Aroma - Matrix Interaction in Food: An APCI Approach

By

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BSc.

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

Division of Food Sciences

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University of Nottingham

November 2014

ACKNOWLEDGEMENTS

I would like to express my heart-felt appreciation and most sincere gratitude to all whom in one way or another contributed to the completion of my PhD 'marathon'. First and foremost, I give thanks to God for His abundant blessings, protection and strength upon me here in Nottingham.

I am so grateful to Nanyang Polytechnic (NYP) for their financial sustenance and the colleagues from School of Chemical and Life Sciences (NYP) for making it possible for me to pursue and focus on my PhD.

My special thanks to my supervisor, Dr. Ian Fisk - you have been an incredible mentor for me. Thank you for your constant reassurance for my work and for allowing me to grow as a research scientist. Your dedication and passion towards research are inspirational and contagious.

To my internal assessor, Dr. David Gray, your advice on my research as well as your fatherly counsel has been priceless. Your words of kindness during my first two years of review made the whole experience very encouraging and enjoyable.

I would also like to extend my wholehearted appreciation to my technical advisors and mentors - Dr Robert Linforth, Dr Bill Macnaughtan and Dr Huw Williams for your expertise and patience in coaching me. If you have not guided me through the usage of the different analytical equipment I needed for this PhD and generously

shared your knowledge with me, this research would not have been possible. Thank you so much!

To my ‘publications-churning Greek machine’, Christos, I cannot express how much I enjoyed working with you. Your enthusiasm and appetite for Science were astounding! You even ignited my interests in PCA and PLS. Thank you for all your help, support and guidance through the chemometrics work.

I would like to thank my friends and colleagues, particularly Christine, Grace, Kay, Chin Ling, Lina, Magarida, Sharon, Nicole and Candy. Thank you for your encouragement and the different gestures of kindness you have shown in my PhD journey

And to single out my dearest buddy and probably my favourite man in SB - Ruben. I cannot thank you enough for all the blessings your friendship have brought into my life here. You have been there to support me since day one- when we burnt our weekends to churn out our reports, and you constantly checked in on me during the tedious days of writing- your text never failed to boost my morale and energize me. Thank you for showering me with so much care, patience and love.

To my precious ‘flat-mates’ – Ifa and Teddy, your company and friendship mean the world to me. Moving in with you was one of the best decisions I have ever made. Your attentiveness and thoughtfulness as a bosom friend have kept me going. Appreciate all the meals you have cooked; the fun and joy you brought into my life that made every day so beautiful. Thank you for putting up with my whims

and fancies, and the ‘racket’ I created either staying up late or waking up early.

James – a big cuddle to you! Thank you for pampering me like a ‘Princess’ during the completion of my studies. Finally, an exceptionally big thank you to my family and friends back in Singapore. You are God’s gifts to me, and I will never exchange your love and care for anything. Your prayers for me were what sustained me thus far. Thank you for believing in me and cheering me on to the end of this race. Thank you for loving me for who I am.

ABSTRACT

An overview is presented of the principle, scope and major applications to date of the use of atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) for monitoring the kinetics of release of flavour volatiles in real time. There are four major areas in this thesis that extend from the measurement of flavour molecular mobility in sugar solutions, to the investigation of APCI-MS as a diagnostic tool for classifying apple cultivars and predicting age of cheese. Headspace techniques are the most extensively employed techniques in food analysis to measure volatile compounds, which play a central role in the perceived quality of food. The use of APCI-MS to measure aroma headspace availability with the addition of solutes was evaluated for two sugars of differing chain lengths (fructose and fructooligosaccharide (FOS)). In vitro aroma release was investigated at equilibrium using APCI-MS. An increase in the concentration of fructose above 25 % (w / w) was shown to significantly ($p < 0.05$) increase the release of ethyl butyrate, ethyl acetate and benzaldehyde into the gas phase above the aqueous solutions. Proton nuclear magnetic resonance (^{1}H NMR) was used to investigate the nature of solvent-sugar-aroma interactions with a view to explain the differential availability of aroma volatiles in sweetened and biopolymer rich solutions. The T1 relaxation times, and diffusion coefficients, provided preliminary clues as to the interactions between water, aroma and biopolymer.

In the case of in vivo measurement, aroma perception was dependent on two key factors – firstly the equilibrium gas phase concentration of the aroma compounds within the oral/ nasal cavity and secondly the dynamic interaction of the compound with food and saliva (persistence). The overall process was influenced by the properties of the flavour compounds (i.e. its hydrophobicity) and the physiological conditions of the mouth, nose and throat during consumption of the food. A comparison was made between the intensities of anisaldehyde, furaneol and guaiacol in the headspace above simple solutions of water, to demonstrate the impact of matrix on flavour release in a frozen dairy dessert. The oral and nasal persistence of aromas following swallowing samples of a model frozen dairy dessert was investigated by collecting expired air from nose and mouth over 60-seconds. The decay curves (which illustrates persistence) of volatiles in breath showed that furaneol was the most persistent aroma, due to its hydrophilic nature and low volatility, that is expressed by the vapour pressure values of the pure compound. It is presumed to interact with the nasal mucosa, thereby prolonging persistence and potentially offering a significantly different perceptual profile to the less persistent compounds (Hodgson, Parker, Linforth, & Taylor, 2004). Thus, real time APCI-MS headspace data could be used to validate mathematical modelling of flavour release (Harvey & Barra, 2003).

The feasibility of APCI-MS volatile compound fingerprinting in conjunction with chemometrics as a new strategy for rapid and non-destructive food classification was demonstrated when 202 clarified

monovarietal juices extracted from apples differing in their botanical and geographical origins were characterised. Partial Least Square-Linear Discriminant Analysis (PLS-LDA) gave 100 % correct classification for the categorization by cultivar. Another PLS regression model was built to interpret and predict the age of Cheddar using headspace data from GC-MS and APCI-MS. The RMSEP and R^2 values for the prediction model were 3.94 and 0.85 respectively. This further established the applicability of multivariate statistical technique as a tool to monitor the quality of foodstuff.

Throughout the thesis, recommendations regarding practical implications for APCI-MS analysis and applications are demonstrated and discussed.

METHODOLOGY

The main objective of this study was to obtain a more extensive application of the APCI-MS headspace technique as a rapid analytical tool in characterisation of different food systems; and while doing so, demonstrates understanding of in vitro and in vivo aroma-matrix interaction.

The overarching research approach is described below:

The influence of molecular mobility on flavour delivery

The effects addition of fructose and fructooligosaccharide at different concentrations had on aroma delivery, followed by the use of ^{1}H-NMR to investigate the molecular mobility of water in these simple model systems to understand the solvent-sugar-aroma interactions.

Development of a method for analysis of flavour compounds in human breath

The optimisation of APCI-MS operating parameters to effectively measure and trend in vivo release of aromas from consumption of frozen desserts.

Classification of apple juices using chemometrics

A case study to evaluate the use of APCI-MS as a novel tool for the classification of apple juices based on their botanical and geographical origins.

Classification and prediction of Cheddar cheese maturity using chemometrics

The use APCI-MS and GC-MS coupled with chemometrics modelling to identify and characterise aroma volatiles in Cheddar cheeses. The predictive model derived using PLS1 was validated to calculate maturity of Cheddar cheese based on their aroma fingerprints.

PUBLICATIONS/ PRESENTATIONS

Peer reviewed published paper

1. Heng Hui Gan, Christos Soukoulis, Ian FIsk. (2014). Atmospheric pressure chemical ionisation mass spectrometry analysis linked with chemometrics for food classification – A case study: Geographical provenance and cultivar classification of monovarietal clarified apple juices. *Food Chemistry*, 146, 149-156.

Oral communications

1. Topic: 'APCI-MS Characterisation of Cheddar Cheese Aroma Using Chemometrics', *In 9th Cheese Symposium, Cork, Ireland, Nov 2014*.

Peer reviewed poster presentations

1. Heng Hui Gan, Christos Soukoulis, Ian FIsk. 'Practical Applications of APCI-MS Headspace Analysis,' *In Mass Spec 2013, Biopolis, Singapore, March 2013*.

2. Heng Hui Gan, Christos Soukoulis, Ian FIsk. 'APCI-MS as a Rapid Analytical Tool in Food Authenticity Testing: A Case Study,' *In 10th Wartburg Symposium, Eisenach, Germany, April 2013.*

3. Heng Hui Gan, Christos Soukoulis, Ian FIsk. 'APCI-MS characterisation of apple juice aroma using rapid chemometrics

screening,' *In 1st Nursten Food and Flavour Research Symposium, Reading, UK, April 2013.*

4. Heng Hui Gan, Yan Jun Tan, Ian FIsk. 'In-vitro and in-vivo measurements of anisaldehyde, benzaldehyde, guaiacol and furaneol release and persistence in ice cream,' *In 2nd Nursten Food and Flavour Research Symposium, Nottingham, UK, April 2014.*

5. Heng Hui Gan, William MacNaughtan, Huw Williams and Ian FIsk. 'Understanding Solvent-Solute-Aroma Interactions in a Simple Sugar System by APCI-MS and NMR,' *In 14th Weurman Flavour Research Symposium, Cambridge, UK, September 2014.*

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionisation-Mass Spectrometry
D_2O	Deuterium Oxide
DOSY	Diffusion Ordered Spectroscopy
DVB/ CAR/ PDMS	Divinylbenzene/ carboxen on polydimethyl siloxane
EC	European Union Council Regulation
ERS	Economic Research Service
FOS	Fructooligosaccharide
GC-MS	Gas Chromatography- Mass Spectrometry
H_2O	Water
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
HPLC	High-Performance Liquid Chromatography
HS-SPME	Headspace-Solid phase microextraction
I_{max}	Maximum Intensity
K_{aw}	Air-water partition coefficient
KNN	K-Nearest Neighbours

LOD	Limit of Detection
LOO	Leave One Out
LOQ	Limit of Quantitation
Log P	Octanol-water partition coefficient, measures lipophilicity
LSD	Lease Significant Difference
NSLAB	Non-starter Lactic Acid Bacteria
PC	Principal Component
PCA	Principal Component Analysis
PDO	Protected Designated of Orgin
PGSE	Pulsed Gradient Spin-Echo
PLS	Partial Least Square
PLS-DA	Partial Least Square Determinant Analysis
RF	Random Forest
R^2	Coefficient of Determination
RV HPLC	Reverse-phase High-Performance Liquid Chromatography
SIMCA	Soft Independent Modelling of Class Analysis
SIR	Selected Ion Resolution
S/ n	Signal to noise
SVM	Support Vector Machine

T1	Spin-Lattice, Longitudinal Relaxation
WSF	Water Soluble Fraction
W/ W	Weight/ weight

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Aroma Release

1.1.1 What is aroma?

Foods are a mixture of volatile and non-volatile components. Flavour compounds can be naturally present in foods or can be added to enhance eating experience. Aroma is an odour and is often referred to as a smell which is sensed by receptors in the nose. Behaviour of aromas in the food matrix is one of the most important parameters involved in a product from its preparation to its consumption. The key features influencing transfer and release of aroma compounds are their chemical nature and the composition and structure of foods. Food matrices are generally multiphasic, containing liquid, solid and gaseous phases. Aroma partition depends on the affinity of the compounds for the different phases and on their availability for release into the gaseous phase (Philippe, Seuvre, Colas, Langendorff, Schippa, & Voilley, 2003). This is the reason why these complicated physicochemical interactions that occur between aroma volatiles and the constituents of the matrix have been so extensively researched. Most of the literature focuses on aroma delivery from simple binary (solvent-aroma) or ternary (solvent-solute-aroma) model systems with low viscosity, in order to have a better understanding of all thermodynamic and kinetic phenomena affecting aroma release.

1.2 Aroma-Matrix Interaction

Understanding matrix parameters that influence the release of aroma compounds from foods provide essential information to control their delivery in food products and allow effective use of these flavour materials (Schober & Peterson, 2004). Aroma-matrix interactions affect flavour intensity, hence quality of foods and consumer overall acceptance. The type of interaction depends on the physicochemical properties of the aroma compounds and the food matrix. The four main groups of interactions include: (i) covalent bonding (such as the interaction between aldehyde or ketone and amino group of proteins), (ii) hydrogen bonding (which occurs between polar or volatile alcohol and heteroatom like nitrogen, sulphur or oxygen, in food components), (iii) hydrophobic bond (a weak and reversible bonding like van der Waals that exist between apolar compounds and fat molecules) and (iv) physical binding (like inclusion complexes, that are common between flavour compounds and starch or its derivatives) (Le Thanh, Thibeaudeau, & Thibaut, 1992; Solms, Osman-Isamail, & Beyeler, 1973).

Log P indicates the relative hydrophobicity (positive value) or hydrophilicity (negative value) of an organic compound. It measures the equilibrium concentration of a compound between n-octanol and water. Compound with high Log P implies that the compound will preferentially partition into organic matter rather than water, and this is inversely related to the solubility of a compound in water. Retention of polar volatiles is expected to be very low. The greater solubility of polar

compounds in water results in greater ease of diffusion through the matrix compared with nonpolar compounds (Terta, Blekes, & Paraskevopoulou, 2006).

Thus it is necessary to understand the behaviour of odorants in food products, considering the physicochemical interactions that exist between aroma compounds and food constituents. The binding that occurs at a molecular level reflects changes, at a macroscopic level, of thermodynamic equilibria, such as volatility and solubility, or changes of kinetic phenomena. Thus, thermodynamic and dynamic approaches can both be used to study the behaviour of aroma compounds in model or complex media that have different microstructures.

1.2.1 Aroma-Carbohydrates

The retention of aroma compounds in systems rich in carbohydrates is more complex than the retention caused by lipids. Simple sugars produce increase in vapour pressure for a number of components at low concentrations and a marked decrease for others (Buttery R.G.; Bomben, 1971; Friel, Linforth, & Taylor, 2000). Carbohydrates are divided into three categories: (i) monosaccharides, (ii) oligosaccharides and (iii) polysaccharides. Carbohydrates change the volatility of aroma compounds relative to water, but the effect depends on the interaction between the particular aroma and carbohydrate molecule.

Generally, mono- and disaccharide exhibit a ‘salting out’ effect in aqueous systems, which cause an increase in volatility of flavour relative to water. In this ‘salting out’ effect, mono- and disaccharides in

solution are known to structure around water molecules such that the amount of free water in the food matrix is reduced. As a result, the concentration of flavour compounds increases in the remaining available free water, which thus upset the apparent partition equilibrium of the volatile compounds in favour of the gas phase.

Small sugars on one hand seem to interfere with the diffusion of volatile compounds and thus slow down release (Delarue, J. and Giampaoli, P, 2006). On the other hand, these sugars were found to increase the vapour pressure of some volatile flavour compounds, in fact higher volatility was observed in higher concentrations of sucrose up to a certain concentration (Godshall, 1997; Friel, Linforth, & Taylor, 2000). Complex carbohydrates offer many more possibilities for chemical interaction than the simple sugar structures since the former would have more diverse functional groups available. Therefore, polysaccharides generally would cause a reduction in aroma release as a result of an increase in viscosity and/ or due to molecular interactions with aroma compounds through vapour pressure reduction or by influencing mass transfer rate. Given such diversity in behaviours, many authors tried investigating the possible driving factors that could explain the headspace concentration of volatile compounds above solutions containing small sugars, after equilibration.

Factors affection retention and release of aroma compounds in food carbohydrates are dependent on: (i) physicochemical properties of aroma compounds such as molecular weight, chemical group and

polarity; (ii) type of carbohydrates and (iii) concentration of carbohydrates acids (Goubet, Le Quere, & Voilley, 1998).

Generally, high molecular weight aroma compounds will retain in carbohydrate matrix more than low molecular flavour compound. When molecular weight of volatile flavour compounds and its molecular size increase, slow diffusion rate is obtained and the aroma compound does not reach the matrix surface as readily (Goubet, Le Quere, & Voilley, 1998). Overall trend recorded by Delarue, J. and Giampaoli, P. (2006) is that polar compounds show an enhanced volatility whereas static headspace concentration of less polar compounds tends to decrease with simple sugars.

Similarly, chain length of flavour compounds also influence retention and release of flavour compounds. Long chain length molecules will be retained more than short chain molecules (Naknean, 2010). When comparing the retention of several classes of aroma compounds, aroma with alcohol functional group are usually the best retained by carbohydrates since they can easily form glycosidic linkage with carbohydrates. Total retention was the greatest for alcohols and lowest for aldehydes. It was found that ketones seem to be less retained than alcohols, similar to esters and more efficiently than acids (Goubet, Le Quere, & Voilley, 1998). Among homologous series, esters showed the highest volatility, followed by aldehydes, ketones, with alcohols as the least volatile series (Buttery, Ling, & Guadagni, 1969).

1.2.2 Aroma-Proteins

Flavour persistence during the consumption of food products has been shown to be increased by the presence of proteins. Proteins and aroma compounds can interact via relatively weak reversible physical adsorption through Van de Waal's or hydrophobic interactions, and strongly covalent or ionic chemical binding. A range of volatiles such as alcohols, aldehydes, ketones, and phenols can bind to protein and this often influence the organoleptic, functional and nutritional properties of the protein. Aldehydes, particularly the unsaturated species, and ketones showed considerable interaction with protein, whereas alcohols were much less reactive (Gremli, 1974). Most research showed that there was an increase in retention of aroma molecules by the protein, as the molecular weight or chain length of the aroma compounds increased (Andriot, Harrison, Fournier, & Guichard, 2000; Guichard & Langourieux, 2000). Aroma binding can induce conformational changes in proteins and can reduce the impact of aroma compounds, affecting the smell and taste of foods (Damandoran & Kinsella, 1980).

1.2.3 Aroma-Fats

Lipids are the food ingredients that have been shown to have the most effect on the partitioning of aroma compounds between product and gaseous phase. The fat content has been shown to affect not only the perceived intensity but also the temporal profile of flavours, as well as their behaviour throughout storage (Hatchwell, 1994). The fat content and the hydrophobicity of the aroma compounds are responsible for

some of the differences in the quality of the aroma of model emulsions, and affect the duration of perception (Vroom, Mojet, Heidema, den Hoed, & Haring, 1996). The type of fat also has an effect on aroma release and increasing solid fat content was found to increase the release (de Roos, 1997; Roberts, Pollien, Antille, Lindinger, & Yeretzian, 2003).

1.3 Techniques used for Analysis and Characterisation

1.3.1 Static Headspace Analysis

Direct mass spectrometry techniques like atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS) have been developed to monitor the concentrations of known volatile compounds in air. Applications range from aroma release in foods to monitoring volatile pollutants in the atmosphere. In fact APCI-MS is now commercially available for the trace analysis of volatile compounds and is fast and sensitive enough to measure breath-by-breath release of a wide range of aroma compounds (A. J. Taylor, 1998; A.J. Taylor & Linforth, 1996). It combines with a specially developed interface to allow continuous analysis of the breath from the nose (nosespace) of a volunteer taster. The many compounds of interests in the exhaled breath on a breath-by-breath basis will be monitored and identified by their different masses. Only a technique having the time resolution capability of APCI-MS can demonstrate the dramatic changes of the relative composition of volatiles in the breath objectively. Such differential temporal release of volatiles from a common matrix is a

general phenomenon and clearly has profound implications for flavour perception (Harvey & Barra, 2003).

A major advantage offered by APCI-MS is that it allows breath to be introduced directly into the MS source. Furthermore, it is a 'soft' ionisation technique, meaning that there is normally insufficient energy to fragment a molecule following ionisation as occurs in electron impact MS and only protonated ion of the flavour molecule will be produced. The main, and usually the only significant ion formed from a vast majority of flavour volatiles, M, is the protonated intact molecule, MH^+. Exceptions are alcohols, which dehydrate, as do aldehydes to a lesser extent. Volatiles can thus be monitored according to their molecular weight and a number of volatiles can be monitored simultaneously. Signal equilibration times when measuring headspace at equilibrium are normally short, owing to the stability of baselines and of the sampling rate into the MS. APCI-MS is thus a high throughput analysis technique to quantify individual compounds in mixtures when an external standard is used for calibration.

Although the method is rapid and simple to use, one drawback that is its major disadvantage, is that molecules which generate identical mass/ charge ions cannot generally be differentiated and quantified.

Another static headspace technique applied is solid phase microextraction (SPME), which is used for the concentration and analysis of volatile organic compounds. This technique is relatively simple and can provide sensitivity similar to dynamic purge and trap analysis. It showed very good repeatability, linearity and accuracy within

the range studied. The popularity for this technique has grown and has gained worldwide acceptance for analyses of alcohols in blood and residual solvents in pharmaceutical products. Other common applications include industrial analyses of monomers in polymers and plastic, flavour compounds in beverages and food products, fragrances in perfumes and cosmetics (Ashraf, Linforth, Bealin-Kelly, Smart, & Taylor, 2010; A.J. Taylor & Linforth, 2003; Wong, Yu, Curran, & Zhou, 2009).

SPME requires only small amount of sample and permits isolation and enrichment of volatile and half-volatile analytes from matrices. It is a conservative technique which allows preservation of the natural contents and composition of the sample. Challenges may be found in the isolation of less-volatile compounds or with high affinity to the matrix, like isolation of polar compounds from matrices of polar nature. In such cases, derivatisation is used or the extraction conditions, such as the type of fibre or temperature, have to be optimised to improve recovery and sensitivity (Plutowska & Wardencki, 2007).

Therefore, the extraction and concentration techniques can become time consuming and costly. Extraction productivity in relation to less volatile components may thus present another disadvantage to SPME technique. Furthermore, in SPME GC-MS – compounds such as acids, alcohols and amines are difficult to analyse because of the presence of reaction hydrogens, which can react with the surface of the injection port or the analytical column and result in tailing peaks and low response. Headspace sample size also can affect time it takes to

transfer sample to the column which can increase peak width and affects chromatographic separations. Potential sample carryover may also occur and there could be possible contamination of septa that may bleed into the headspace of the vial during equilibration.

APCI-MS headspace sampling is hence the fastest and cleanest method for analyzing volatile organic compounds. Even if APCI-MS cannot provide, in general, unambiguous identification of compounds, literature data and comparison with other data set allows tentative identification of many peaks.

1.3.2 Aroma Characterisation –modelling aroma release

Flavour release from food has been recognised as an important factor in determining the perceived flavour quality in many food products. Flavours could be released at different rates from food, due to the breakdown of the food matrix and the different extent flavour compounds bind to the mucus lining in the mouth during eating, therefore resulting in the flavour profile and intensity changing with time. Taste perception is enhanced as the temperature of food and beverage products increases. In the case of ice cream and frozen yogurt, temperature effect increases the surface area of the food exposed to saliva and air, facilitating dissolution of taste compounds in saliva and the release of volatiles in the ‘mouth space’ gas phase, leading to flavour sensations. While the pattern of stimulation varies with food type, the contribution to flavour perception from volatile molecules in the breath is usually the dominant factor, as demonstrated by the

commonly experienced difficulty in differentiating between flavours when the nose is blocked.

Flavour perception is determined by several processes which can be categorized as (1) food breakdown and flavour release in the mouth, (2) transport of flavours through the nasal cavity, and (3) flavour perception at the olfactory epithelium. A very large number of parameters are involved in these processes (Plug & Haring, 1994). Overall flavour of a food comprises of taste (i.e. sensed by the tongue), odour (sensed by the nose) and pain (sensed by the trigeminal receptors in the mouth and nose). To be perceptible, aroma compounds have to be released into the headspace in the mouth and transferred to the nose, where aroma perception results.

Real time APCI-MS headspace data may be used for monitoring the kinetics of release of flavour volatiles in real time, principally from breath during eating in order to develop a mathematical model for flavour release (Harvey & Barra, 2003). The technique is rapid, quantitative, sensitive to the ppb level and can be used to monitor the vast majority of flavour volatiles. Advances made during the last 5 years in our understanding of factors affecting flavour release, particularly when conducted simultaneously with sensory evaluation, are contributing increasingly to more efficient product development in the food and flavour industry and to the design of flavour systems with desired dynamic flavour characteristics.

There was an extensive use of GC-olfactometry to evaluate the contribution of individual flavour compounds to overall flavour and

methods for measuring real time in vivo aroma release. There are also a number of emerging rapid non-destructive methods for chemical grouping of foods such as the direct injection mass spectrometric techniques (DIMS) (Davies, Linforth, Wilkinson, Smart, & Cook, 2011), proton transfer reaction mass spectrometry (PTR-MS) (Biasioli, Yeretzian, Gasperi, & Mark, 2011) and selected ion flow tube mass spectrometry (SIFT-MS) (Langford, Reed, Milligan, McEwan, Barringer, & Harper, 2012) which have gained the attention of the researchers working in the field for classification and authenticity, due to their ability to perform real time non-invasive analysis with high sensitivity and limited sample pre-treatment. PTR in combination with a time-of-flight mass spectrometer (PTR-ToF-MS) have been extensively used for classification studies of a broad range of food products including PDO cheese, olive oil and dry cured hams, intact fruits and their derivatives (Aprea, Biasioli, Sani, Cantini, Mark, & Gasperi, 2006; Biasioli, Gasperi, Aprea, Colato, Boscaini, & Märk, 2003; Cappellin, Soukoulis, Aprea, Granitto, Dallabetta, Costa, et al., 2012; Del Pulgar, Soukoulis, Biasioli, Cappellin, García, Gasperi, et al., 2011; Galle, Koot, Soukoulis, Cappellin, Biasioli, Alewijn, et al., 2011).

In this research, the focus will be using APCI-MS technique to examine simple ternary model systems with low to high viscosity in attempt to understand the intermolecular interactions that exist in the solvent-solute-aroma system. This will be followed by case studies on monitoring the aroma release in various complex systems like, frozen

desserts, apple juice and Cheddar cheese, containing proteins, fats and carbohydrates to explore aroma-interaction and characterisation.

CHAPTER 2: MATERIALS & METHODS

2.1 Chemicals

2.1.1 Volatile Compounds

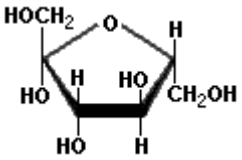
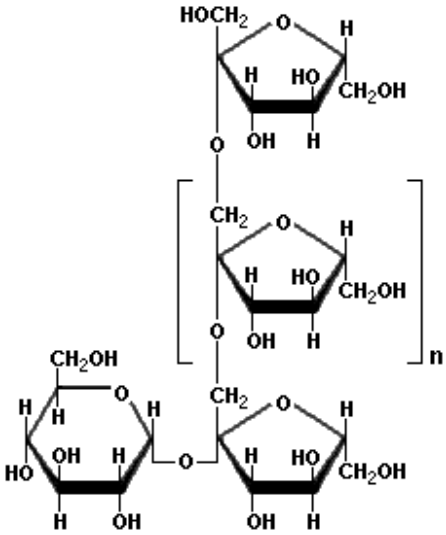
Table 2.1 lists all the volatile aroma compounds used, along with their physic-chemical data. All reagents used were of analytical grade with 90 % purity or greater. Logarithm of partition coefficient P (Log P) or lipophilicity refers to the tendency of the compound to partition between lipophilic organic phase (immiscible with water) and polar aqueous phase. A more positive Log P means that the compound is more lipophilic or 'water-hating'.

Table 2.1 Volatile Compounds used with Physico-chemical Data (Chemspider 2013)

Volatile Aroma Compound	Molecular weight	Solubility (mg / L at 25°C)	Vapour pressure (mm Hg, 25 °C)	Log P
Ethyl butyrate	116	$2.745e^{3}$	14.6	1.85
Ethyl acetate	88	$2.993e^{4}$	98.3	0.86
Benzaldehyde	106	$6.100e^{3}$	1.0	1.71
Hexanal	100	$3.527e^{3}$	9.6	1.80

Table 2.2 lists all non-volatile compounds used. All chemicals were obtained from Sigma-Aldrich, Dorset, UK or Sensus B.V, Zuidwest-Nederland, The Netherlands.

