	0.2 % (w/w) Tween 80 + 1:2 (v/v) dilution	1	5.02	2.49
		2	3.48	1.70
	1:2 (v/v) dilution	1	4.52	2.95
		2	3.27	1.49

LSD (P = 0.05) = 0.6912

7.3.1.5 Concluding evaluations with additional red onion cultivars

The PDH biosensor was consequently evaluated with additional red cultivars Red Globe and PX21 with the original Tween concentration (0.5% v/v) without diluting. It is difficult to explain why both PDH and PyOx-based biosensor performances consistently had a greater deviation away from colorometric pyruvate values when tested with freshly extracted juices of cv. Hired without analysing the exact constitution of the juice complex. However, expected values of pyruvate for these very pungent cultivars did fall within acceptable levels compared with conventional analysis (Table 7.5). Being at the upper end of the pungency scale where perceptible differences in taste cannot be realised, this value was well within acceptable limits compared with the current assay. Moreover, the PDH biosensor would be capable of objectively classifying these onions as 'strong' or cooking onions.

Table 7.5. PDH biosensor assessment of pyruvate in freshly extracted red onion (cvs. Red Globe and PX21) juices pretreated with Tween 80 (0.5%; v/v) and verified against conventional colorimetric analysis.

		Pyruvate concentration (mM; µmol g^{-1} FW)	
Cultivar	**Treatment**	**Spectrophotometric pyruvate**	**PDH Biosensor**
Red Globe	Control	10.15	6.10
	0.5% (w/w) Tween 80	10.22	10.17
PX21	Control	14.77	10.60
	0.5% (w/w) Tween 80	15.36	13.70

LSD ($P = 0.05$) = 0.298

For both biosensors, the coefficient of variation with pyruvate standards were between 5 and 10% (n = 3), but up to 22% for onions samples for both PDH and PyOx-based biosensor respectively, presumably owing to matrix effects. It is also expected that batch to batch variation would be considerably reduced if sensors were constructed using automated techniques. Irregularity of the enzyme on the working sensor surface was observed following manual deposition and drying.

7.3.2 PyOx and PDH-based biosensor detection limits and calibration profiles

The calibration plot for the PyOx biosensor is shown in Figure 7.1. Using pyruvate standards in buffer, the linear range and detection limit of PDH-based biosensor was almost two-fold that of PyOx-based biosensor, thus making it more applicable for very pungent bulbs or where greater precision is required. It can be seen that the linear range of the (PyOx) biosensor is 7.5 mM (≡ 7 μmol g^{-1} FW in onion), with a saturation point at around 15 mM. The limits of detection (LOD) and other performance characteristics (Table 7.6) for the pyruvate biosensors were calculated from the linear calibration graphs at 95% confidence limit as follows:

LOD = 2 x standard deviation of signal from blank electrodes, where $y = 16x$ and $y = 61x$ for PyOx and PDH biosensors respectively. Hence LOD for the PyOx:

2 x 0.201(Std. error) = 16.5x, so LOD = 71 μM and for PDH, 2 x 0.059 (Std. error) = 60.6x, giving an LOD of 6.62 μM. These limits of detection were very low and comparable with some other pyruvate biosensor developments (Mascini and Mazzei, 1987; Kulys *et al.,* 1992; Bergmann *et al.,* 1999).

With regards to resolution, ±1.0 mM would be favourable, being a requirement for both onion breeders and marketers alike, assisting the selection process and also allowing differentiation between mild/sweet (< 4.0 mM) and Intermediate (> 5 mM) strength types. Both biosensors had resolutions of 0.5 mM.

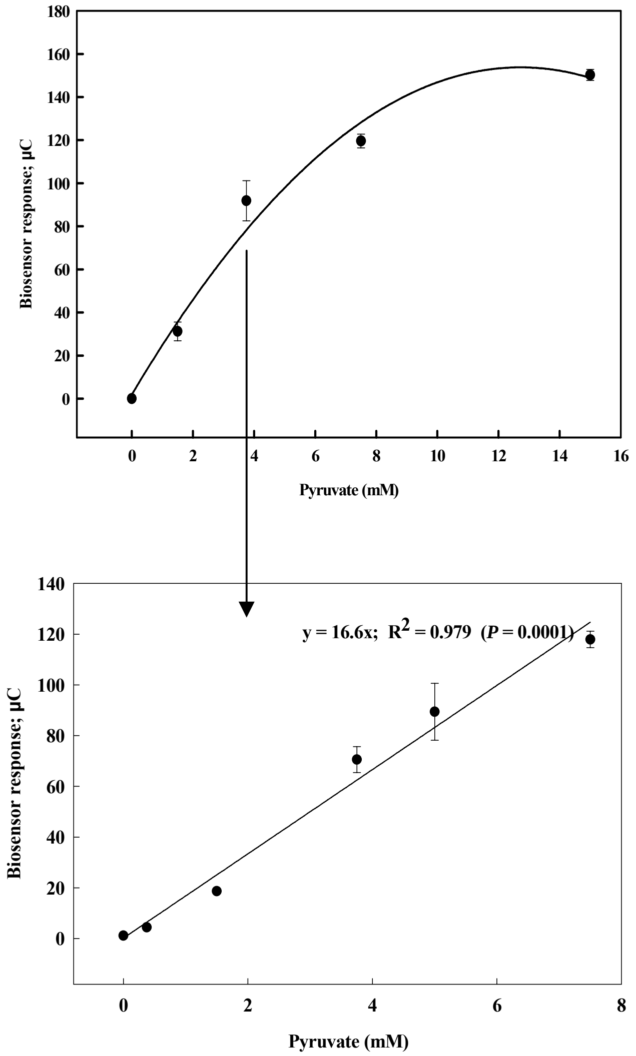

Figure 7.1. PyOx-based biosensor calibration plot with 0 – 15 mM pyruvate standards (≡ 0 to 15 μmol g^{-1} FW in onion) verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). Standard error bars are from the mean of three experiments. +150 mV; phosphate buffer pH 5.7; 0.1M KCl; 21°C.

Table 7.6. Some comparative characteristics for PyOx and PDH-based biosensors against conventional pyruvate analysis

	Amperometric detection		**Spectrophotometric assay (Schwimmer and Weston, 1961)**
Attribute	**PDH**	**PyOx**	
Background residual currents	4.259 μC	1.153 μC	-
Linear range (using standards)	15 mM	7.5 mM	Up to 20 mM
Time to reach steady state	60 s	100s	-
Assay time	120 s	180 s	3600 s
Limit of detection	6.62 μM	71.46 μM	40 μM
Unit cost per unit enzyme	4 x PyOx		-

Calibration with onion juices yielded slightly different profiles. Albeit at 200 mV, the PyOx biosensor was able to measure pyruvate concentrations up to 12 mM (Chapter 4, Figure 4.3), and so it would also be expected that the limit of detection would also give higher values for an onion calibration due to background contribution.

7.3.3 Other analytical considerations

Currents arising from blank sensors can be subtracted from overall currents, leaving that which arises from the catalytic reaction between enzyme and substrate alone. On analysing background interference as a contribution to gross signal, it was evident that currents from the enzyme-substrate reaction for the PDH biosensor were five-fold greater than for the PyOx biosensor. Enzyme activity for PDH calculated from activity assays was found to be 5-fold greater than for PyOx (Appendix A). Therefore reduced quantities may be utilised than was adopted in this work and still give good performance, reducing also unit costs. Similarly the calibration graphs for the PDH and PyOx reflected a four-fold difference in catalytic currents. Background signal arising with freshly extracted juices did not contribute more than 10%, whereas for the PyOx biosensor, contributions to overall signal could be as much as 70% for some cultivars. However, for freshly extracted juices from mild cultivars where background current is generally higher, background current can be quantified using blank sensors.

7.4. Conclusion

Both PyOx and PDH-based pyruvate biosensors have been optimised and validated against a wide range of onion bulbs. Background interference problems and biosensor performance have been improved by various pre-treatments, the best of which were the addition of Tween 80 (0.5% (v/v) or dilution of onion juice samples before application of the juice onto the electrode. Either strategy represents a simple operation which significantly extends the range of cultivars the biosensors can be used upon. Consequently, both pyruvate biosensors have now been adapted so that they are now directly comparable to the original Schwimmer and Weston-based colorimetric assay in terms of resolution, accuracy and versatility for quantification of enzymatically produced pyruvate, whilst being superior by reduced total assay time required and cost.

CHAPTER 8

Towards a multi-analyte glucose and pyruvate biosensor for onions (*Allium cepa* L.)

8.1 Introduction

Sweetness in many vegetables is a desirable attribute that is often governed, in part, by tissue sugar concentration. Carbohydrates in onion constitute the bulk of dry matter (Henry and Darbyshire, 1978). The major non-structural carbohydrates identified in onions are fructose, glucose and sucrose and fructans, but proportions vary with cultivar and maturity. Low pungency, low dry matter cultivars tend to have greater concentrations of fructose and glucose than more pungent, high dry matter onions (Kahane *et al.,* 2001; Terry *et al.,* 2005; Chope *et al.,* 2007)). Recent work has suggested that higher concentration of both glucose and fructose in certain onion cultivars is positively correlated to perceived sweetness and taste preference (Davis *et al.,* 2007). In addition, the presence of organic acids and phenylpropanoids in onion has also been shown to influence differences in taste preference. Hence, onion flavour and taste preference are complex. It is generally recognised that the measurement of pyruvate in onion bulbs is equivalent to pungency (Schwimmer and Weston, 1961; Wall and Corgan, 1992). Furthermore, it has been suggested that pungency masked the perception of sweetness once pyruvate exceeded *ca*. 4 μmol g^{-1} FW (Crowther *et al.,* 2005).

Electrochemical (amperometric) transducers have been dominated by blood glucose measurement. The simultaneous determination of multiple analytes facilitated by the formulation of biosensor arrays has been the result of the success of single analyte biosensors (Newman *et al.,* 2004) such as the glucose sensor marketed by Yellow Springs Instruments (Ohio, USA). The rapid detection and accurate quantification of both glucose and pyruvate has been of increasing clinical importance. Numerous configurations of glucose, and more recently, those coupled with pyruvate biosensors have been reported. These are shown to vary widely in their construction complexity, and hence cost. A lactate biosensor may be used for example to indirectly quantify pyruvate (Sprules *et al.,* 1995; Warriner *et al.,* 1997). Below, Table 8.1 summarises the progress of some of these biosensors, including those incorporating the mediator meldolas blue used in the present study. The majority are fabricated in a three-electrode arrangement.

The market for sweet, low pungency onions is increasing. In the UK, the breeding of sweet onion varieties is constrained by the cost of the existing pyruvate test, considering that large numbers of bulbs need to be screened. If this were resolved the current variability (bulb to bulb) would be reduced and input to new varietal development significantly improved. In addition to the shortcomings associated with spectrophotometric methods for measuring pyruvate, organoleptic assessments undertaken for onion breeding is time consuming, expensive and requires trained taste panels.

Either fructose or glucose could have been chosen as the target analyte for biosensor development. However, glucose was chosen as the most appropriate and feasible analyte to develop a biosensor for quantifying perceived sweetness in onions. Fructose dehydrogenase is expensive (Jawaheer *et al.,* 2003) and there have been previously reported difficulties in the amperometric measurement of fructose (Terry *et al.,* 2005). A glucose biosensor utilising the identical electrode format for both pyruvate biosensors described in Chapters 4 and 5 was optimised and validated for determining glucose in onion juice. A multi-analyte glucose/pyruvate biosensor would allow simultaneous and rapid quantification of pungency and sweetness in onion. Improved quality control would also abet the onion breeding process by providing an estimate of overall flavour as perceived by taste panel assessment. Another objective of this study was to ascertain to what extent a common fabrication format (Meldolas Blue, MB) could be used in order to simplify manufacture and allow future integration of individual (pyruvate and glucose) sensors. The reaction scheme for glucose oxidase on mediated meldolas blue electrodes is shown in Eqts 8.1-8.3, where FAD(H) represents the enzyme's oxidised and reduced active site.

$\text{Glucose} \xrightarrow{\text{GOx}} \text{gluconolactone} + \text{GOx}\ (\text{FADH}_2)$ Equation 8.1

$\text{GOx}\ (\text{FADH}_2) + \text{MB}^+ \rightarrow \text{GOx}\ (\text{FAD}) + \text{MBH} + 2\text{H}^+$ Equation 8.2

$\text{MBH} \rightarrow \text{MB}^+ + \text{H}^+ + 2\text{H}^+ + 2e^-$ Equation 8.3

Table 8.1. Selected mediated multi-analyte biosensor developments for glucose, pyruvate and lactate

Analyte	Electron transfer facilitator	Construction Format	Measurable range	Applied potential (V)	Reference
Glucose	Meldolas blue	Immobilised glucose oxidase (GOx) on oxygen electrode	0 - 25 mM	+50 mV (versus saturated calomel electrode; vs. SCE)	Kulys *et al.*, 1994b
Glucose and choline	Meldolas blue	Immobilised HRP/polymerised MB on glassy carbon electrode	Glucose: to 500µM Choline: to 100 µM	-300 mV (vs. Ag/Ag/Cl)	Mao and Yamamoto, 2000
Glucose, pyruvate and lactate	Cationinic osmium redox polymer	Photolithographic on gold microelectrode array using nano-composite thin film	Glucose: 0 – 20mM Pyruvate: 0 - 2 mM Lactate: 0 - 10 mM	Not specified	Revzin *et al.*, 2002
Glucose	Cobalt Phthalocyanine	Disposable Screen-printed GOx in carbon mediated paste	0.025 - 2 mM	+500 mV (vs. SCE)	Crouch *et al.*, 2005

Table 8.1 continued

Glucose	Polypyrrole	Four electrode configuration-GOx immobilised on Polypyrrole film	0.005 - 20 mM	+500 mV (vs. SCE)	Chen *et al.*, 2006
Glucose and pyruvate	Poly(neutral) red	Amperometric detection Neutral red polymerised on carbon film, enzyme modified electrode surface	Glucose: 90 µM - 1.8 mM Pyruvate: 90 - 600 µM	Glucose: -350 mV (vs. Ag/Ag/Cl) Pyruvate: -250 mV (vs. SCE)	Ghica and Brett, 2006
Glucose	Hexacyanoferrate (III)-chitosan oligomer	Disposable; screen-printed; two electrode configuration; GOx in carbon mediated paste	up to 33 mM	+300 mV Working electrode type not specified	Lee *et al.*, 2006
Glucose	Prussian blue	Amperometric detection Immobilised GOx in chitosan-gold hybrid film on glassy carbon Prussian blue- modified	1 µM - 1.6 mM	+300 mV	Xue *et al.*, 2006

8.2 Materials and methods

8.2.1 Reagents and plant material

Glucose oxidase (EC 1.1.3.4; GOx) derived from *Aspergillus niger*, D- glucose, D-fructose and sucrose were purchased from Sigma (Dorset, UK). D-glucose enzyme test kits were purchased from Megazyme International Ltd., C. Wicklow, Republic of Ireland). Glucose standard solutions were prepared fresh daily and made up in 0.1 M sodium phosphate buffer, pH 5.7. All other chemicals were as described in section 4.2.1.

A range of onions expected to have relatively low pungency were grown to provide material for both glucose and pyruvate biosensor testing. Onion cultivars were grown in the UK using conventional practices. Single 25m rows of each variety were planted as sets in a light sandy soil at G's Marketing Ltd. Cultivars were harvested in 2005 and consisted of cvs. SS1, Buffalo and a commercially bought cv. SS1. Bulbs were cured following standard industry practise.

8.2.2 Onion HPLC sample preparation and extraction

Individual 0.5 cm thick equatorial slices (dry scales removed) were taken from ten randomly selected onions bulbs for each cultivar and immediately snap frozen in liquid nitrogen. Samples were stored briefly at -40°C before being freeze-dried in a Edwards Modulyo freeze drier (W. Sussex, UK) and then milled to a fine powder, before being returned to the freezer until use. Non-structural carbohydrates were extracted according to O'Donoghue *et al*. (2004) with slight modifications (Davis *et al.,* 2007). Freeze-dried onion powder (150 mg) was combined with 3 ml of 62.5:37.5 HPLC grade methanol:water (v/v) and mixed well. Vials of the slurry were placed in a shaking water bath at 55°C for 15 mins, removing briefly and vortexing for 20s every 5 mins. The cooled samples were centrifuged, filtered through a 0.2 m Millex-GV syringe driven filter unit (Millipore Corporation, MA, USA) and stored at -40 until HPLC analysis.