Table 2.2 Non-volatile Compounds used with Structural Data

Non-volatile Compound	Degree of Polymerisation (DP)	Molecular structure
Fructose	1	
Fructooligosaccharide (FOS)	n = 2 to 10	

2.2 Sample Preparation

2.2.1 Preparation of Batch Solutions of Volatile Aroma Compounds and Non-volatile Compounds

Batch solutions of volatile and non-volatile compounds were made up in volumetric flasks, and were then diluted down 10- or 100- fold to make up the desired concentrations for each of the mixtures in subsequent experiments detailed in the following chapters. Dilutions were made up using distilled water, mixed in beakers using magnetic stirrers, at 500 oscillations min^{-1} for at least 30 min. At least three replicates of each mixture and concentration were prepared for the various trials.

2.2.2 Mixing of Final Solutions

Solutions of volatile aroma compounds were mixed in appropriate amounts with the non-volatile solutions to make up the desired concentrations for the different analytical measurements. The final solutions were agitated for an hour on tube roller Spiramix 10 mixer (Thermo Scientific,UK) for thorough mixing. Samples were then equilibrated for at least 30 min at 20 ˚C prior to any analysis.

2.3 Physicochemical Properties Measurements

Soluble solids contents of the sugar solutions used were determined in ^{0}Brix, using PAL-1 hand-held refractometer (Atago, Tokyo, Japan) at room temperature (20 °C) to validate complete solution of sugars in water. A drop of the sugar solution was placed on the sensor disc and measurement was taken when ‘GO’ button was triggered. Water activity

(A_w), which is a measure of the availability or mobility of water in a food, is defined as the equilibrium relative humidity divided by 100. A_w describes the amount of pure water molecules in a system that is not bound to other molecules. It was quantified using a water activity instrument (Aqua Lab Series 31E, Decagon Devices, Inc., USA). Viscosity measurement was carried out with RVA-Super4 (Newport Scientific, USA) at 25 ºC. Approximately 20 g of fructose solutions was poured into the sample cell and measurement was carried out at paddle speed of 160 rpm for 3 min.

2.4 Headspace Measurement

2.4.1 Static Headspace Partitioning of Volatile Aroma Compounds using Atmospheric Pressure Chemical Ionization-Mass Spectrometer (APCI-MS)

Atmospheric Pressure Chemical Ionisation-Mass Spectrometer (APCI-MS) used here is a technique which creates ions at atmospheric pressure. APCI-MS volatilizes solvent and sample molecules by vaporizing the sample solution into a heated corona discharge tube (source of electrons) with the aid of nitrogen nebulization. Ions are produced in the discharge and extracted into the mass spectrometer. Analyte types suitable for APCI-MS analysis include small molecules that are moderately polar to non-polar, samples that contain heteroatoms, samples that are not charged in solution and those that are very thermally unstable or photosensitive. The APCI-MS mass spectrum usually contained the quasi-molecular ion, [M + H] +.

Protons are transferred between these reaction ions and sample molecules (ion-molecule reaction) to ionize sample molecules by either adding or removing a proton. Ionization of the substrate is very efficient as it occurs at atmospheric pressure, and thus has a high collision frequency. For most compounds, the voltage that gives the greatest ion intensity for the MH ion is used. Additionally, APCI-MS considerably reduces the thermal decomposition of the analyte because of the rapid desolvation and vaporization of the droplets in the initial stages of the ionization. This combination of factors most typically results in the production of singly charged ions of the molecular species with fewer fragmentations than many other ionization methods, making it a soft ionization method.

The technique has been used to measure flavour release from strawberries (Grab & Gfeller 2000), from model confectionery gels (RST Linforth, Baek, & Taylor, 1999), biscuits (Brauss, Balders, Linforth, Avison, & Taylor, 1999), static headspace analysis of the aroma-sugar samples (A.J. Taylor, Linforth, Harvey, & Blake, 2000) and real-time flavour release from people eating foods (Friel, Linforth, & Taylor, 2000).

2.4.1.1 Static Headspace Partitioning Measurement

Aroma partitioning was analysed using APCI-MS. The MS Nose interface (Micromass, Wythenshawe, UK) was fitted to a Quattro Ultima mass spectrometer (Micromass, Wythenshawe, UK). Half of the 100 mL Schott bottles (Fisher Scientific, Loughborough, UK), which were fitted with a one-port lid (Figure 2.1), were filled with the solutions. After a 30

min equilibration period at room temperature (20 ^{0}C), static equilibrium was assumed to be established between the gas phase (headspace) and the unstirred aqueous phase in the sealed bottle, the headspace was then drawn into the APCI-MS interface through 60 cm (100 ˚C) heated transfer line at a rate of 10 mL min-^{1}. Volatile molecules were ionized by a 3.5 kV corona discharge using a cone voltage of 60 V. The APCI-MS was operated in a positive ion mode with a source block temperature of 75 ^{0}C in Selected Ion mode (SIR), with dwell time of 0.05 s and an interscan delay of 0.02 s. Intensities of each compound were recorded as peak height ion counts. The ion trace $[M-H]^{+}$ displayed a rapid increase as the headspace was sampled for about 30 s period, followed by a plateau value. All analyses were randomized and run in triplicates. m/z = 89, 101, 107 and 117 protonated ions were used to measure the four aroma compounds: ethyl acetate, hexanal, benzaldehyde and ethyl butyrate respectively.

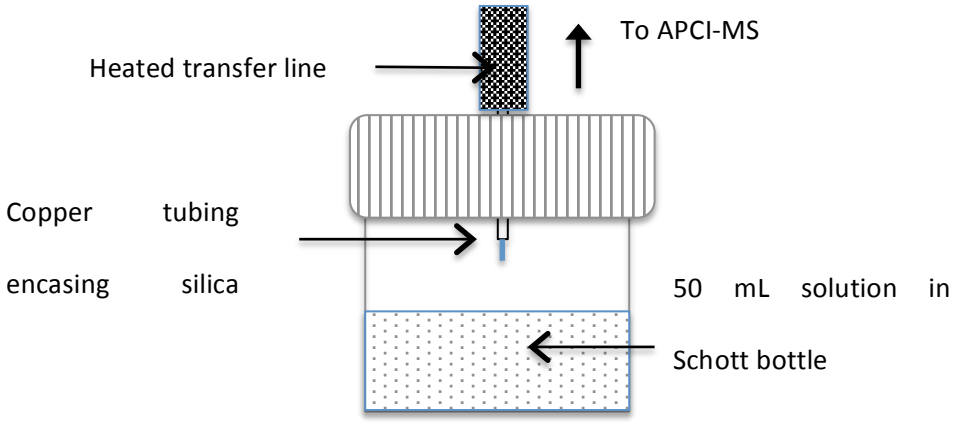


Figure 2.1 Sampling vessel used in static headspace analysis

2.4.2 Dynamic Headspace Partitioning of Volatile Aroma Compounds using APCI-MS

2.4.2.1 Sample Preparation

Low, medium and high concentrations of sugar were prepared by mixing 7.5 %, 25 % and 50 % (w / w) fructose and FOS respectively in water. The final concentrations for ethyl butyrate, ethyl acetate, benzaldehyde and hexanal used were similar to that used in static headspace measurement. However in dynamic headspace measurements, three-quarters of the 100 mL Schott bottles, which were fitted with two-port lid (Figure 2.2), were filled.

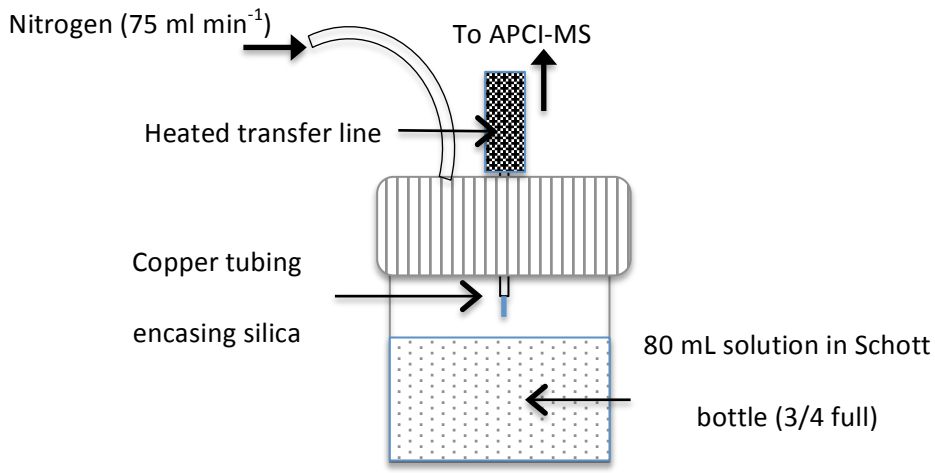


Figure 2.2 Sampling vessel used in dynamic headspace analysis

2.4.2.2 Dynamic Aroma Release Measurements

Nitrogen gas flow was introduced, the headspace was diluted as nitrogen swept through the sample headspace at a flow rate of 75 ml

min^{-1} for 8 min. Aromas were detected in settings similar to that of static headspace, but the APCI-MS was scanning with a dwell time of 0.5 s.

2.5 Data Processing and Statistical Analysis

2.5.1 APCI-MS Data Handling

Changes in headspace concentrations in the experimental system were expressed as changes on a relative percentage scale of the signal heights where a standard solution was considered as 100 %. Semi-quantification of APCI-MS data was achieved by comparison of heights of the signals to that of an external standard used. Conversion of the raw data to headspace concentrations was done using 'MassLynx v. 4.1' software followed by Microsoft Excel 2010 spread sheet. Upper limit of sensitivity was set when there was no increase in signal with increasing volatile concentration. The lower limit was set when the signal to noise ratio (s/n) decreased below a ratio of 3:1. S/n of 0.5 ppm ethyl butyrate standard solution was checked before and during the headspace analysis to determine the sensitivity of APCI-MS.

2.5.2 Statistical Analysis

The headspace data of the aroma compounds in different matrices were averaged and subjected to analysis of variance (ANOVA) using SPSS software version 19 (IBM, Hampshire, UK), at $P < 0.05$. Significant differences in the signal peaks of each aroma compound in the matrices were calculated using Post Hoc Tukey's Lease Significant Difference (LSD, $\alpha = 0.05$).

2.5.3 Multivariate Data Analysis

Principal Component Analysis (PCA) and Partial Least Square-Determinant Analysis (PLS-DA) of APCI-MS fingerprinting of food systems were conducted using Unscrambler version 9.7 (Camo, Oslo, Norway). PCA investigates the discrimination capability of the food systems. It studies the main source of variability present in the data set; detects differences or similarities between the different food samples. In order to study the relationship between a single physical attribute and instrumental parameters (aroma headspace), the method of partial Least Square Analysis 1 (PLS-1) was used. It is a regression model where one single y-dependent variable is related to two or more x independent variables. The model is estimated maximising the covariance between the X matrix and the single-column. PLS-1 models can be validated by are-sampling leave-one-out method (Esbensen, 2009). Thus, in PLS-1 results, R^2 was measured on the set of data used to implement the model, measuring how much the model fit the data.

CHAPTER 3: THE INFLUENCE OF MOLECULAR MOBILITY ON FLAVOUR DELIVERY

3.1 Introduction

Carbohydrates, in particular sugars, are used broadly by the food industry to provide palatability, mouthfeel and taste enhancement within beverages. In response to a growing consumer interest in sugar reduction, knowledge of how sugar impact flavour and aroma release will assist the food industry to develop sugar-reduced or sugar-free food products with minimal compromise to taste when compared to that of conventional sugar-containing foods. Sugars with different numbers of equatorial hydroxyl groups were said to show very different change in headspace concentration, through the interaction within their solvents to modify the static partitioning of volatile aroma compounds from a beverage into the headspace. The hydroxyl groups or the hydration number of sugars was suspected to play a role in the effect of each individual sugar on aroma release (Kabayama 1958), this change in headspace concentration is proposed to be due to a re-structuring of solvent (Evageliou & Patsiakou, 2014).

The influence of different types of sugars on aroma headspace concentrations have been previously reported in the literature (Wientjes 1968; Goubet and others 1998; Rabe 2003), but some of these earlier studies appeared to contradict one another, offering inconsistent data with flavour release being either enhanced or suppressed upon addition of sugars. No authors had offered explicit reasons for the behaviour of

volatiles in sugar solutions and no one attempted to predict the effect of sugars on the wide range of volatile compounds (Friel and others 2000). Substantial work has been carried out on the release of volatile aroma compounds from aqueous systems because this is a main route for flavour release before, and during eating (Taylor 1998). However flavour release from complex food matrices are difficult to predict. Thus, interactions between aroma molecules and food ingredients are often examined in binary and tertiary model systems such as water, aroma compounds and carbohydrates to understand the release behaviour and mechanisms.

3.2 Proton Nuclear Magnetic Resonance (^{1}H-NMR)

Proton nuclear magnetic resonance (^{1}H-NMR) has been used to provide information on the state of water in sugar and carbohydrate biopolymer solutions. Using high resolution methods and measuring the decay or relaxation times (T_1) and diffusion constants, at specific chemically shifted sites, information as to the mobility of protons in the water, aroma and biopolymer/ sugar fractions can be obtained. These methods are being assessed for their ability to investigate the nature of solvent-sugar-aroma interactions with a view to explain the differential availability of aroma volatiles in sugar and long chain carbohydrate solutions. Longitudinal relaxation rates were measured to investigate the kinetics of proton exchange for the sugar chain lengths across a concentration range. ^{1}H-NMR could be used to explain the behaviour of

water within each sugar system and to extract parameters for average mobility and binding.

Molecular dynamics and spin-lattice (T_1) relaxation of the protons result in local magnetic field fluctuations and molecular tumbling plays a dominant role in NMR relaxation. Diffusion ordered spectroscopy (DOSY) permits a fingerprint of complex mixtures to be obtained based on the measure of the translational diffusion of the different compounds present. This work includes measuring the self-diffusion coefficient of the aroma compounds diffusing in the fructose solution.

It has been acknowledged that nuclear magnetic resonance (NMR) using three magnetic water nuclei, the proton, deuteron and oxygen-17 is a very powerful method for probing structural and, especially the dynamic state of water in aqueous systems (Lai & Schmidt, 1993). The behaviour of aroma molecules in the carbohydrate solution is governed by the interactions between the solvent and water molecules that they bind. A range of techniques has been used in an attempt to better understand these hydration properties, including differential scanning calorimeter, differential thermal analysis, sorption/ desorption experiments and nuclear magnetic resonance (NMR). ^{1}H-NMR has the potential to characterize water binding and mobility in a quantifiable manner, and has been shown to be effective in previous studies of water-polymer interaction (Hills, 1991).

Therefore, the aim of our study was to first investigate how the addition of fructose and fructooligosaccharides (FOS) at different concentrations affects the release of four different aroma compounds (ethyl acetate,

ethyl butyrate, benzaldehyde and hexanal). Atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS) would be the primary technique used to measure real-time aroma partitioning and release by static headspace and dynamic headspace approaches, this method had been previously shown to be reliable and reproducible (Taylor 1998).

The second aim of the study was to explain the finding of the first aim using ^{1}H-NMR to investigate the molecular mobility of water in the simple model system. The working hypothesis followed findings of (Aroulmoji, Mathlouthi, Feruglio, Murano, & Grassi, 2012) which showed that water-sugar interaction was preferred to water-aroma binding due to the higher number of equatorial hydroxyl groups which give rise to higher relaxation rate, hence resulting in more aroma molecules being released into the headspace in solutions with lower water activity. The T_1 relaxation times and diffusion coefficients would provide preliminary clues as to the interactions between water, aroma and the biopolymer. For two compounds, one that showed significant headspace release (like ethyl butyrate) and a second with, similarity to hexanal, that have no effect on headspace on both low and high concentrations of sugar solutions.

3.3 Materials and Methods

Static and dynamic headspace analysis of sugar-aroma solutions were carried out using APCI-MS, as described in Chapter Two of this thesis.

3.3.1 Sample Preparation

3.3.1.1 Preparation of Stock Solutions of Volatile Aroma Compounds

Aliquots of pure volatile aroma compounds were dissolved in water and diluted; made up to the stock concentrations of 50 ppm of ethyl butyrate, 50 ppm of ethyl acetate, 1000 ppm of benzaldehyde and 1000 ppm of hexanal. These solutions were transferred to 250 mL Schott bottles (Fisher Scientific, Loughborough, UK) and shaken at about 400 oscillations min^{-1} for 2 hours on a roller mixer (Thermo Scientific, tube roller Spiramix 10).

3.3.1.2 Preparation of Stock Solutions of Non-volatile Compounds

Fructose was purchased from Sigma-Aldrich, Dorset, UK and fructooligosaccharide or oligosaccharide (FOS) was provided by Sensus B.V, Zuidwest-Nederland, The Netherlands. Aqueous solutions of non-volatile compounds of fructose and FOS were made up to 0 %, 7.5 %, 12.5 %, 25 %, 37.5 % and 50 % (w / w) in 500-mL volumetric flasks. All these solutions were separately mixed in beakers, using magnetic stirrers, at 500 oscillations min^{-1} for at least 30 min. Three replicates of each sugar type for each concentration were prepared.

3.3.1.3 Mixing of Final Solutions

Volatile aroma solutions were added to appropriate amounts of the non-volatile sugar solutions to make up to desired concentrations such that the gas phase concentrations for the headspace signals (measured using atmospheric pressure chemical ionization – mass spectrometer (APCI-MS)) was approximately 10 % of full scale (Rob Linforth, Martin, Carey, Davidson, & Taylor, 2002), so, 1 % (w / w) stock volatile was made up in each of the different concentrations of sugar solutions. The final mixtures were sealed in 100 mL Schott bottles (Fischer Scientific, Loughbourough, UK) and agitated for an hour on roller mixer (Thermo Scientific, tube roller Spiramix 10). These samples were then equilibrated for at least 30 min at 20 °C prior to analysis.

3.3.1.4 Preparation of Samples for NMR Analysis

A 5 % (w / w) deuterium oxide (D_2O) solution was made up by mixing 5 mL D_2O (Goss Scientific Instruments Ltd, Cheshire, UK) in 95 mL water. This was used as the solvent in all the samples prepared for NMR measurement. The polysaccharide matrices were then prepared by mixing 1 % (w / w) aroma stock solutions in 5 % (v / v) D_2O/ H_2O with different sugar concentrations [10 % and 30 % (w / w) of fructose]. Each matrix was stirred for 10 min. The aroma-fructose-D_2O solutions were finally poured into 5 mm NMR glass test tubes and equilibrated for about 60 min at the required temperature (20° C) before measurement.

The following samples were studied: 10 % (w / w) fructose, 30 % (w / w) fructose, 10 % (w / w) FOS, 10 ppm ethyl butyrate in 0 %, 10 % and 30 % fructose-D_2O and 10 ppm hexanal in 0 %, 10 % and 30 % fructose-D_2O.

3.3.2 NMR Procedure

High resolution ^1H NMR relaxation measurements were carried out using a Bruker (Coventry, UK) 800 MHz Avance III Spectrometer equipped with a QCI cryoprobe and at a temperature of 25°C. The inversion recovery sequence, 180 °– тau –90 ° was used for (T_1) measurements. For each measurement, the magnetic field was checked and the relaxation times were measured (Aroulmoji, Mathlouthi, Feruglio, Murano, & Grassi, 2012).

Self-diffusion constants were measured on a Bruker 600 MHz spectrometer. Pulsed gradient spin-echo (PGSE) sequences with convection compensation from the Bruker standard library were used to measure the aroma (ethyl butyrate) and fructose self-diffusion coefficients. A total of 64 scans was collected using the PGSE sequence with a recycle delay of 10 s. Diffusion measurements were using the delays Δ of 200 ms and δ of 2.2 ms. Echo intensity was reduced as a function of gradient strength with delta values optimised to give 90 % reduction between the start and end values. In total 10 values were recorded with signal averaging 64 transients. Temperature was maintained at 25°C. Diffusion constants for each resonance were obtained from optimally fitted decay curves.

All data were processed using Bruker Topspin 3.1 software.

Diffusion ordered spectroscopy (DOSY) spectra are presented with chemical shifts on the horizontal axis and self-diffusion coefficients on the vertical axis. The diffusion axis is logarithmically sampled and self-diffusion coefficient values are expressed in $m^2\ s^{-1}$. Peaks were picked on the 2D DOSY spectra and diffusion coefficients were determined from the peak heights (Gostan, Moreau, Juteau, Guichard, & Delsuc, 2004).

3.4 Results and Discussion

3.4.1 Static Headspace Measurements

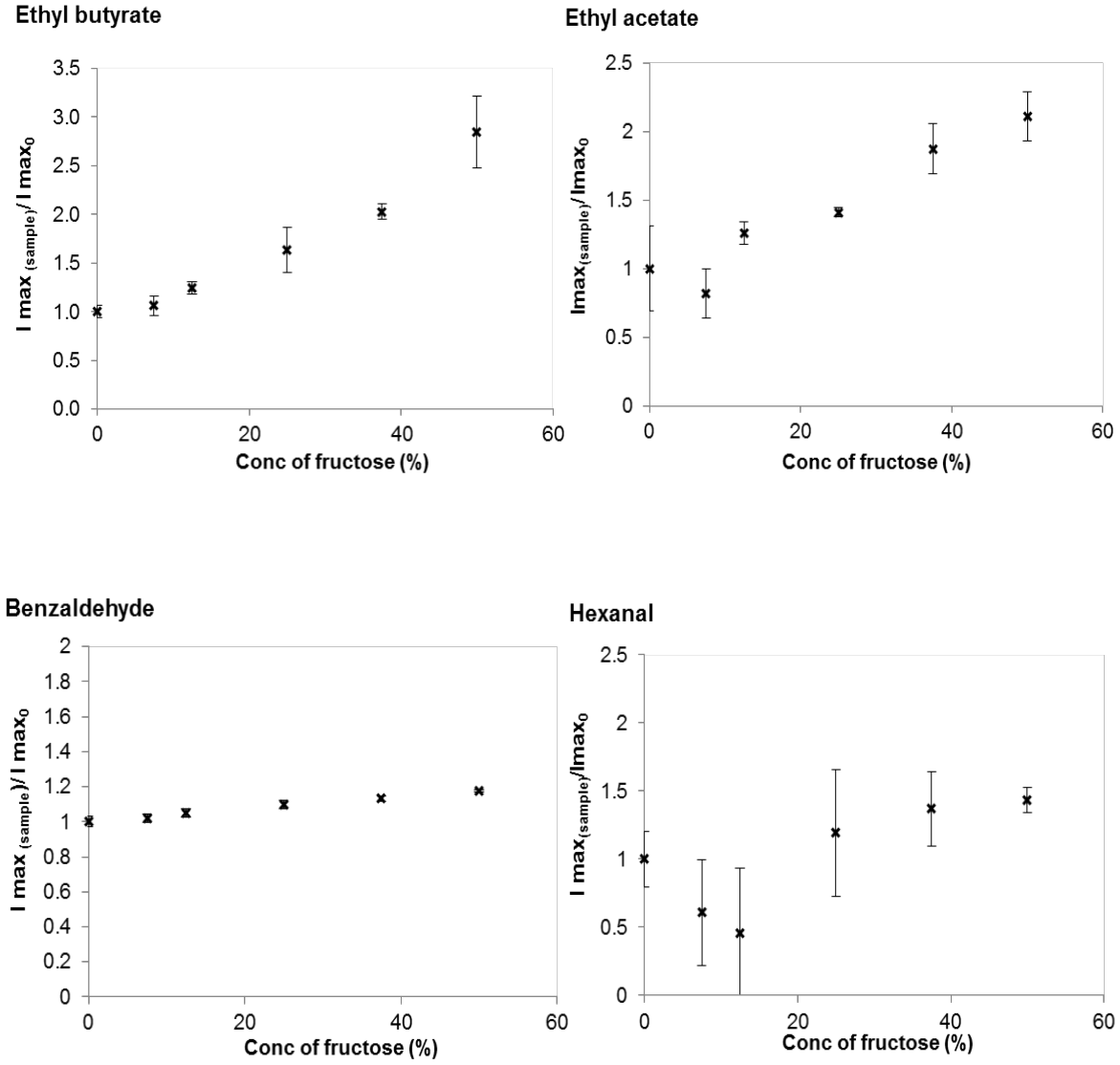


Figure 1. Relative static headspace of aroma ($Imax_{sample}$ / $Imax_0$) in different % of fructose solutions

The ratios of the headspace concentrations above the carbohydrate solutions and above water were calculated and these normalised values were used for ease of comparison of all the compounds (Figure 1). An

increase in the concentration of fructose above 25 % (w / w) was shown to significantly ($p < 0.05$) increase the release of ethyl butyrate, ethyl acetate and benzaldehyde into the gas phase above the aqueous solutions (Table 1).

Table 1. Static headspace (in mV) for volatile aroma compounds in different fructose concentrations % (w / w)

Fructose Conc (%)	Ethyl butyrate (mV)	Ethyl acetate (mV)	Benzaldehyde (mV)	Hexanal (mV)
0	1.34 (0.09)×10^{7a}	1.50 (0.50)×10^{6ab}	8.91 (0.30)×10^{7a}	1.25 (0.30)×10^{6ab}
7.5	1.42 (0.10)×10^{7ab}	1.23 (0.30)×10^{6a}	9.09 (0.20)×10^{7a}	7.60 (5.00)×10^{5ab}
12.5	1.67 (0.09)×10^{7ab}	1.72 (0.30)×10^{6ab}	9.37 (0.20)×10^{7ab}	5.68 (6.00)×10^{5a}
25	2.19 (0.30)×10^{7bc}	2.12 (0.05)×10^{6bc}	9.77 (0.20)×10^{7bc}	1.49 (0.40)×10^{6ab}
37.5	2.72 (0.10)×10^{7c}	2.81(0.30)×10^{6cd}	1.01 (0.01)×10^{8cd}	1.71 (0.30)×10^{6b}
50	3.82 (0.50)×10^{7d}	3.16 (0.30)×10^{6d}	1.05 (0.01)×10^{8d}	1.79 (0.10)×10^{6b}

[a]Samples with the same letter are not significantly different (at $p < 0.05$) within each aroma group. Values in parentheses are standard deviations

Rabe et al (2003) attributed a decrease in ratio of free to bound water in sucrose solutions, as the reason for increased flavour release in the systems containing increasing levels of dissolved solute. Likewise, the increase in static headspace observed here at fructose concentration above 25 % could be due to a 'salting out' effect of fructose, as a result of the development of hydrogen bonds between the water and the fructose with itself. This is when fructose interacted with water, decreasing the ratio between the free and bound water, thus increasing the concentration of aroma compounds in the remaining 'free' water. Therefore the aroma compound would be partitioned out into the headspace in order to maintain equilibrium in the static environment.

As seen from Table 2, the solubility of ethyl butyrate and benzaldehyde in water are lower than ethyl acetate due to their hydrophobic characters (indicated by log P values), therefore these compounds had less interactions with water, resulting in the significant increase in headspace from 25 % to 50 % (w / w) fructose solutions. The reason ethyl butyrate showed a greater gradient of headspace increase than benzaldehyde (Figure 1), could be because the vapour pressure of ethyl butyrate is about 14 times more than the latter, and thus has more likely to break away from water.

Some researchers attributed the retention behaviour of non-polar compounds in carbohydrate-containing aqueous solutions to the formation of hydrophobic inclusion complexes through hydrogen bonding of water molecules with the equatorial hydroxyl groups of polysaccharide macromolecules (Terta, Blekes, & Paraskevopoulou,

2006). In other words, fructose-fructose interactions may occur, forming hydrophobic regions entrapping non-polar aroma compounds. This might explain the behaviour of hexanal and benzaldehyde in fructose solutions, since there were no distinctive changes in the headspace of these aroma compounds in response to the increased fructose concentration (Fig 1).