8.2.3 HPLC Glucose analysis

Glucose, fructose and sucrose were determined according to O'Donoghue *et al.* (2004) using a HPLC system comprising a P580 pump, Dionex STH column thermostat and GINA 50 autosampler (Dionex, CA). Diluted 1:10 (v/v) crude onion extract (20 µl) in HPLC grade water, or standard sugar solution was injected into a Rezex RCM monosaccharide Ca^+ size exclusion 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+} security guard cartridge of 4 mm x 3mm diameter (Phenomenex; Part no. AJ0-4493). The mobile phase was HPLC grade water at a flow rate of 0.6 ml min^{-1}. Column temperature was held at 75°C using a Dionex STH column thermostat. Eluted carbohydrates were monitored by an evaporative light scattering detector (ELSD 2420, Waters, MA) connected to the Dionex system using a UCI-50 universal chromatography interface. ELSD was chosen as the preferred method of detection due to greater baseline stability and sensitivity as compared with conventional detection by refractive index (Terry *et al.,* 2005). The presence and abundance of fructose, glucose and sucrose were automatically calculated by comparison of peak area with peak area of external standards using Chromeleon version 4.6 software (Dionex). Assays (n=370) were performed in triplicate.

8.2.4 Onion pyruvate analysis

Total pyruvate was measured according to Schwimmer and Weston (1961) and Crowther *et al.* (2005) with slight modifications as described in section 4.2.2.

8.2.5 Electrodes and electrochemical measurements

To assess the potential for adopting a common format for multi-analyte testing, the same electrodes (Meldolas Blue) used for pyruvate biosensors (*cf.* chapters 4 and 5) were used for the GOx biosensor. Electrochemical measurements were measured as described in sections 4.1. Enzyme immobilization was achieved by depositing 10 µl of solution containing 8 units of GOx (no co-factors required) in sodium buffer (pH 5.7) onto the surface of the electrode. Electrodes were left to air dry for 4 h and then subsequently stored at 4°C overnight until use. Measurements were made by depositing 0.1M 20 µl KCl electrolyte in phosphate buffer on the electrode surface prior the addition of onion juice or standards.

8.2.6 *GOx optimisation and calibration studies*

Optimisation studies were carried out using a 5 mM glucose standard in buffer. These included the effect of applied potential (-50 to +250 mV), GOx loading (0 to 10 units) and operating temperature (5 to 40°C). The best conditions identified for optimum GOx biosensor performance were used thereafter. The GOx-mediated biosensor was calibrated against glucose standards ranging from 0 to 60 mM (0 to 12 mg ml^{-1}) according to the procedure described in section 5.2.6. Selectivity was tested by also measuring current density produced from fructose and sucrose standards ranging from 0 to 12 mg ml^{-1}.

8.2.7 *Real sample testing*

The concentration of glucose (mg g^{-1} FW) and pyruvate (mM) in previously frozen and then thawed juices of the same (n=3) individual onion cvs. SS1, commercial SS1 and Buffalo bulbs was measured using optimised glucose oxidase (GOx)-based and pyruvate oxidase (PyOx)-based mediated meldolas blue biosensors, respectively. Following GOx biosensor calibration studies, it was found necessary to dilute onion juice samples. A 1:10 (v/v) dilution procedure in phosphate buffer was adopted.

Interference for all freshly extracted and previously frozen thawed bulb juice samples was quantified by examining the background currents produced on bare-mediated electrodes. The catalytic currents arising solely from the GOx/glucose reaction were calculated by subtracting the background signal evolved from bare-mediated working electrodes from the charge density arising from GOx-mediated sensors. Blank or dummy electrodes provide a good estimate of the background contribution to overall signals from an individual test (Jawaheer *et al.,* 2003). Results were compared against conventional analysis.

8.2.8 Statistical analysis

Data were subjected to ANOVA using Genstat for Windows Version 7.1.0.198 (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. All measurements were made in triplicate.

8.3 Results and discussion

8.3.1 GOx optimisation

The optimal applied potential was recorded as 0 mV (Fig. 8.1). None of the previously highlighted glucose biosensors (see Table 8.1) operated at such a low potential, which is favourable for minimising the potential for positive bias in output encountered from potential interferents within biological matrices. The maximum current output was obtained with an enzyme loading of between 6 to 8 units GOx (Fig. 8.2). Eight units was adopted for all further development work as a precaution owing to large standard error with 6 units GOx. However, using 8 would not affect performance. Current response was shown to increase with increasing operating temperatures (Fig. 8.3). Xue *et al.* (2006) and other workers (Chen *et al.* (2006) have also shown GOx reaches maximum activity around 40 ˚C albeit with differently constructed electrode formats.

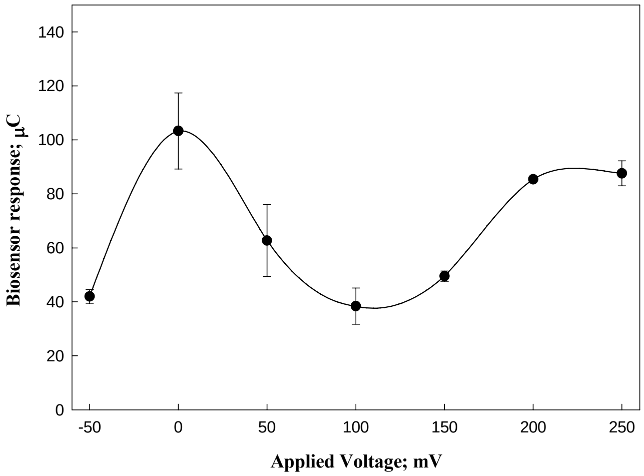

Figure 8.1. Glucose oxidase biosensor optimisation of applied potential (V) -50 mV to +250 mV; 5 mM glucose standard in phosphate buffer pH 5.7; 0.1M KCl; 10 units GOx. Standard error bars represent the mean of three replications.

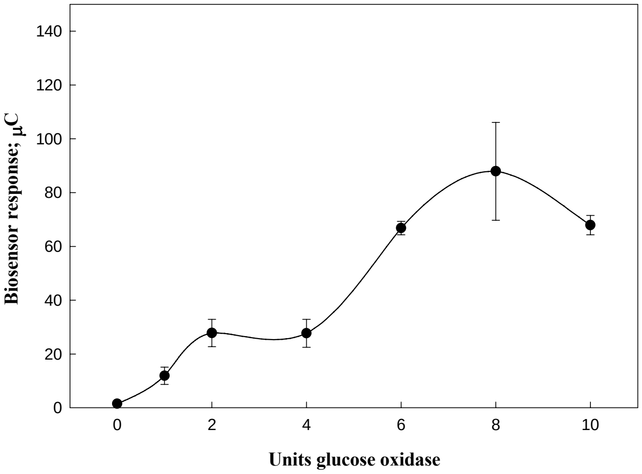

Figure 8.2. Glucose oxidase (GOx) optimisation 0 to 10 units; 0 mV; 5 mM glucose standard in phosphate buffer pH 5.7; 0.1M KCl; 21°C. Standard error bars are from the mean of three experiments.

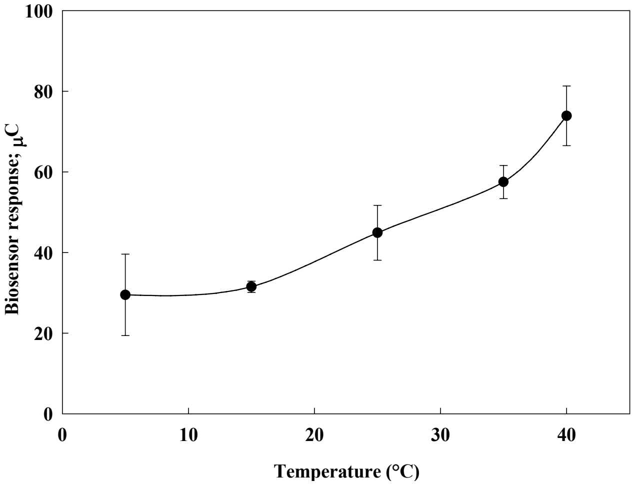

Figure 8.3. Influence of operating temperature (5 to 40 °C) using 5 mM glucose standard in phosphate buffer pH 5.7; 0.1M KCl; 0 mV; 8 units GOx. Standard error bars are from the mean of three experiments.

8.3.2 Linear range and selectivity

The GOx biosensor linear range (Fig. 8.4) was defined as 1-4 mg glucose g^{-1} FW (0-22 mM) and thus a 5-fold dilution of onion juice samples was necessary. This measurable range was comparable to that developed by Kulys (1994b) also using meldolas blue as a mediator although performance was not evaluated against real biological samples.

Virtually no response was observed with the addition of fructose or sucrose (Fig. 8.5) confirming the GOx-based biosensor selectivity towards the substrate glucose in the presence of the other two major simple sugars within the onion juice matrix,

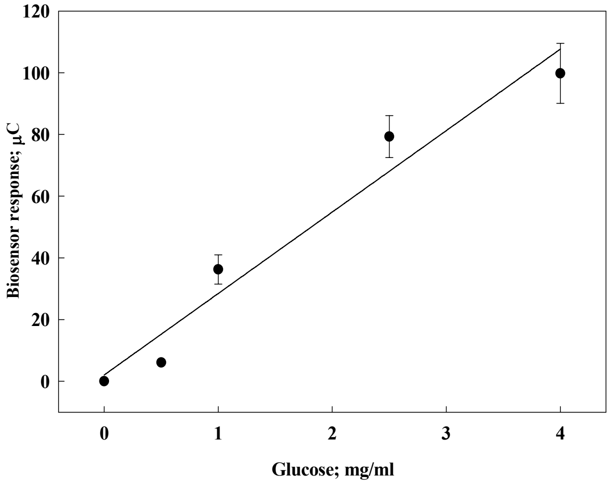

Figure 8.4. Calibration of GOx-mediated biosensor using glucose standards in phosphate buffer pH 5.7; 0.1M KCl. 0 mV; 8 units GOx. Standard error bars are from the mean of three experiments. Biosensor response (µC) = 5 x glucose concentration (mM) + 2; P < 0.001. R^2 = 0.957 (0 to 4 mg ml^{-1} glucose; 0 to 22.2 mM; *P* = 0.0039).

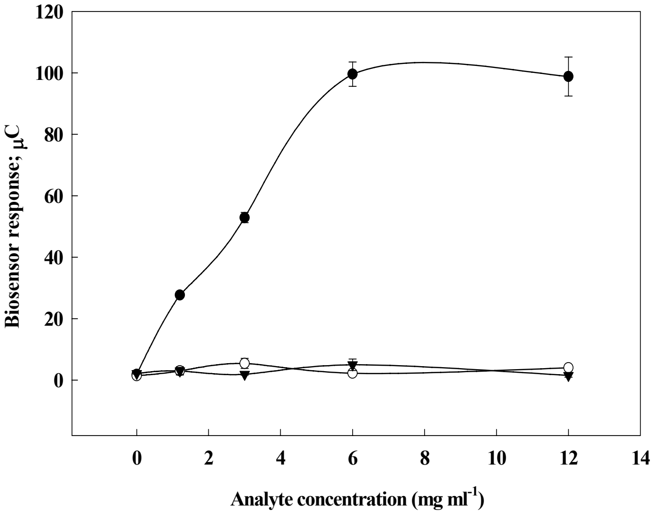

Figure 8.5. Selectivity of GOx-mediated biosensor using 0 to 12 mg ml $^{-1}$ standards of glucose (●), fructose (○) and sucrose (▼) in phosphate buffer pH 5.7; 0.1M KCl; 0 mV; 8 units GOx. Standard error bars are from the mean of three experiments.

8.3.3 Glucose and pyruvate oxidase-based biosensor performances with real onion juice samples

No issues with the glucose biosensor's ability to accurately measure glucose were encountered with the cultivars examined in this study. It is possible though that interferences may present a problem when tested against a wider range of cultivars, as was encountered with the pyruvate biosensors when tested with some red onion cultivars. However, the difference in background current on bare mediated electrodes from freshly extracted juices across or within these mild or sweet cultivars (Table 8.2) was negligible. The only other amperometric glucose and pyruvate biosensors tested on onions were those subsequently reported on by Ghica and Brett (2006) who highlighted no issues with interference. However, the authors did not validate performance with individual onion bulbs, specify the cultivars tested, and more importantly were not able to assess both pyruvate and glucose with the same sample preparation protocol or biosensor format, as their pyruvate and glucose biosensors required different buffer types and pH for optimum performance.

Table 8.2. Background interference on bare electrodes from freshly extracted juices of cvs SS1, commercial SS1 and Buffalo; Operating potential 0V for glucose biosensing.

Cultivar	Bulb	Background biosensor response (μC)
SS1	1	11.8
	2	36.4
	3	15.8
Buffalo	1	12.4
	2	19.4
	3	16.1
Commercial SS1	1	22.2
	2	9.5
	3	15.6

LSD (0.05) = 21.83, *P* = 0.016

Both GOx- and PyOx-based biosensors using the same electrode format were shown to be able to adequately quantify glucose and pyruvate on the same individual onion samples (Table 8.3).

Table 8.3. Concentration of pyruvateand glucose in previously frozen and then thawed juice of individual onion cvs. SS1, commercial SS1 and Buffalo bulbs measured using glucose oxidase (GOx)-based and pyruvate oxidase (PyOx)-based mediated biosensors, respectively, and verified against O'Donoghue et al. (2004)-based HPLC and Schwimmer and Weston (1961) colorimetric assays, respectively.

Cultivar	Bulb	Colorimetric pyruvate	PyOx biosensor	HPLC glucose	GOx biosensor
		mM		mg g^{-1} FW	
SS1	1	3.6	4.3	27.8	28.1
	2	3.2	4.2	22.6	24.3
	3	4.6	5.2	23.9	29.7
Buffalo	1	6.2	5.2	30.3	27.3
	2	5.0	3.8	26.4	34.9
	3	4.7	4.2	25.6	32.9
Commercial SS1	1	5.0	4.6	22.2	31.2
	2	5.1	4.4	18.8	25.7
	3	5.5	4.5	19.0	26.9

Colorimetric pyruvate; LSD (0.05) = 0.169, $P < 0.001$. PyOx biosensor pyruvate; LSD (0.05) = 0.822, $P = 0.036$). HPLC glucose; LSD (0.05) = 0.237, $P < 0.001$. GOx biosensor glucose; LSD (0.05) = 21.83, $P = 0.016$.

With respect to multi-analyte testing, results revealed pyruvate and glucose analysis would have to be performed sequentially as both biosensors operated at different applied potentials, 0 mV, +50mV and +150mV for the glucose, pyruvate dehydrogenase and pyruvate oxidase-based biosensors, respectively. Nevertheless, overall testing time would not exceed 5 minutes. Of the numerous amperometric glucose biosensors researched, none have operated at lower applied potentials than in the present study (*cf*. Table 8.1). In the current research, both prototype pyruvate biosensors performed satisfactorily well when onion juice was diluted ten-fold (Chapter 7). Hence, a common sample preparation would be feasible for glucose and pyruvate quantification in onion juice.

8.4 Conclusion

For the first time, a prototype glucose biosensor was developed, optimised and validated against real onion samples using the same electrode format as used previously for pyruvate biosensors. No extraction or incubation procedure was required so that glucose concentration could be quantified directly from freshly extracted onion juices. Both the glucose and pyruvate biosensors were shown to be able to satisfactory quantify glucose and pyruvate in the same onion juice samples. Analysis time was reduced to as much as 40-fold as compared to conventional HPLC.

CHAPTER 9

General discussion and conclusions

9.1 Introduction

The UK onion crop shows strong potential for growth through new product development as consumers become increasingly aware of the health and dietary benefits of fresh onion consumption. Prospects for the production of new, more palatable sweet onions will be boosted by the development of grower diagnostics for flavour standardisation. A previous Horticulture LINK project identified a market for sweet, low pungency onions to be eaten fresh and uncooked. However, grading and accepted standards are required to support the market. Consequently, growers require simple-to-use on-farm diagnostics to assure flavour quality. The introduction of pungency tests has led to increased mild onion sales, but tests are currently out-sourced and costly. Through this research study, hand-held biosensor technology adapted from the medical sector has been developed for improved pungency and sweetness analysis in onions at lower cost.

9.2 Discussion

9.2.1 Bulb sampling

The following discussion is based on the research objectives stated in Chapter 1 and reviews issues raised during the course of biosensor development work. In chapter 3, it was reported that pungency tends to fluctuate during storage (Randle *et al.*, 1998; Uddin and MacTavish, 2002). It was also necessary to investigate known variability within onion bulbs such that a sampling technique would be representative of both strength and sweetness. Although some work had demonstrated a flavour gradient either across or down onion bulbs (Freeman, 1975; Bacon *et al.*, 1999), no other reports were cited on three-dimensional profiling of flavour. This is important as numerous sampling methods for onion bulbs are currently being adopted. Results from the present study showed that equatorial sections consistently underestimated overall bulb pungency in both onion (cvs. SS1 and Renate) cultivars by as much as +2 μmol g^{-1} FW. This is a potentially a significant error in the marketing of mild or sweet (<5 μmol g^{-1} FW) onions. It was suggested therefore that sampling should be revised using wedges or quarters in order to obtain representative results.

9.2.2 *Pyruvate biosensors for onion pungency*

Research described in Chapters 4 and 5 led to two disposable prototype pyruvate biosensors being constructed using either pyruvate oxidase (PyOx) or pyruvate dehydrogenase (PDH) immobilised on mediated meldolas blue electrodes to determine pungency in onions. The outcome from this work demonstrated values for pyruvate determined via biosensors were within ±1.0 mM (≡ 1.0 μmol g^{-1} FW in onion) of those depicted by the industry standard (Schwimmer and Weston, 1961) assay. Breeding programmes by Pike at the Vegetable and Fruit Improvements Centre (V.F.I.C), Texas have shown that pyruvate can be reduced 0.5 to 1.0 μmolg^{-1} FW (≡ 0.5 to 1.0 mM) in a single generation. Therefore it is required that the resolution of an onion pyruvate biosensor is also comparable with the current method. For both PyOx and PDH biosensors, detection limits for pyruvate in onion were <10 μM with a resolution was *ca.* 0.5 mM. Reduction of overall pyruvate and bulb to bulb variation is fundamental to future progression of a market place for flavour in onions. Analysis time, however, is a serious constraint to universal uptake, as also is a discrepancy in results between different labs. One of the problems with the current industry assay is the number and variation of steps within the analytical method adopted around different labs. This leads to inconsistent results. It is envisaged that a commercial pyruvate biosensor will reduce error as the number of steps with this assay will be reduced to a minimum. Availability of the biosensor will be of major significance to breeders in that individual bulb selection can be easily and economically progressed. The availability of rapid on site testing will have a major impact in finally promoting and implementing an international standard. Both biosensors developed in the present research were able to detect up to 15 mM pyruvate, providing the capability of classifying strong onions which have also seen a growth in sales. The only other pyruvate biosensor with similar linear range (1 – 10 mM) was that described by Mascini and Mazzei (1987). The glucose-pyruvate biosensor film developed by Ghica and Brett (2006) for onions only measured up to 0.6 mM pyruvate. Although Keusgen *et al.* (2003) investigated a potentiometric biosensor for the quantification of total cysteine sulphoxides, shown to correlate with pyruvate in onion (Bacon *et al.*, 1999; Resemann *et al.*, 2003), where the analysis took 10 minutes.

From the extensive field trials conducted (Chapter 6), it is clear why there are no commercial biosensors available for routine quality control within the fresh produce industry. When developing a biosensor for fresh food applications, biochemical changes that occur in fresh produce are not researched and tested alongside biosensor development. Hence, many biosensors fail when tested with real food samples (Prodromidis and Karayannis, 2002). As found in this study, it is crucial to examine these potential influences and devise strategies to minimise this negative influence on biosensor performance (Chapter 7). Some of these methods were more practical and thus more preferable than others. The addition of surfactant directly to onion samples was established as a novel way for improving onion pyruvate biosensor performance by aiding distribution of sample on the sensor surface. An alternative, but more expensive strategy adopted by Abbott Laboratories, U.S. would be incorporate the surfactant within a mesh and integrate this on the sensor.

9.2.3 *Glucose biosensors for onion sweetness*

Following the establishment of markers that generally indicate onion flavour which was required to facilitate simultaneous multi-analyte testing of both pungency and sweetness, a prototype glucose biosensor was developed (Chapter 8) using the same meldolas blue-mediated sensor format. The glucose biosensor performance was shown to be comparable with standard methods of quantification (*viz.* HPLC and test kit), and in some cases, superior to other glucose biosensors (Revzin *et al.,* 2002; Crouch *et al.,* 2005; Ghica and Brett, 2006) reported in terms of simplicity, operating potential, detection limits and analytical range. The glucose biosensor developed was again validated using real onion samples. The problems encountered with sample coverage on the sensor surface with pyruvate biosensors did not appear to be an issue here, presumably because of the low fructan content of these mild cultivars coupled with the dilution pretreatment which reduces juice viscosity.

9.2.4 *Multi-analyte testing of pyruvate biosensors and glucose for assessing onion flavour*

The refractometer which measures total soluble solids (% TSS) is the only hand-held instrument currently used by industry to routinely assess organoleptic quality for a wide range of fruits. TSS however does not correlate well with sugar content in onions (Chope *et al.,* 2006). Furthermore, sweetness in onion can only be perceived when pungency is <4.0 μmol g^{-1} FW (Crowther *et al*., 2005). Therefore, for objective assessment of onion flavour, a multi analyte sweetness/pungency indicator is necessary. With the exception of the present study and work subsequently undertaken by Ghica and Brett (2006), the only research on multi-analyte testing of both pyruvate and glucose has been for clinical applications and thus may not be suitable for highly variable onion bulbs.

Multi-analyte testing would be facilitated where a common sample pre-treatment is carried out as was the case in this study. Although different (0, +50 and +150 mV vs. Ag/AgCl for GOx, PDH and PyOx-based biosensors respectively) applied potentials yielded the optimum currents, this does not suggest a common applied potential could not still be adopted for integrated pyruvate-glucose biosensors and still give acceptable performance levels. An example of a portable device being marketed by GEM is shown below (Plate 9.1) also showing individual sensors housed within a plastic casing.

Plate 9.1. A Prototype transducer with disposable sensors. Reproduced with permission (Gwent Electronics Ltd, Pontypool, Wales, UK).

9.2.5 Biosensor shelf life

There is a need for sensors to be stable for a given shelf life as compared to freshly prepared sensors used in this project. Shelf life is an important consideration which also determines commercialization. This requires the sensor to have a stable life of at least six months. Shelf life tests on the PyOx biosensor were carried out using a number of stabilisation kits supplied by AET. Results are detailed in Appendix A. Other workers have also investigated the PyOx stability. Pyruvate oxidase (derived from *Lactobacillus plantarum*) immobilized electrodes in carbon paste retained 95% activity after 2 months at 4˚C (Kulys *et al.,* 1992) and 100% at 3 months at 4˚C (Situmorang *et al.,* 2001). Situmorang and co-workers (2001) demonstrated immobilization of PyOx in humid conditions was critical to retaining activity. These results demonstrate the potential for constructing sensors with a shelf life of the required six months.

9.3 Conclusion

To conclude, this research project represents an international breakthrough in the quality assurance for flavour in onions. This research has demonstrated for the first time the possibility of replacing the standard colorimetric assay used ubiquitously by the world onion industry for determining pyruvate concentration with a more rapid method using a mediated amperometric biosensor. Introduction of a pyruvate biosensor for onions will transform quality control, now being possible in minutes and on site, (rather than outsourcing pungency analysis). This will drive improvements in cultivars and in turn consumer confidence with better and internationally acceptable quality assurance, providing consistent assurance to the UK consumer.

9.4 Recommendations for future work

Initially, as previously detailed the prototype biosensor has to be developed into a commercial system which can be supplied as a kit to give guaranteed reliable performance. Developmental issues are the subject of a Link Development Grant now submitted "Optimising and Validating Rapid Biosensor Analysis for Cost Effective Quality Assurance". Further research and development will also be necessary to validate the glucose biosensor and integrate with pyruvate biosensor. The aim is to combine strength and sweetness as the market develops. The following specifics are proposed:

- Improve/confirm shelf life of the sensors by using accelerated shelf life procedures on sensors produced by several automated biosensor construction processes (i.e. screen-printing, non-pipetting enzyme deposition, drying).
- Develop a standard user friendly sample preparation technique designed to minimize interference, sample dilution and other treatments to improve the accuracy of the biosensor and operate in the desired linear range for the enzyme system.
- In order for consumers to gain confidence in the labelling classification of onions, a common protocol must also be adopted for tissue sampling and juice extraction.

- Using blank or dummy sensors and re-evaluate pyruvate biosensor performance on known high interferent cultivars with the addition of surfactant to freshly extracted samples.
- The employment of a correction factor should be explored for assessing pyruvate in certain problematic Red cultivars.
- The PyOx and PDH biosensor calibration should be re-examined using the standard addition method where pyruvate standards are spiked with onion juice so as to compensate for the matrix effects.
- Resolution of the GOx biosensor should be investigated with a range of cultivars known to vary widely in glucose content.
- Re-evaluate PDH performance with a reduced enzyme loading to further reduce unit costs.

9.5 Industry Usage

9.5.1 Portability and sampling methods

The initial target was for a rapid system which can be used in-field, to the point of using a garlic press (or similar) to extract onion juice which could then be directly measured.

Onion juice incubation trials (Chapter 6), including tests within the consortium using prototype sensors produced by G.E.M. confirmed however that a time interval of at least 15 minutes was necessary for the enzyme reaction (extraction of juice disrupts cells and releases the enzyme – allinase from vacuoles (Lancaster and Collin, 1981) which then reacts with sulphur compounds within the cells) to be completed, and initial labile transient sulphur compounds to settle. From this aspect, it seems unlikely at this stage for in-field sampling to be a practical proposition. Additionally, whilst individual bulbs may be tested, it is likely in practice that composite samples of 5 bulbs will be more appropriate. It will then be necessary to macerate tissue for juice extraction with a household blender prior to testing. This then will require a simple bench procedure such that testing can be close to field sites and acceptable for all expected use. Due to current costs of lab analysis 10 bulbs are tested as a composite per filed per drilling at harvest and when onions are dried prior to first sales. When testing is more straightforward sampling can be more intensive and readily extended to field testing internationally prior to harvest. The opportunities for use of this technology will extend 'flavour related sales'.

9.5.2 *Potential number of tests*

Full Quality Assurance for sweet onions will encompass field checks before harvest, bulbs in long-term storage, including imports. A reliable 12 month supply can then be established which will significantly increase sales and further benefit UK production, currently from late June to October.

Despite variability in strengths, sales have been increasing but not as rapidly as anticipated, considering the consumer surveys carried out in HL0186. Including imports the present total ca 1,000 tonnes pa can be expected to increase over 5 years to 10,000 tonnes with a potential level in order of 25,000 tonnes or around 3-4% of total consumption. Sweet onions command a ca 100% price premium at present hence added value up to £27m is not an unrealistic target. Sales of 10,000 tonnes per annum (pa) would require in order of 1,500 tests based on sampling 20 tonne lots twice before dispatch and once on arrival.

9.5.3 *Market classification of onions*

Whilst some retailers have abandoned the term "Mild", now replaced by "Large Onions" the market place is still confused and has not universally accepted the findings of HL0186. Whilst some imports (of a total ca 100,000 tonnes or 15% total consumption) are <5 μmol g^{-1} FW much is above this, hence should not be labelled as Mild. Rapid sensing will enable both promotion and acceptance of standards more readily than at present. Either elimination of "Mild" labels will more clearly differentiate "Sweet" onions or if in line with standards then consumer confidence will be further reinforced.

Validation of sensors for the full range of strengths will offer possibilities of "extra strong" onions and also of extending technology to sales of shallots and eschalions. There is little doubt that consortium members will use the biosensor extensively to survey sales categories in the formulation of future development strategy.

9.5.4 *Market trends*

Further areas of development will be possible. Red onions have increased up to 20% pa in recent years leading to sales ca 50,000 tonnes (7.6% of total consumption). Often used fresh in salads, there is perceived market potential for sales of Mild or Sweet Red Onions in the future. It is clear that HL0186 (Completed August 2001) set the basis for Mild Onion sales, having dispelled the previous acceptance that 100,000 tonnes Spanish / Chilean imports were necessarily Mild. Sweet onions, of no importance at the time, have since received much press coverage and established a market which has since, gradually gathered momentum.

9.5.5 *Development of an international standard*

Analysis time however is a serious constraint to universal uptake, as also is a discrepancy in results between different labs (Havey *et al*., 2002). The availability of rapid on site testing will have a major impact in finally implementing standards which were envisaged within HLO186 and this project. In addition it will be possible to promote an international standard with use of the biosensor as a common system. It is, with onions being produced in every country of the world and consumption ca 50m tonnes (F.A.O.), in prospect, of major international interest.

CHAPTER 10

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APPENDIX A

Investigating the Stability of Pyruvate Oxidase

A.1 Introduction

In order to produce an affordable commercial disposable biosensor, it is imperative the biological element be stabilised for at least six months to allow mass, batch production. The stability of pyruvate oxidase (PyOX) was examined in two separate experiments.

A.2 Material and methods

A.2.1 Reagents

All of the chemicals used were of analytical grade. Pyruvate oxidase (E.C.1.2.3.3) derived from bacterial spp. (~10 unit/mg), thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), aminoantipyrine, N-ethyl-N-(2-hydroxy-3-sulpfopropyl)-m-toluidine, peroxidase NaCl and ethylenediaminetetraacetic acid, $C_3H_3O_3Na$ and $MgSO_4$, KOH, NaOH and KCl, Na_2HPO_4, NaH_2PO_4 and KH_2PO_4 were purchased from Sigma (Dorset, UK), Fisher Scientific Chemicals (Dorset, UK), BDH. Ltd. (Leics., UK) and Fluka Chemika (Switzerland) respectively. All reagents were made up in reverse osmosis water. For electrochemical experiments, FAD and TPP co-factors were made up as 3 mM and 6 mM stock solutions, respectively, and stored at -20˚C until use. $MgSO_4$ was prepared as a 0.9 M stock solution and stored at 4˚C. Pyruvate oxidase was made up in the co-factor mix. Pyruvic acid sodium salt for deriving calibration standards was made up as a 5 mM stock solution and stored at 4˚C. Reagents TPP, FAD and pyruvate oxidase for stabiliser trials were prepared immediately before use.

A.2.2 Experiment 1: Shelf life of immobilised sensors

A.2.2.1 Electrochemical measurements

Generic carbon mediated with meldolas blue (C2030519D5, Gwent Electronic Materials Ltd. GEM, UK) working electrodes (28 mm^2) were screen printed with a Ag/AgCl reference/counter electrode onto a PVC substrate in a two electrode configuration. Electrochemical measurements were carried out using a PalmSense potentiostat (Palm Instruments BV, The Netherlands). Electrochemical measurements were carried out at +150 mV for the oxidase biosensor at 21˚C. All measurements were made by depositing 20 µl KCl electrolyte on the electrode surface, applying the potential then allowing a steady state current to be reached before adding 20 µl pyruvate standard.

A.2.2.2 Pyruvate oxidase immobilisation

Enzyme immobilisation was achieved by amalgamating an enzyme cocktail containing 1 unit PyOx, 0.04 mM TPP, 0.1 mM FAD and 30 mM $MgSO_4$ (optimised final concentrations), and depositing onto the surface of the working electrode. Electrodes were left to air dry for 4 hours, then subsequently stored in three relative humidity (0, 75 & 100%) and temperature regimes (4, 10 and 20˚C). Prepared sensors were placed in sealed containers and kept in an incubator. Humidity within the containers was maintained by lining with silica (0% RH) and by placing a beaker containing 75% sodium chloride or distilled water (100%) representing dry to fully saturated conditions. Sensors were stored for 7 days. Biosensor performance was evaluated each day by removing sensors from the containers, leaving at room temperature for 30 min to reacclimatise before testing with a 5 mM pyruvate standard.