In the dilute regime, wherein macromolecules or the fructose molecules were isolated from each other, hexanal being an electrophile with a carbonyl functional group R-C=O was able to participate in hexanal-water interactions, whereas interactions between the fructose species remained negligible. At higher fructose concentrations (> 25 %), fructose, which has a better fit with the quasi-tetrahedral structure of water, since it has a high number of equatorial hydroxyl groups, impose a greater degree of order on the solution and this hydration water is strongly bound (Portmann & Berch, 1995). Fructose, being more soluble than other sugars like glucose, tends to hold onto water more strongly. With the formation of fructose-water and fructose-fructose complexes, the small hexanal molecules may be entrapped in the cavity of the helix of these complexes (Conde-Petit, Escher, & Nuessli, 2006; C. Jouquand, Ducruet, & Bail, 2006).

In addition, hexanal has low vapour pressure and partition coefficient (K_{aw}) (Table 2), therefore no significant release of hexanal into the headspace was observed with the change in fructose concentration (Table 1), indicating that hexanal had been mainly retained.

On the other hand, ethyl acetate solubility is greatest among the four aroma compounds, meaning there could be more hydrophilic interactions with water molecules in the solutions, which explains why the significant increase in headspace occurred at a higher fructose concentration of 37.5 % (w / w) than that observed in ethyl butyrate and benzaldehyde (Table 1).

Table 2. Aroma compounds and their physicochemical properties (Chemspider 2013)

Physicochemical Properties	Ethyl butyrate	Ethyl acetate	Benzaldehyde	Hexanal
Solubility (mg / L at 25°C)	2745	29900	6100	3527
Partition coefficient (K_{aw})	1.63×10^{-2}	5.48×10^{-3}	1.09×10^{-3}	8.71×10^{-3}
Vapour pressure (VP) (mm Hg, 25 °C)	14.6	98.3	1.01	9.57
Molecular mass (MW)	116.16	88.11	106.12	100.16
Log P	1.85	0.86	1.71	1.8

Vapour pressure appeared to be the principal factor that discriminates the release behaviour of the esters (ethyl acetate and ethyl butyrate) from that of the carbonyls (benzaldehyde and hexanal) (Table 2). The increase in static headspace observed in esters, with the increasing fructose concentration, was more apparent that that observed in carbonyls (Figure 1). Esters with higher vapour pressures had highest

tendency to break away from water surface compared to the carbonyls, the ‘salting out’ behaviour is in agreement with the studies by Buttery, et al (1969). Furthermore the carbonyl functional groups, when compared to the ester groups, are considered to interact better with the fructose matrices at higher concentration by way of hydrogen bonds (Goubet, Le Quere, & Voilley, 1998).

Volatile release appeared to be much dependent on free water volume and solute / volatile interactions. The different concentrations of fructose are therefore directly impacting water activity, low concentrations of fructose gave high water activity values and vice versa. Increasing total solutes in the solution reduced the water activity, consequently increased the partitioning out of the aroma volatiles (Figure 2).

Moreover, in the presence of fructose, there exists a competition to bind water molecules occurred with the polar molecule, like ethyl acetate, which led to a release of the aroma compound in the gas phase. The same behaviour was reported by (Kieckbusch & King, 1979) for polar acetates. The physicochemical interactions that occur between aroma compounds and other constituents of the food matrix, in particular fructose in this study play an important role in the retention of volatile substances during food processing and consumption.

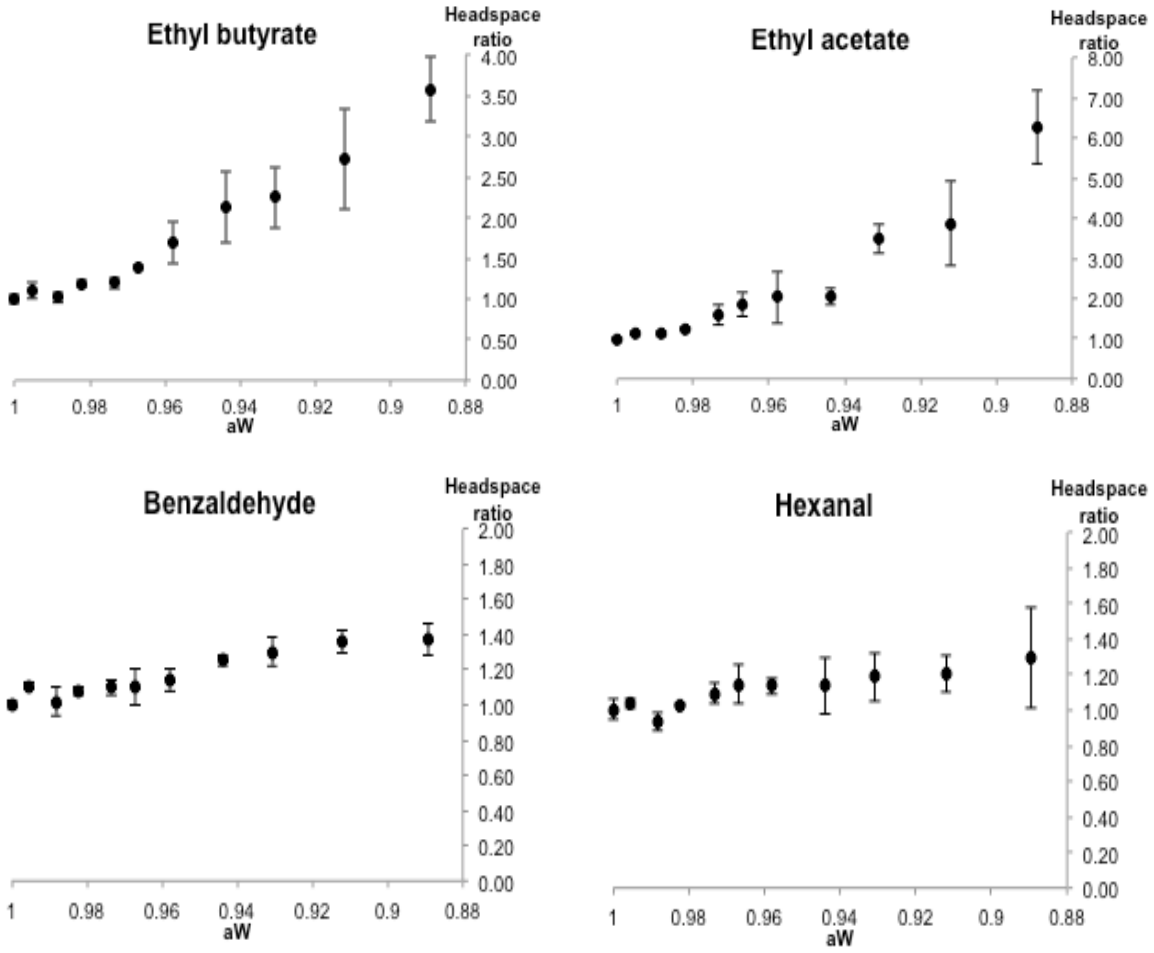


Figure 2. Relative static headspace of aroma ($Imax_{sample}$ / $Imax_0$) in solutions with different water activity (aW). Points presented are the mean of three replicates; error bars represent standard deviation of the mean.

Table 3. Static headspace (in AU) for volatile aroma compounds in different fructooligosaccharide (FOS) concentrations % (w / w)

FOS Conc (%)	Ethyl butyrate (AU)	Ethyl acetate (AU)	Benzaldehyde (AU)	Hexanal (AU)
0	2.33 (0.50)×10^{7a}	1.56 (0.20)×10^{6a}	2.08 (0.20)×10^{7a}	1.25 (0.30)×10^{6a}
7.5	2.17 (0.08)×10^{7a}	1.78 (0.20)×10^{6ab}	2.07 (0.20)×10^{7a}	1.44 (0.20)×10^{6a}
12.5	1.91 (0.10)×10^{7a}	1.84 (0.10)×10^{6ab}	2.02 (0.07)×10^{7a}	1.22 (0.20)×10^{6a}
25	2.11 (0.07)×10^{7a}	2.16 (0.08)×10^{6bc}	2.10 (0.04)×10^{7a}	1.31 (0.40)×10^{6a}
37.5	2.52 (0.30)×10^{7a}	2.60 (0.20)×10^{6c}	2.10 (0.08)×10^{7a}	1.18 (0.20)×10^{6a}
50	2.27 (0.40)×10^{7a}	3.28 (0.30)×10^{6d}	2.20 (0.09)×10^{7a}	1.36 (0.30)×10^{6a}

[a]Samples with the same letter are not significantly different (at $p < 0.05$) within each aroma group.

At the molecular level, polysaccharides like FOS, may interact in various ways with the aroma compounds. Molecular interactions include hydrogen bonding, Van der Waals forces, hydrophobic interactions. Hydrogen bonds could result from electrostatic interactions between electronegative atoms such as oxygen (or acceptors of hydrogen bonds)

and hydrogen atoms (or hydrogen donors) involved in covalent bonding. The presence of weak, temporarily induced dipole moments in non-polar molecules (Van der Waals forces) tend to gather and associate these aroma solutes to each other, while being surrounded by water molecules (held together through hydrogen bonds). Hence resulting in retention of these aroma solutes in the FOS solutions (Delarue, J. and Giampaoli, P, 2006).

However ethyl acetate was the only aroma volatile compounds that showed salting out at higher concentrations of FOS (Table 3). This result could be explained by the high vapour pressure and lowest molecular weight of this aroma compound and hence the greater ability to partition into the headspace and not be affected by the potential inclusion complexes (Table 2). Furthermore, the low stability of FOS complexes with small compounds like ethyl acetate could also be explained by the fact that they do not provide sufficient contact with the wall of the cavity and the strength of the binding is subsequently lowered (Eftink, 1989)

3.4.2 Dynamic Headspace measurements

In dynamic systems, where the boundary layers are often not stagnant, the most important mechanism is the eddy or convection diffusion. Eddy diffusion transports elements or eddies of the fluid of gas from one location to another, carrying with them the dissolved aroma molecules. The rate of eddy diffusion is completely independent of aroma compound type and is not much affected by binding to lipids or

macromolecules because the bonded aroma molecules are usually transported at the same rate as the free molecules (de Roos and Wolswinkel, 1994). Headspace depletion of aroma in fructose showed comparable qualitative behaviour as that in FOS, hence only results in fructose were reported. Depletion of headspace was most obvious in ethyl butyrate-fructose solutions, since ethyl butyrate has the highest value of K_{aw} among the four aromas (Figure 3). As such, the mass transfer in the aqueous phase becomes the main driving factor; therefore the surface depletion of volatiles from solution decreases the headspace concentration (de Roos and Wolswinkel, 1994).

Due to the large variability found when running dynamic headspace analysis, there were noticeable differences only in the depletion of headspace between low and high concentrations of fructose for the ethyl esters. Changes in mass transport in both the aqueous and gas phase affected the differences in the headspace as viscosity increased with increasing fructose concentrations. Rate of aroma depletion was greater at high fructose concentrations compared to the lower concentrations for the ethyl esters but this was not evident for benzaldehyde and hexanal (Figure 3).

With the addition of more fructose molecules at higher concentrations, there would be less free water available in the aqueous phase; more aroma volatiles would be partitioned into the gas phase. The concentration gradients generated at the aqueous surface increased with the increase of the aroma volatility, the mass transport rate in gas phase and the resistance to mass transfer in water. So, it became more

difficult to replenish the depleted concentrations at the aqueous surface (Marin, Baek & Taylor, 1999).

When comparing the rates of depletion between the ethyl butyrate and ethyl acetate at high fructose concentrations, the gradient of ethyl butyrate depletion curve was found to be steeper than that of ethyl acetate. This can be explained by the fact that ethyl butyrate exhibited higher K_{aw} and higher log-P values; hence had less interaction with the polar water molecules and resulting in a high initial rate of depletion (Naknean, 2010). Mass transfer was more impeded for ethyl butyrate than ethyl acetate as fructose concentrations increased, since the former molecule is bigger, so there was more and faster depletion of headspace (Celine Jouquand, Drucruet, & Giampaoli, 2004).

At low sugar concentrations the partition coefficient primarily controls the flavour release, whereas at higher sugar concentrations the mass transfer coefficient has more influence (Nahon, Harrison, & Roozen, 2000).

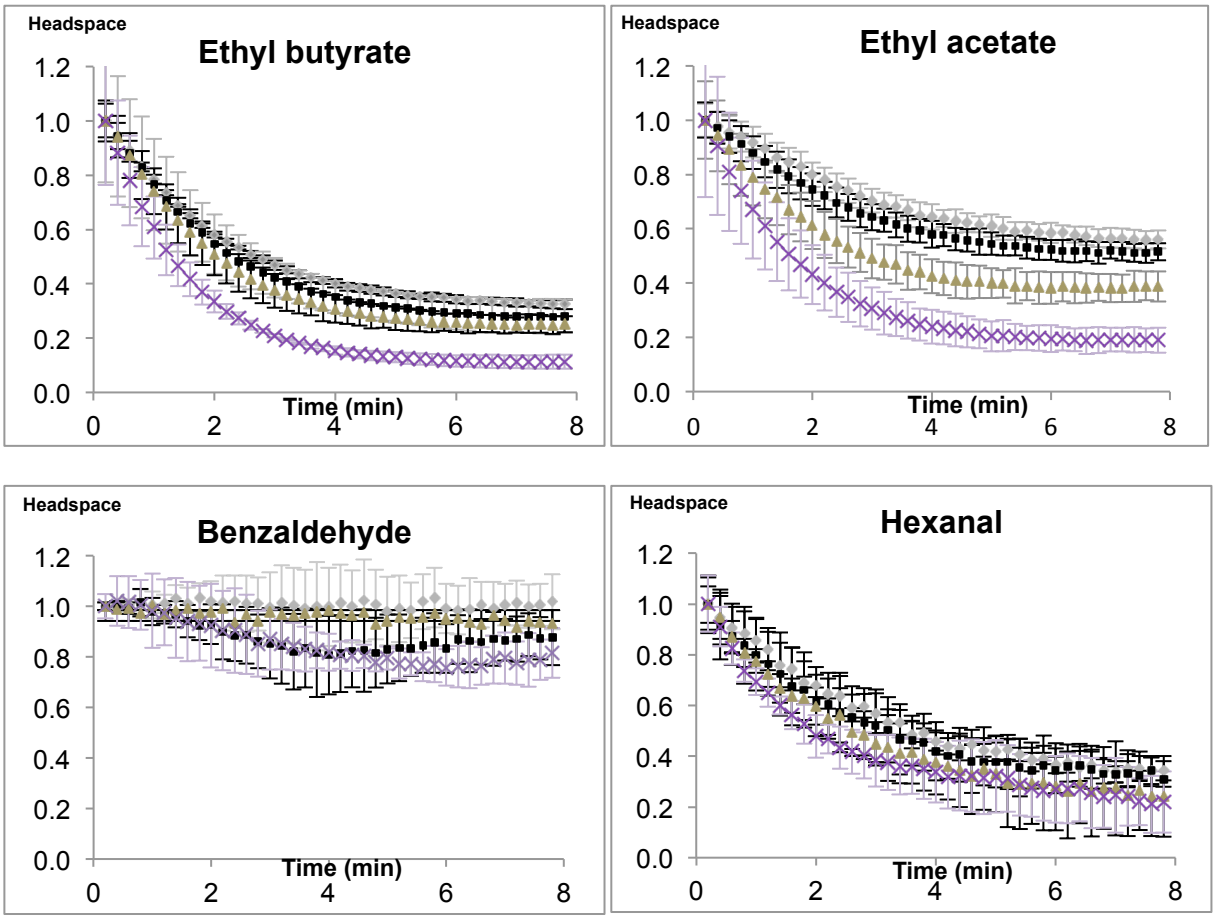


Figure 3. Effects of different concentrations of sugars: ♦ = 0%, ■ = low, 7.5 %, ▲ = med, 25 %, × = high, 50 % (w / w) on headspace depletion of the four aroma compounds in dynamic environment. Points presented are the mean of three replicates; error bars represent standard deviation of the mean.

3.4.3 NMR T1 Measurements

T_1 values, which reflect the tumbling rate of the ethyl butyrate (EB), hexanal (HEX) and fructose, were measured in water and fructose solutions. The relaxation rates of EB in fructose and fructose in EB

solution and water increased, (T_1 decreased), as the concentration of the sugar increased (Figure 4). Similar behaviour was observed for hexanal in water and fructose solutions (Figure 5). This could be explained as the decrease in water mobility at high fructose concentrations due to the formation of intra- and inter-molecular hydrogen bonds between the water and the sugar with itself; and the increase in proton exchange between sugar and water. Fructose and ethyl butyrate molecules gave substantially different T_1 values, with the change in T_1 as a function of sugar concentration being identical. This suggests that the EB and fructose molecules are behaving independently with minimal long-lived interactions between them. On the other hand, fructose and hexanal molecules appeared to have less different T_1 values among them, which could imply more interactions between HEX and fructose. This could also simply reflect different motions in the HEX compared with the EB molecule.

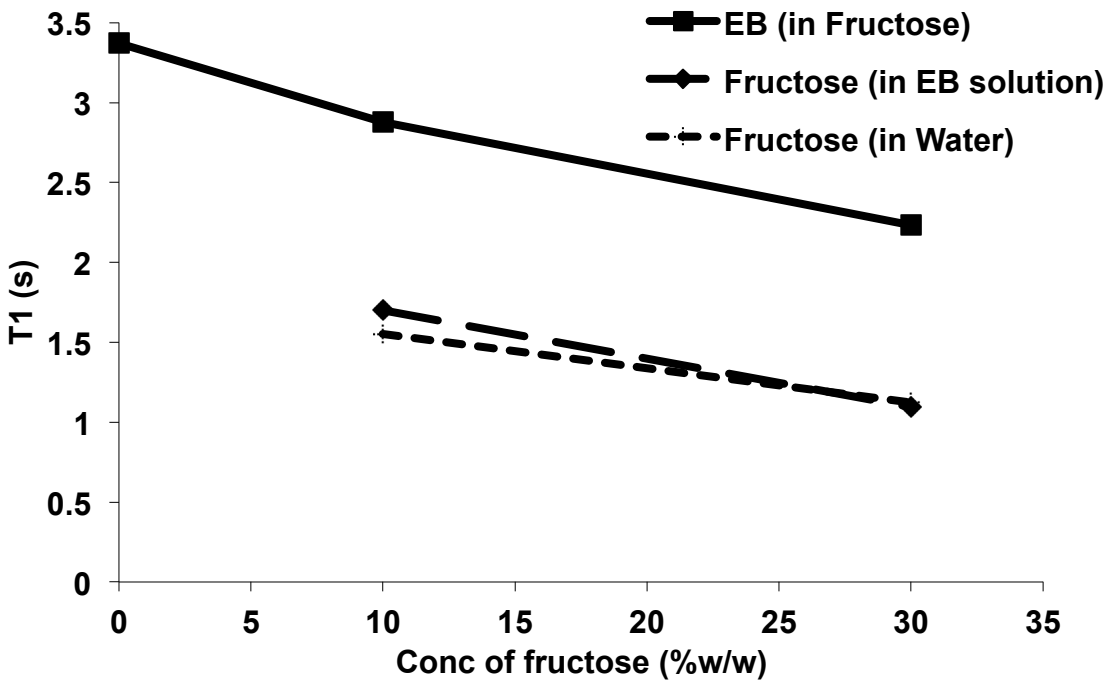


Figure 4. T_1 values of Ethyl butyrate (EB) and fructose vs [fructose]

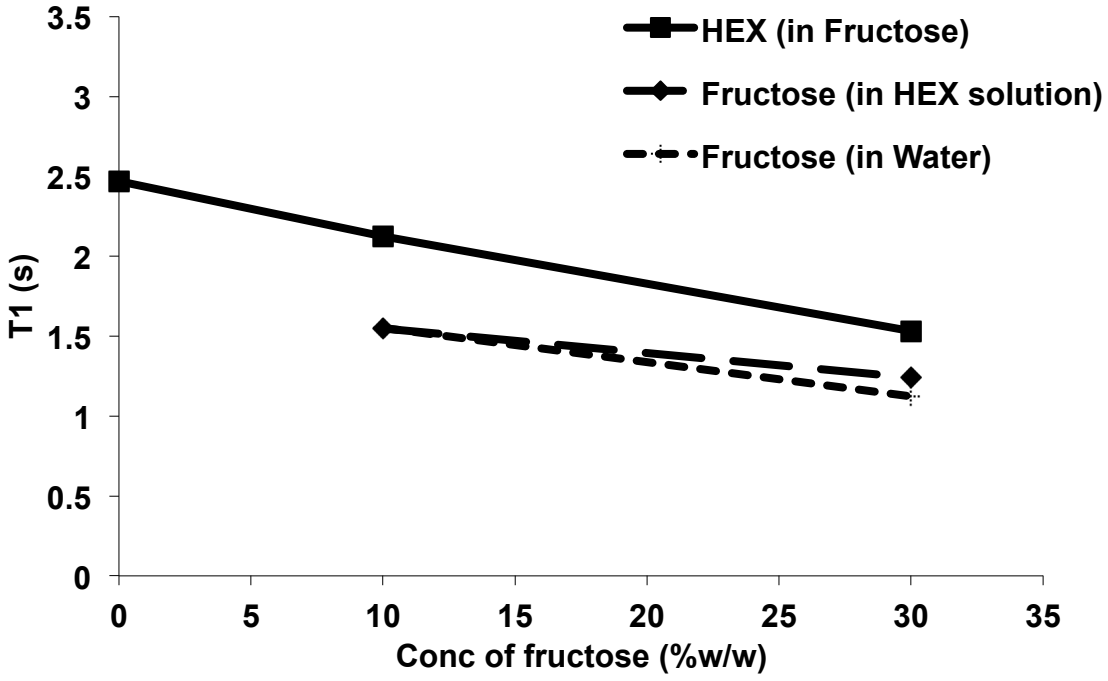


Figure 5. T_1 values of Hexanal (HEX) and fructose vs [fructose]

3.4.4 NMR Diffusion Measurements

The water self-diffusion coefficient was measured in EB and fructose solutions. The diffusion constant of EB in fructose solution was higher than for the fructose itself (Figure 6). Whilst the EB diffusion constant was reduced as the fructose content and hence the viscosity was increased, the reduction was not as large as that of the surrounding sugar molecules. Neither of these observations is completely explained by the changes in molecular mass or viscosity based on simple theory. The gradient of the change in diffusion coefficient as a function of fructose concentration of EB (15%) was less than that seen in fructose (44%) and suggests that the EB motion is somehow detached from the matrix.

The NMR results show that it is possible to measure motional properties of all components simultaneously in complex systems. The aroma molecules showed higher tumbling rates and diffusion constants than the sugar and from this limited data set the tentative conclusion can be drawn that there appear to be no long-lived interactions between ethyl butyrate and the fructose. Explanations for the ‘salting out’ phenomenon of aromas must be sought elsewhere such as in the reduction of free water, which is capable of behaving as a solvent towards aroma compounds.

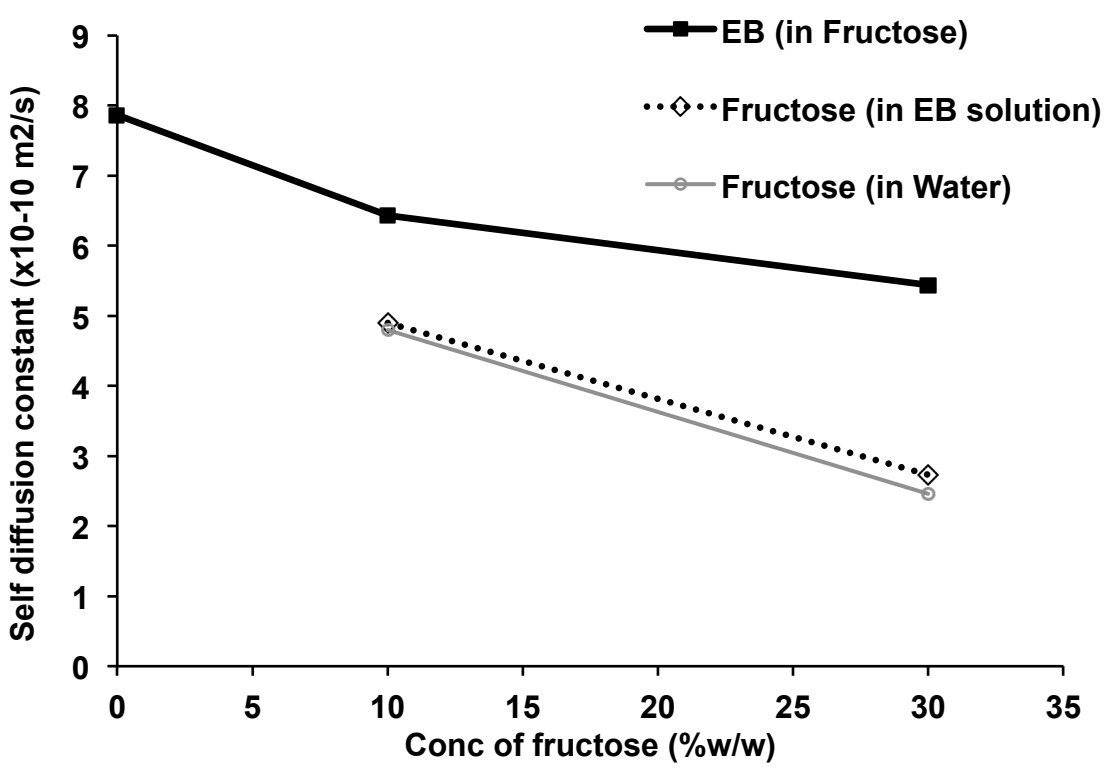


Figure 6. Plot of diffusion coefficient vs [fructose]

3.5 Conclusion

Higher concentrations of simple fructose (above 25 % w/ w) resulted in increased gas/ liquid partition coefficients, in agreement with prior research (Hansson, Andersson, & Leufven, 2001; Nahon, Harrison, & Roozen, 2000). Presence of fructose and FOS appeared to change the delivery of aroma compounds, but the extent of this effect was carbohydrate and aroma compound dependent. Competing effects of intermolecular attractions among the fructose, aroma molecules and water made it difficult to formulate a general theory for sugar-aroma interactions. The effect of polysaccharides, like FOS, on the other hand caused a decrease in aroma release, more likely due of its physical structure limiting aroma diffusion, rather than the presence of any molecular interactions. From the NMR results, binding of aroma compounds to the simple sugars fructose did not seem probable, since their T_1 values and diffusion constants were different, implying that interaction between them would be minimal.

CHAPTER 4: DEVELOPMENT OF A METHOD FOR ANALYSIS OF FLAVOUR COMPOUNDS IN HUMAN BREATH

4.1 Flavour Release

One major theme within flavour science is the development of an understanding of volatile release mechanisms and transport routes to receptors for perception, this is particularly important as the rate of delivery of flavour compounds impacts not only perception but also dietary behaviour and that oral processing patterns significantly impact release kinetics (A.J. Taylor, 2002). A true understanding of the mechanisms behind flavour release such as the binding or volatilization of aroma compounds or the release of tastants from food during eating will therefore enable a greater degree of control over perception (Deborah D Roberts & Taylor, 2000).

Aroma delivery is dependent on the availability of volatile compounds in the gas phase, the release of which is often reduced by binding to large molecules in food systems or solubilisation within solvent phases (water, ethanol or lipid). Binding of flavour compounds in food systems can take place in the form of adsorption, absorption, physicochemical binding and chemical binding (A. J. Taylor & Linforth, 2010). Interactions of volatile compounds and the food matrix may also occur with the lining of the mouth, throat and nose and saliva thus directly impacting *in-vivo* release behaviour. Various physicochemical properties of the volatile such as molecular size, vapour pressure, polarity and gas/ product

partition coefficient, strongly influence the ability of the aroma compounds to enter the vapour phase, that is. its release into the headspace (Friel, Linforth, & Taylor, 2000; Le Thanh, Thibeaudeau, & Thibaut, 1992; van Ruth, O'Connor, & Delahunty, 2000).

Basic composition of foods impacts aroma release through dissolution, adsorption, binding and entrapment/ encapsulation. When food is eaten, it undergoes many physical and chemical modifications due to mastication, the addition of saliva and temperature variation, which ultimately impact flavour release and flavour binding. Such modifications trigger significant changes in the partition of aroma compounds between the matrix and the air phase (Cayot, Dury-Brun, Karbowiak, Savary, & Voilley, 2008).

Since the release of aroma is widely assumed to be responsible for the characteristics flavour of food, many research works focus on the release of volatile constituents; this is shown by van Ruth et al (2000) who confirmed the impact of matrix composition and mastication on aroma release for seven aroma compounds she studied. Mechanistic approach for the development of an aroma release model, with experimental in vivo release curves to describe the consumption process of a food matrix had been validated by (Trelea, Atlan, Deleris, Saint-Eve, Marin, & Souchon, 2008). This constituted a first step toward accurate prediction of volatile concentration in the nasal cavity of subjects consuming flavoured food.

Headspace analysis using soft ionisation mass spectrometer APCI-MS has been widely used for the direct measurement of volatiles released

from foods and in human breath during eating. In a near ideal state of infinite dilution, Henry's law prevails and, at equilibrium, the partial pressure of the volatile in the gas phase above the solution is proportional to the volatile concentration in the liquid phase of the food. Partition coefficients provide information on flavour-matrix interactions under equilibrium conditions (van Ruth, O'Connor, & Delahunty, 2000). It is therefore of a great interest to have qualitative and quantitative descriptions of in vivo aroma release since they provide understanding of the role of product (composition and structure), the type of aroma and of the consumer in the perceived flavour. Although real time determination and monitoring of the aroma compound concentrations in the oral and nasal cavities can now be possible with sufficiently sensitive and rapid in vivo measurement techniques like APCI-MS, it is not always easy to detect and follow the in vivo behaviour of aroma volatiles, especially with hydrophilic compounds.

Other approaches for tracing volatile compounds include a method for the determination of the food aldehydes was developed by adsorptive stripping voltammetry (Yardim, Gulcan, & Senturk, 2013) and an electronic nose consisting of a headspace sampling that could bring aroma to a chamber fitted with a gas sensor array had been used to monitor flavour released in the headspace (Rodriguez & Bernik, 2014).

Proteins and aldehydes are known to form covalent bonds through a Schiff's base and it has been found that aldehydes can bind proteins through hydrogen bonds (Wang, Xie, Ren, Yang, Xu, & Chen, 2011). A proportion of food aldehyde hence becomes unavailable for release

during consumption. During mastication of frozen desert, the presence of mucins in saliva (which are heavily glycosylated proteins) and alpha-amylases was shown to result in a reduction in the release of aldehydes. The exact effect depends on saliva flow and composition, which in turn is partly determined by the nature of the stimulus that generates extra saliva on mastication (Guinard, Zoumas-Morse, Walchak, & Simpson, 1997). Water-soluble aldehydes are absorbed to a high degree by moist tissue of the upper airways and their effective concentration profiles in the nose may both be attenuated in magnitude and shifted in time. Not only are flavour compounds released from a product into the air which is described by a product-air distribution, but absorption to the (moist) nasal tissues introduces an additional aqueous phase (Plug & Haring, 1994).

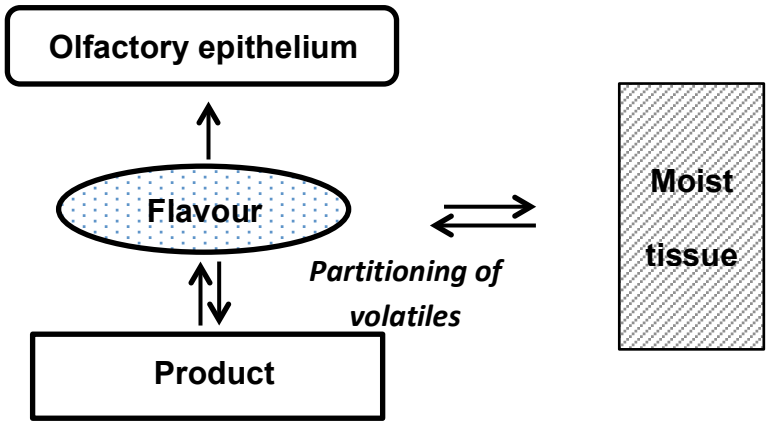


Figure 4.1 Illustration depicting absorption of water soluble flavour by moist tissues of upper airways (Plug & Haring, 1994; Seuvre, Diaz, & Voilley, 2000)

It was observed that up to 49 % of the aldehyde in an aldehyde-protein system was bound and the flavour perception was directly proportional to the amount of free aldehyde (Ng, Hoehn, & Bushuk, 1989). This study also showed that the concentration of aldehyde and protein and the conformation of the protein all contributed to the binding of the flavour by the protein (Ng, Hoehn, & Bushuk, 1989). Common volatile flavour compounds tend to be comparatively non-polar and are therefore relatively incompatible with highly polar, aqueous solutions thus likely to have very large activity coefficients, in terms of intermolecular forces, unlike the aldehyde, which was hydrophilic in nature. For compound with low partition coefficient (K_{aw}), the release is limited by the evaporation of the compound from the surface of solutions. Addition of fresh air will therefore significantly increase this evaporation in a dynamic system and the release rate will be enhanced.

4.2 Method Development

Monitoring flavour release from foods during eating is one example, requiring a sensitive (g / kg), rapid (millisecond) response to a wide range of compounds like the aldehyde chosen for this work. To overcome the slow analysis times associated with chromatographic–MS methods, an alternative is to introduce the mixture of volatile compounds directly into the MS and resolve them entirely by mass. This provides real time analysis but at the expense of structural information and identification (A.J. Taylor & Linforth, 2003).

The volatile concentrations in the breath during the consumption of standard foods like ice cream, cheese, biscuits etc. can be considerably lower relative to those in the headspace (Deibler, Lavin, Linforth, Taylor, & Acree, 2001). The relationship between volatile release into the breath and headspace is dependent on its air/ water coefficient (K_{aw}), compounds with high K_{aw} values (10^{-2}) show poor release relative to those with lower K_{aw} values (10^{-5}) compared with their thermodynamic equilibrium, similar to their headspace concentration stability during dilution. The reason being compounds with high K_{aw} values have to transfer a high proportion of molecules into the gas phase in order to maintain the equilibrium, and the surface layer is constantly depleted. Likewise in the case of in vivo, the limited surface area available for volatile transfer into the upper airways and inaccessibility of the food matrix will also result in surface depletion of volatiles with high K_{aw} values. The single pulse of hydrophilic compounds entering the gas flow of the nose-piece would be retarded and smeared as they partitioned in and out of the mucous membranes, unlike the hydrophobic compounds that would show much less retention and peak broadening. However, no experimental evidence was available to confirm the predictions of these behaviour (R. S. T. Linforth, 2010).

There are a number of factors that should to be considered when conducting quantitative analysis, particularly when the analyte is present at low concentrations or the release of analyte into the headspace is limited. These include limits of detection, specificity, speed and cost of each analysis. The specificity of a method ensures

that the analyte could be distinguished from other compounds (Kalra, 2011).

The detection limit is estimated as three times the standard deviation in the background measurements. The lower the limits of detection (LOD) the higher is the sensitivity of the mass spectrometer. Limit of quantitation (LOQ) is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision in chromatography, typically requiring peak heights 10 to 20 times higher than the baseline noise. Linearity then refers to the directly proportional relationship between the x- and y- quantities (Figure 4.2).

Sensitivity of the method is defined as the capability of the method to discriminate small differences in concentration of the test analyte, and it is determined from the signal/ noise (s/ n) ratio of the headspace concentration indicated in the APCI chromatogram.

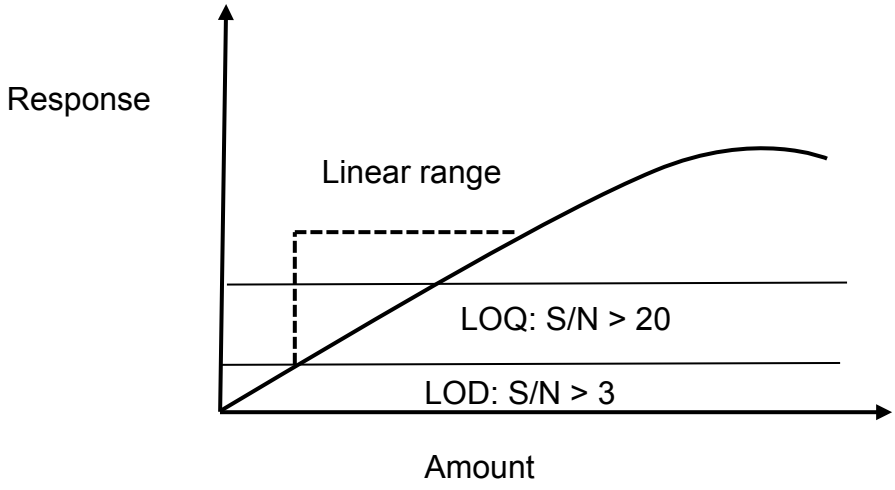


Figure 4.2 Definitions for linearity, LOD and LOQ

This chapter aims to develop and optimise the APCI-MS technique to measure in vivo aroma release from a frozen dessert.

4.3 Materials and Methods

4.3.1 Frozen desert formulation

The following documented procedure was the recipe of the frozen desert used in this research:

Ingredients

284 ml double cream

300 ml full fat milk

115 g caster sugar

3 large egg yolks

The canister from Gelato Chef 2000 (Magimix, Italy) was kept in the freezer the day before making the frozen desert. Half the amount of sugar was added to the cream and milk and heated over low heat, with

occasional stirring until the mixture was about to boil. Egg yolks and rest of the sugar was beaten with an electric hand mixer (Kenwood, UK) until the mixture turned pale in colour and thickened. The cream mixture was mixed into the egg yolk mixture and heated at low heat until the custard was thick enough to coat the back of the spoon. The desired amount of flavour was stirred into this mixture and then cooled overnight. The cold custard was churned for 20 min before storing in the freezer for a minimum of three hours.

4.3.2 Quantification of aldehyde X

1-g sample was weighed for extraction. Every sample was extracted with 9 g of methanol (Sigma Aldrich, UK). The mixed samples were placed on a roller mixer (Thermo Scientific, tube roller Spiramix 10) for 30 min, and then centrifuged at 1300 g for 20 min at 5 °C (Thermo CR3i multifunction centrifuge, KeyWrite-DTM). The upper solvent layer was isolated and 1 ml of the extract was filtered through nylon syringe filter of 4 mm diameter and 0.4 µm pore size (Thermo Scientific Nalgene, UK), into 2-ml amber vials, capped with Teflon-coated lids and analysed by HPLC. 100 ppm standard was also filtered through the syringe filter. All filtrate was removed from the centrifuge tubes; methanol was used again for second extraction from the remaining residue and the mixture placed on the roller bed for thorough mixing before filtering through the nylon syringe filters, collected and analysed in HPLC. Second extraction was carried out to ensure full extraction.

An Agilent 1000 Series HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a diode array detector (G1315B DAD, Agilent Technologies, Waldbronn, Germany) was used. Compounds were measured at an absorption wavelength of 279nm and separated by a C18 column (Agilent Zorbax RX-C18, L: 250 mm x ID: 4.6 mm; Agilent Technologies, Kansas, USA). The instrument settings were as follows: injection volume 10 µL; flow rate 1 ml per min; isocratic elution with 35% (A) water (1 % acetic acid) and (B) methanol over 10 min at 30 °C The chromatography data were analysed by ChemStation software (Agilent Technologies, Waldbronn, Germany).

Samples were analysed in triplicates. Concentration was calculated from the ratio of the peak area of the compound of interest to the peak area of the 100 ppm standard in both samples and standards.

4.3.3 Breath-by-breath analysis using APCI-MS

A MS Nose interface (Micromass, Manchester, UK) fitted to a Micromass Quattro Ultima Pt mass spectrometer (Waters, UK) operating in the APCI positive ion mode was used in the in vitro and in vivo analysis of volatile flavour compounds.

The intensity of the ions of interests was measured at default capillary energy of 3 kV, cone voltage of 60 V, source temperature of 75 ^{0}C, and.optimised APCI source parameters, determined in the earlier series of experiments, using 10 cm transfer line with temperature set at 150 ^{0}C and air flow rate at 400 ml / min (see details in ‘Results and Discussion’). Five panellists were trained for breath-by-breath analysis and asked to

consume 10 g of frozen desert containing 50, 100, 200 and 400 ppm aldehyde and chew with their mouths closed. Before the sample had completely dissolved in the mouth, panellist had to rest one nostril in the end of a T-piece plastic sampling tube (12 mm - 50 mm) which was attached to the MS-nose, and make a swallow event while breathing normally into the end of the tube. Subsequent swallow events were made every 10 s for 1 min. Air from the nose was sampled into the APCI positive ion source at 400 ml min^{-1}. A higher flow rate is required for breath-by-breath analysis due to the smaller concentrations of volatiles in the respective gas phases (Friel, Linforth, & Taylor, 2000). Each panellist evaluated three replicates with rest periods (15 – 20 min) between each sample during which they would cleanse their palates with mineral water and crackers. Exhalations were tracked for a minute after consumption, so that temporal changes in breath aroma compounds could be followed. Prior to consuming the sample, the exhaled air from the nose of each panellist was monitored for 20 s to ensure that there was no carry-over of aroma from the previous experiment and that the signal had returned to baseline levels. Acetone (m/z = 59) ions were also traced to check that the panellist was breathing normally.

Adopting similar in vivo measurement conditions as above, the same five panellists were asked to breathe into the APCI-MS sampling tube through their mouths for 20 s to check baseline levels. Following a clean baseline level, they would then eat 10 g (a teaspoon) of sample, chew with their mouths closed, swallow the sample then exhale through

their mouths into the sampling tube for 10 s, alternating each exhalation with 10 s rest away from the tube. This process was monitored for one minute. Each panellist evaluated the three replicates of sample with rest periods (15 – 20 min) between each sample during which they would cleanse their palates with mineral water and crackers.

Dwell time was maintained at 0.05 s, similar to that in static headspace analysis, for both the in-nose and in-mouth breath-by-breath analyses. The arbitrary quantity associated with the corresponding peak

4.3.4 Statistical Analysis

'Design Expert' software version 7 (Stat-Ease, Minnesota, USA) was employed to build a series of experiments in order to establish prime operating conditions.

4.4 Results and Discussion

4.4.1 Optimising APCI Source Parameters for Static Headspace Analysis

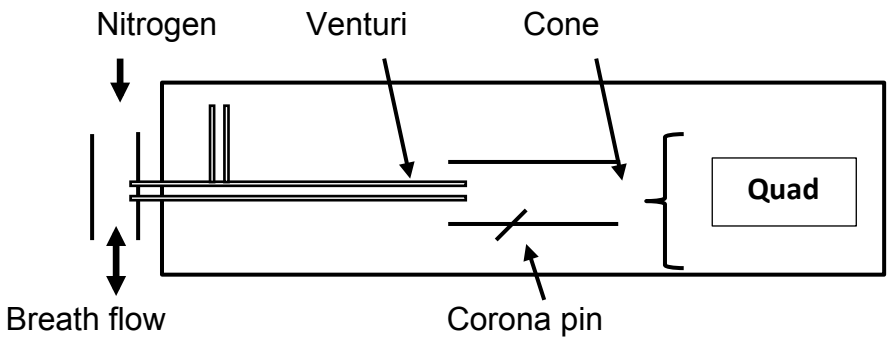


Figure 4.3 APCI inlet designed by (A.J. Taylor, Linforth, Harvey, & Blake, 2000)

In APCI-MS cone voltage is a major factor in fragmentation and it helps draw ions into the first vacuum region, if optimised it will enhance sensitivity and stability. Optimal cone voltage, which typically lies in 20 – 70 V range, has to be defined to obtain 'clean' spectra so that fragmentation is limited such that only the protonated molecular ion is detected for maximum sensitivity and spectral clarity (A.J. Taylor, 2002). Capillary voltage enhances or suppresses ion density and the suggested optimal voltage for positive ions is 2 – 4 kV. Extractor voltage focuses ions toward the hexapole and RF lens voltage focuses ions towards the centre of the quadrupole. Temperature and flow rate parameters control the extent of solvent evaporation and adduct formation.

The concentration of aldehyde in breath or the signal to noise ratio (s/ n) of the protonated aldehyde ion will be low; therefore high frequency sampling will be required for the monitoring of the physiological events during consumption, when aromas are transmitted from the mouth to the nose. The operating parameters of the APCI source had to be first optimized to ensure that s/ n of the selected protonated ion of the chosen analyte (100 ppm, Sigma Aldrich, Dorset, UK) could achieve the LOQ for both static headspace and in-nose analysis.

Frequency sampling time or dwell time for static headspace analysis was maintained at 0.05 s in order to get reliable mass spectral data. Flow rate, transfer line temperature, source temperature, cone energy, capillary energy, hexapole energy, ion energy and length of transfer line

were subsequently modified based on experimental plan constructed by 'Design Expert' in a randomised order, for the optimisation of the static headspace analysis (Table 4.1).

Table 4.1 List of operating parameters for method optimisation

Operating Parameters	Range of values
Flow rate (ml / min)	70 – 130
Transfer line temperature (˚C)	100 - 180
Source temperature (˚C)	25 - 75
Capillary voltage (kV)	2.5 – 3.75
Cone voltage (V)	25 - 90
Hexapole energy (V)	40 - 70
Ion energy (V)	0.2 – 1.5
Length of transfer line (cm)	10 – 60

4.4.2 In vivo Release Measurement of Volatile Aroma Compounds from a Frozen Desert

Since the effects of declustering analyte species require more attention with APCI, as many of the gas phase analyte ions produced are clustered with water molecules and reagent ions derived from the eluent system; a heated transfer capillary line (Figure 4.4) was essential to reduce the background noise and enhanced the analyte signal. A range

of transfer line temperatures from 100 ˚C to 180 ˚C was selected by 'Design Expert' software version 7 (Stat-Ease, Minnesota, USA). The increase of transfer line temperature from 100 ˚C to 180 ˚C had shown to improve temporal response time of the aldehyde ion, resulting in a quicker response and better resolution of the signal. The latter ion peak reached its plateau more quickly. Optimum response was observed at 150 ˚C (Figure 4.5a), as further increase in temperature from 150 ˚C to 180 ˚C did not appear to significantly improve the signal response. On the other hand, source temperature set at 25 ˚C, 50 ˚C and 75 ˚C did not show significant impact on the aldehyde signal response.

Gas or air flow is highly interrelated to the temperature, although not as critical. Sample volumes of aldehyde (50 ml) were analysed at different air sampling flow rates (70 ml / min, 90 ml / min, 110 mml / min, 125 ml / min, up to 130 ml / min) to study the effective impacts on volatile headspace (Figure 4.5b). Varying the flow significantly changed the s/ n ratio of the static headspace measurement of the aqueous aldehyde, s/ n improved from 27 with a flow rate of 70 ml / min to 71 at 130 ml / min. High air flow rate was able to drive the aldehyde volatiles out of the column to enhance the maximum intensity of the aroma signal.

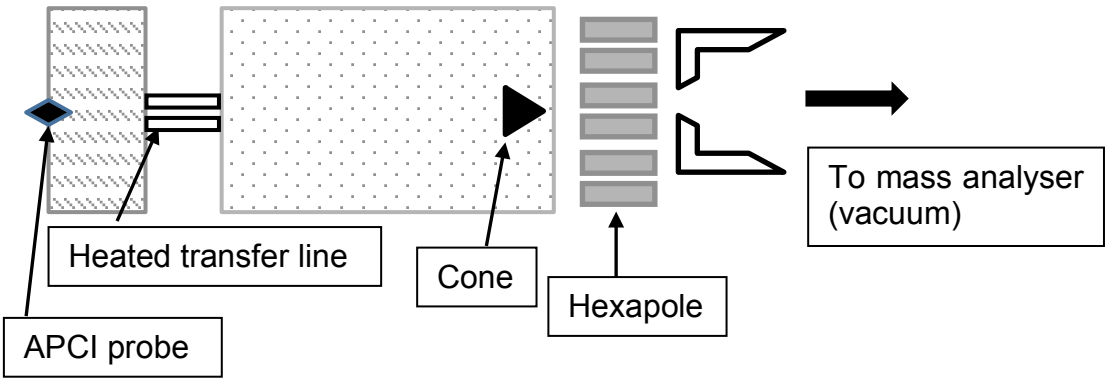


Figure 4.4 Diagram of APCI-MS highlighting parts that require optimisation

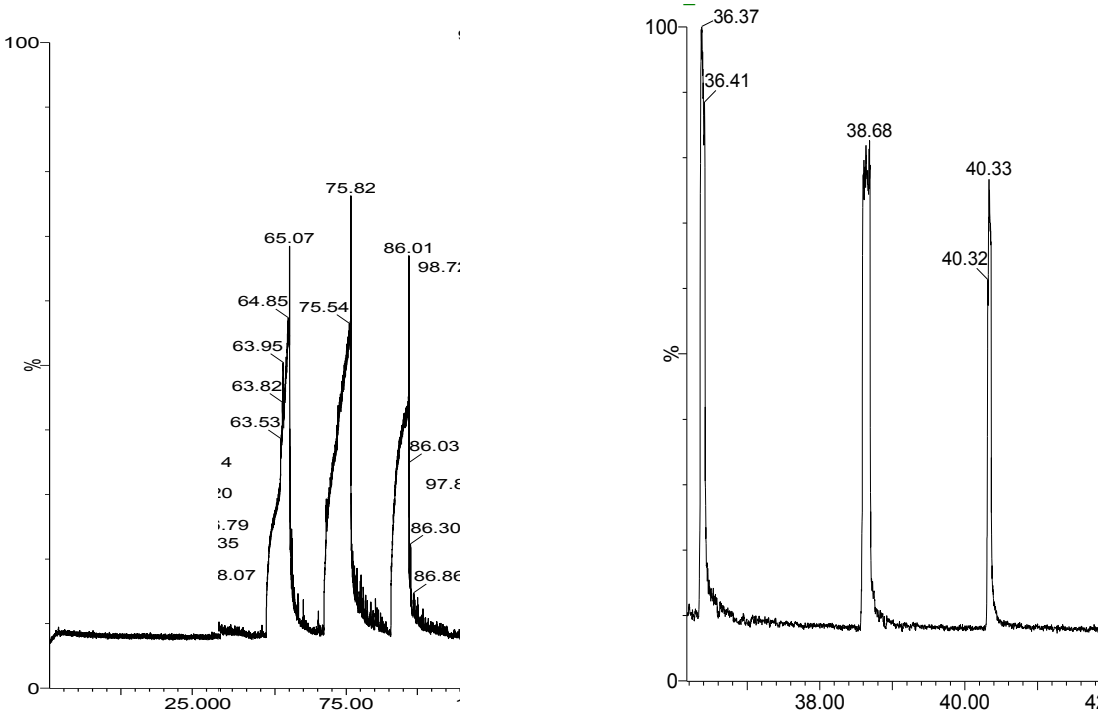


Figure 4.5a Spectral peaks with different transfer line temperatures: 120 ˚C (left) and 150 ˚C (right)

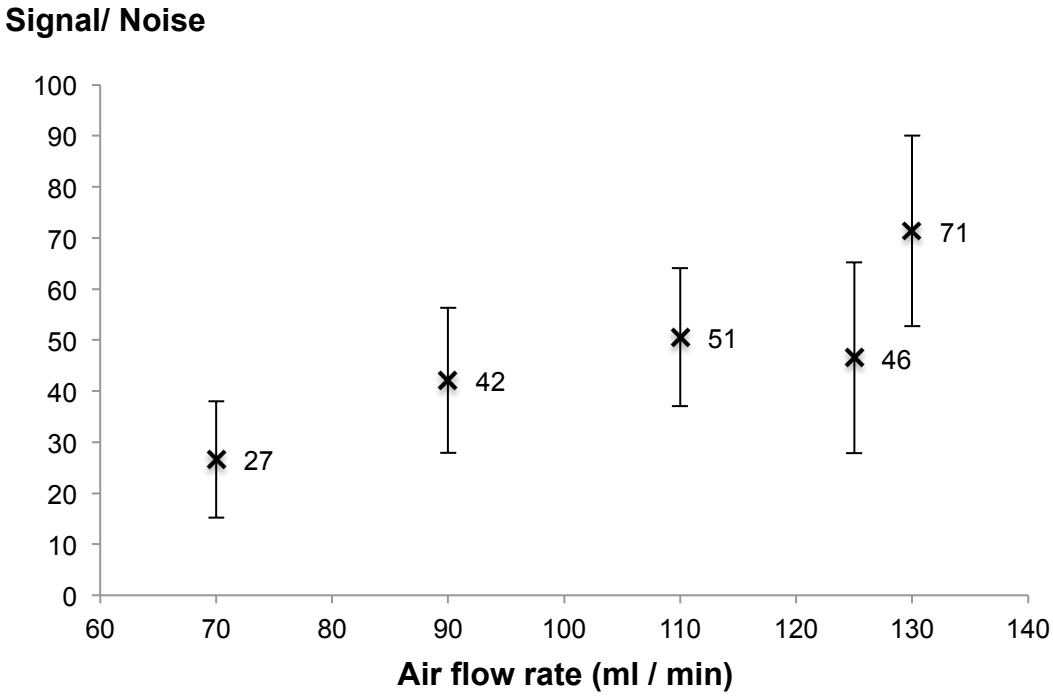


Figure 4.5b Changes in signal/ noise ratios with varying air flow rates (ml / min) in static headspace measurement

Capillary energy programmed at 2.5 kV, 3.0 kV and 3.75 kV did not show significant impact on the signal of aldehyde. Varying cone energy from 25 V to 90 V was not effective in improving the signal of the aldehyde peak. Likewise different hexapole energies (40 – 70 V) used also failed to significantly impact the aldehyde signal. Neither did ion energy tested at 0.2 V to 1.5 V brought noticeable difference to the signal response.

Shortening the transfer line from 60 cm to 10 cm not only significantly increased the signals of the headspace of aqueous aldehyde (Figure 4.5c); it inevitably allowed the air flow rate to be increased to 400 ml / min. As a result, the s/ n was improved from 41 with a 60 cm transfer line to an optimized s/ n ratio of 123 when the transfer line was

shortened to 10 cm (Figure 4.6). The combination of higher flow rate and higher transfer line temperature had effectively and efficiently driven the less volatile aldehyde out of the transfer line into the mass spectrometry resulting in detection of higher static headspace signals.