A.2.3 Experiment 2: Stability solutions

In a subsequent experiment, four stabilisation solutions (ST02, ST03, ST05& ST14) supplied by AET were evaluated against a control for their ability to ensure stability of pyruvate oxidase and hence biosensor performance over a period of 6 months. Pyruvate oxidase activity was assayed according to the protocol set out by Sigma-Aldrich (Sedewitz *et al.,* 1984) with minor modifications. The reaction cocktail was prepared by mixing 10ml 150 mM potassium phosphate buffer solution pH 5.9, 2 ml 7.4 mM 4-aminoantipyrine, 0.3% w/v N-ethyl-N-(2-hydroxy-3-sulpfopropyl)-m-toluidine, 3 mM cocarboxylase, 0.15 mM flavin adenine dinucleotide, 15 mM ethylenediaminetetraacetic acid, 150 mM magnesium sulphate and 3 ml peroxidase enzyme solution (50 units/ml). A volume (2.50 ml) of the reaction cocktail and 0.50 ml of 300 mM pyruvate was mixed by inversion and equilibrated at 37˚C. The cuvette within the spectrophotometer was maintained at 37˚C using a water bath and peristaltic pump set-up. The absorbance was monitored with a 1 cm light path at A_{550nm} before 0.10 ml of enzyme solution (0.2-0.4 units/ml) was added. The final solution was immediately mixed by inversion and the increase in absorbance recorded for 5 min. Using the maximum linear rate for the test and a blank (50 mM potassium phosphate buffer solution pH 5.7), the change ΔA_{550nm} per min was obtained and used to estimate pyruvate oxidase activity.

The pH of each stabilisation solution was tested. A number of serial dilutions (1:1, 1:2 and 1:4) of the stabilisation solutions were then made with 50 mM phosphate buffer pH 5.7 and their final pH recorded.

The shelf life trial was set up as follows, three methods consisting of either 0.2-0.4 (A) or 0.6-1.2 units/ml PyOX (B) in a 1:8 dilution (final concentration) with stabilisation solutions and (0.6-1.2 units/ml) dissolved in a 1:4 dilution of stabilisation solutions (C). 0.1 ml of all solutions containing either 0.2-0.4 or 0.6-1.2 units/ml were transferred into 1.5 ml ependorf tubes. Pyruvate oxidase activity was tested for each treatment prior to freeze drying following the described method. Next, tubes were held in liquid nitrogen until the solution was visibly frozen then freeze-dried for 24h. With methods A and B, eppendorf tubes were not capped during the freeze drying process. A pinhole was made in the caps of tubes in method C. Enzyme activity was again tested for each treatment post freeze drying by diluting with either 0.1 (methods A and B) or 0.3 ml (method C) 50 mM potassium phosphate buffer solution pH 5.7. Tubes were stored in a sealed container and removed only for assaying again at 1, 2, 4 and 6 month intervals.

A.3 Results and discussion

A.3.1 Stability of pyruvate oxidase mediated meldolas blue sensors

Irrespective of temperature, there was no significant difference in biosensor stability between dry and fully saturated conditions (Fig A1. i). After one week the biosensor retained 75% original response with no additional stabilisers. It would not however be practical to store and transport sensors in fully saturated conditions as the biological element cannot be contained on the surface of the sensors. Furthermore, saturated conditions would eventually favour microbial contamination of the biological component. Sensors held in 75% relative humidity at 10˚C (Fig A1. ii) and particularly 20˚C (FigA1. iii) provided the worst conditions for enzyme stability. With the pyruvate oxidase stability trials undertaken by Ghica and Brett (2006) on polyneutral red carbon film biosensors, a 19% loss of activity occurred after 7 weeks of storage in dry conditions at 4˚C. Again, PyOx-mediated methylene green biosensors immobilised in carbon paste retained its activity following two months storage in similar conditions (Kulys *et al.,* 1992).

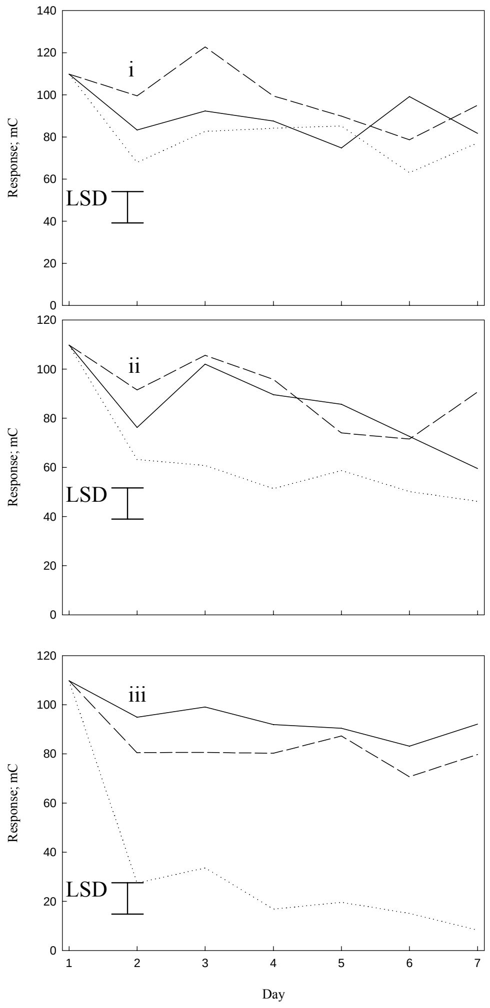


Figure A.1. Mediated meldolas blue sensor response at 0, 75 and 95% relative humidities over one week storage at 4˚C (i), 10˚C (ii) and 20˚C (iii). Solid, small and large dashed lines represent dry (0% relative humidity), semi-humid (~75% relative humidty) and fully saturated (~95% relative humidity) conditions respectively. LSD =18.85; $P = 0.001; 0.05$). (Appendix B, Fig. A.1).

A.3.2 Effect of stabilisers on pyruvate oxidase stability

Below (Table A.1) shows the pH of the stabilisers before and following serial dilutions. For some of the stabilisers, these dilutions were necessary to adjust the pH for optimum pyruvate oxidase activity.

Table A.1. pH of stabilisers ST02, ST03, ST05 AND ST14 prior to and following dilutions with 50 mM potassium phosphate buffer pH 5.7.

	Stabiliser			
	ST02	ST03	ST05	ST14
Normal pH	4.30	5.65	5.63	9.50
pH after 1:2 dilution with 50mM buffer pH 5.7	5.46	5.52	5.53	6.55
pH after 1:4 dilution with 50mM buffer pH 5.7	5.45	5.52	5.52	6.55

(Appendix B, Table A.1).

The following table (Table A2) shows the pyruvate oxidase activity with different methods and added stabilisers prior to and post freeze drying. Freeze drying had a significant ($P = 0.001; 0.05$) effect on enzyme activity. Maximum activity was 7 units/mg not 10 units/mg as stated by Sigma. However this is not unusual. Other studies have demonstrated reduced activity from that listed by manufacturers. It may be due to error introduced during the numerous steps required for performing the assay.

Over the 6 month storage period, with methods A and B, ST02, PyOX activity fell by 50% over the first month and a further 90% by the second month of storage, remaining constant thereafter. However, with method C, activity did not change significantly from its initial value during the 6 months.

For stabiliser ST03, a similar reduction profile was witnessed for ST02 method A. However, there was a 50% loss in activity for method B by the end of the first month which remained stable for the remainder of the storage period. With method C, there was a 40% loss in activity at the end of the second month. This was not found to have changed by the end of the fourth month but by the end of the trial activity was less than 1 unit/mg from 4.6 unit/mg.

Table A.2. The effect of stabilisers ST02, ST03, ST05 AND ST14 and preparation methods prior to and following freeze drying for 24h.

	Pyruvate oxidase activity (units/mg)				
	Stabiliser				
Method	Control	ST02	ST03	ST05	ST14
Before freeze drying (Method A)	7.01	6.89	5.49	5.89	4.93
A (1:8 dilution, 0.2 - 0.4 units/ml) uncapped during freeze drying	0.08	0.45	4.56	3.38	1.29
B (1:8 dilution, 0.6 - 1.2 units/ml) uncapped during freeze drying	1.08	0.99	4.82	6.61	4.88
C (1:4 dilution, 0.6 - 1.2 units/ml Pinholed caps)	1.82	1.33	4.60	7.06	4.78

LSD, 1.28, *P*=0.001, (Appendix B, Table A.1).

With stabiliser ST05, PyOX activity with both methods A and B was approximately 20% of the initial value by the end of the first month, representing the largest reduction over that period. There was no further change by the end of the trial period. Conversely, with method C, after one month activity fell from 7 units/mg to 4.7 units/mg (33%) and further to 2.7 units/mg at the end of the second month. No significant change was observed thereafter.

For stabiliser ST14 there was no change in activity throughout the storage trial. With method B, there was over a 70% reduction at the end of the first month. This was stable until the fourth month where there was a further 90% loss.

Method C gave higher enzyme activity values followed by B then A. This may have been due to loss of enzyme during freeze drying. Also, all the enzyme solution cannot be completely pipetted out of the tube. The type of stabiliser plays an important role during the freeze drying process. There were significantly lower (*P* = 0.001; 0.05) activity levels with the control and ST02 for all methods compared with the other stabilisers just after freeze drying, with ST05 giving the highest activity (7 units/mg). With method C, stabilisers ST03, ST05 and ST14 demonstrated similar performances (Table A3) during the 6 month storage period.

Table A.3. Comparative performances of stabilisers ST03, ST05 and ST14 over 6 months with 1:4 dilutions (final concentrations) and 0.6 - 1.2 units/ml pyruvate oxidase.

	Pyruvate oxidase activity (units/mg)		
		Stabiliser	
Month	ST03	ST05	ST14
0	4.6	7.0	4.8
1	5.3	4.7	1.4
2	2.8	2.7	2.8
4	2.0	1.9	1.5
6	0.7	0.6	1.3

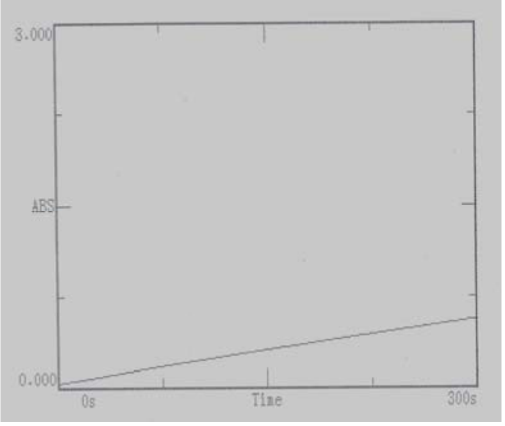
LSD, 1.28, *P*=0.001 (Appendix B, Table A.1).

A.4 Conclusion

From the current study, pyruvate oxidase activity was not sufficiently retained to allow mass batch production of these sensors. Clearly, the results demonstrate a stabiliser is required for pyruvate oxidase if a freeze drying process is to be adopted during the manufacturing of these sensors. Alternative methods for freeze drying should be examined as well as well as the incorporation of additional FAD to the solution. Manufacturers currently add FAD as a stabiliser to pyruvate oxidase, however this concentration may have been diluted during the preparation for freeze drying and a gradual loss may also occur during long term storage. This is supported by the results from *experiment 1* where in dry conditions after one week the biosensor still retained most of its original response. There where no stabilisers present and no freeze drying process had taken place. Pre-freeze drying results from Table A2 in *experiment 2* also support this idea. Subsequent storage practices whereby vials are not disturbed during observations should also be examined. In addition, alternative or modified stabilisers may improve the stability of the enzyme as some of the stabiliser properties may be reduced during the dilution process. Once this has been achieved, optimum storage of sensors would be in dry non-humid conditions at room temperature.

A.5 Pyruvate oxidase and pyruvate dehydrogenase activities determined via constant rate spectrophotometric method (Sigma-Aldrich)

Pyruvate oxidase: I.U.: +1.040



Pyruvate dehydrogenase: I.U.: +5.555

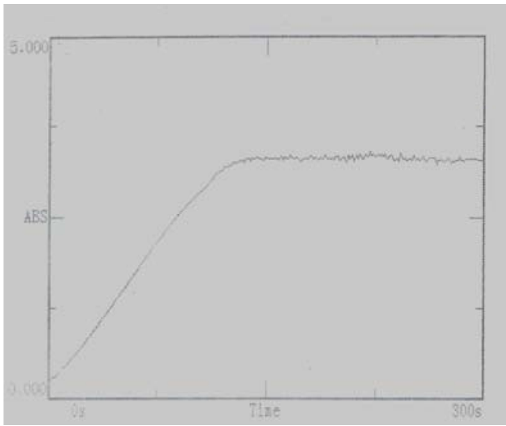


Plate A.1. Comparative enzyme activity profiles for pyruvate oxidase and pyruvate dehydrogenase.

APPENDIX B

Anova tables

CHAPTER 3

Figure 3.1

Figure B3.1.1 cv. Renate: Pyruvate (mg g^{-1} FW) temporal changes

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		579.59	115.918	17.13	<.001
Residual	425	55	2876.148	6.767		
Total	430	55	3348.453			

Figure B3.1.2 cv. SS1: Pyruvate (mg g^{-1} FW) temporal changes

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		892.887	178.577	64.86	<.001
Residual	398	82	1095.737	2.753		
Total	403	82	1520.664			

Table B3.1.3 cv. Renate: Vertical Pyruvate profiling: Top to bottom

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		580.082	116.016	19.88	<.001
LOCATION	2		143.642	71.821	12.31	<.001
TIME.LOCATION	10		348.172	34.817	5.97	<.001
Residual	413	55	2410.307	5.836		
Total	430	55	3348.453			

Figure B3.1.4 cv. SS1: Vertical Pyruvate profiling: Top to bottom

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		892.887	178.577	64.86	<.001
Residual	398	82	1095.737	2.753		
Total	403	82	1520.664			

Figure 3.2

Figure B3.2.1 cv. Renate: Horizontal Pyruvate profiling: Inner to outer scales

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		580.62	116.124	20.9	<.001
LOCATION	2		198.054	99.027	17.82	<.001
TIME.LOCATION	10		431.737	43.174	7.77	<.001
Residual	413	55	2294.498	5.556		
Total	430	55	3348.453			

Figure B3.2.2 cv. SS1: Horizontal Pyruvate profiling: Inner to outer scales

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		934.657	186.931	78.17	<.001
LOCATION	2		207.871	103.935	43.46	<.001
TIME.LOCATION	10		134.416	13.442	5.62	<.001
Residual	386	82	923.029	2.391		
Total	403	82	1520.664			

Figure 3.3

Figure B3.3.1 cv. Renate: Glucose (mg g^{-1} FW) temporal changes

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		490.18	98.04	8.87	<.001
Residual	461	19	5095.73	11.05		
Total	466	19	5582.47			

Figure 3.3 cotinued

Figure B3.3.2 cv. SS1: Glucose (mg g^{-1} FW) temporal changes

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		5012.11	1002.11	36.08	<.001
Residual	417	63	11585.40	27.78		
Total	422	63	15704.94			

Figure B3.3.3 cv. Renate: Vertical Glucose profiling: Top to bottom

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		493.42	98.68	9.19	<.001
LOCATION	2		195.04	97.52	9.08	<.001
TIME.LOCATION	10		85.49	8.55	0.8	0.633
Residual	449	19	4823.7	10.74		
Total	466	19	5582.47			

Figure B3.3.4 cv. SS1: Vertical Glucose profiling: Top to bottom

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		4767.79	953.56	41.36	<.001
LOCATION	2		1479.46	739.73	32.08	<.001
TIME.LOCATION	10		1252.61	125.26	5.43	<.001
Residual	405	63	9337.55	23.06		
Total	422	63	15704.94			

Figure 3.4

Figure B3.4.1 cv. Renate: Horizontal Glucose profiling: Inner to outer scales

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		510.909	102.182	14.1	<.001
LOCATION	2		1455.771	727.886	100.45	<.001
TIME.LOCATION	10		501.671	50.167	6.92	<.001
Residual	449	19	3253.592	7.246		
Total	466	19	5582.473			

Figure B3.4.2 cv. SS1: Horizontal Glucose profiling: Inner to outer scales

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
TIME	5		5030.33	1006.07	40.48	<.001
LOCATION	2		839.52	419.76	16.89	<.001
TIME.LOCATION	10		1163.13	116.31	4.68	<.001
Residual	405	63	10064.42	24.85		
Total	422	63	15704.94			

Figure B3.4.3 cv. Renate: Firmness

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Blocks	2		1479.6	739.8	3.94	
TIME	7		16508	2358.4	12.57	<.001
Residual	58	12	10883.0	187.6		

Figure B3.4.4 cv. SS1: Firmness

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Blocks	2		549.71	274.85	3.13	
TIME	8		630.83	78.85	0.90	0.523
Residual	68	2	5966.4	87.74		
Total	78	2	7083.33			

CHAPTER 4

Section 4.3

Interference at different applied potentials for cvs. Red Baron, Spanish Pandero and Hyfort

Table B4.3.1 cv. Red Baron

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
±PyOx	1	13620.3	13620.3	77.81	<.001
Applied voltage	1	3516.3	3516.3	20.09	<.001
Cultivar	1	1370.5	1370.5	7.83	0.013
±PyOx. Applied voltage	1	130.1	130.1	0.74	0.401
±PyOx. Cultivar	1	35.5	35.5	0.20	0.659
Applied voltage. Cultivar	1	481.2	481.2	2.75	0.117
±PyOx. Applied voltage. Cultivar	1	309.6	309.6	1.77	0.202
Residual	16	2800.8	175.0		
Total	23	22264.1			

Table B4.3.2 cvs. Spanish Pandero and Hyfort

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
±PyOx	1	6222.65	6222.65	75.89	<.001
Applied voltage	1	240.22	240.22	2.93	0.106
Cultivar	1	5763.83	5763.83	70.29	<.001
±PyOx. Applied voltage	1	302.82	302.82	3.69	0.073
±PyOx. Cultivar	1	2747.12	2747.12	33.50	<.001
Applied voltage. Cultivar	1	792.93	792.93	9.67	0.007
±PyOx. Applied voltage. Cultivar	1	9.09	9.09	0.11	0.743
Residual	16	1311.99	82.00		
Total	23	17390.64			

CHAPTER 5

Table B5.3.3 Classification of onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	2		454798	227399	128.78	<.001
Cultivar	5		134485	26897	15.23	<.001
± PDH	1		17438989	17438989	9876.19	<.001
Bulbs	4		17729	4432	2.51	0.042
Time. Cultivar	10		111349	11135	6.31	<.001
Time. ±PDH	2		304037	152019	86.09	<.001
Cultivar. ± PDH	5		206820	41364	23.43	<.001
Time. Bulb	8		11273	1409	0.80	0.605
Cultivar. Bulb	20		101609	5080	2.88	<.001
± PDH. Bulb	4		15297	3824	2.17	0.072
Time. Cultivar. ± PDH	10		142178	14218	8.05	<.001
Time. Cultivar. Bulb	40		157891	3947	2.24	<.001
Time. ± PDH . Bulb	8		16100	2012	1.14	0.336
Cultivar. ± PDH. Bulb	20		95930	4796	2.72	<.001
Time. Cultivar. ± PDH. Bulb	39	1	137838	3534	2.00	<.001
Residual	345	15	609187	1766		
Total	523	16	19436729			

CHAPTER 6

Table 6.1

Table B6.1.1 Spectrophotometric (Schwimmer and Weston, 1961) pyruvate assay with frozen-thawed (cv. SS1) juices.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	5		11900.71	2380.14	50.98	<.001
Bulb	4		773.57	193.39	4.14	0.004
+PyOx	1		201026.42	201026.42	4305.65	<.001
Cultivar. Bulb	20		1961.76	98.09	2.10	0.008
Cultivar. +PyOx	5		3794.59	758.92	16.25	<.001
Bulb. +PyOx	4		163.62	40.91	0.88	0.481
Cultivar. Bulb. +PyOx	20		2367.40	118.37	2.54	0.001
Residual	113	7	5275.84	46.69		
Total	172	7	217869.16			

Table B6.1.2 Expected pyruvate values from pyruvate oxidase-based biosensor with cv. SS1 verified against standard industry (Schwimmer and Weston, 1961) analysis

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	2	460.73081	230.36541	3258.18	<.001
Cultivar	5	724.33126	144.86625	2048.92	<.001
Bulb	4	26.04370	6.51093	92.09	<.001
±PyOx	1	0.00000	0.00000	0.00	1.000
Time. Cultivar	10	77.47985	7.74799	109.58	<.001
Time. Bulb	8	18.46252	2.30781	32.64	<.001
Cultivar. Bulb	20	67.99985	3.39999	48.09	<.001
Time. ±PyOx	2	0.00000	0.00000	0.00	1.000
Cultivar. ±PyOx	5	0.00000	0.00000	0.00	1.000
Bulb. ±PyOx	4	0.00000	0.00000	0.00	1.000
Time. Cultivar. ±PyOx	40	193.71793	4.84295	68.50	<.001
Residual	360	25.45333	0.07070		
Total	539	1594.21926			

Table 6.2

Table B6.2.1 Spectrophotometric (Schwimmer and Weston, 1961) pyruvate assay with freshly extracted onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	2	460.731	230.365	4072.73	<.001
Cultivar	5	724.331	144.866	2561.15	<.001
Bulbs	4	26.044	6.511	115.11	<.001
Time. Cultivar	10	77.480	7.748	136.98	<.001
Time. Bulbs	8	18.462	2.308	40.80	<.001
Cultivar. Bulbs	20	67.999	3.399	60.11	<.001
Time. Cultivar. Bulbs	40	193.717	4.843	85.62	<.001
Residual	450	25.453	0.056		
Total	539	1594.219			

Table B6.2.2 Spectrophotometric (Schwimmer and Weston, 1961) pyruvate assay with frozen-thawed onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	5		11900.71	2380.14	50.98	<.001
Bulb	4		773.57	193.39	4.14	0.004
+PyOx	1		201026.42	201026.42	4305.65	<.001
Cultivar. Bulb	20		1961.76	98.09	2.10	0.008
Cultivar. +PyOx	5		3794.59	758.92	16.25	<.001
Bulb. +PyOx	4		163.62	40.91	0.88	0.481
Cultivar. Bulb. +PyOx	20		2367.40	118.37	2.54	0.001
Residual	113	7	5275.84	46.69		
Total	172	7	217869.16			

Table B6.2.3 Pyruvate oxidase-based biosensor response (µC). Juices from freshly extracted onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	2		5171.9	2585.9	14.22	<.001
Cultivar	5		32763.7	6552.7	36.03	<.001
±PyOx	1		685163.0	685163.0	3766.84	<.001
Bulbs	4		6008.9	1502.2	8.26	<.001
Time. Cultivar	10		12356.4	1235.6	6.79	<.001
Time. ±PyOx	2		947.7	473.8	2.61	0.075
Cultivar. ±PyOx	5		17361.7	3472.3	19.09	<.001
Time. Bulb	8		3070.8	383.9	2.11	0.034
Cultivar. Bulb	20		19592.3	979.6	5.39	<.001
±PyOx. Bulb	4		1299.6	324.9	1.79	0.131
Time. Cultivar. ±PyOx	10		18961.8	1896.2	10.42	<.001
Time. Cultivar. Bulb	40		24144.7	603.6	3.32	<.001
Time. ±PyOx . Bulb	8		3327.3	415.9	2.29	0.021
Cultivar. ±PyOx. Bulb	20		8580.6	429.0	2.36	<.001
Time. Cultivar. ±PyOx. Bulb	40		21961.9	549.0	3.02	<.001
Residual	350	10	63662.6	181.9		
Total	529	10	906484.1			

Table B6.2.4 Pyruvate dehydrogenase-based biosensor response (µC). Juices from freshly extracted onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	2		454798	227399	128.78	<.001
Cultivar	5		134485	26897	15.23	<.001
± PDH	1		17438989	17438989	9876.19	<.001
Bulbs	4		17729	4432	2.51	0.042
Time. Cultivar	10		111349	11135	6.31	<.001
Time. ±PDH	2		304037	152019	86.09	<.001
Cultivar. ± PDH	5		206820	41364	23.43	<.001
Time. Bulb	8		11273	1409	0.80	0.605
Cultivar. Bulb	20		101609	5080	2.88	<.001
± PDH. Bulb	4		15297	3824	2.17	0.072
Time. Cultivar. ± PDH	10		142178	14218	8.05	<.001
Time. Cultivar. Bulb	40		157891	3947	2.24	<.001
Time. ± PDH . Bulb	8		16100	2012	1.14	0.336
Cultivar. ± PDH. Bulb	20		95930	4796	2.72	<.001
Time. Cultivar. ± PDH. Bulb	39	1	137838	3534	2.00	<.001
Residual	345	15	609187	1766		
Total	523	16	19436729			

Table B6.2.5 Pyruvate dehydrogenase-based biosensor response (µC). Juices from frozen-thawed onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	5	126021	25204	13.30	<.001
Bulbs	4	40134	10033	5.29	<.001
±PDH	1	14384999	14384999	7591.06	<.001
Cultivar. Bulbs	20	144473	7224	3.81	<.001
Cultivar. ±PDH	5	135275	27055	14.28	<.001
Bulbs. ±PDH	4	35820	8955	4.73	0.001
Cultivar. Bulbs. ±PDH	20	146039	7302	3.85	<.001
Residual	111	210344	1895		
Total	170	14494458			

Figure 6.1

Figure B6.1.1 Time-course studies: Pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	6	9.329	1.555	12.88	<.001
Residual	35	4.227	0.1208		
Total	41	13.556			

Figure 6.2

Figure B6.2.1 Pyruvate in onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs stored over 18 weeks (Schwimmer and Weston, 1961).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	2	460.731	230.365	4072.73	<.001
Cultivar	5	724.331	144.866	2561.15	<.001
Bulbs	4	26.044	6.511	115.11	<.001
Time. Cultivar	10	77.480	7.748	136.98	<.001
Time. Bulbs	8	18.462	2.308	40.80	<.001
Cultivar. Bulbs	20	67.999	3.399	60.11	<.001
Time. Cultivar. Bulbs	40	193.717	4.843	85.62	<.001
Residual	450	25.453	0.056		
Total	539	1594.219			

Figure B6.2.2 Background interference over 18 weeks. Juices from freshly extracted onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs, on blank-mediated sensors. +150mV.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	2		5171.9	2585.9	14.22	<.001
Cultivar	5		32763.7	6552.7	36.03	<.001
±PyOx	1		685163.0	685163.0	3766.84	<.001
Bulbs	4		6008.9	1502.2	8.26	<.001
Time. Cultivar	10		12356.4	1235.6	6.79	<.001
Time. ±PyOx	2		947.7	473.8	2.61	0.075
Cultivar. ±PyOx	5		17361.7	3472.3	19.09	<.001
Time. Bulb	8		3070.8	383.9	2.11	0.034
Cultivar. Bulb	20		19592.3	979.6	5.39	<.001
±PyOx. Bulb	4		1299.6	324.9	1.79	0.131
Time. Cultivar. ±PyOx	10		18961.8	1896.2	10.42	<.001
Time. Cultivar. Bulb	40		24144.7	603.6	3.32	<.001
Time. ±PyOx. Bulb	8		3327.3	415.9	2.29	0.021
Cultivar. ±PyOx. Bulb	20		8580.6	429.0	2.36	<.001
Time. Cultivar. ±PyOx. Bulb	40		21961.9	549.0	3.02	<.001
Residual	350	10	63662.6	181.9		
Total	529	10	906484.1			

Figure B6.2.3 Background interference over 18 weeks. Juices from freshly extracted onion (cvs. SS1, Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs, on blank-mediated sensors. +50mV.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	2		454798	227399	128.78	<.001
Cultivar	5		134485	26897	15.23	<.001
±PyOx	1		17438989	17438989	9876.19	<.001
Bulbs	4		17729	4432	2.51	0.042
Time. Cultivar	10		111349	11135	6.31	<.001
Time. ±PyOx	2		304037	152019	86.09	<.001
Cultivar. ±PyOx	5		206820	41364	23.43	<.001
Time. Bulb	8		11273	1409	0.80	0.605
Cultivar. Bulb	20		101609	5080	2.88	<.001
±PyOx. Bulb	4		15297	3824	2.17	0.072
Time. Cultivar. ±PyOx	10		142178	14218	8.05	<.001
Time. Cultivar. Bulb	40		157891	3947	2.24	<.001
Time. ±PyOx . Bulb	8		16100	2012	1.14	0.336
Cultivar. ±PyOx. Bulb	20		95930	4796	2.72	<.001
Time. Cultivar. ±PyOx. Bulb	39	1	137838	3534	2.00	<.001
Residual	345	15	609187	1766		
Total	523	16	19436729			

Section 6.3.4

B6.3.4.1 PyOx biosensor response (µC) to organic acids

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Organic acid type	4		23610.80	5902.70	123.45	<.001
Residual	8	2	382.50	47.81		
Total	12	2	18809.30			

Table 6.3

Hydrogen peroxide, ascorbic acid and pyruvate content in uncured onion (cvs. SupaSweet (SS1), Renate, Musika, Red Baron, Rose de Roscoff and Rumba) bulbs.

Table B6.3.1 Hydrogen peroxide

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	5	7.55e-06	1.51e-06	6.3	0.0004
Replicate	1	1.70e-07	1.70e-07	0.71	0.416
Cultivar. Replicate	5	7.89e-06	1.58e-06	6.58	0.004
Residual	12	2.88e-06	2.40e-06		
Total	23	1.85e-05			

Table B6.3.2 Ascorbate

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Cultivar	5		28.204	5.641	38.3	<.001
Replicate	1		2.302	2.302	15.63	0.002
Cultivar. Replicate	4	-1	4.226	1.056	7.17	0.004
Residual	11	-1	1.620	0.147		
Total	21	-2	35.672			

Table B6.3.3 Pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	5	57.254	11.451	10.41	<.001
Residual	18	19.792	1.1		
Total	23	77.046			

Table 6.4

PyOx biosensor response (μC) to the removal of ascorbic acid in onion cv. Sprinter juice.

Table B6.4.1 Pyruvate

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Treatment	4		3129.321	782.330	954.20	<.001
Residual	85	2	69.689	0.819		
Total	89	2	3199.010			

Table B6.4.2 Response

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
±PyOx	1		62809.8	62809.8	478.48	<.001
Treatment	4		108063.7	27015.9	205.81	<.001
±PyOx. Treatment	4		43202.1	10800.5	82.28	<.001
Residual	68	12	8926.2	131.3		
Total	77	12	223001.8			

Table 6.5

Biosensor response (μC) to onion cvs. Hyfort, Red Baron and Spanish Pandero bulb extracts on bare and PyOx-meditated sensors before and after freezing.