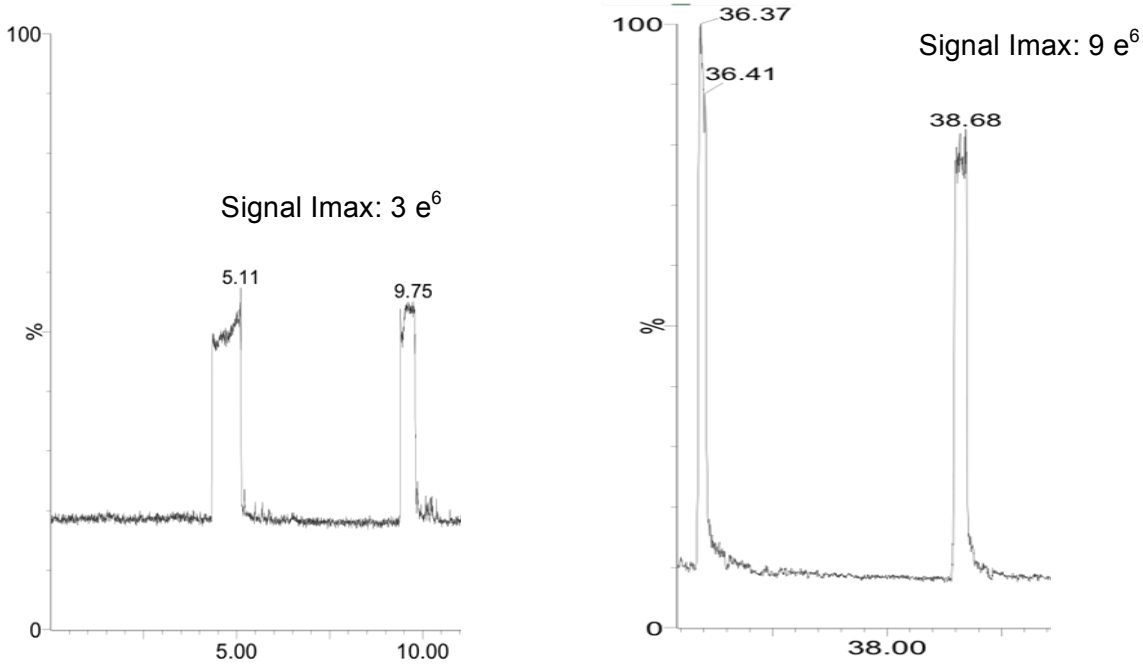


Figure 4.5c Spectral peaks using 60 cm heated (150 °C) transfer line (left) and 10 cm heated (150 °C) transfer line (right)

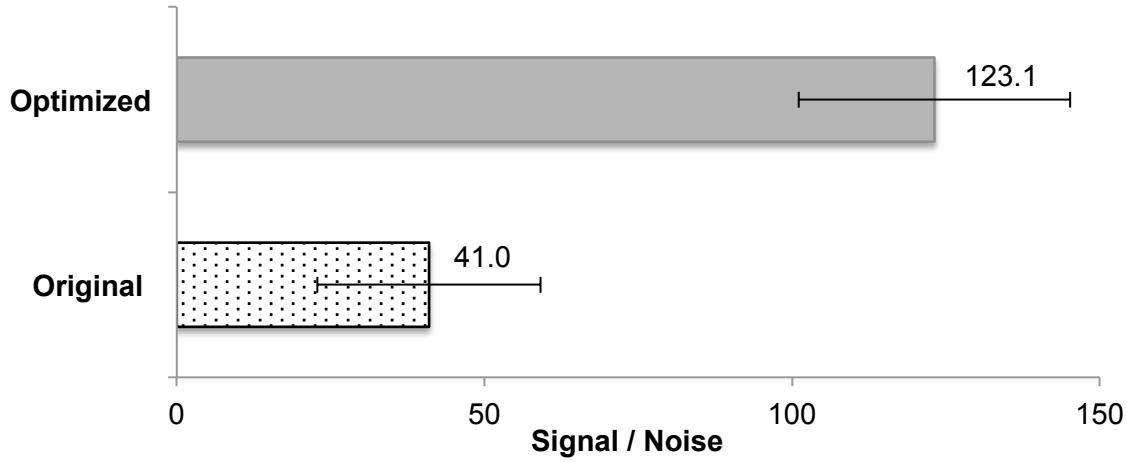


Figure 4.6 Comparison of original static headspace signal/ noise (s/ n) with optimized s/ n values for aqueous solutions

Table 4.2 summarises the parameters that showed significant impact on the s/ n ratio of the aldehyde headspace. With the optimized settings in place, static headspace measurement of the aldehyde in the frozen desert and even in-vivo monitoring of the aldehyde during consumption of sample could be more efficiently demonstrated.

Table 4.2 Parameters that affect static headspace signal/ noise (s/ n) values of aqueous solutions

Operating parameters	Original	Optimized
Transfer line (˚C)	120	150
Length of transfer line (cm)	60	10
Air flow rate (ml / min)	70	400

The length and temperature of transfer line appeared to have greatest impact on the signal/ noise ratio. Sensitivity of APCI appeared to have increased with the increase in air flow rate; hence the limit of detection of the static headspace of aldehyde could be reduced to 50 ppm. This improvement proved beneficial for subsequent work on investigation of persistence of aldehyde and flavour compounds (of similar physicochemical properties as that of aldehyde), during consumption.

Analysis of the static headspace of aldehyde in melting sample was improved when the optimised operating parameters of APCI-MS were employed; and there was an approximate five-fold improvement in s/n ratios of the aldehyde headspace as compared to that using the original settings (Figure 4.7). The hydrophilic aldehyde was released into the headspace when the sample structures collapsed during melting and this s/n was further enhanced when the optimised operating parameters were used. This also effectively facilitated the in-mouth and in-nose monitoring of aldehyde during consumption of the sample.

With the newly optimised APCI settings, the detection limits of breath signals of the aldehyde in water could be more easily determined at relevant aldehyde concentrations of approximately 200 ppm for in-mouth with an r^2 value of 0.998 (Figure 4.8a) and 600 ppm for in-nose with an r^2 value of 0.834 (Figure 4.8b).

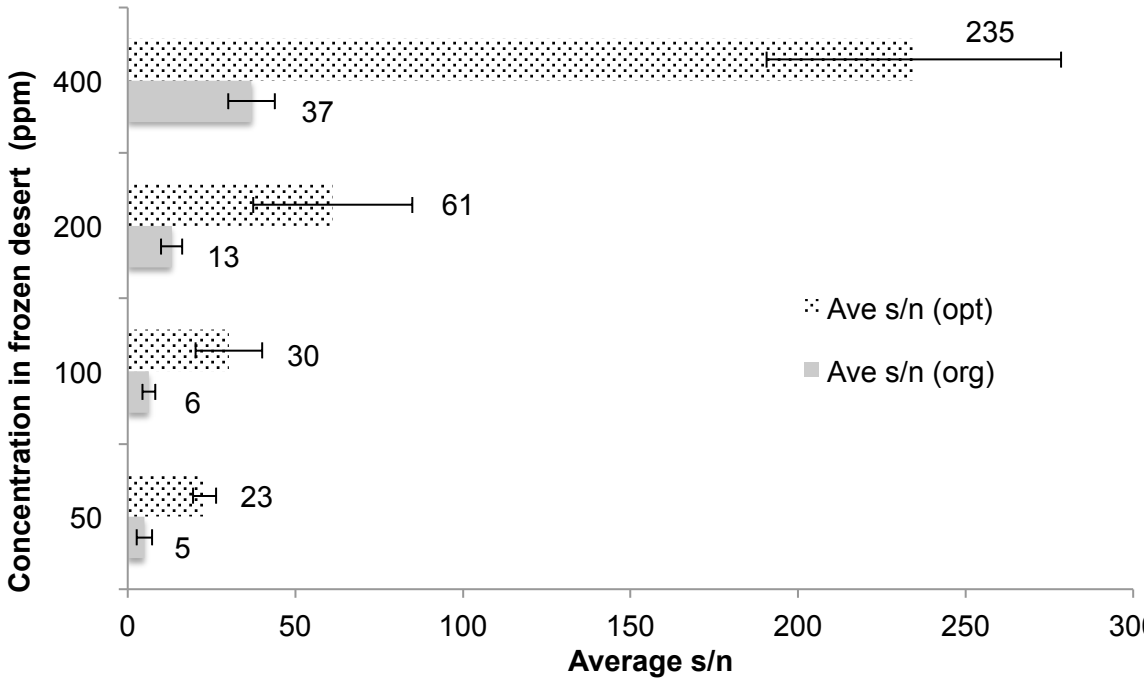


Figure 4.7 Comparison of original static headspace s/ n ratio (org) with optimized s/ n ratio (opt) in frozen desert

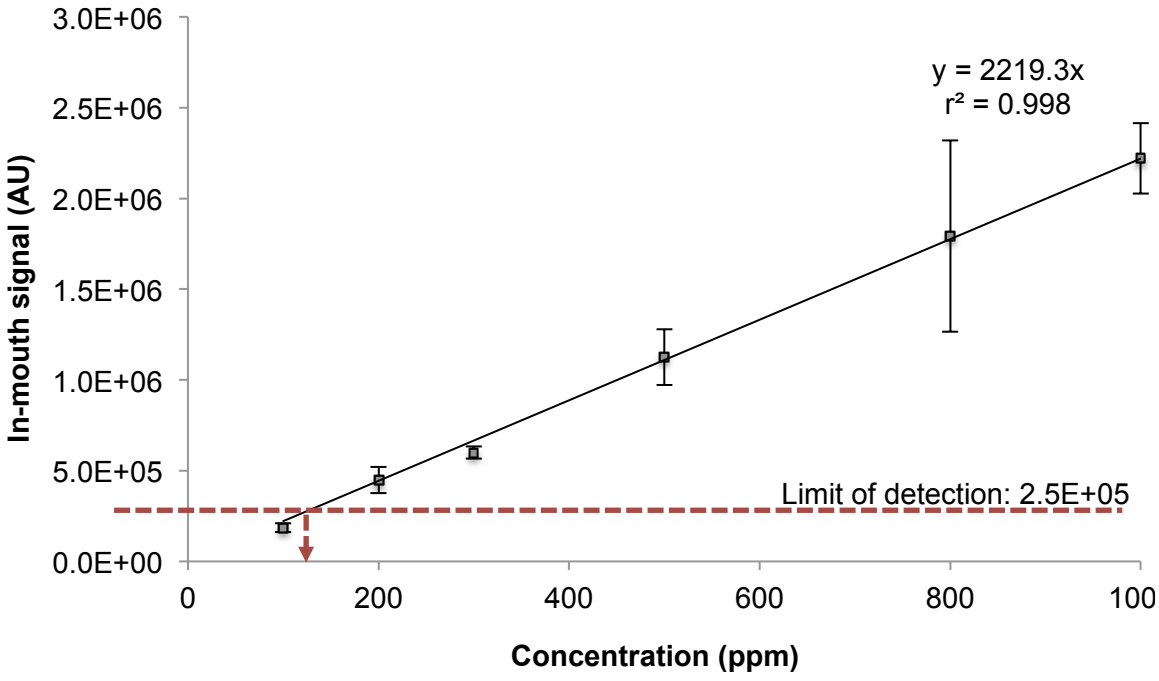


Figure 4.8a In-mouth signal in water

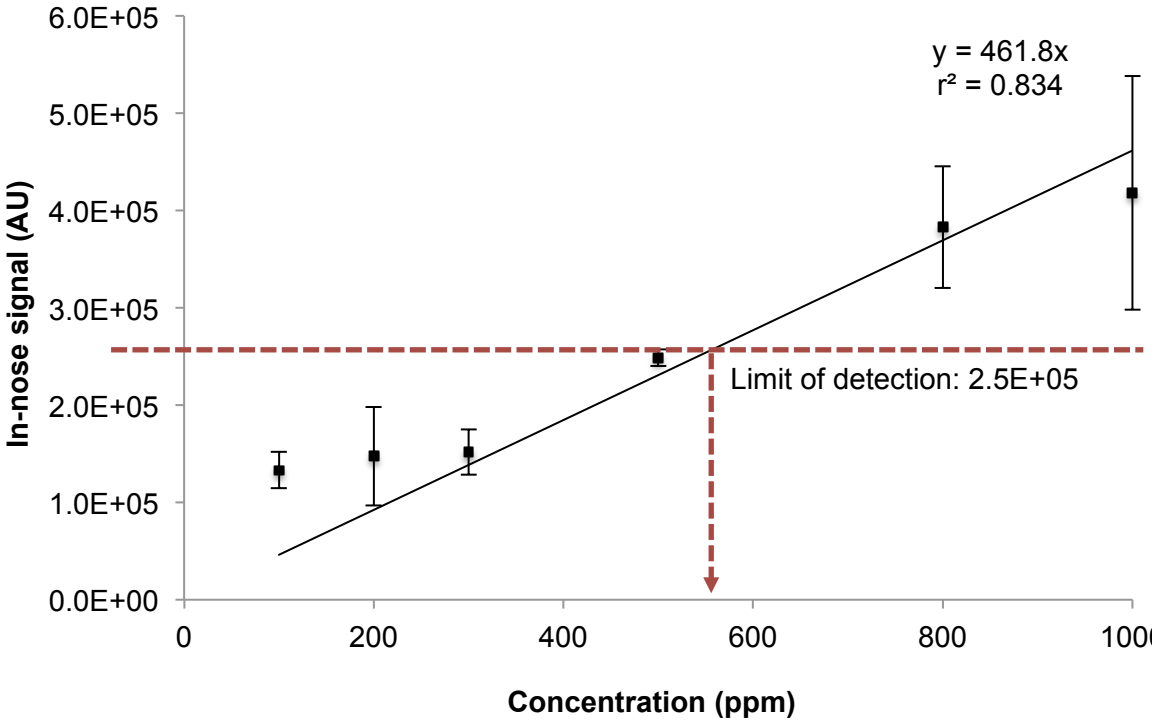


Figure 4.8b In-mouth signal in water

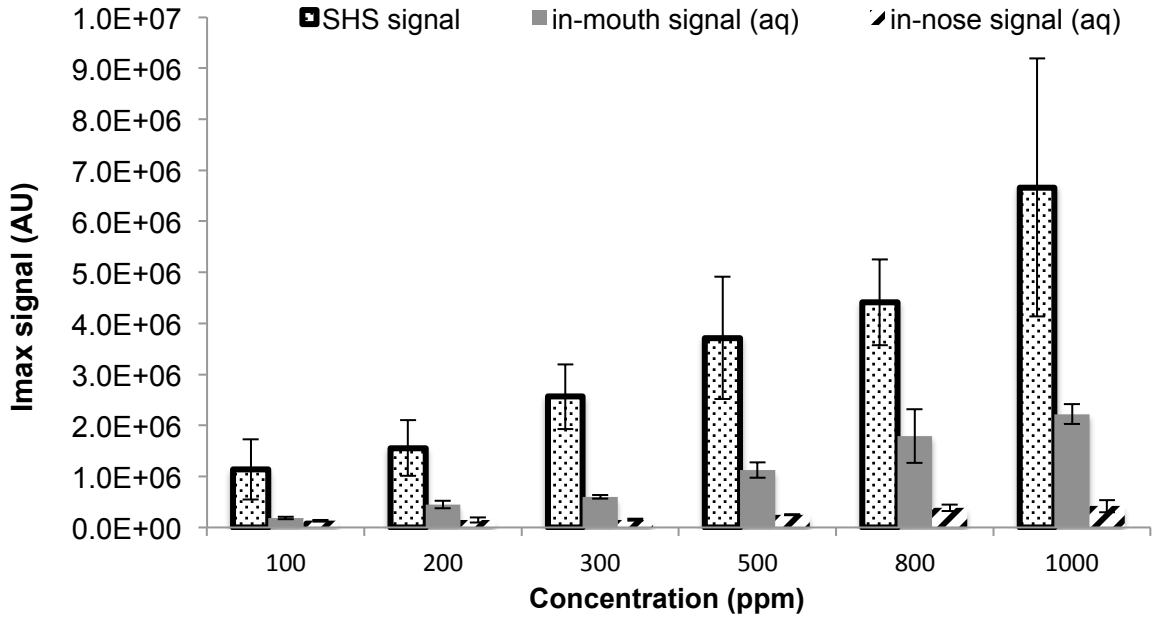


Figure 4.9 Comparison of static headspace, in-mouth and in-nose signals (Imax) in water

During eating, hydrogen bonding and inter-molecular interactions between the mucin proteins in the mucus lining and the aldehyde volatiles resulted in less aldehyde being released into the headspace as compared to the static headspace measurements (Figure 4.9) (Guinard, Zoumas-Morse, Walchak, & Simpson, 1997) .

4.4.3 Validation of Measurement Capability: Release of Aldehyde in frozen deserts

Quantification of aldehyde using HPLC showed that aldehyde compound was not trapped within the nylon syringe filter and that differences observed between the measured and the actual quantity would be possibly due to interaction with the matrix. No significant differences was perceived in the aldehyde concentrations between the filtered and unfiltered aldehyde standards (Figure 4.10a), implying that the aldehyde compounds were able to pass through the mesh of the nylon filter as there was no physical impediment locking in the flavour compounds. Comparing concentration of aldehyde added and measured in the frozen desert gave a correlation coefficient (r^2) of 0.904, inferring and validating that most if not, all the aldehyde compounds had been released from the matrix (Figure 4.10b). Matrix of the frozen desert being hydrophobic was not able to interact with the hydrophilic aldehyde compounds, resulting in release of the compound.

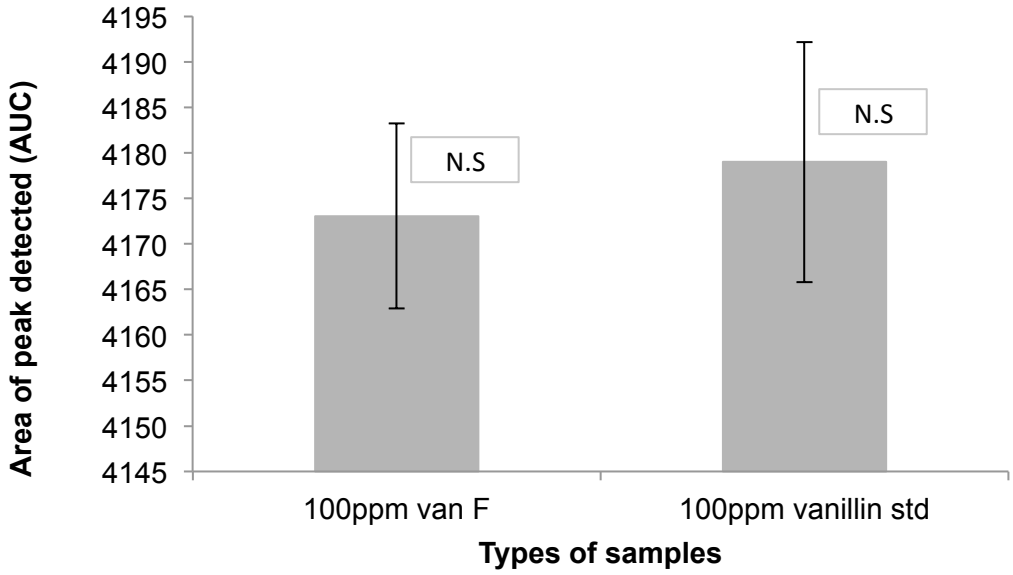


Figure 4.10a Comparison of intensity (AUC) of aldehyde in aldehyde standard: filtered (left) and unfiltered (right)

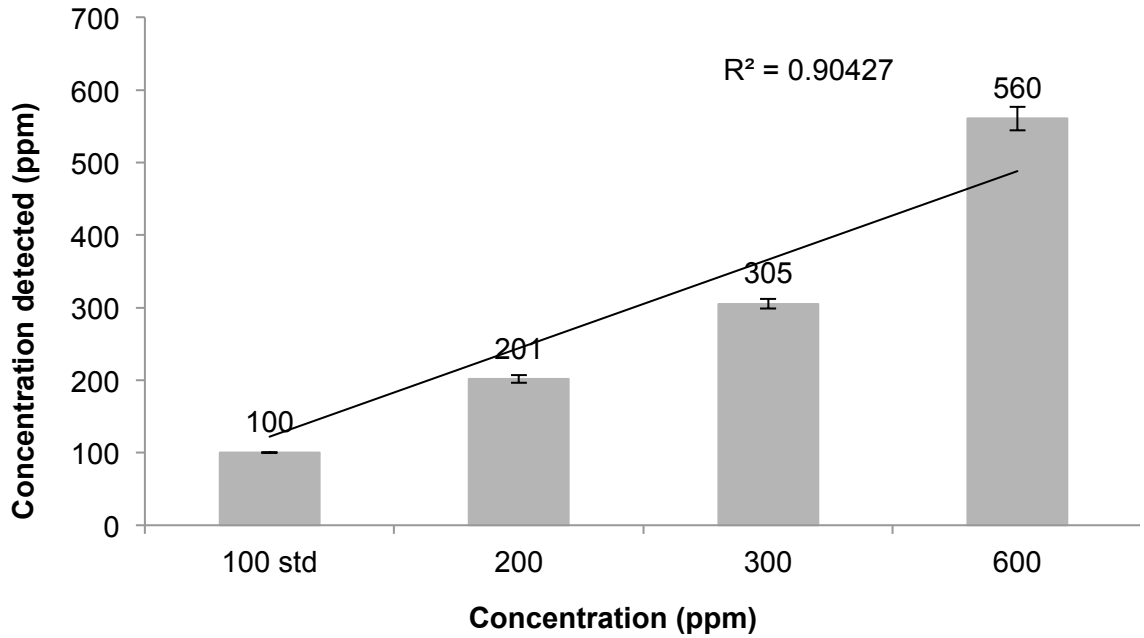


Figure 4.10b Concentration of aldehyde added (ppm) against actual aldehyde detected (ppm)

It has been generally observed that the composition of volatiles in the headspace of any food differs, from that measured in the nose space when the food is eaten. The nose space volatile concentrations are usually much lower than those found in the corresponding headspace (Figure 4.11). It was found that the dominance of the mass transfer coefficient between aqueous and gas phases in vitro under conditions of air dilution was applicable in vivo; nose space/ headspace concentration ratios were compound specific and could be as large as two or even three orders of magnitude (Marin, M., Baek, I., Taylor, A.J., 2000). Further reductions in concentrations of volatiles released during eating when exhaled retronasally were shown to result from gas phase dilution in the upper airway and absorption by the nasal epithelia. In addition, measured volatile concentrations in nose space were significantly lower than when exhaled through the mouth.

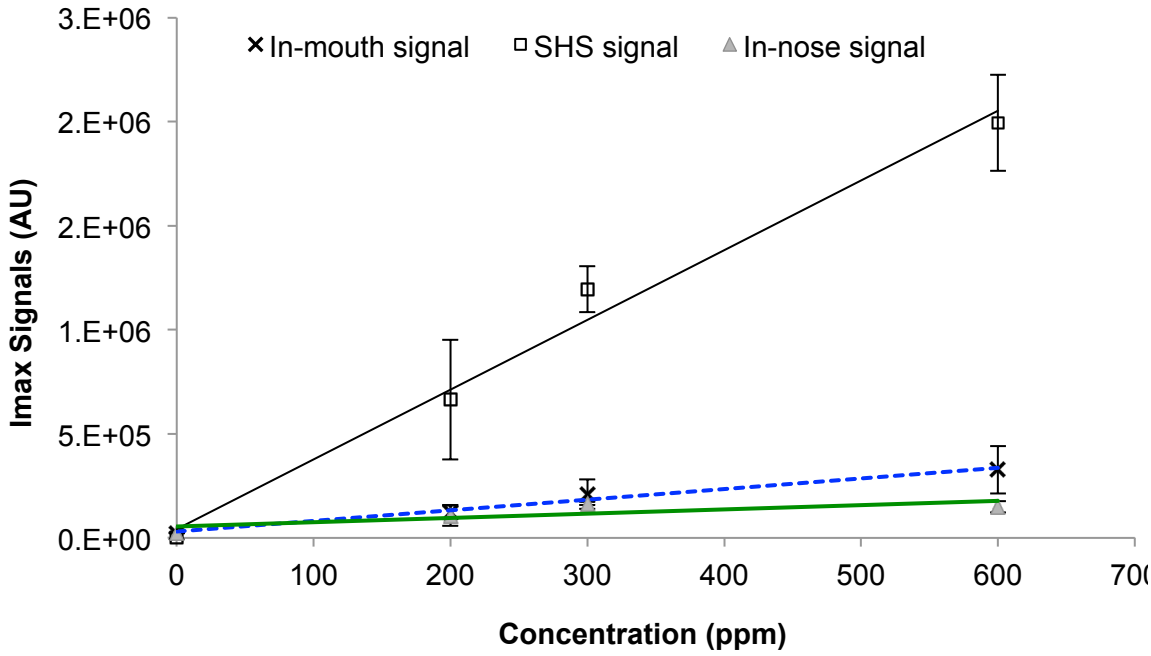
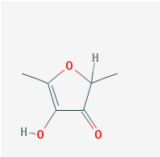
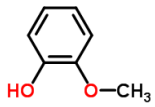
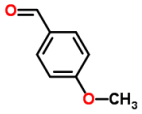


Figure 4.11 Release of aldehyde containing different concentrations (ppm) of aldehyde from frozen desert

4.4.4 Release of other Flavour Compounds

Three volatile compounds of different physical properties were tested, the physicochemical properties of these vanilla-like flavour compounds were tabulated (Table 4.3), and the *in-vivo* release of these compounds was monitored and compared against each other (Figure 4.12).

Table 4.3 Physicochemical properties of furaneol, guiacol and anisaldehyde

Physicochemical Properties	Furaneol 	Guaiacol 	Anisaldehyde 
Solubility (mg / L at 25 °C)	1.85×10^{4}	1.87×10^{4}	2728
Partition coefficient Kaw	6.01×10^{-4}	1.09×10^{-3}	3.25×10^{-5}
Vapour pressure (VP) (mm Hg, 25 °C)	9.36×10^{-4}	0.113	0.0303
Molecular mass (MW)	128	124	136
Log P	0.82	1.34	1.79

Furaneol was the most persistent aroma compound during in-nose consumption when compared to anisaldehyde and guaiacol. Furaneol has a low vapour pressure when compared to the other two volatiles and is more hydrophilic, therefore it is presumed to interact with the nasal mucosa, thereby prolonging persistence and potentially offering a significantly different perceptual profile to the less persistent compounds (Hodgson, M., Parker, A., Linforth, R.S.T., Taylor, A.J., 2004).

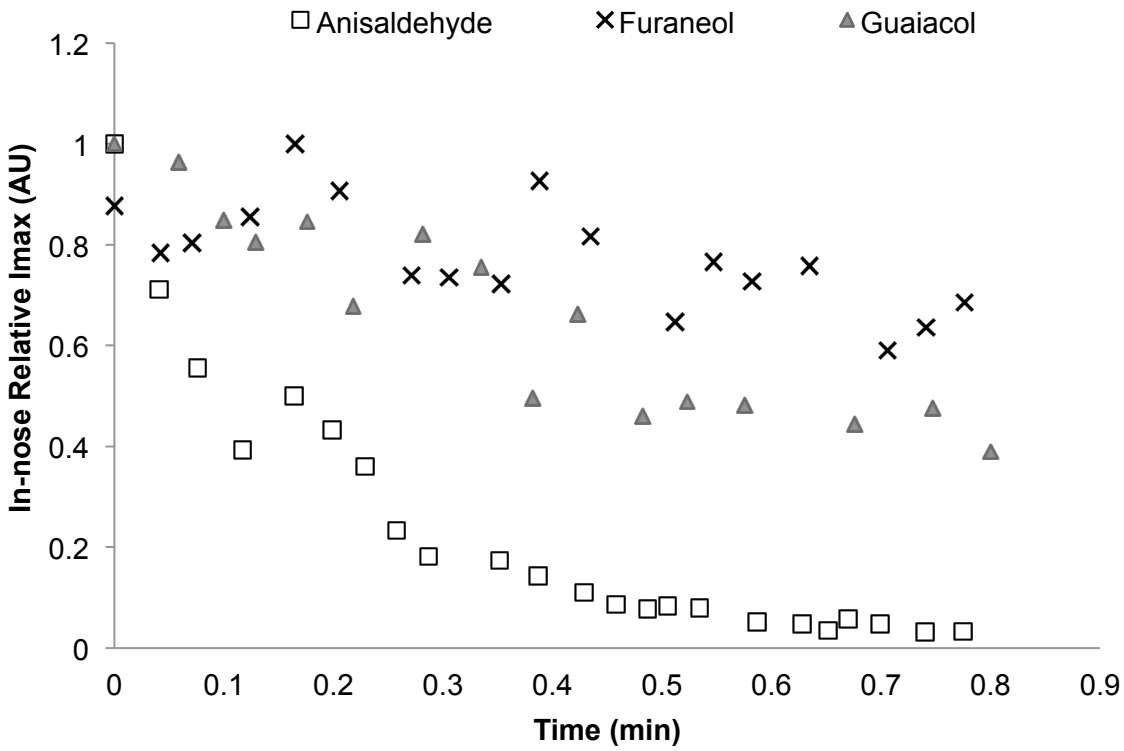


Figure 4.12 In-nose releases of furaneol, guiacol and anisaldehyde

4.4.5 Application of Measurement Capability: Persistence of Flavour Compounds in Human Breath during Consumption of Frozen Desert

Volatile persistence was shown to vary greatly among the volatile test set. Hydrophobicity and vapour pressure of the volatiles were found to be major components, although not the only components, of a QSPR model, which was then shown to have some predictive capacity. Mechanisms of flavour release from foods are in general not well understood. A number of theoretical models of volatile release from foods have been proposed, depending on the food type and primary release process (e.g. melting, solubilisation), but these generally lack

experimental validation, which is now possible to provide using APCI-MS (Harvey, B.A, J. Barra, J., 2003).