Table B6.5.1 Colorimetric pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	0.490	0.490	0.92	0.346
Cultivar	2	89.556	44.778	83.82	<.001
Time. Cultivar	2	2.107	1.053	1.97	0.157
Residual	30	16.027	0.534		
Total	35	108.179			

Table B6.5.2 Biosensor Response

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Time			1550.65	1550.65	19.64	<.001
±PyOx	1		31099.91	31099.91	393.85	<.001
Cultivar	2		10498.19	5249.10	66.48	<.001
Time. ±PyOx	1		2036.87	2036.87	25.80	<.001
Time. Cultivar	2		546.45	273.22	3.46	0.049
±PyOx. Cultivar	2		1296.51	648.25	8.21	0.002
Time. ±PyOx. Cultivar	2		2067.41	1033.70	13.09	<.001
Residual	23	1	1816.16	78.96		<.001
Total	34	1	48536.36			

CHAPTER 7

Table 7.1

Table B7.1.1 Biosensor (PyOx and PDH) response (µc): Fresh onion (cv. Renate) juice dilution, PVPP, Tween 80 and filtering

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	4	923.5665	230.892	1061.52	<.001
Cultivar	2	12.195	6.097	28.03	<.001
Time. Cultivar	8	24.37	3.046	14.01	<.001
Residual	30	6.523	0.218		
Total	44	966.657			

Table B7.1.2 PyOx response

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Time	4		547.6	136.9	170	<.001
Cultivar	2		7.448	3.724	4.62	0.019
Time. Cultivar	8		18.02	2.253	2.8	0.021
Residual	27	-3	21.75	0.806		
Total	41	-3	519.5			

Table B7.1.3 PDH response

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Time	4		907.589	226.897	130	<.001
Cultivar	2		4.584	2.292	1.31	0.286
Time. Cultivar	8		28.177	3.522	2.02	0.084
Residual	26	-4	45.373	1.745		
Total	40	-4	921.833			

Table 7.2

Table B7.2.1 Biosensor (PyOx and PDH) response (µC): Frozen onion (cvs. Renate, Hired, Rumba and SS1) juice with dilution and Tween 80.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Time	1		3.171	3.171	4.89	0.029
Cultivar	2		1229.49	614.747	947.47	<.001
±PyOx	3		236.745	78.915	121.63	<.001
Bulbs	2		3.648	1.824	2.81	0.064
Time. Cultivar	2		4.589	2.299	3.54	0.032
Time. ±PyOx	3		4.806	1.602	2.47	0.065
Cultivar. ±PyOx	6		178.63	29.772	45.89	<.001
Time. Bulb	2		6.823	3.412	5.26	0.006
Cultivar. Bulb	4		3.403	0.851	1.31	0.27
±PyOx. Bulb	6		3.040	0.507	0.78	0.586
Time. Cultivar. ±PyOx	6		7.485	1.308	2.02	0.069
Time. Cultivar. Bulb	4		14.329	30582	5.52	<.001
Time. ±PyOx . Bulb	6		2.482	0.414	0.64	0.7
Cultivar. ±PyOx. Bulb	12		9.834	0.819	1.26	0.249
Time. Cultivar. ±PyOx. Bulb	12		3.697	0.308	0.47	0.926
Residual	118	-26	76.562	0.649		
Total	189	-26	1527.87			

Table 7.3

Biosensor (PyOx and PDH) response (µC): Fresh onion (cvs. Rumba and Hired) juice dilution and Tween 80

Table B7.3.1 Colorimetric pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	1	0.0067	0.0067	0.02	0.882
Cultivar	1	4.86	4.86	16.47	<.001
Bulb	1	0.3067	1.3067	4.43	0.051
Treatment. Cultivar	1	0.06	0.06	0.2	0.658
Treatment. Bulb	1	0.0067	0.0067	0.02	0.882
Cultivar. Bulb	1	0.1667	0.1667	0.56	0.463
Treatment. Cultivar. Bulb	1	0.3267	0.3267	1.11	0.308
Residual	16	4.72	0.295		
Total	23	11.453			

Table B7.3.2 Pyruvate biosensor pyruvate

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Treatment	1		52.555	52.555	26.13	<.001
Cultivar	1		70.572	70.572	35.09	<.001
Bulb	1		0.163	0.163	0.08	0.782
Treatment. Cultivar	1		7.656	7.656	3.81	0.08
Treatment. Bulb	1		0.004	0.004	0	0.967
Cultivar. Bulb	1		3.561	3.561	1.77	0.213
Treatment. Cultivar. Bulb	1		5.709	5.709	2.84	0.123
Residual	10	-6	20.112	2.011		
Total	17	-6	121.955			

Table 7.4

Biosensor (PDH) response (µC): Untreated, surfactant and combined surfactant/dilution on fresh onion (cvs. Rumba and Hired) juice.

Table B7.4.1 Colorimetric pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	665.692	332.846	9532.86	<.001
Cultivar 1	1	11.438	11.438	327.6	<.001
Cultivar 2	1	15.310	15.310	438.49	<.001
Treatment. Cultivar 1	2	1.969	0.984	28.19	<.001
Treatment. Cultivar 2	2	3.042	1.521	43.57	<.001
Cultivar 1. Cultivar 2	1	8.522	8.522	244.09	<.001
Treatment. Cultivar 1. Cultivar 2	2	4.789	2.395	68.59	<.001
Residual	24	0.838	0.035		
Total	35	711.602			

Table B7.4.2 Biosensor pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	0.389	0.195	1.16	0.331
Cultivar 1	1	0.184	0.184	1.09	0.306
Cultivar 2	1	0.426	0.426	2.53	0.125
Treatment. Cultivar 1	2	0.309	0.155	0.92	0.412
Treatment. Cultivar 2	2	0.776	0.388	2.31	0.121
Cultivar 1. Cultivar 2	1	0.619	0.619	3.68	0.067
Treatment. Cultivar 1. Cultivar 2	2	3.869	1.935	11.5	<.001
Residual	24	4.037	0.168		
Total	35	10.612			

Table 7.5

Biosensor (PyOx and PDH) response (µC): Optimised pre-treatments with juice from red onions (cvs. Red Globe and PX21)

Table B7.5.1 Colorimetric pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	1	71.492	71.492	2862.54	<.001
Treatment	1	0.337	0.337	13.48	0.006
Cultivar. Treatment	1	0.205	0.205	8.22	0.021
Residual	8	0.199	0.025		
Total	11	72.234			

Table B7.5.2 Biosensor pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	1	48.401	48.401	24.1	0.001
Treatment	1	38.521	38.521	19.18	0.002
Cultivar. Treatment	1	0.701	0.701	0.35	0.571
Residual	8	16.067	2.008		
Total	11	103.689			

CHAPTER 8

Table 8.3

Table B8.3.1 Glucose oxidase biosensor: background interference

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Bulb	4		1930.9	482.7	2.7	0.039
Cultivar	2		77.4	38.7	0.22	0.806
±PyOx	1		380316	380316	2129.64	<.001
Bulb. Cultivar	8		6807.1	850.9	4.76	<.001
Bulb. ±PyOx	4		979.5	244.9	1.37	0.255
Cultivar. ±PyOx	2		8.8	4.4	0.02	0.976
Bulb. Cultivar. ±PyOx	8		3734.2	466.8	2.61	0.016
Residual	59	-1	10536.3	178.6		
Total	88	-1	399746			

Table 8.4

Table B8.4.1 Colorimetric pyruvate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	12.276	6.138	625.87	<.001
Bulb	2	1.606	0.802	81.85	<.001
Cultivar. Bulb	4	5.724	1.431	145.92	<.001
Residual	18	0.177	0.009		
Total	26	19.783			

Table B8.4.2 Pyruvate content via PyOx biosensor

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Cultivar	2		0.128	0.0642	0.29	0.752
Bulb	2		1.885	0.943	4.28	0.035
Cultivar. Bulb	4		3.064	0.767	3.48	0.036
Residual	14	4	8.023	0.220		
Total	22	4				

Table B8.4.3 HPLC glucose

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	2	256.824	128.412	6723.14	<.001
Bulb	2	98.944	49.472	2590.16	<.001
Cultivar. Bulb	4	4.939	1.235	64.66	<.001
Residual	18	0.343	0.019		
Total	26	361.052			

Table B8.4.4 Glucose content via GOx biosensor

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Bulb	4		1930.9	482.7	2.7	0.039
Cultivar	2		77.4	38.7	0.22	0.806
±PyOx	1		380316	380316	2129.64	<.001
Bulb. Cultivar	8		6807.1	850.9	4.76	<.001
Bulb. ±PyOx	4		979.5	244.9	1.37	0.255
Cultivar. ±PyOx	2		8.8	4.4	0.02	0.976
Bulb. Cultivar. ±PyOx	8		3734.2	466.8	2.61	0.016
Residual	59	-1	10536.3	178.6		
Total	88	-1	399746			

APENDIX A

Figure A1

A1 Pyruvate oxidase biosensor one week shelf life at different temperatures and % relative humidity

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Day	6		33849.6	5641.6	41.47	<.001
% humidity	2		42047.2	21023.6	154.54	<.001
Temperature (˚C)	2		12158.2	6079.1	44.69	<.001
Day. % humidity	12		8867.7	739.0	5.43	<.001
Day. Temperature (˚C)	12		3120.4	260.0	1.91	0.039
% humidity. Temperature (˚C)	4		16282.7	4070.7	29.92	<.001
Day. % humidity. Temperature (˚C)	24		7766.7	323.6	2.38	0.001
Residual	120	6	16324.8	136.0		
Total	182	6	139730.1			

Tables A1 & A2

A.1 Enzyme stability over 6 months using stabilising solutions

Source of variation	d.f.	m.v.	s.s.	m.s.	v.r.	F pr.
Month	5		232.812	46.562	73.59	<.001
Method	2		20.382	10.191	16.11	<.001
Treatment	4		78.438	19.609	30.99	<.001
Month. Method	10		431.442	43.144	68.19	<.001
Month. Treatment	20		173.43	8.672	13.71	<.001
Method. Treatment	8		56.073	7.001	11.08	<.001
Month. Method. Treatment	40		118.241	2.956	4.67	<.001
Residual	158	-22	99.968	0.633		
Total	247	-22	1035.69			

APPENDIX C

Pyruvate biosensor field trial standard operating procedure

Onion sample preparation

1. Select onions from representative batch.
2. Place an onion bulb on chopping board or similar surface.
3. Remove and discard neck, stem and dry outer onion skin with sharp knife.
4. Cut the bulb into approximately equally sized top, middle and bottom horizontal sections.
5. Discard top and bottom portions of bulb.
6. Remove and discard 2 outermost scales from middle section.
7. Chop up remaining onion tissue into *ca*. 2 cm^2 pieces and place into a 100 mL glass beaker.
8. Plug in domestic blender (Braun, Type 4192, Spain) power cable to mains supply.
9. Immerse and hold the hand blender in the beaker within the onion sample
10. To switch on the blender, slide the power switch down.
11. When the onion is completely homogenised after approximately 2 minutes, switch off the blender by sliding the power switch back up.
12. Remove the blender and immerse bottom half in another beaker containing water.
13. Switch on to clean.
14. Switch off when finished.
15. Disconnect power cable from mains supply.
16. Leave homogenate to stand for 10 minutes.
17. Filter the homogenised onion extract through Whatman No.1 filter paper into another beaker.
18. Transfer 3 x 1.5 mL filtered undiluted homogenate into vials and store at -20 for traceability
19. For homogenising additional onion samples, repeat steps 1 to 18.

20. Connect Palmsens instrument to PC.

Connecting Palmsens instrument to PC

1. Switch on lap top or desk top computer. When fully charged, the lap top has an internal battery and can operate several hours without the power cable. However, for long runs it is recommended the power cable remains connected.
2. Similarly, the batteries must be fully charged before the Palmsens instrument can be used without an adaptor. Open the lid at the left-hand side of Palmsens by pressing the hinge downwards and then turning the lid.
3. Connect the adapter to the ‘analogue i/o’ miniDIN-connector. When the adaptor is connected the green LED is shown
4. Switch on Palmsens by pressing the power key until the display shows ‘self test’
5. When the display shows ‘PwrOK’ the batteries are charged and the adapter can be disconnected
6. The USB cable is used to connect the PC to Palmsens.
7. Switch on PC and run the Palmsens PC program: From the Windows StartMenu (left-bottom of your screen), click “Programs” and “PalmsensPC”
8. Select ‘Load method’ in the file menu.
9. Select the PalmTime method file type and choose ‘PalmTime’ from the list.
10. From the Method tab, select ‘amperometric detection’
11. Check the parameters on the Method tab on the parameter sheet. These should read: E_begin (0.15V); interval (1s); t_run (200s); E_cond (0V); E_dep (0V); t_cond (0s); t_dep (0s); t_equi (0s).
    The ‘cell on after measurement’ box should be clear.
    A current range of 10 nA to 10 µA should be highlighted.
12. Start onion pyruvate measurements with PalmSens.

Conducting pyruvate measurements

1. Remove one sensor from its packaging and insert horizontally into the slot on the PalmSens instrument.
2. Using a micropipette, drop 30 µl of electrolyte solution over the whole area of the sensor surface.
3. Start the measurements by selecting the ‘Start’ icon on the bottom of the screen.
4. After measurement has been running for 100 secs, using a micropipette, drop 30 µl of filtered onion extract on the sensor of the surface and wait until measurement has finished (at 200 secs).
5. Remove and dispose of sensor.
6. Select ‘Save data’ from the file Menu.
7. Repeat steps 1 to 6 for additional pyruvate measurements.
8. The calculated onion pyruvate concentration(s) are shown within the ‘Results data’ window.
9. Palmsens is switched off by pressing the power key until the display shows ‘Shutting down’.
10. Close the Palmsens program by clicking the top right-hand cross.

APPENDIX D

Defra project assessment

David O'Connor
Allium and Brassica Centre Ltd
Wash Road
Kirton
Lincs PE20 1AA

20th February 2007

Dear David,

LINK Collaborative research into biosensors for sweet onions

Thank you to you and your colleagues for presenting your final report and new proposal to the Programme Management Committee last week. I am able to offer feedback as follows.

Final report HL0164: Defining quality assurance for sweet onions with rapid biosensor analysis

The project has delivered on most of its objectives and the Committee were pleased at the cohesive way that the partners had worked together. There are good prospects of commercial outcomes from this work that can make a difference in industry and the knowledge transfer activities carried out so far were commended. The project was awarded a score of 8 *ex* 10 which I think all partners should be pleased with. The project performance assessment guide followed by the PMC is attached to the LINK Completion Form proforma which you completed.

Development proposal HL0181: Optimising rapid biosensor analysis for cost effective quality assurance of onion

The Committee accepted the case that Development LINK was an appropriate vehicle to help exploit the science done in HL0164 and that the practical outcomes justified this support. The Committee have given technical approval for this project but there are further processes to be gone through before Defra can make funding commitments in the present round.

Yours sincerely

David Cole

Co-ordinator, Horticulture LINK Programme

APPENDIX E

Abstracts, presentations and conferences

E.1 Seventh Postgraduate Research Conference, Cranfield University. (Oral presentation). June, 2006, UK.

The introduction of onion (*Allium cepa* L.) pungency assays has led to better quality assurance. However, the assay which measures onion pyruvate content (Schwimmer and Weston, 1961) as an indicator of pungency or strength is currently out-sourced by growers. Also been found is a positive correlation between non-structural carbohydrates fructose and glucose concentration, with sweetness and likeability when eating uncooked onions (Crowther et al., 2005). Hence, taste panel evaluation is a complementary tool for determining taste preference across onion cultivars. This study examines the feasibility of a multi-analyte biosensor for assessing onion flavour using pyruvate and glucose as selected markers.

The application of biosensor technology is still commercially dominated by the medical diagnostics market (Newman *et al*., 2004). One of the major reasons for the lack of commercial biosensors within food and environmental industries is their failure when applied to real samples (Prodromidis & Karayannis, 2002). Optimised disposable laboratory prototype pyruvate oxidase (PyOx), dehydrogenase (PDH) and glucose oxidase (GOX) immobilised biosensors have been produced for assessing onion flavour, giving rise to the potential of a commercial, integrated and rapid on-farm quality control test. Both PyOx and PDH-based biosensors have been calibrated using pyruvate standards over a linear range of 0-10 µmol (R^2 = 0.94 and 0.97, respectively).

The pyruvate biosensors were tested against a wider range of onion cultivars and gave good precision and resolution as the conventional Schwimmer and Weston-based assay currently employed by industry. The glucose biosensor was calibrated with glucose standards over a linear range of 0-10 mg/ml. The glucose biosensor was tested with juice from a range of mild cultivars. Extensive assessments were made with the same onions by taste panels. Results were verified against HPLC analysis for non-structural carbohydrates. Developments with the glucose biosensor are still ongoing and details will be presented.

E.2 Second International Workshop on Biosensors for Food Safety and Environmental Monitoring. (Oral presentation). November, 2005, Morocco.

The common onion (*Allium cepa* L.) is a major world crop grown mainly for their bulbs and is highly valued for their flavour and nutritional qualities. The UK onion crop shows strong growth potential, owing to the increasing awareness of health and dietary benefits (Grffiths *et al*., 2002). The introduction of pungency assays has led to better quality assurance. However, pungency analysis is currently out-sourced by growers. The application of biosensor technology is still commercially dominated by the medical diagnostics market (Newman *et al*., 2004). One of the major reasons for the lack of commercial biosensors within food and environmental industries is their failure when applied to real samples (Prodromidis & Karayannis, 2002). An optimised laboratory prototype oxidase immobilised biosensor has been produced for assessing pungency in onions giving rise to the potential of a commercial, rapid, cheap, on-farm quality control test. Progress is also being made on the development of an alternative dehydrogenase immobilised onion biosensor. The biosensor performance was assessed and validated with real onion juice samples. Results were verified against the standard colorimetric industry assay established by Schwimmer and Weston, 1961.

E.3 Postgraduate Conference, Cranfield University. (Poster presentation 2nd prize). June, 2005, UK.

An optimised laboratory prototype pyruvate biosensor has been produced for assessing pungency in onions (Allium cepa L.) giving rise to the potential of a commercial, rapid, cheap, on-farm quality control test. The common onion is a major world crop grown mainly for their bulbs and are highly valued for their flavour and nutritional qualities. Since the introduction of pungency assays, UK sales of mild onions have increased through better quality assurance. Pungency analyses are currently outsourced and require skilled personnel. A 12-fold reduction is assay time has been achieved and a 30-fold reduction in assay cost has been realised.

Research demonstrated that a mediated biosensor was the best biosensor format for detection of pyruvate in onion tissue. There was a strong correlation between the biosensor response and known pyruvate concentrations (2-10 µmol g-1 fresh weight) in onion assayed with the conventional method established in 1961 by Schwimmer and Weston. Progress is being made on the development of a sweetness onion biosensor which will ultimately form part of the final biosensor array thus leading to an improved measure of onion flavour as perceived by taste panels.

E.4 AAB Centenary - Association of Applied Biologists: Advances in Applied Biology – Providing New Opportunities for Consumers and Producers in the 21st Century. (Poster presentation). December, 2004, UK.

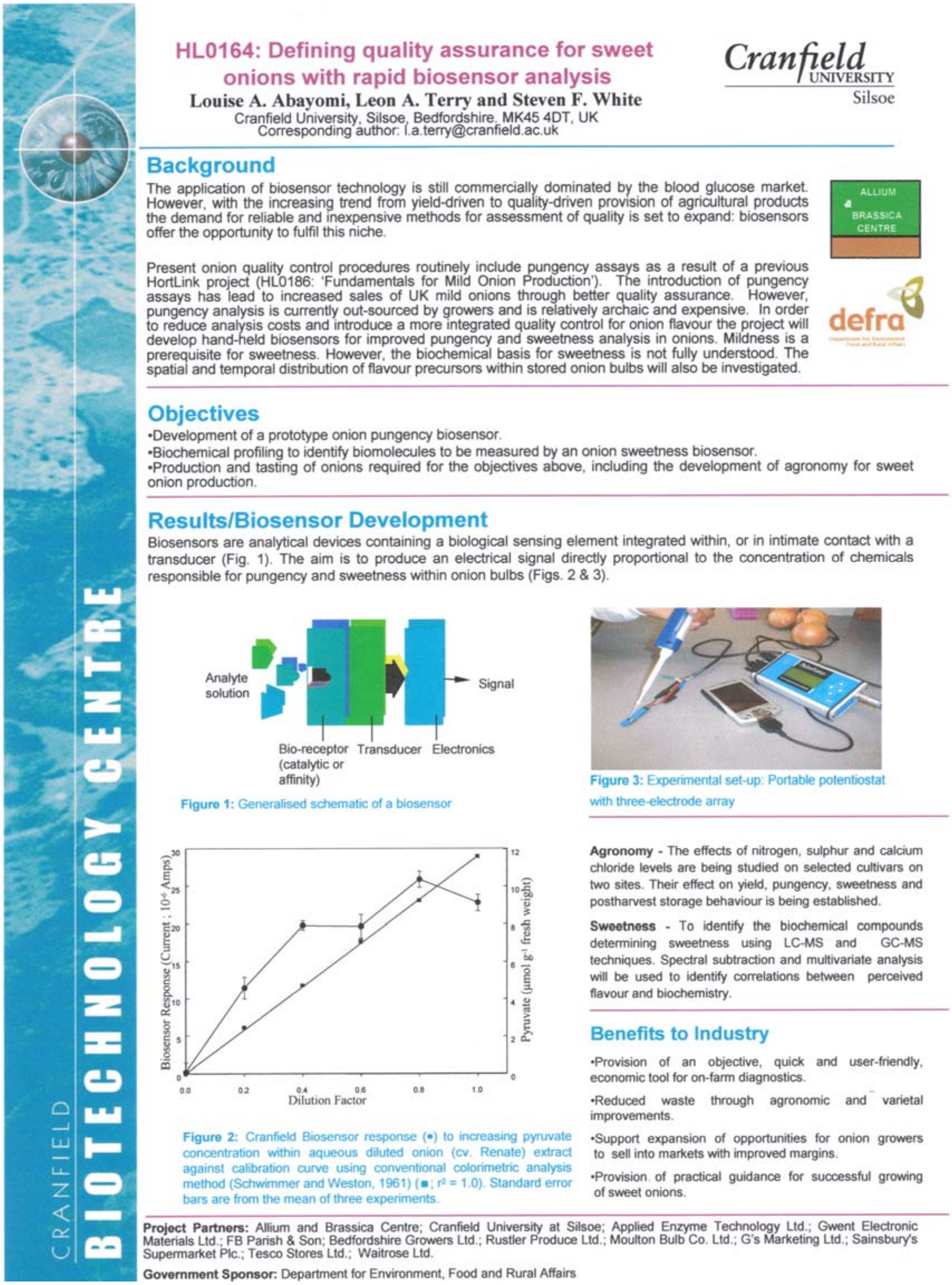
With the increasing trend from yield-driven to quality-driven provision of agricultural products the demand for reliable and inexpensive methods for assessment of fresh produce quality is set to expand. The UK onion crop (*Allium cepa* L.) shows strong growth potential, owing to the increasing awareness of health and dietary benefits. The introduction of pungency assays has led to better quality assurance. However pungency analysis is currently out-sourced by growers and is relatively archaic and expensive. In order to reduce analyses costs and produce a more integrated quality control for onion flavour an individual enzyme based biosensor involving a three electrode array was developed. A linear correlation between biosensor response and increasing concentration of onion pungency markers within aqueous diluted onion bulb extract was demonstrated against conventional colorimetric analysis.

Ongoing developments on biosensor optimisation, including temporal and spatial profiling of pyruvate will be reported.

E.5 Horticulture Link - Sensors and Analytical Techniques through the Food Chain (Poster presentation). June, 2004, UK.

With the increasing trend from yield-driven to quality-driven provision of agricultural products the demand for reliable and inexpensive methods for the assessment of fresh produce quality is set to expand. The UK market for onion crop (*Allium cepa* L.) shows strong growth potential, owing to the increasing awareness of health and dietary benefits. The introduction of assays for pungency has led to better quality assurance. However pungency analysis is currently out-sourced by growers and is relatively archaic and expensive. In order to reduce analysis costs and produce a more rapid, inexpensive and integrated quality control for onion flavour an individual enzyme-based biosensor, involving a three-electrode array, is under development. Initial results have shown a linear correlation between biosensor response and increasing concentration of an onion pungency marker within aqueous diluted onion bulb extract. Accuracy was confirmed using conventional colorimetric analysis. Future developments will include a multi-analyte array to ascertain both onion pungency and sweetness. These will be reported in this presentation, together with more recent data on biosensor optimisation.

E.6 The Eighth World Congress on Biosensors (Poster presentation). May, 2004, Granada, Spain.



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HL0164: Defining quality assurance for sweet onions with rapid biosensor analysis

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Background

The application of biosensor technology is still commercially dominated by the blood glucose market. However, with the increasing trend from yield-driven to quality-driven provision of agricultural products the demand for reliable and inexpensive methods for assessment of quality is set to expand: biosensors offer the opportunity to fulfil this niche.

Present onion quality control procedures routinely include pungency assays as a result of a previous HortLink project (HL0186: 'Fundamentals for Mild Onion Production'). The introduction of pungency assays has lead to increased sales of UK mild onions through better quality assurance. However, pungency analysis is currently out-sourced by growers and is relatively archaic and expensive. In order to reduce analysis costs and introduce a more integrated quality control for onion flavour the project will develop hand-held biosensors for improved pungency and sweetness analysis in onions. Mildness is a prerequisite for sweetness. However, the biochemical basis for sweetness is not fully understood. The spatial and temporal distribution of flavour precursors within stored onion bulbs will also be investigated.

Objectives

- Development of a prototype onion pungency biosensor.
- Biochemical profiling to identify biomolecules to be measured by an onion sweetness biosensor.
- Production and tasting of onions required for the objectives above, including the development of agronomy for sweet onion production.

Results/Biosensor Development

Biosensors are analytical devices containing a biological sensing element integrated within, or in intimate contact with a transducer (Fig. 1). The aim is to produce an electrical signal directly proportional to the concentration of chemicals responsible for pungency and sweetness within onion bulbs (Figs. 2 & 3).

Figure 1: Generalised schematic of a biosensor

Figure 3: Experimental set-up: Portable potentiostat with three-electrode array

Figure 2: Cranfield Biosensor response (●) to increasing pyruvate concentration within aqueous diluted onion (cv. Renate) extract against calibration curve using conventional colorimetric analysis method (Schwimmer and Weston, 1961) (■; $r^2 = 1.0$). Standard error bars are from the mean of three experiments.

Agronomy - The effects of nitrogen, sulphur and calcium chloride levels are being studied on selected cultivars on two sites. Their effect on yield, pungency, sweetness and postharvest storage behaviour is being established.

Sweetness - To identify the biochemical compounds determining sweetness using LC-MS and GC-MS techniques. Spectral subtraction and multivariate analysis will be used to identify correlations between perceived flavour and biochemistry.

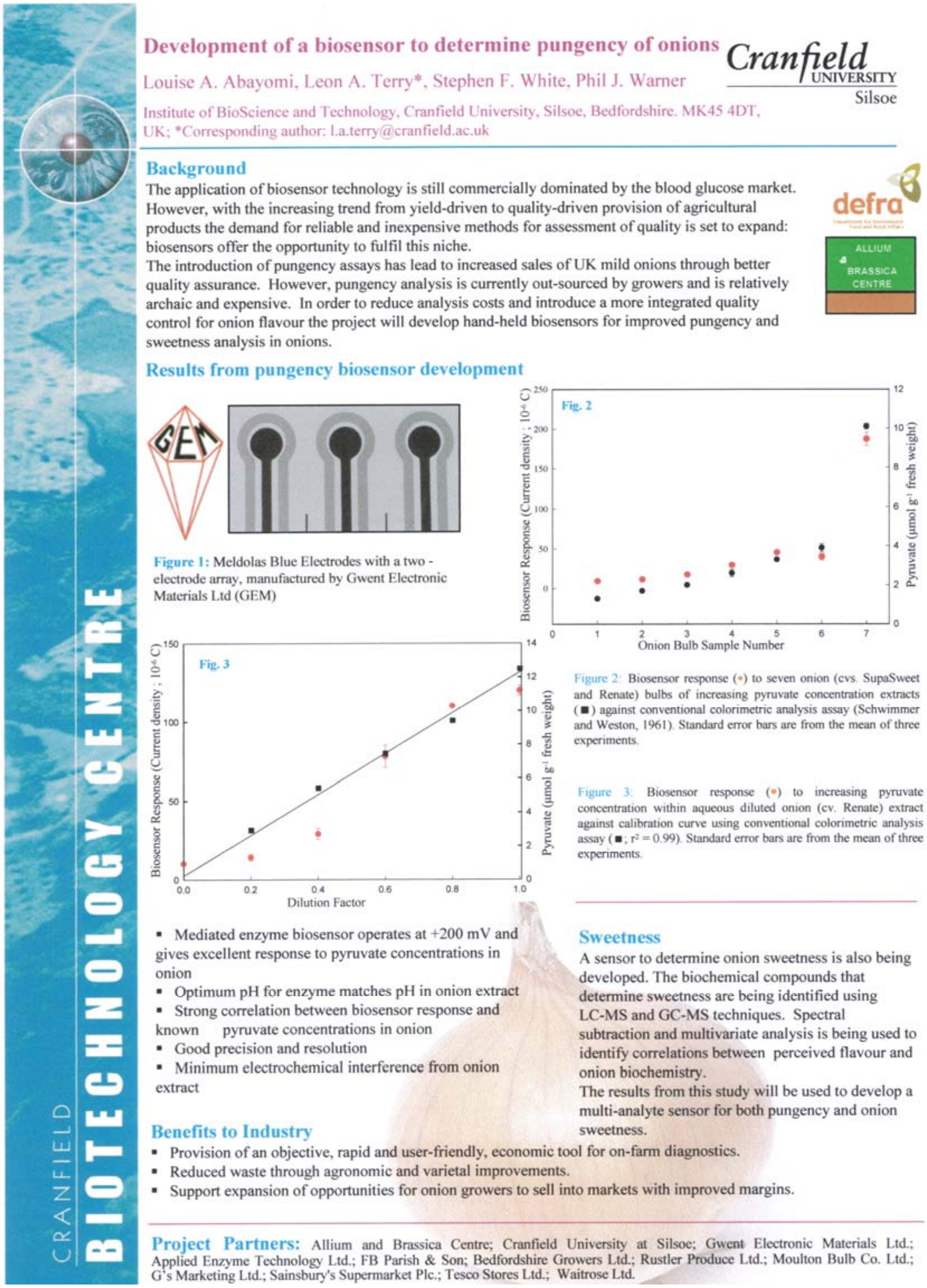
Benefits to Industry

- Provision of an objective, quick and user-friendly, economic tool for on-farm diagnostics.
- Reduced waste through agronomic and varietal improvements.
- Support expansion of opportunities for onion growers to sell into markets with improved margins.
- Provision of practical guidance for successful growing of sweet onions.

Project Partners: Allium and Brassica Centre; Cranfield University at Silsoe; Applied Enzyme Technology Ltd.; Gwent Electronic Materials Ltd.; FB Parish & Son; Bedfordshire Growers Ltd.; Rustler Produce Ltd.; Moulton Bulb Co. Ltd.; G's Marketing Ltd.; Sainsbury's Supermarket Plc.; Tesco Stores Ltd.; Waitrose Ltd.

Government Sponsor: Department for Environment, Food and Rural Affairs

E.7 International British Onion and Potato Association (Poster presentation). December, 2003, UK.



Development of a biosensor to determine pungency of onions

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Background

The application of biosensor technology is still commercially dominated by the blood glucose market. However, with the increasing trend from yield-driven to quality-driven provision of agricultural products the demand for reliable and inexpensive methods for assessment of quality is set to expand: biosensors offer the opportunity to fulfil this niche.

The introduction of pungency assays has lead to increased sales of UK mild onions through better quality assurance. However, pungency analysis is currently out-sourced by growers and is relatively archaic and expensive. In order to reduce analysis costs and introduce a more integrated quality control for onion flavour the project will develop hand-held biosensors for improved pungency and sweetness analysis in onions.

Results from pungency biosensor development

Figure 1: Meldolas Blue Electrodes with a two - electrode array, manufactured by Gwent Electronic Materials Ltd (GEM)

Figure 2: Biosensor response (●) to seven onion (cvs. SupaSweet and Renate) bulbs of increasing pyruvate concentration extracts (■) against conventional colorimetric analysis assay (Schwimmer and Weston, 1961). Standard error bars are from the mean of three experiments.

Figure 3: Biosensor response (●) to increasing pyruvate concentration within aqueous diluted onion (cv. Renate) extract against calibration curve using conventional colorimetric analysis assay (■; $r^2 = 0.99$). Standard error bars are from the mean of three experiments.

- Mediated enzyme biosensor operates at +200 mV and gives excellent response to pyruvate concentrations in onion
- Optimum pH for enzyme matches pH in onion extract
- Strong correlation between biosensor response and known pyruvate concentrations in onion
- Good precision and resolution
- Minimum electrochemical interference from onion extract

Sweetness

A sensor to determine onion sweetness is also being developed. The biochemical compounds that determine sweetness are being identified using LC-MS and GC-MS techniques. Spectral subtraction and multivariate analysis is being used to identify correlations between perceived flavour and onion biochemistry.

The results from this study will be used to develop a multi-analyte sensor for both pungency and onion sweetness.

Benefits to Industry

- Provision of an objective, rapid and user-friendly, economic tool for on-farm diagnostics.
- Reduced waste through agronomic and varietal improvements.
- Support expansion of opportunities for onion growers to sell into markets with improved margins.

Project Partners: Allium and Brassica Centre; Cranfield University at Silsoe; Gwent Electronic Materials Ltd.; Applied Enzyme Technology Ltd.; FB Parish & Son; Bedfordshire Growers Ltd.; Rustler Produce Ltd.; Moulton Bulb Co. Ltd.; G's Marketing Ltd.; Sainsbury's Supermarket Plc.; Tesco Stores Ltd.; Waitrose Ltd.

APPENDIX F

Peer-reviewed research papers

Abayomi, L.A. Terry, L.A., White, S.F. and Warner, P.J. (2006). Development of a disposable pyruvate biosensor to determine pungency in onions (*Allium cepa* L.). *Biosensors and Bioelectronics* 21, 2176-2179 1316.

Abayomi, L.A. Terry, L.A., White, S.F. and Warner, P.J. (2006). A pyruvate dehydrogenase-based amperometric biosensor for assessing postharvest pungency in onions (*Allium cepa* L.). *Postharvest Biology and Technology* (submitted).