From the release curves of both the in-mouth (Figure 4.13a) and in-nose (Figure 4.13b) measurements, the maximum aroma ion intensity (*Imax*) for each sample, for each panellist and for each replicate was recorded as ion count. The mean maximum values (*Imax*) and the persistence of the aroma in breaths for all the panellists were calculated and modelled using an exponential decay function: $Y = a * Exp\ (b*X)$.

The original aldehyde signal was observed to linger in the headspace for the in-mouth and the in-nose whereas Ethyl butyrate signal was reduced to zero almost instantaneously. Ethyl butyrate having a higher K_{aw} (air-water partition coefficient) when compared to the aldehdye had therefore decayed at a faster rate, which resulted in a more rapid depletion of this volatile from the mucus**.**

As explained by Guinard et al (1997), the presence of saliva components, mucins (which are heavily glycosylated proteins) could have formed non-covalent interaction via reversible physical adsorption. Flavour compounds bind to protein only when binding sites are available; that is, if the sites are not engaged in protein-protein or other interactions. The aldehyde could likely be absorbed to a higher degree than the more hydrophobic Ethyl butyrate by moist tissue of the upper airways and thereby enhancing the persistence of the volatile aldehyde in the mouth and especially in the nose.

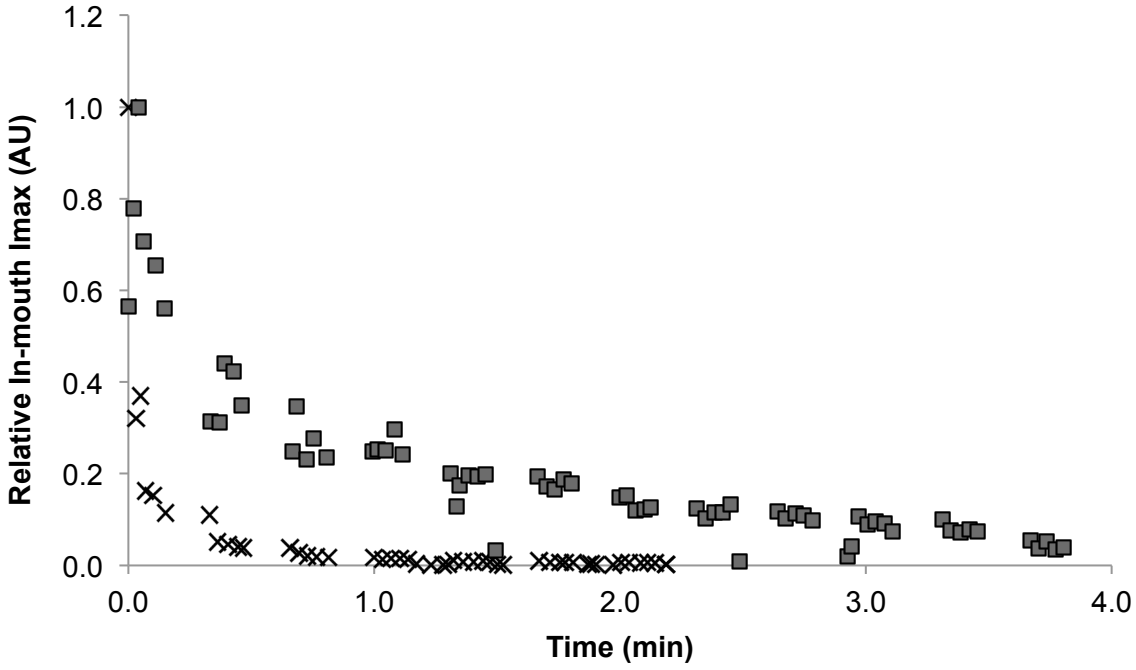


Figure 4.13a In-mouth persistence of aqueous aldehyde X (■) and aqueous ethyl butyrate (x) during consumption

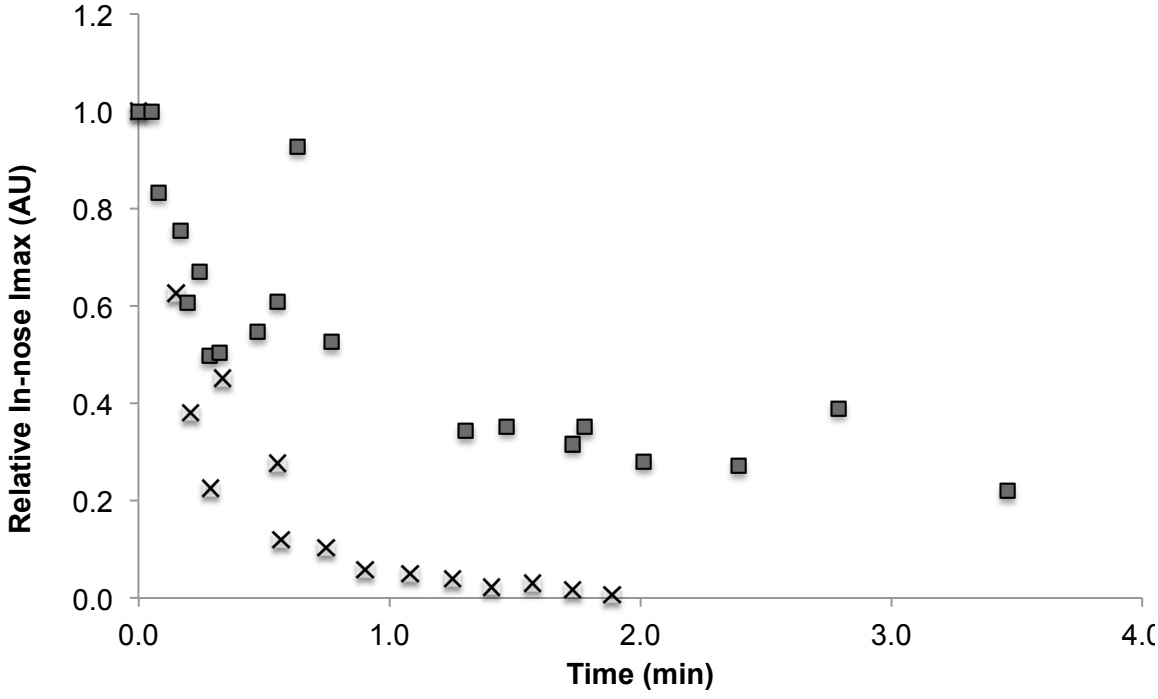


Figure 4.13b In-nose persistence of aqueous aldehdye X (■) and aqueous ethyl butyrate (x) during consumption

4.6 Conclusion

In conclusion, modern food and flavour industry today is no longer only interested in the composition but also in the performance and release of the food flavours to imitate the properties of natural food with manufactured food. The flavour release from food is influenced by two different principles: a static distribution of the flavour into the different phases of a product: solid matrix, hydrophilic or hydrophobic liquid phase and the gas phase. This behaviour is controlled by the partition coefficients of the molecules. The second principle is a dynamic factor, controlled by the mass transfer through a matrix and interfaces. Both factors influence the perception of the food (Grab & Gfeller, 2000).

While the kinetics of volatile release can be readily measured and compared from solid or liquid systems of varying formulation or under different conditions using APCI-MS, the capacity to monitor the kinetics of volatile release into the headspace systematically in model systems can also provide a valuable tool in helping to elucidate the most important factors controlling volatile release. The development and validation of mathematical models of flavour release with greater predictive power can thus be greatly facilitated using the technique, which should then enable reductions in the resources required for product development.

CHAPTER 5: CLASSIFICATION OF APPLE JUICES USING CHEMOMETRICS

5.1 Introduction

Apple juice is one of the most common fruit juices in the world, with world production led by China, Poland, the United States of America and Germany. In fact, the Economic Research Service (ERS), USDA, 2013 reported that apples are the second most consumed fruit (fresh and processed uses combined), following oranges. Factors contributing to increased apple and apple product consumption include new varieties, production expansion, products that better meet consumer lifestyles and also their unique flavour characteristics. The most abundant volatile elements in apples that are associated with the intrinsic aroma are esters, alcohols, aldehydes, ketones and ethers (Rita, Zanda, Daina, & Dalija, 2011). Aroma of food products, including fruits, has become an important quality parameter for consumers. The analysis of the volatile aroma constituents of food and beverages is one of the main parameters to be assessed when a new product is developed because of their influence on the final sensory properties.

It is widely known that the production of aroma compounds is highly influenced by several different factors such as environment, apple cultivar, ripeness, ageing and processing condition. Thus, the flavour profile and quality of apple juice may be inconsistent when fruits at different stages of maturation are used. Chemometrics is defined as

‘the art of extracting chemically relevant information from data produced in chemical experiments’. The chromatographic fingerprint of the aromatic compounds obtained could possibly classify the apple juices in order to monitor and standardise the final product quality. For this reason, the aroma profile can be investigated using chemometric approach coupled with GC data to differentiate the volatile compounds between the large numbers of apple juice samples (Braga, Zielinski, Marques da Silva, Fernandes de Souza, Pietrowski, Couto, et al., 2013).

Furthermore, with recent cases of food producers’ malpractices resulting in authenticity scares and lack of Protected Designation of Origin (PDO) traceability, there is growing consumer awareness for traceable authenticity of foods. Food authenticity issues may be classified into four main groups: adulteration; mislabelling associated with geographical provenance, botanical or species origin; implementation of non-authorised practices and non-compliance to legislative standards (Carcea, Brereton, Hsu, Kelly, Marmiroli, Melini, et al., 2009). Profiling methods will then offer powerful tools for forensic applications. The complex nature of chemical profiles provides a specific fingerprint for food fraud prevention if reproducible. The use of interpretable data capture techniques ensures that simplified methods can be developed when specific authenticity markers are detected. One response to these could be done through legislation, the European Union Council Regulation (EC) 510/2006 exists to identify and protect geographical indications and designations of origin for agricultural products and foods across Europe, which ensures easier traceability of

issues associated with food authenticity, allowing more efficient quality and safety control of the food market.

There is therefore clearly a need for rapid non-destructive analytical methods to support the consumers' rights for confidence in authenticity; these approaches must allow rapid monitoring of food origins, quality and safety, with the minimum processing time and cost per sample; reducing sample pre-treatment and simple measurement protocols are also of paramount importance (Reid, O'Donnell, & Downey, 2006).

For fruit juices, the main authenticity issues are related with false labelling of products in terms of their cultivar or geographical origin, blending of expensive fruit juices with juices extracted from lower value fruits, adulteration of juice with pulp wash and peel derived by-products, addition of unauthorised sugars and the use of juice concentrates of undeclared origin (Singhal, Kulkarni, & Rege, 1997).

To date several techniques have been used for the authentication and classification of apple juices and similar beverages, these include chemical profiling (Souza, Cruz, Walter, Faria, Celeghini, Ferreira, et al., 2011) stable isotopes analysis (Magdas & Puscas, 2011), infrared spectroscopy e.g. NIR, MIR, FT-IR (Kelly & Downey, 2005; León, Daniel Kelly, & Downey, 2005; Sivakesava, Irudayaraj, & Korach, 2001), chromatographic techniques e.g. GC-MS (Fisk, Kettle, Hofmeister, Virdie, & Silanes Kenny, 2012; Guo, Yue, & Yuan, 2012; Lignou, Parker, Oruna-Concha, & Mottram, 2013; Montero-Prado, Bantayeb, & Nerin, 2013), HPLC (Yamamoto, Kawai, Miwa, Tsukamoto, Kodama, &

Hayakawa, 2008) and direct injection spectrometric techniques such as PTR-MS (Biasioli, Gasperi, Aprea, Colato, Boscaini, & Märk, 2003; Biasioli, Yeretzian, Gasperi, & Mark, 2011). In these cases, classification typically uses the data matrix resulting from the entire mass spectrum (spectral fingerprint) and statistical treatment to identify clusters, trends or correlations, appropriate data mining techniques may include partial least squares discriminant analysis (PLS-DA), K-nearest neighbours (KNN), soft independent modelling of class analogies (SIMCA) (Fisk, Virdie, Kenny, & Ullrich, 2010) support vector machine (SVM) and random forest (RF) (Cappellin, et al., 2012)

Whist direct injection mass spectrometric techniques are rapid and information rich, gas phase chemometric classification approaches should always take into consideration the availability of volatile compounds in the gas-phase and the equilibrium concentration difference between the product and its gas phase. The chemical potential of a volatile component is dependent firstly on the physicochemical properties of the analyte, the physical structure of the matrix (Yang, Fisk, Linforth, Brown, Walsh, Mooney, et al., 2012; Yu, Macnaughtan, Boyer, Linfoith, Dinsdale, & Fisk, 2012), the presence of multiple phases (Fernández-Vázquez, Linforth, Hort, Hewson, Vila, Heredia Mira, et al., 2013; Fisk, Linforth, Taylor, & Gray, 2011) and chemical composition of the product being analysed (Fisk, Boyer, & Linforth, 2012).

Notwithstanding its use as tool for real time aroma analysis, APCI-MS can also provide a rapid and informative mass spectral fingerprint of a

foods volatile compliment; it can therefore be hypothesised that APCI-MS could be used for the monitoring of food authenticity with the validation using GC-MS. The aim of the present work was to evaluate APCI-MS as a novel tool for the classification (based on geographical and botanical origin) of a foods volatile compliment, using a real food (clarified apple juice) with broad commercial diversity as an exemplar.

5.2 Materials and Methods

5.2.1 Chemicals

The chemical standards used in the GC analysis were of analytical grade with 90% purity or greater. 2-methyl-butyraldehyde, methyl butyrate, ethyl butyrate, trans-2-hexenal, hexanal, ethyl hexanoate, hexyl acetate, hexanal and cis-3-hexenol were all acquired from SAFC (Loughborough, UK). 2-methyl-butanol was sourced from Fluka (Poole, UK). 3-methyl butyraldehyde and n-butyl acetate were purchased from Acros Organics (Loughborough, UK)

5.2.2 Sampling and Juice Preparation

The five cultivars (Figure 5.1): Malus domestica ‘Braeburn’ (B), Golden Delicious (GD), Malus ‘Granny Zazzle Smith’ (GS), Jazz (J), trademarked brand of Malus domestic ‘Scifresh’ and Pink Lady (PL), trademark name of Cripps Pink, were bought in four separate instances from four different retailers in August and September 2012.

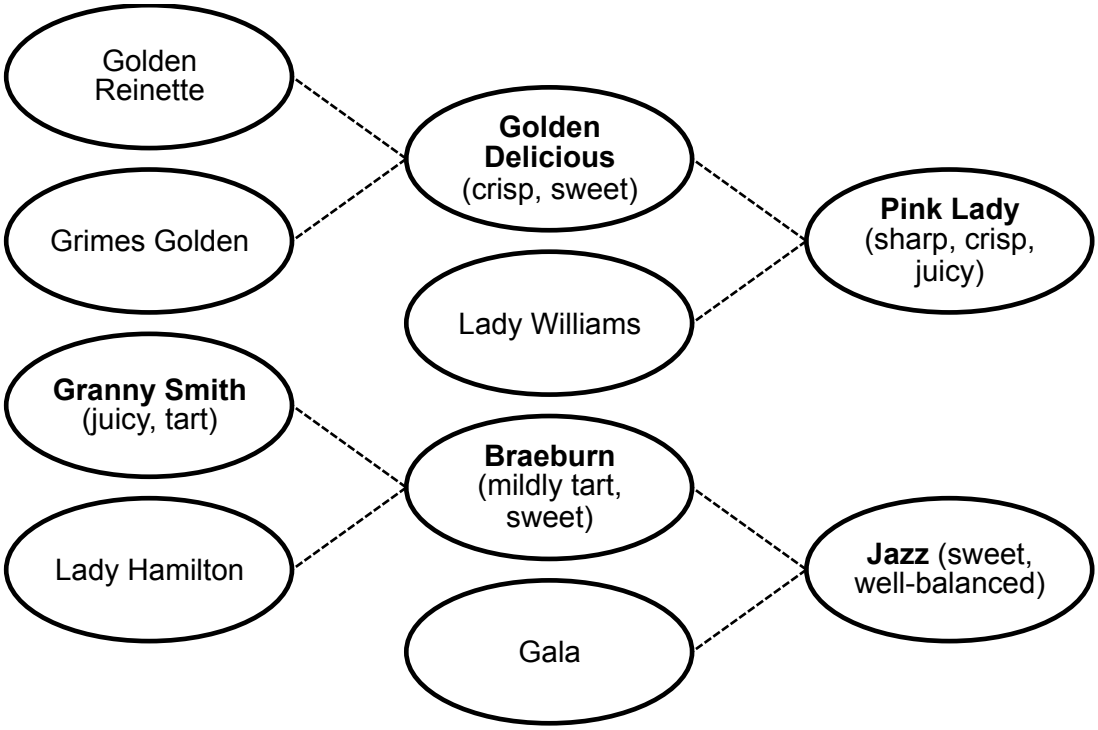


Figure 5.1 Chart showing classification of apple cultivars (names in bold are cultivars selected for this research) (Borrie, 2005a, 2005b)

For each cultivar, 12 apples were randomly selected and used in the preparation of apple juice for the analysis of static headspace, pH, soluble solids and titratable acidity (expressed as % malic acid). Apples were peeled, cored, sliced and placed in an antioxidant solution (0.05 % citric acid, 0.02 % ascorbic acid, 0.02 % calcium chloride) to retard enzymatic browning, as previously described by (Ting, Soukoulis, Silcock, Cappellin, Romano, Aprea, et al., 2012). Apple flesh was squeezed using a domestic kitchen juicer (Philips, UK) and the freshly extracted apple juice was immediately heat treated at 60 °C for 30 s using a water bath to retard any further enzyme activity. Excessive pulp and foam were removed from the juice by filtering through a 100-mesh

cloth filter. Clarification of the apple juice was conducted by pectinase (Sigma-Aldrich, Dorset, UK) treatment at 37 °C for 60 min and subsequent centrifugation of the juices at 5000 rpm (Beckman Ltd., J2-21M, UK) for 10 min. A total of 240 apple juices (triplicates of four batches of juices made out of the five different cultivars from the four retailers) were prepared.

5.2.3 Measurement of Physical-Chemical Properties

Physicochemical properties of the pure apple juices were quantified in triplicates. Soluble solids content was determined in ^{0} Brix, using PAL-1 hand-held refractometer (Atago, Tokyo, Japan) at room temperature. A drop of sample was placed on prism surface before pressing the START key. The Brix value (indicating sugar content/ concentration) would be displayed. The Titratable acidity measurement was done in compliance to AOAC Official Methods of Analysis, by titrating 10 mL of juice with 0.1 N of aqueous sodium hydroxide (NaOH) to pH 8.1 using 1 % phenolphthalein (Fisher Scientific, UK) as an indicator and the acidity was expressed as g / L malic acid (Mehinagic, Prost, & Demaimay, 2004). The amount of malic acid was calculated using the following equation:

Malic acid (g / L) = [0.1 N x titre (ml) x 6.7] / (Vol of sample ml)

Equation 5.1

pH was measured with Five-Go portable meter (Mettler Toledo, UK) at room temperature (20 ^{0}C). Calibration of the meter was achieved using

pH 4 and pH 7 solutions before samples were measured. Electrode probe was submerged into the juice samples and pH readings were shown on the display screen.

5.2.4 GC-MS Analysis

Headspace solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) was applied to analyse the volatile compounds of apple juices. An automated SPME sampling unit (CombiPal. Zwingen, Switzerland) was used with a SPME StableFlex fibre with 50 / 30 µm divinylbenzene/carboxen on polydimethylsiloxane coating (DVB/ CAR/ PDMS) purchased from Supelco (Sigma Aldrich, Dorset, UK). Five mL of juice sample was transferred to a 20 mL vial crimp-sealed with 23 mm diameter aluminium seal and a Teflon septum. After 10 min equilibration at 20 ^{0}C, the SPME fibre was exposed to the sample headspace for 15 min. The fibre was then removed from the vial and immediately inserted into the injector port of the GC-MS system for thermal desorption at 220 ^{0}C for 10 min.

Analysis of the aroma components were performed on a Trace GC Ultra (Thermo Scientific, USA) that was attached to a DSQ series mass spectrometer (Thermo Scientific, USA). The gas chromatograph was equipped with ZB-Wax capillary column (100% polyethylene glycol phase, 30 m x 0.25 mm x 1.0 µm) (Phenomenex, UK). Helium was the carrier gas with a constant flow rate of 1.5 ml / min. The GC oven was held for 2 min at 40 ^{0}C and heated to 220 ^{0}C at a rate of 8 ^{0}C / min. The

GC to MS transfer line was maintained at 250 ^{0}C. The mass spectrometer was operated in the electron impact mode with a source temperature of 230 ^{0}C and an ionizing voltage of 70 eV and scanned mass range of m/z = 50 to 200. Six individual apple juices samples per cultivar referring to different market suppliers and geographical origin were selected. The pure apple juices were run in triplicates. Compounds were identified by comparison of their retention times with those in the standard solutions and using the NIST Library.

5.2.5 APCI-MS Analysis

A MS Nose interface (Micromass, Manchester, UK) fitted to a Micromass Quattro Ultima Pt mass spectrometer (Waters, UK) was used in the static headspace analysis of the apple juice samples. The mass spectrometer was operated in positive ion mode. 50 mL aliquots of samples were placed in 100 mL flasks fitted with a one-port lid. After 30 min equilibration period at room temperature (20 ^{0}C), the headspace was drawn into the APCI-MS source at a rate of 5 mL / min. The samples were analysed in full scan mode, monitoring ions of mass to charge (m/z) ratios from 40 – 200. The intensity of these ions was measured at cone voltage of 20 V, source temperature of 75 ^{0}C and dwell time of 0.5 s. All analyses were run in triplicates (Ashraf, Linforth, Bealin-Kelly, Smart, & Taylor, 2010).

5.2.6 Statistical Analysis

Data was presented as mean ± standard deviation. The GC data was subjected to one-way ANOVA followed by Post Hoc Tukey test (SPSS v. 19, IBM, US) to determine the samples with significant differences. A significance level of $P < 0.05$ was applied throughout the study. A chemometric approach composed of several multivariate techniques were implemented to analyse the aroma profile and to try to classify the juices on the basis of the aroma compounds.

Principal component analysis (PCA) is a powerful statistical tool that has been widely used to help understand the interrelationships among the apple juices in terms of their volatile compounds composition. PCA involves a mathematical procedure that identifies patterns in data, reduces the large number of correlated variables represented in the original data set to a few uncorrelated variables or principal components to explain most of the variation in the original data set. It was used to highlight relationships between variables and samples or demonstrate the lack of any relationship (Coker, Crawford, Johnston, Singh, & Creamer, 2005).

The APCI-MS dataset matrix consisted of 240 samples and 120 variables (m/z 40-160), these were log transformed, auto-scaled and principal component analysis (PCA) was consequently performed on them. The unsupervised PCA was used to identify potential outliers (according to Hotelling's ellipse and Leverage plot) and natural clusters (Tres, Ruiz-Samblas, van der Veer, & van Ruth, 2013). PCA revealed the presence of natural clusters for both geographical origin and cultivar

type. 12 samples from France and 26 samples classified as outliers were removed from further analyses.

Partial least square-discriminant analysis (PLS-DA) classification model was conducted using the APCI-MS fingerprint (matrix comprised of 202 samples and 120 variables) to construct the classification models for the verification of the cultivar and geographical origins of the clarified apple juices. PLS was therefore used to determine the relationship between the multiple dependent predictor variables and the dependent variable, as an exploratory analysis tool to select suitable predictor variables for predictive linear modelling (Coker, Crawford, Johnston, Singh, & Creamer, 2005). Log transformation, mean centering and auto-scaling of the spectral dataset was applied prior to conducting the PLS-DA analysis. The performance of the fitted model was evaluated with external validation, for this 30 % of the samples were removed at random from the complete dataset. A leave-one-out (LOO) full cross validation was also used to evaluate the performance of the models constructed using the training dataset and the optimal number of principle components (PCs) required to achieve the best classification from the constructed models was also calculated. All statistical analysis was performed using Unscrambler v10.0 (Camo Process, AS., Norway).

5.3 Results and Discussion

5.3.1 Headspace Analysis by GC-MS

Table 5.1 Volatile compounds identified in the headspace above monocultivar apple juices using SPME-GC-MS. Data refers to the normalized peak area of the identified compounds relative to the intensity of cis-3-hexenol (25 µL / L). Results are reported as means of 6 individual measurements for each apple cultivar

	Aroma descriptors[1]	Braeburn	Golden Delicious	Granny Smith	Jazz	Pink Lady	p-va
Carbonyl compounds							
2-methyl-butanal	Chocolate, sweet	$1.77X10^{-03a}$	$2.35X10^{-03a}$	$2.84X10^{-03a}$	$1.40X10^{-03a}$	$1.69X10^{-03a}$	0.3
3-methyl-butanal	Caramel	$7.77X10^{-05a}$	$6.21X10^{-05a}$	$9.12X10^{-05a}$	$2.28X10^{-05a}$	$4.10X10^{-05a}$	0.1
hexanal	Green, grassy	$1.86X10^{-01a}$	$7.39X10^{-01c}$	$3.11X10^{-01b}$	$1.12X10^{-01a}$	$1.28X10^{-01a}$	<0.0
trans-2-hexenal	Green, grassy	$3.45X10^{-01b}$	$3.11X10^{-01b}$	$3.87X10^{-01b}$	$1.57X10^{-01a}$	$1.60X10^{-01a}$	<0.0
Alcohols							
1-butanol	Light-fruity	$2.91X10^{-01b}$	$4.02X10^{-02a}$	$2.05X10^{-02a}$	$5.02X10^{-01c}$	$3.11X10^{-01b}$	<0.0
2-methyl-1-butanol	Alcohol, solvent	$6.43X10^{-02bc}$	$4.60X10^{-02a}$	$7.93X10^{-02b}$	$1.25X10^{-01c}$	$6.82X10^{-02bc}$	<0.0
1-hexanol	Light-apple	$1.79X10^{-02b}$	$1.75X10^{-02b}$	$6.96X10^{-03a}$	$3.39X10^{-02d}$	$2.55X10^{-02c}$	<0.0
cis-3-hexenol	Fresh, green, grassy	$4.42X10^{-05ab}$	$1.12X10^{-05a}$	$6.35X10^{-05b}$	$4.56X10^{-05b}$	$7.98X10^{-06a}$	0.0
Esters							
n-butyl acetate	Sweet, fruity	$4.76X10^{-01b}$	$1.29X10^{-02a}$	$6.00X10^{-03a}$	$1.66X10^{+00c}$	$4.65X10^{-01b}$	<0.0
2-methyl-propyl acetate	Sweet, fresh	$1.22X10^{-02c}$	$2.75X10^{-04a}$	$1.36X10^{-04a}$	$9.69X10^{-03bc}$	$6.73X10^{-03b}$	<0.0
2-methylbutyl-acetate	Fresh, banana	$4.80X10^{-02b}$	$3.38X10^{-03a}$	$9.29X10^{-05a}$	$1.30X10^{-01c}$	$3.50X10^{-02b}$	<0.0
hexyl acetate	Sweet, fruity	$2.99X10^{-01a}$	$4.79X10^{-02a}$	$3.50X10^{-03a}$	$1.93X10^{+00c}$	$6.59X10^{-01b}$	<0.0
methyl butyrate	Fruity, apple	$3.53X10^{-03a}$	$7.16X10^{-05a}$	$1.76X10^{-03a}$	$2.10X10^{-04a}$	$3.17X10^{-02b}$	<0.0
ethyl butyrate	Sweet, fruity	$1.38X10^{-03a}$	$4.35X10^{-05a}$	$3.27X10^{-04a}$	$2.75X10^{-04a}$	$1.54X10^{-02b}$	<0.0
ethyl hexanoate	Fruity	$2.61X10^{-02b}$	$2.32X10^{-02b}$	$2.87X10^{-02b}$	$1.09X10^{-02a}$	$1.12X10^{-02a}$	<0.0

[1] Aprea et al., (2012); Aprea et al., (2011); Dimick and Hoskin (1983); Komthong et al., (2006); López et al. (2007); Burdock (2009); Lignou et al., (2013)

[a] The same letter within a row indicates no significant difference according to Duncan's mean post hoc comparison test ($p<0.05$)

Sixteen volatile compounds were detected and identified in the headspace of the fresh monocultivar apple juices (Table 5.1), the identified aromatic composition found were mainly aldehydes, alkyl-esters, alcohols and carboxylic acids. The characteristic flavour profile of the apple juices was found to be in accordance with previously published data in apple juices and fresh cut apple samples (E. Aprea, Corollaro, Betta, Endrizzi, Dematte, Biasioli, et al., 2012; E. Aprea, Gika, Carlin, Theodoridis, Vrhovsek, & Mattivi, 2011; Dimick, Hoskin, & Acree, 1983; Pongsuriya Komthong, Igura, & Shimoda, 2007; M. López, C. Villatoro, T. Fuentes, J. Graell, I. Lara, & G. Echeverría, 2007).

Granny Smith apple juices were profiled as having the lowest alkyl-esters concentration (with the exception of ethyl hexanoate) and the highest concentration of cis-3-hexen-1-ol and trans-2-hexenal and intermediate concentrations of hexanal, and 2-methylbutanol. Cis-3-hexen-1-ol and trans-2-hexenal are both related with strong green – grassy flavour notes which together with hexanal are considered the main contributor of green flavour in apples and their derivatives (Pongsuriya Komthong, Hayakawa, Katoh, Igura, & Shimoda, 2006; P. Komthong, Katoh, Igura, & Shimoda, 2006). Aprea et al., (2011) showed that apple cultivars such as Granny Smith, Topaz, Pilot or Reinette, that are generally known to emit lower amounts of esters, are

characterised as having higher concentrations of alcohols such as cis-3-hexen-1-ol.

As is detailed in the PCA bi-plot (Figure 5.2) the apple juice extracted from Golden Delicious exhibited a similar flavour profile to that of Granny Smith with high concentrations of volatile compounds related with green – grassy notes (trans-2-hexenal and 1-hexanal and cis-3-hexenol) and low concentrations of acetates. The latter has been also confirmed by Ting et al., (2012) who reported lower concentrations of acetates in the headspace of fresh cut Golden Delicious samples compared to other apple cultivars i.e. Red Delicious, Jonagold or Fuji. Moreover, the aldehyde to alcohol ratio is indicative of ripeness, as aldehydes can be metabolised to alcohols and subsequently esterified with the present carboxylic acids (Defilippi, Dandekar, & Kader, 2005). Based on GC-MS data, the ratio of aldehydes to their corresponding alcohols were higher for Golden Delicious and Granny Smith juices implying a lower level of ripeness for these apple samples. Pink Lady and Braeburn were characterised as having moderate concentrations of most of the identified flavour compounds, apart from a marked elevation in concentration for trans-2-hexenal in Braeburn. Jazz had the greatest fruity-ethereal-flowery flavour type compounds as indicated by the higher concentration of acetates (2-methylpropyl, butyl, 2-methylbutyl, and hexyl acetates) and the low green-grassy odour related compounds (cis-3-hexen-1-ol and trans-2-hexanal).

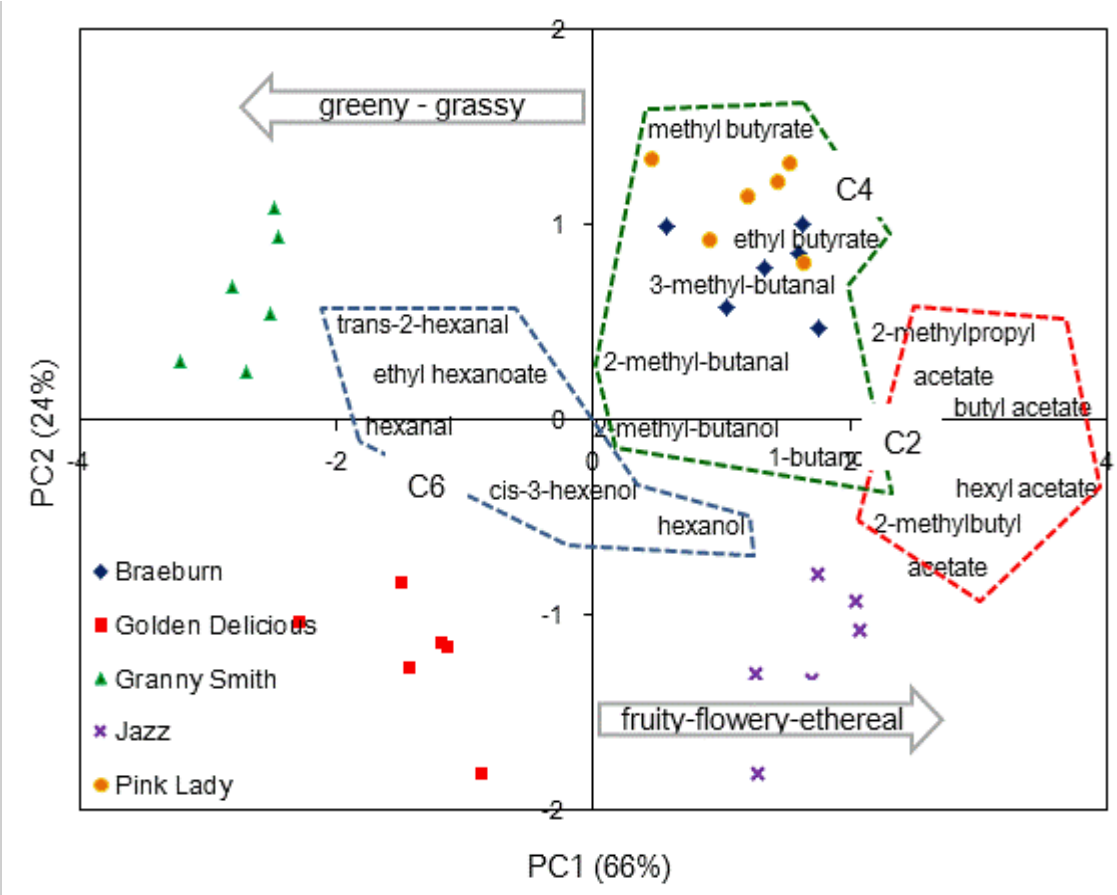


Figure 5.2: Principal components analysis (PCA) biplot on the data (averaged, mean centered and auto-scaled) obtained by GC-MS headspace analysis of the clarified fresh apple juices.

Regardless the cultivar type, acetates and more specifically butyl and hexyl acetate were the dominant esters in the headspace of the apple juices, this has previously been reported in other studies (E. Aprea, et al., 2012; Crook & Boylston, 2004; Kato, Shimoda, Suzuki, Kawaraya, Igura, & Hayakawa, 2003; Pongsuriya Komthong, Igura, & Shimoda, 2007; Ting, et al., 2012). 1-butanol was the most abundant alcohol in the headspace of the juices followed by 1-hexanol. In contrast to esters

and aldehydes, alcohols are generally characterised as having higher odour threshold and thus they are considered as secondary contributors to apple flavour perception (Echeverrıa, Graell, López, & Lara, 2004). It is also interesting that 1-butanol was highly correlated (according to Pearson's test) with butyl acetate ($r = 0.926$, $p<0.001$), hexyl acetate ($r=0.898$, $p<0.001$), trans-2-hexenal ($r = -0.777$, $p<0.001$) and hexanal ($r = -0.748$, $p<0.01$) and it could be surmised that these compounds are generated by a similar metabolic pathway during apple ripening. Finally, it should be noted that in the present work the major sesquiterpene found in the headspace of apples such as alpha-farnesene was not detected. This could be attributed either to the adopted protocol for the identification and quantification of the volatiles by GC or to the post juice extraction treatments e.g. enzymatic clarification and pectinase inactivation by heating. Su and Wiley (2006) investigated the impact of enzymatic clarification and pasteurisation on the major flavour volatile compounds of clarified apple juices and reported significant changes in concentration with processing.

No significant differences were observed for 2-methylbutanal and 3-methylbutanal. Although, the latter is well known as a precursor of the esters formed via the alcohol esterification pathway, the first has been rarely identified in fresh cut apple samples. However, both aldehydes have been previously identified in processed fruit juices, including apple juice (Burdock, 2009; Sapers, Abbott, Massie, Watada, & Finney, 1977). Due to the presence of 2-methylbutanol at relatively high levels the

presence of the former aldehydes is possibly related to the activity of enzymatic induced oxidation of alcohols.

5.3.2 Cultivars Classification of Apple Juices by APCI-MS

The volatiles were analysed by principal components analysis (PCA) in order to reduce the dimensions and investigate which differences of the apple cultivars could be visualized. The data were log transformed, mean centered and auto-scaled to facilitate the formation of clusters and subsequently subjected to the supervised classification technique PLS-DA. No specific pre-treatment of the data e.g. dimensionality reduction using PCA, was carried out apart from the log transformation of data in order to avoid the over fitting problems that have previously been reported by Granitto, Biasioli, Aprea, Mott, Furlanello, Märk, et al (2007).

The scores and the X-loadings plots are represented in Figure 5.3 for principle component one (PC1) and principle component two (PC2), PC1 and PC2 account for the 53 % of total variance of the spectral data. For the PLS-DA models, seven principle components were used which accounted for 81 % of the total variability According to the PLS-DA scores plots, very good clustering was observed for the monocultivar apple juices used in the present study, with juices extracted from Jazz apples showing the largest distance from Granny Smith, Golden Delicious and Pink Lady.

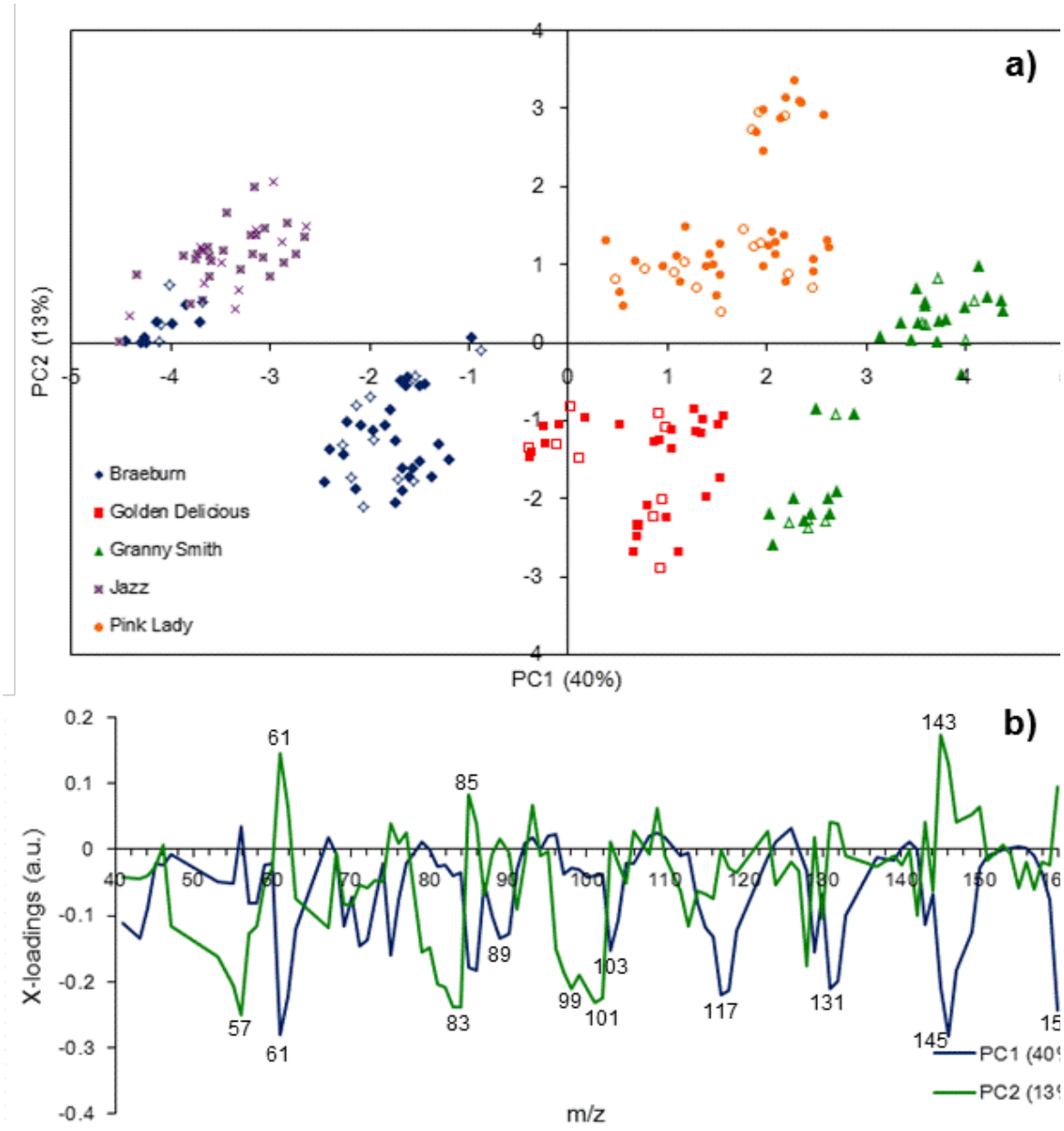


Figure 5.3 PLS-DA scores (a) and loadings (b) for the first two factors of the classification models based on the APCI-MS data obtained by the headspace analysis of the clarified apples juices made of different apple cultivars. (Symbols in bold = classification samples and empty symbols = test samples)

As is illustrated in the classification matrix for the calibration and validation (test set) datasets (Table 5.2), juices produced from Golden Delicious, Jazz, Granny Smith, and Pink Lady apples were 100 % correctly classified whilst in the case of the Braeburn extracted juices only one sample was misclassified. In both cases the total classification percentage was excellent (99.3 % and 100 % for internal and external validation) which indicates the robustness of the PLS-DA predictive models. Moreover, with an RMSE value ranging from 0.10 to 0.23 representing a total error of less than 5 %, the predictive power of the herein constructed models is very good.

The individual masses were also evaluated to gain an insight into the chemistry that is driving the multivariate discrimination of the apple juices (cultivar). The X-loading plot for the first two axes was constructed and is shown in Figure 5.3b. PC-1 was mainly correlated with m/z 61, 75, 85, 89, 103, 117, 131, 145 and 159, these are well known parent and fragment ions of common alkylesters (Aprea, Biasioli, Märk, & Gasperi, 2007; E. Aprea, Biasioli, Mark, & Gasperi, 2007). Similar fragments have also been reported in other DIMS studies. PC1 can therefore be tentatively identified as being related to the relative abundance of esters, and therefore the axis would be correlated to flavour notes such as fruity, ethereal, and fresh. Indeed, Jazz and Braeburn samples were clustered in the left side of the PCA map whilst the Granny Smith and Golden Delicious in the right. The second PC axis was also correlated with fragments of esters and alcohols, of which m/z 61 or 85 are tentatively attributed as fragments of acetates and 1-

hexanol (Soukoulis, Cappellin, Aprea, Costa, Viola, Mark, et al.), and 101 and 99 are proposed to be the parent ions of carbonyl compounds e.g. 1-hexanal (m/z 101) or trans-2-hexenal (m/z 99). Furthermore, m/z 83 was strongly discriminating and could be attributed to a dehydration product of 1-hexanal.

Table 5.2: Results of PLS-DA analysis (based on 7 PCs accounting for the 81% of the total variance) applied for the classification of clarified apple juices by means of cultivar

	Correctly classified	Misclassified	% correct classification
Internal validation			
Braeburn	32	1	96.7
Golden Delicious	25	0	100
Granny Smith	26	0	100
Jazz	26	0	100
Pink Lady	34	0	100
Total	**143**	**1**	**99.3**
External validation			
Braeburn	15	0	100
Golden Delicious	10	0	100
Granny Smith	10	0	100
Jazz	10	0	100
Pink Lady	14	0	100
Total	**59**	**0**	**100**

According to the X-loading plot for PC-1 and PC-3 (Figure 5.4b) the peaks at m/z 47 and 45, which correspond to ethanol and

acetaldehyde respectively (Davies, Linforth, Wilkinson, Smart, & Cook, 2011), allowed the discrimination between Jazz and Braeburn apples

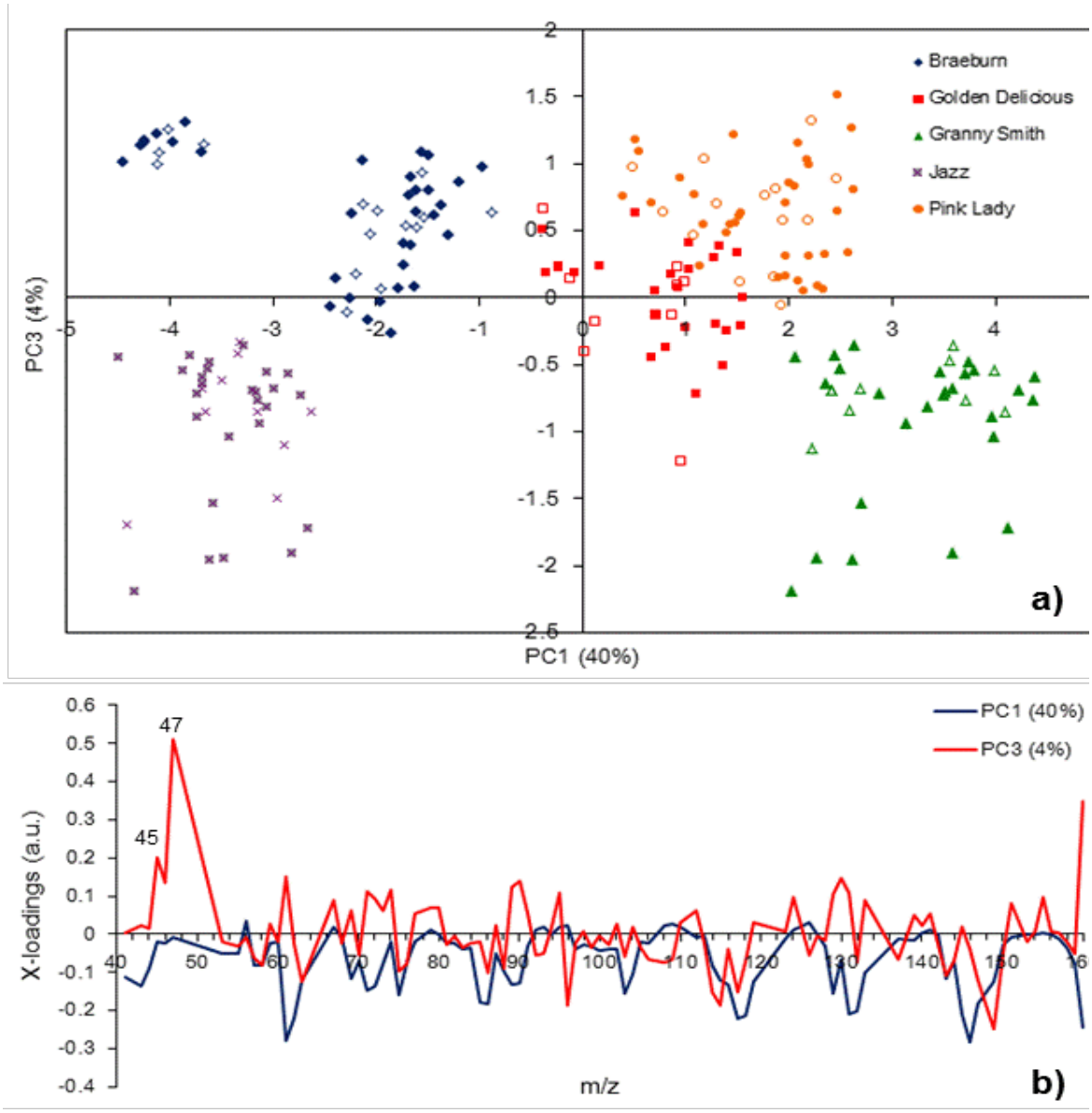


Figure 5.4: PLS-DA scores (a) and loadings (b) for the first and third factors of the classification models based on the APCI-MS data obtained by the headspace analysis of the clarified apples juices made from different apple cultivars. Symbols in bold = classification samples and empty symbols = test samples)

supporting the classification data displayed in Table 5.2. Acetaldehyde (m/z = 45) is one of the most abundant volatile compounds present in the headspace of fresh cut apples (Ting, et al., 2012). Apples juices extracted from Braeburn, Golden Delicious and Pink Lady were characterised by higher levels of acetaldehyde and ethanol which is in accordance with previously published data (Ting, et al., 2012). Ethanol is considered as an indicator of post harvesting conditions such as exposure to hypoxia, stage of climacteric ripening (Dixon, 1999). According to Figure 5.4a, juices extracted from Braeburn and Pink Lady had higher amounts of ethanol compared to Jazz and Granny Smith.

5.3.3 Geographical Provenance Determination by APCI-MS

For the further evaluation of APCI-MS as a viable method for food authenticity testing and classification, the geographical provenance of the apples tested previously was also modeled. As it can be seen in Figure 5.5a, effective clustering for the three apple juices was obtained, with New Zealand and South Africa being most clearly discriminated. The first two principle component axes accounted for 48 % of total variability. For the PLS-DA models, five principle components were used which accounted for 79.7 % of the total variability). The most robust classification performance was obtained in the case of internally validated PLS-DA models (97.1 %) although the externally validated models were also successful (94.2 %); this is further shown in Table 5.3. However, it should be further noted that in both cases the performance of the APCI-MS as a tool for geographical provenance determination

was very good considering the high intrinsic variability due to the use of commercial samples. Whilst the use of commercial samples does allow the inclusion of true sample variability, it does not permit strict control of process parameters that support a mechanistic explanation of the model (e.g. cultivation and irrigation practices, environmental factors, edaphological parameters, post-harvesting practices). In both internal and external validation datasets the samples originating from New Zealand were all successfully classified, and of the total 135 samples only 4 were misclassified, resulting in an error rate of <3 %.

Table 5.3: Results of PLS-DA analysis (based on 5 PCs accounting for 79.7% of the total variance) applied for the classification of clarified apple juices by means of geographical origin

	Correctly classified	Misclassified	% correct classification
Internal validation			
Chile	16	0	100
New Zealand	32	0	100
South Africa	87	4	95.6
Total	135	4	97.1
External validation			
Chile	6	2	75
New Zealand	17	0	100
South Africa	44	2	95.6
Total	67	4	94.2

There was a similar correlation of m/z to principle components, to that observed previously (Figure 5.5). More specifically, the first axis is proposed to be related to alkyl-esters (m/z 61, 75, 85, 89, 103, 117, 131, 145) and dehydrated alcohols (i.e. m/z 85 for 1-hexanol, m/z 57 for 1-butanol) in the form of fragments or parent ions. The second most powerful discriminating factor is shown on PC 2 and was found to associated with the green-grassy odor like volatiles such as 1-hexanal and trans-2-hexenal (m/z 101, and 99 respectively), or 1-hexanal and cis-hex-3-en-ol (m/z 83). Thus, complete discrimination between New Zealand and South Africa juices appear to be dependent on the ester-related flavor notes (fruity – flowery), whilst the Chilean samples appear to be discriminated by moderate ester concentration and low amounts of green-grassy flavor type volatiles.

Fruit aroma is a complex mixture of a large number of volatile compounds that contribute to the overall sensory quality of fruit specific to species and cultivar. Only a handful of these chemicals are ‘character impact’ compounds, which have a range of aroma thresholds. Some are present in very low concentrations and contribute potent aroma characteristics typical of apple aroma such as ethyl butyrate). Others contribute to aroma intensity (e.g. trans-hexenal) or are related to aroma quality (e.g. ethanol) (Dürr & Schobinger, 1981). Most aroma compounds, in variable proportions, are present in volatile emissions from most apple cultivars but there appear to be no key characteristic compound for any given cultivar (Paillard, 1990). Notwithstanding this, large sensory differences in flavour and aroma exist among cultivars

and taste panel could distinguish cultivars with strong aroma with ‘characteristic’ apple taste from weak and ‘uncharacteristic’ apple taste (Cunningham, Acree, Barnard, Butts & Breall, 1986). Esters are quantitatively and qualitatively the most important autonomously produced volatile compounds contributing to apple aroma (Dixon and Hewett, 2000). Ester production in apple fruit is an ethylene-dependent process and the majority of the esters are synthesized during the climacteric phase of ripening (Fan et al, 1998; Song and Bangerth, 1996). In fact, the effect of exposure to hypoxia on apple quality at warm temperatures induces substantial quality and quantitative changes in concentration of volatiles thought to be important in apple aroma. The type of compounds enhanced included low odour threshold volatiles such as ethyl butyrate, which is of commercial significance to the apple juice processing industry (Ampun, 1997). Hypoxic conditions consistently enhance acetaldehyde and ethanol concentrations that may affect fruit metabolism by stimulating or inhibiting various biochemical pathways involved in ripening. Therefore, it should be noted that the variability of the New Zealand and South Africa labeled juices based on the green – grassy flavor criterion was quite high indicating differences in the ripening level of the sampled apples.