Abayomi, L.A. Terry, L.A. (2007). Detailed spatial and temporal profiling of pyruvate and glucose within onion (*Allium cepa* L) bulbs. *J. Sci. Food Agri* (to be submitted).

Abayomi, L.A. Terry, L.A., White, S.F. and Warner, P.J. (2007). Comparative performances of pyruvate dehydrogenase and oxidase-based biosensors during onion field trials. *Biosensors and Bioelectronics* (to be submitted).

Abayomi, L.A. Terry, L.A., White, S.F. and Warner, P.J. (2007). Developments towards multi-analyte glucose and pyruvate testing to determine onion flavour (to be submitted).

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Short communication

Development of a disposable pyruvate biosensor to determine pungency in onions (*Allium cepa* L.)

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Abstract

A disposable prototype pyruvate biosensor was constructed using pyruvate oxidase immobilised on mediated meldolas blue electrodes to determine pungency in onions (*Allium cepa* L.). The optimum operating potential was +150 mV (versus Ag/AgCl). A strong correlation between the biosensor response and untreated onion juice of known pyruvate concentration 2–12 µmol/g fresh weight (FW) was demonstrated. The biosensor was able to differentiate between low and high pungency onions. The detection limit using 1 unit of pyruvate oxidase was 1–2 µmol/g FW. Optimum concentrations of co-factors TPP, FAD and $MgSO_4$ comprising the enzyme cocktail were determined as being 0.04, 0.1 and 30 mM, respectively.

Keywords: Meldolas blue; Fresh produce; Quality assurance; Sweet onions

1. Introduction

Bulb onions are the second most important horticultural crop after tomatoes (Griffiths et al., 2002) and are consumed worldwide for their unique flavour. Increasingly, low pungency bulbs (often referred to colloquially as mild and/or sweet onions) are consumed raw in the USA and elsewhere. Pyruvate concentration (µmol/g FW) in macerated onion tissue is used as a quality assurance indicator of pungency (Schwimmer and Weston, 1961; Wall and Corgan, 1992; Crowther et al., 2005) or flavour intensity in most onion producing countries. Typically, low pungency onions have a pyruvate concentration of ca. <5 µmol/g FW and command a price premium.

Despite improvements to the original Schwimmer and Weston (1961) method over the last four decades, current quality assurance assays for onion pungency (e.g. Randle and Bussard, 1993; Yoo and Pike, 2001) are still relatively time-consuming and expensive. Confidence in the accuracy of pyruvate measurements is becoming more important, particularly as the popularity of low pungency onions increases (Yoo and Pike, 2001; Havey et al., 2002). Pungency tests are currently out-sourced. Decentralising the current pyruvate assay will empower growers and packers marketing low pungency onions to improve their quality assurance procedures.

The demand for reliable and inexpensive methods for the assessment of fresh produce quality is set to expand; biosensors offer a viable opportunity to fulfil this niche (Terry et al., 2005). Over the last 20 years, research has been carried out to produce a pyruvate biosensor, mainly for clinical applications (Table 1). The present study describes the development of an amperometric biosensor to detect and quantify the pyruvate concentration in juice from macerated onion tissue based on the following enzyme reaction:

$$\text{Pyruvate} + HPO_4^{2-} + O_2 \xrightarrow{\text{pyruvate oxidase (PyOx)}} \text{acetylphosphate} + H_2O_2 + CO_2$$

Mediators were used to reduce the effects of electrochemically active species, found in many food matrices (Terry et al., 2005). Meldolas blue was the preferred mediator used for this study, the reaction of which is as follows:

$$\text{Pyruvate} + HPO_4^{2-} + O_2 \rightarrow \text{acetylphosphate} + \text{PyOx}(FADH_2) \qquad (1)$$

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doi:10.1016/j.bios.2005.10.024

Table 1
A selection of biosensor formats used to detect pyruvate

Enzyme(s)	Detection	Detection range	Construction format	Reference
PyOx[a]	H_2O_2 (V not stated)	1–10 mM	Chemical bonding. Polyazetidine prepolymer, nylon membrane	Mascini and Mazzei (1987)
PyOx	0.2–0.5 V	0.38–1.03 mM	Modified carbon, methylene green	Kulys et al. (1992)
PyOx	0.3 V	1 µM–1.8 mM	Electropolymerisation, conductive redox polymer, glassy carbon	Arai et al. (1999)
PyOx/HRP[b]	H_2O_2 (−0.05 V)	0.1–3 mM	Modified carbon, methylene green	Bergmann et al. (1999)
PyOx	H_2O_2 (+0.65 V)	5 µM–5 mM	Covalent attachment to polytyramine	Situmorang et al. (2002)

[a] Pyruvate oxidase.
[b] Horseradish peroxidase.

$$\mathrm{PyOx(FADH_2)} + \mathrm{MB}^+ \rightarrow \mathrm{PyOx(FAD)} + \mathrm{MBH} + 2\mathrm{H}^+ \qquad (2)$$

$$\mathrm{MBH} \rightarrow \mathrm{MB}^+ + \mathrm{H}^+ + 2e^- \qquad (3)$$

2. Materials and methods

2.1. Reagents, standards and plant material

All of the chemicals used were of analytical grade. Pyruvate oxidase (E.C. 1.2.3.3.; PyOx) derived from *Pediococcus* spp., thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), hydrochloric acid (HCl) and 2,4-dinitrophenyl hydrazine (2,4-DNPH); pyruvic acid sodium salt ($C_3H_3O_3Na$), magnesium sulphate ($MgSO_4$), sodium hydroxide (NaOH) and trichloroacetic acid (TCA); potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma (Dorset, UK), Fisher Scientific Chemicals (Dorset, UK), and BDH. Ltd. (Leics., UK), respectively. All reagents were made up in reverse osmosis water. FAD and TPP co-factors were made up as 3 and 6 mM stock solutions, respectively, and stored at −20 °C until required. $MgSO_4$ was prepared as a 0.9 M stock solution and stored at 4 °C. Pyruvate oxidase solution was made up in the co-factor mix. Pyruvic acid sodium salt for deriving calibration standards was made up as a 5 mM stock solution and stored at 4 °C.

Commercially grown onion cvs. SupaSweet (SS1), Renate, Hyfort, Red Baron, UK Sturon, Crystal, Marimba and Spanish Pandero bulbs were donated by F.B. Parrish and Son (Beds., UK), Moulton Bulb Co. Ltd. (Lincs., UK) or Bedfordshire Growers Ltd. (Beds., UK).

2.2. Onion pyruvate analysis

Total pyruvate was measured according to Schwimmer and Weston (1961) and Crowther et al. (2005) with slight modifications. Whole onion bulbs were homogenised using a domestic blender (Braun, Type 4192, Spain) (Yoo and Pike, 1999). The juice was left to incubate for 1 h at room temperature. Aliquots (1.5 ml) were transferred to Eppendorf tubes and centrifuged at 16,060 × *g* (rotor 3325) for 10 min (Biofuge Pico, Kendro Laboratory Products, Germany). Some samples were subsequently stored at −20 °C prior to analysis. Juices were thawed at room temperature for 30 min and diluted 15-fold in deionised water. Filtrates (0.5 ml) were added to 1 ml aliquots of 0.0125% (v/v) (2,4-DNPH) in 2 M HCl and 1.5 ml deionised water in boiling tubes. The mixture was briefly vortexed and incubated at 37 °C for 10 min. Five millilitres of 0.6 M NaOH was added and the absorbance at 420 nm recorded (Camspec M501, Camspec Ltd., Cambs., UK). A standard curve to allow calculation of pyruvate concentrations from onion samples was produced by taking 10 ml of 5 mM pyruvic acid stock solution and diluting to 1 mM, followed by serial dilutions giving a concentration range of standards of 0.04–0.4 mM. Pyruvate concentrations (µmol/g FW) in onion were determined from the equation of the straight line on the standard curve.

2.3. Unmediated electrodes

Screen printed disposable plain carbon electrodes were manufactured by Cranfield University, Silsoe, UK. The electrodes comprised of a central carbon working electrode (10 mm^2), a counter electrode and an Ag/AgCl reference electrode. The electrodes were printed using a DEK 247 screen-printer (DEK Printing Machines Ltd., Dorset, UK). Sensors were connected to an Autolab workstation (Echochemi, Utrecht, The Netherlands) via custom-made electrical connectors (RS Components, Northhants., UK). The Autolab was controlled by the Autolab General Purpose Electrochemical System (GPES) software. Measurements were initially carried out at +800 mV at 21 °C. All experiments were undertaken in triplicate. All electrodes were only used once before disposal.

Initially, the response of unmediated carbon electrodes without enzyme and co-factors to onion cv. Renate juice was examined. Reagents included 50 mM sodium phosphate buffer, pH 6.9, and co-factor mix A—2 units PyOx, 0.2 mM TPP, 0.01 mM FAD and 10 mM $MgSO_4$ (final concentrations). The electrochemical response to increasing pyruvate concentrations in previously frozen undiluted onion juice was also compared against a calibration curve using the modified Schwimmer and Weston (1961) assay. All measurements were made by depositing 20 µl KCl electrolyte on the electrode surface, applying the potential, then allowing a steady-state current to be reached before adding 20 µl onion juice.

2.4. Mediated electrodes

Generic carbon, mediated with meldolas blue (C2030519D5, Gwent Electronic Materials Ltd., Gwent, UK), comprised the working electrode substrate (28 mm^2), and were screen-printed with a Ag/AgCl reference/counter electrode onto a PVC platform in a two-electrode configuration. Electrochemical measurements were carried out using a PalmSense potentiostat (Palm Instruments BV, The Netherlands). Electrochemical measurements were carried out at either +150 or +200 mV at 21 °C.

Enzyme immobilisation was achieved by depositing an enzyme cocktail containing PyOx, TPP, FAD and $MgSO_4$ made up in 0.1 M sodium phosphate buffer, pH 5.7, onto the surface of the working electrode. Electrodes were left to air dry for 4 h and then subsequently stored at 4 °C prior to use. The optimum pH for hydrogen peroxide liberation from the reaction of pyruvate oxidase and pyruvate in the presence of phosphate and oxygen is 5.7. Irrespective of pyruvate content (2.3–6.6 µmol/g FW), the pH of onion juices for cvs. Red Baron, SS1, Marimba, Crystal, UK Sturon and Spanish Pandero, marketed in the UK, corresponded with the optimum for PyOx activity.

The electrochemical response to undiluted juices from individual onion cvs. SS1 and Renate bulbs of increasing pyruvate concentration were examined in two separate experiments using meldolas blue sensors.

2.5. Interference

The effect of operating potential on interference in juices from different onion (cvs. Red Baron, Spanish Pandero and Hyfort) cultivars were compared at +150 and +200 mV using co-factor mix B—1 unit PyOx, 0.04 mM TPP, 0.1 mM FAD and 30 mM $MgSO_4$ (final concentrations).

3. Results and discussion

3.1. Response of unmediated carbon electrodes with and without PyOx and co-factors to onion juice

The electrochemical biosensor response to onion cv. Renate juice was considerably amplified with the addition of enzyme and co-factors (data not shown). However, a significant response was also evident with the addition of juice on bare electrodes due to other electrochemically active species in onion inevitably being oxidised at +800 mV (data not shown). Nevertheless, these preliminary results demonstrated the feasibility of measuring pyruvate concentration in onion juice using an amperometric biosensor format, but in order to reduce the biosensor operating potential, mediated sensors were adopted for further biosensor development.

3.2. Response of mediated meldolas blue electrodes to undiluted onion juice

Meldolas blue biosensors responded positively to increasing pyruvate concentrations in onion juice at a significantly reduced potential of +200 mV compared with unmediated electrodes at +800 mV. An operating potential of +200 mV was adopted following preliminary experiments where a constant operating range between +50 and +250 mV was demonstrated (data not shown). Furthermore, the meldolas blue biosensor gave considerably enhanced signals compared with the standard carbon format. A good correlation between the mediated biosensor response to known pyruvate concentrations within juices of onion cvs. SS1 (low pungency) and Renate (high pungency) are shown (Figs. 1 and 2).

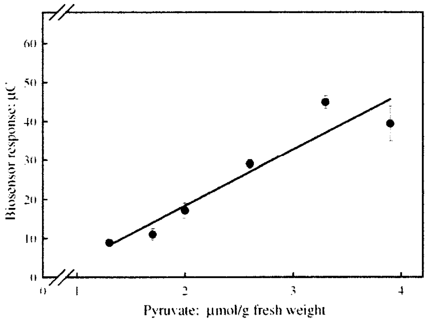

Fig. 1. Mediated biosensor response to onion juices from six individual low pungency bulbs (cv. SS1) of increasing pyruvate concentration verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). $R^2 = 0.83$; $y = 14x - 9$; $P < 0.001$. Standard error bars are from the mean of three experiments. +200 mV; phosphate buffer pH 5.7; co-factor mix B.

3.3. Interference experiments

There was, with some cultivars, a significant response on bare compared with enzyme immobilised mediated electrodes, indicating the presence of interference compounds. There was also a clear variation in background current across and within onion cultivars at +150 and +200 mV. This background response

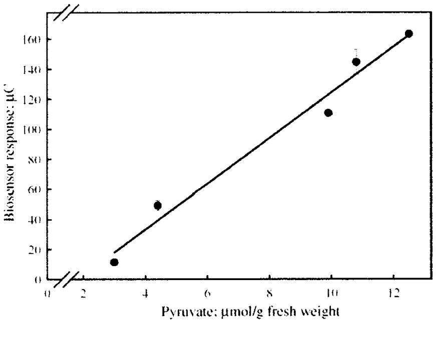

Fig. 2. Mediated biosensor response to onion juices from two individual low pungency (cv. SS1) and three high pungency (cv. Renate) bulbs of increasing pyruvate concentration verified against conventional colorimetric analysis (Schwimmer and Weston, 1961). $R^2 = 0.97$; $y = 15x - 27$; $P = 0.001$. Standard error bars are from the mean of three experiments. +200 mV; phosphate buffer pH 5.7; co-factor mix B.

was reduced at +150 mV without any deterioration in performance. Levels of interference were 4.7% ± 1.8, 80.3 ± 5.5 and 32.0 ± 4.4% at +150 mV and 31.0 ± 3.8, 89.3 ± 1.9 and 42.7 ± 8.1% at +200 mV for cvs. Red Baron, Spanish Pandero and Hyfort, respectively. The corresponding pyruvate contents ranged between 4.3–6.6, 7.7–9.5 and 10–10.7 μmol/g FW for cvs. Red Baron, Spanish Pandero and Hyfort, respectively. These results suggested that major polyphenols in red-skinned onions (cv. Red Baron; Price et al., 1997), were unlikely to be a major contributing factor given the low interference response.

4. Conclusion

Meldolas blue mediated electrodes were shown to be the best biosensor format tested for the amperometric detection of pyruvate in the juice from macerated onion tissue. There was a strong correlation between the biosensor response and known pyruvate concentrations (2–12 μmol/g FW) in onion as measured using the modified Schwimmer and Weston (1961) colorimetric assay. No sample dilution was necessary as found with some other pyruvate biosensors developed for clinical applications (cf. Table 1).

This preliminary study has demonstrated for the first time the possibility of replacing the standard colorimetric assay used ubiquitously by the world onion industry for determining pyruvate concentration with a more rapid method using a mediated amperometric biosensor. Introduction of a pyruvate biosensor for onions will empower growers to undertake their own quality control, rather than out-sourcing pungency analysis. Ongoing work aims to further improve biosensor performance and elucidate possible variations in cultivar interference.

Acknowledgements

This work forms part of a larger HortLink project (HL0164LFV; defining quality assurance for sweet onions with rapid biosensor analysis) and is financially supported by the UK Government (Department for Environment, Food and Rural Affairs, DEFRA) and UK industry representatives (Allium and Brassica Centre Ltd. [ABC], Applied Enzyme Technology Ltd., Bedfordshire Growers Ltd., F.B. Parrish and Son, G's Marketing Ltd., Gwent Electronic Materials Ltd. [GEM], Moulton Bulb Co., Ltd., Rustler Produce Ltd., Sainsbury's Supermarkets Ltd., Tesco Stores Ltd. and Waitrose Ltd.). Special appreciation is extended to David O'Connor [ABC], for organising plant material and Robin Pittson [GEM] for the supply of sensors. The technical support of Allen Hilton, Cranfield University is gratefully acknowledged.

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