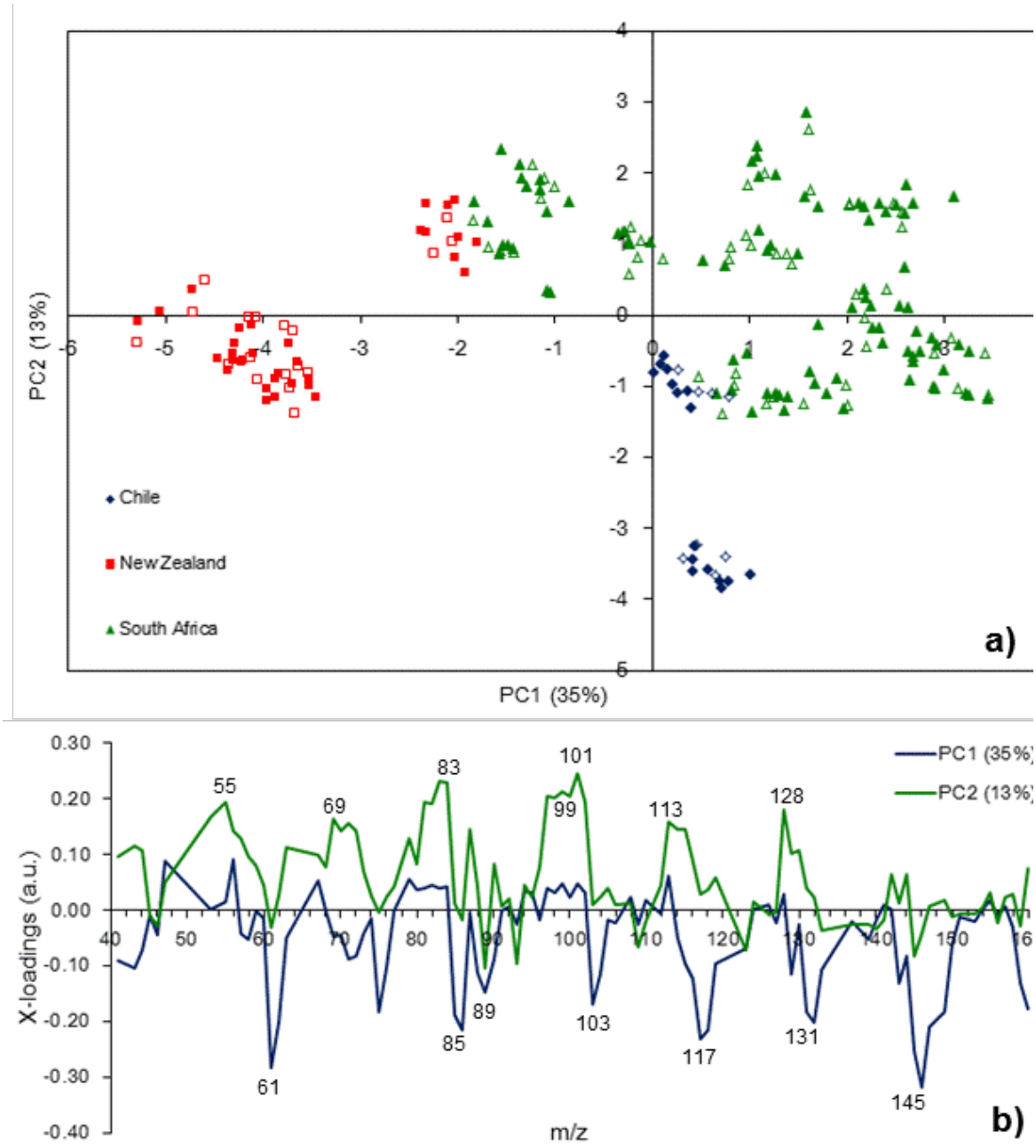


Figure 5.5: PLS-DA scores (a) and loadings (b) for the first two factors of the classification models based on the APCI-MS data obtained by the headspace analysis of the clarified apples juices made from apples differing in their geographical origin. (Symbols in bold = classification samples and empty symbols = test samples)

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5.4 Conclusion

In conclusion, using a PLS-DA chemometric approach, it was possible to classify apple juices on the basis of aromatic compounds detected from raw APCI-MS data. The models generated were robust enough to reliably discriminate (100 % correct classification with external validation set) apple juices prepared from Braeburn, Golden Delicious, Granny Smith, Jazz and Pink Lady varieties, furthermore developments on the model allowed the reliable (94.2 % correct classification with external validation set) discrimination of the geographical provenance of monovarietal clarified apples from Chile, New Zealand and South Africa. This demonstrated the applicability of multivariate statistical technique as tools to monitor the quality of apple juices.

CHAPTER 6: CLASSIFICATION AND PREDICTION OF CHEDDAR CHEESE MATURITY USING CHEMOMETRICS

6.1 Introduction

Cheese is comprised of a casein matrix in which fat, water, lactose, minerals, vitamins, bacteria and enzymes are interspersed. The basic principles for the production of all types of cheese are the same for most cheese types, with relatively small changes resulting in significant flavour differences in the final cheese. Cheese-making comprises five key variable: milk composition; key cheese manufacturing processes (that include the use of different starter cultures and adjuncts); moisture content; curd manipulation and storage conditions (temperature and length of time) (C. J. Coker, R. A. Crawford, K. A. Johnston, H. Singh, & L. K. Creamer, 2005, Bachmann, Butikofer, & Meyer, 1999; Chen, Irudayaraj, & McMahon, 1998). These factors lead to the diversity in texture and flavour of the many hundreds of cheese types produced around the world. Therefore maintaining cheese consistency throughout the manufacturing season is a particular problem for cheese makers where seasonal and lactational variations in milk composition and chemistry exist. Furthermore, many cheese manufacturers develop their own strains of nonstarter-lactic acid bacteria (NSLAB) with time, significantly influence the final composition and flavour profiles of the cheese.

Cheddar cheese is an important food commodity and the subject of international trade of substantial value. It is the most widely purchased and eaten cheese in the world, and is always made from cow's milk. The Cheddaring process leads to the formation of fibrous protein molecules that produce a very close textured cheese. Cheddar is a hard cheese with a little crumbly texture if appropriately cured. Cheddar cheese flavour is composed of a complex mixture of at least 180 compounds, including alcohols, aldehydes, ketones, esters, lactones, sulfides, free fatty acids and pyrazines (Curionia & Bosset, 2002). Throughout manufacture, cheese production represents a series of finely coordinated biochemical events, which, if synchronised and balanced, lead to products with highly desirable aromas and flavours, but when balance is absent, off-flavours and odours result.

During maturation, bacteria and enzymes act on the fat, protein and carbohydrate in the cheese to produce the body, texture and flavour characteristic of mature Cheddar. The changes in the body and texture that transform the rubbery, elastic mass of curd to a cheese with a firm close texture are the result of hydrolysis of one of the main structural proteins in bovine milk (α_{s1}-casein) to the soluble casein α_{s1}-1 casein fractions (Hort & Le Grys, 2001) and a sharper taste develops in Cheddar as it matures. Volatiles play an important role in the flavour of cheese. The generation of volatile components from the curd during ripening contributes aroma and associated flavours to the cheese. Typical cheese aroma is in fact the result of a complex mix of biochemical processes that include glycolysis, lipolysis, proteolysis; as

well as secondary fermentations that take place when the products of these processes are catabolised (Coker, 2003).

6.1.1 Proteolysis and Lipolysis in Cheddar

Proteolysis is recognised as one of the most important and complex of these biochemical processes that affect cheese texture and physical properties. It involves the hydrolysis of caseins that form the cheese matrix to progressively smaller peptides and free amino acids by proteinases including chymosin (a type of animal rennet), plasmin (an indigenous milk enzymes) and the cell-envelope proteinases and peptidases of the starter and non-starter lactic acid bacteria (NSLAB) (Coker, 2003). During cheese ripening, the enzymatic degradations of proteins and peptides lead ultimately to the formation of flavour-impact volatiles (Papetti & Carelli, 2013).

Cheese fat affects cheese flavour in two ways: firstly it acts as a source of aromatic compound and secondly as a solvent for the aromatic compounds. Lipolysis results in the formation of free fatty acids, which are key constituents of Cheddar cheese flavour and can also be precursors of flavour compounds such as methylketones, alcohols and lactones (Smit, Vileg, Smit, Ayad, & Engels, 2002). Fatty acids are important, or even predominant, components of the flavour of many cheese types. During ripening, free fatty acids containing four or more carbon atoms may originate from lipolysis of milk fat or the breakdown of triglyceride substrates due to the action of the indigenous lipases in raw milk. Lactic acid bacteria present in starter cultures are generally

only weakly lipolytic, however, most of the fatty acids, having between 4 and 20 carbon atoms, come from the lipolysis of triglycerides by moulds. Short and moderate-chain, even numbered fatty acids contribute to the characteristic note of Cheddar (Curionia & Bosset, 2002). The principal volatile fatty acids present during maturation include acetic, propionic, butyric, pentanoic, and hexanoic (De Wit, Osthoff, Viljoen, & Hugo, 2005). Therefore, most cheese flavour is generated during lipolysis, proteolysis and catabolisation of lactose and citrate by cheese micro flora.

Cheese maturation is a continuous process whereby cheese texture, flavour and aroma develop over a period of time. For some cheese types, the ripening temperature protocol is never altered and therefore the length of time of ripening is correlated with the particular attributes such as aroma and texture that are used to categorize the cheese so that it can be sold as mild, medium, mature, extra mature or vintage.

6.1.2 Use of Chemometrics for Dairy Classification

There are manufacturing processes (e.g. addition of adjunct cultures) that can be applied to alter the rate of ripening. In these circumstances, methods for determining or predicting maturity are particularly useful (C. Coker, R. Crawford, K. Johnston, H. Singh, & L. Creamer, 2005). Pham & Nakai (1984) used Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) peak data to match cheese to their maturity categories. Classification of Cheddar cheese on the basis of maturity and quality has also been attempted by various authors.

O'Shea, Uniacke-Lowe, & Fox (1996) used RP-HPLC to analyse the retentate and permeate of the water soluble fractions (WSF) of 60 Cheddar cheese samples that varied in age and quality as determined by cheese graders but received poor differentiation of maturity. Recent attempts using casein, peptide and amino acid data produced from electrophoretic and chromatographic methods to show correlations between sensory and instrumental textural information. Much work has also been carried out using multivariate statistics for determining the influence of different manufacturing processes, types of raw milk, or the incorporation of adventitious NSLAB strains on proteolysis and maturation (Ardo, Thage, & Madsen, 2002; Furtula, Nakai, Amantea, & Laleye, 1994; Skeie, Lindberg, & Narvhus, 2001), which could provide information of the characteristics of the specific Cheddar cheese. Changes in sensory properties of Cheddar cheese during maturation were profiled by Muir, Hunter, Williams, & Brennan (1998) and should give manufacturers understanding of the quality of their cheese. However there is no ‘best’ combination of analytical and statistical methods that can be used for every situation. Sensory analysis techniques have over the years developed into powerful tools for understanding how the appearance, flavour and texture attributes of Cheddar cheese products drive consumer preferences. These tools can help determine variations in sensory attributes associated with processing variables, geographical origin, production processes and season.

Traditionally, judging and grading cheese normally involves one or two trained ‘experts’ or cheese graders who will assign quality scores on the appearance, flavour and texture of the products based on the presence or absence of predetermined defects. Unfortunately, these traditional judging methods have several shortcomings: they cannot predict consumer acceptance; quality scores are subjective and do not combine analytical oriented attribute ratings with affectively oriented quality scores (Claassen & Lawless, 1992). On top of that human subjects have limited analytical capacity in identifying chemical compounds. Coupling sensory analysis with chemical analysis data can provide even more insights than using either technique alone. Identifying specific volatile compounds associated with particular significant aroma in Cheddar cheese during maturation improves the understanding of how aroma changes with ripening, and the effect on consumer preference.

The aim of this research was to evaluate chromatographic technique such as GC-MS and direct injection APCI-MS for the ability to identify and characterise the aroma volatiles of commercial Cheddar cheese manufactured in UK. The second aim was to predict the age of Cheddar cheese using proposed PLS models, derived from the analytical headspace data to replace or complement expensive and time-consuming sensory panels or cheese graders. The Initial work relating to the development of a methodology in the classification of a food volatile compliment using APCI-MS have recently been published (Gan, Soukoulis, & Fisk, 2014) and is shown in Chapter 5. This is an

extension to evaluate the soft modelling approach based on chemometric analyses of the chromatographic profiles of another real food with broad commercial diversity, cheese volatiles during different stages of ripening.

6.2 Materials and Methods

6.2.1 Cheddar Cheese Samples

Five different commercial Cheddar cheese brands (coded V, W, X, Y, Z) comprising of five maturity grades: mild (MI), medium (ME), mature (M), extra mature (EM) and vintage (V) were ripened at 3 – 4 months, 10 – 12 months, 14 months and 18 – 36 months respectively. Cheeses were grated and placed into glass bottles for headspace analysis. A total of 52 Cheddar cheese samples (with triplicates) were prepared and stored at 4 ˚C until they were analysed.

6.2.2 Measurement of Physicochemical Properties

All the Cheddar cheese samples were grated by handheld stainless steel cheese grater. 10 g of each sample were poured into labeled 100 ml beakers made up to 100 g with distilled water. The solutions were thoroughly mixed using the magnetic stirrers for 5 min. pH was measured with Five-Go portable meter (Mettler Toledo, UK) at room temperature (20 ^{0}C). Calibration of the meter achieved using pH 4 and pH 7 solutions before samples of cheese were measured. The electrode probe was immersed into the cheese solutions and pH readings were shown on the display screen. For the salt measurement,

a drop of the solution was dispensed onto the measuring sensor of the Pocket salt instrument (Atago, Japan) and the results were displayed on the screen. Triplicates were taken for all above measurements.

The colour instrument (Hunter LAB, UK) was calibrated and all the blocks of cheese samples were measured for their L, a, b readings.

6.2.3 GC-MS Analysis

Headspace solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) was applied to analyse the volatile compounds of Cheddar cheese samples. An automated SPME sampling unit (CombiPal. Zwingen, Switzerland) was used with a SPME StableFlex fibre with 50 / 30 µm divinylbenzene/carboxen on polydimethylsiloxane coating (DVB/ CAR/ PDMS) purchased from Supelco (Sigma Aldrich, Dorset, UK). This was exposed to the headspace for 30 min. The cheese samples were stirred for 30 min at 60 °C to accelerate the equilibrium of headspace volatile compounds between the cheese matrix and the headspace. Analysis of the volatiles was performed on a Trace GC Ultra (Thermo Scientific, USA) that was attached to a ISQ series mass spectrometer (Thermo Scientific, USA), carried out in the electron impact mode with a source temperature of 200 ^{0}C, and a scanned mass range of m/z = 15 – 200. The gas chromatograph was equipped with ZB-Wax capillary column (100% polyethylene glycol phase, 30 m x 0.25 mm x 1.0 µm) (Phenomenex, UK). GC oven was held at 40 ^{0}C for 2 min then heated up to 250 ^{0}C at a rate of 4 ^{0}C / min. The GC to MS transfer line was maintained at 250

^{0}C. Helium was the carrier gas with a constant flow rate of 1.0 ml / min in the GC-MS. Cheddar cheeses were analysed in triplicates. Aroma compounds were identified by comparison of their retention times with those found in NIST Library and published journals.

6.2.4 APCI-MS Analysis

The cheese samples were analysed in full scan mode, using MS Nose interface (Micromass, Manchester, UK) fitted to a Quattro Ultima mass spectrometer (Milford, Waters). Following the method described by (Gan, Soukoulis, & Fisk, 2014), ions of mass to charge (m/z) ratios from 40 – 200 were monitored. The intensity of these ions was measured at cone voltage of 20 V, source temperature of 75 ^{0}C and dwell time of 0.5 sec. All analyses were run in triplicates (Ashraf, Linforth, Bealin-Kelly, Smart, & Taylor, 2010).

6.2.5 Statistical Analysis

Data from MS nose were exported using the Waters Masslynx^{TM} Software version 4.1(Hertfordshire, United Kingdom), whereas data from GC-MS were processed with Thermo Scientific^{TM} Xcalibur^{TM} Software (United Kingdom) for chemometric analysis into The Unscrambler software (version 9.7, CAMO AS, Norway). The chemometric approach composed of principal component analysis (PCA) and partial least square regression (PLS1) performed with full cross validation, used to analyse the aroma profile and classify the maturity of the cheese on the basis of these aroma compounds. Full

cross validation is an evaluation tool to check calibration model based on systematically removing groups of samples in the modeling and testing the performance of the model using the remaining data set. The maximum number of factors in both PCA and PLS models were selected by the criterion of the lowest number of factors that gave the closest to minimum value of predicted residual error sum of squares function in order to avoid over fitting of the data (Cozzolino, Smyth, Cynkar, Janik, Dambergs, & Gishen, 2008). All variables were autoscaled and weighted (1/ standard deviation) prior to chemometrics application so that drifts and baseline effects were removed. PLS regression was used to determine the relationship between multiple dependent predictor variables (such as the colour, salt %, GC and APCI data) and the maturity/ age of cheese. Furthermore it was used as an exploratory analysis tool to select suitable predictor variables for predictive linear modelling. PLS1 was applied to model the maturity/ age of cheese using the aroma volatiles with predictive ability.

6.3 Results and Discussion

6.3.1 Headspace Analysis by GC-MS and APCI-MS

Twenty-three volatile flavour compounds were detected and identified in the headspace of the grated Cheddar cheeses (Table 6.1), the identified aromatic composition found were mainly aldehydes, ketones and carboxylic acids. The characteristic flavour profile of Cheddar was found to be in accordance with previously published data in Cheddar cheese (Biasioli, Gasperi, Aprea, Endrizzi, Framondino, Marini, et al.,

2006; C. Coker, R. Crawford, K. Johnston, H. Singh, & L. Creamer, 2005; Muir, Hunter, Williams, & Brennan, 1998; Whetstine, Drake, Nelson, & Barbano, 2006)

Table 6.1 Volatile compounds identified in the headspace above grated Cheddar cheese using SPME-GC-MS.

Aroma Volatiles (Molecular weight)	**Description[1]**
Acetonitrile (41)	Solvent-like
Acetic acid (60)	Vinegar
Butyric acid (88)	Rancid cheese
Diacetyl (86)	Buttery flavour
2-heptanone (114)	Banana; cinnamon; spicy
Methional (104)	Beef; cheese; creamy; meaty; oily
Heptanal (114)	Fatty; oily
Octanal (128)	Fatty; citrus
Hexanoic acid (116)	Cheese; fatty; sour
Acetoin (88)	Butter; creamy
Ethyl-octanoate (172)	Apricot; banana; floral; pear; wine-like
n-decanoic acid (172)	Fatty; citrus
Dodecanoic acid / Lauric acid (200)	Fatty

2-ethyl-1-hexanol (130)	Oily; rose
δ-Nonalactone (156)	Butter; meaty; nutty; sweet
2-decenal (154)	Fatty; green; meaty; oily
2-undecanone (170)	Citrus; fruity; rose
Pentanoic acid / Valeric acid (102)	Fatty; earthy
Heptanoic acid (130)	Cheese
Octanoic acid / Caprylic acid (144)	Cheese; oily
2-propanol-1-ethoxy (104)	Solvent-like
9-decenoic acid (170)	Soapy
2-methyl-2-buten-1-ol (86)	Green; fruity

[1] (Whetstine, Drake, Nelson, & Barbano, 2006)

The Cheddar cheese samples were randomly grouped into 'calibration' and 'prediction' set. The different brands were indicated by the different prefixes: 'V', 'W', 'X', 'Y' and 'Z', whereas the different maturity of cheese was marked by the different suffixes: -*MI* for mild, -*ME* for medium, -*M* for mature, -*EM* for extra mature and -*V* for vintage. (Maturity grading was based on labelling printed on packaging).

A Principal Component Analysis (PCA) was performed to evaluate the variance associated with the samples by the instrumental data from GC-MS and APCI-MS. 42 calibration Cheddar cheese samples and 47 key aroma volatiles that exhibited significance in classifying Cheddar

maturity were used. Interpretation of the dimensions of the PCA was facilitated by inspection of the vector loadings and of the correlation coefficients of the scores of the cheese samples. As shown in Figure 6.1, the first principal component (PC1) accounted for 28 % of the aroma compounds and was mainly associated with maturity.

As the Cheddar mature, more aroma volatiles, especially acids (such as butanoic, pentanoic, hexanoic acids) and ketones like 2-heptanone, 2-undecanone, were produced (respectively marked by green circle, purple hexagon in Figure 6.2). The more aged cheese also displayed cheesier, meatier and/ or more fatty profiles with higher concentration of characteristic aromas like octanal and methional (the most common sulfur-compound in cheese), as described in Table 6.1.

The biplot (Figure 6.2) provided an insight in the role played by APCI-MS peaks. It indicated that there was positive relationship among the ester peaks (m/z = 117, m/z = 131, m/z =85) that were associated with the vintage series (V) (marked by grey oval in Figure 6.2). Even though APCI-MS could not provide unambiguous identification of aroma compounds, literature data and comparison with other data enabled the tentative identification of many peaks. The protonated masses m/z = 89 (butyric acid), m/z = 101 (2-hexanone), m/z = 155 (decanoic acid) and m/z = 157 (2-decanone) appeared to be the main volatiles that contribute to cheese profiles (Biasioli, Gasperi, Aprea, Endrizzi, Framondino, Marini, et al., 2006). Overall effect of ripening resulted in a progressive increase in aroma in the headspace of the Cheddar cheeses.

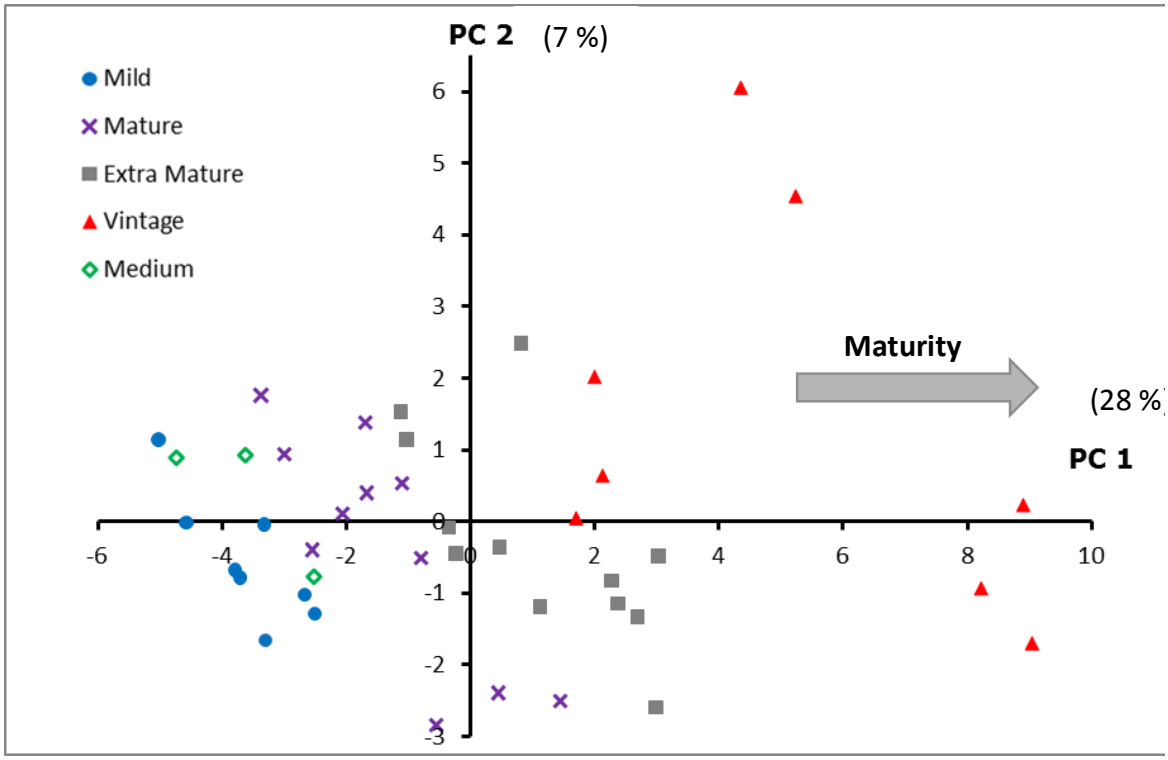

Figure 6.1: Principal components analysis (PCA) on the data (averaged, mean centered and auto-scaled) obtained by GC-MS and APCI-MS headspace analysis of the 42 grated Cheddar cheeses ('calibration' set).

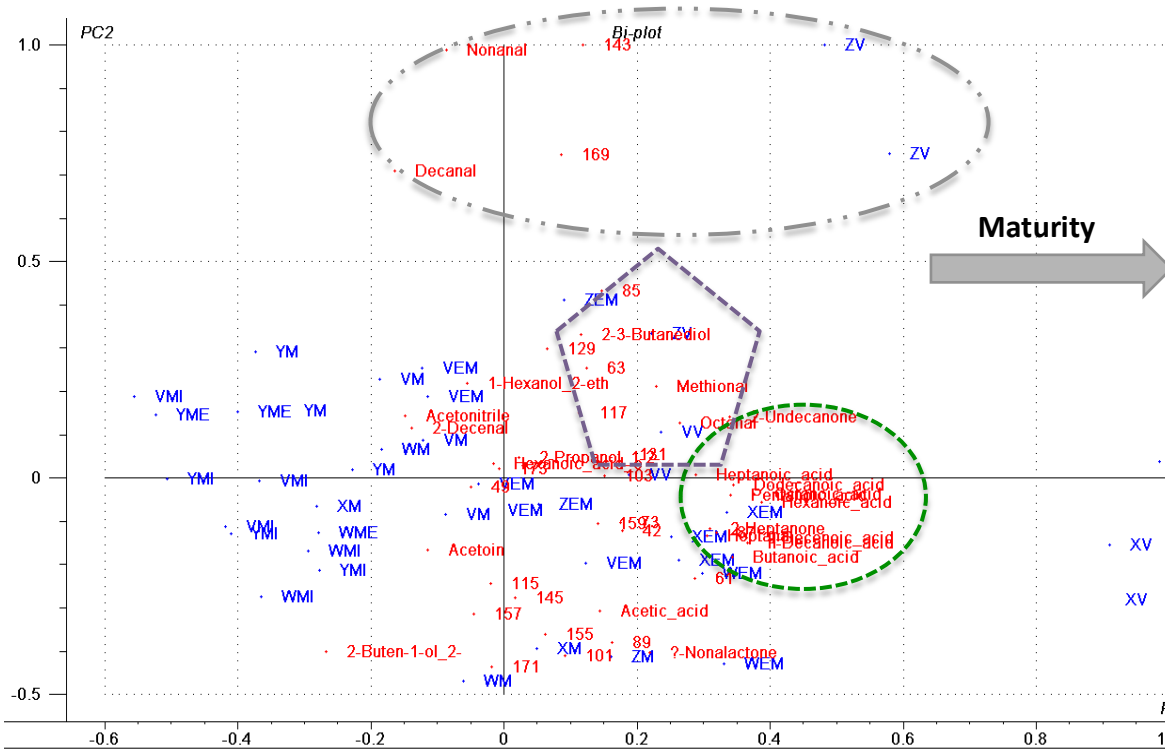

Figure 6.2: Principal components analysis (PCA) biplot on the obtained by GC-MS and APCI-MS headspace analysis of the grated Cheddar cheeses.

Estimated regression coefficients provided evidence of the cumulative importance of each of the aroma volatile to the age of Cheddar cheese. Butanoic, pentanoic, hexanoic, heptanoic, octanoic, dodecanoic, n-decanoic, 9-decenoic acids, 2-heptanone, heptanal, octanal, methional, 2-undecanone and 3-methylbutanal (m/z = 87) on PC1; nonanal and 2-methyl-2-butenol on PC2 were statistically significant in predicting age of the Cheddar cheese (Figure 6.3). As observed in Figure 6.2, ZV cheeses were rich in nonanal. These volatiles had positive correlation to the cheese age. On the same note, Whetstine, Drake, Nelson, & Barbano (2006) had also identified these acids as predominant odorants in aged Cheddar. These were probably the headspace

compounds that differentiate the mild Cheddar from the more matured ones. Similarly, Curionia & Bosset (2002) identified 3-Methyl-butanal, ethyl butyrate (m/z = 117) and 1-octen-3-ol (m/z = 129) as potent odorants in aged Cheddar and these aroma were seen having close relationships with vintage (V) Cheddar on Figure 6.2. 2-methyl-2-butenol and δ-nonalactone on PC2 were also significant, but associating more with the mature (M) categories of cheese.

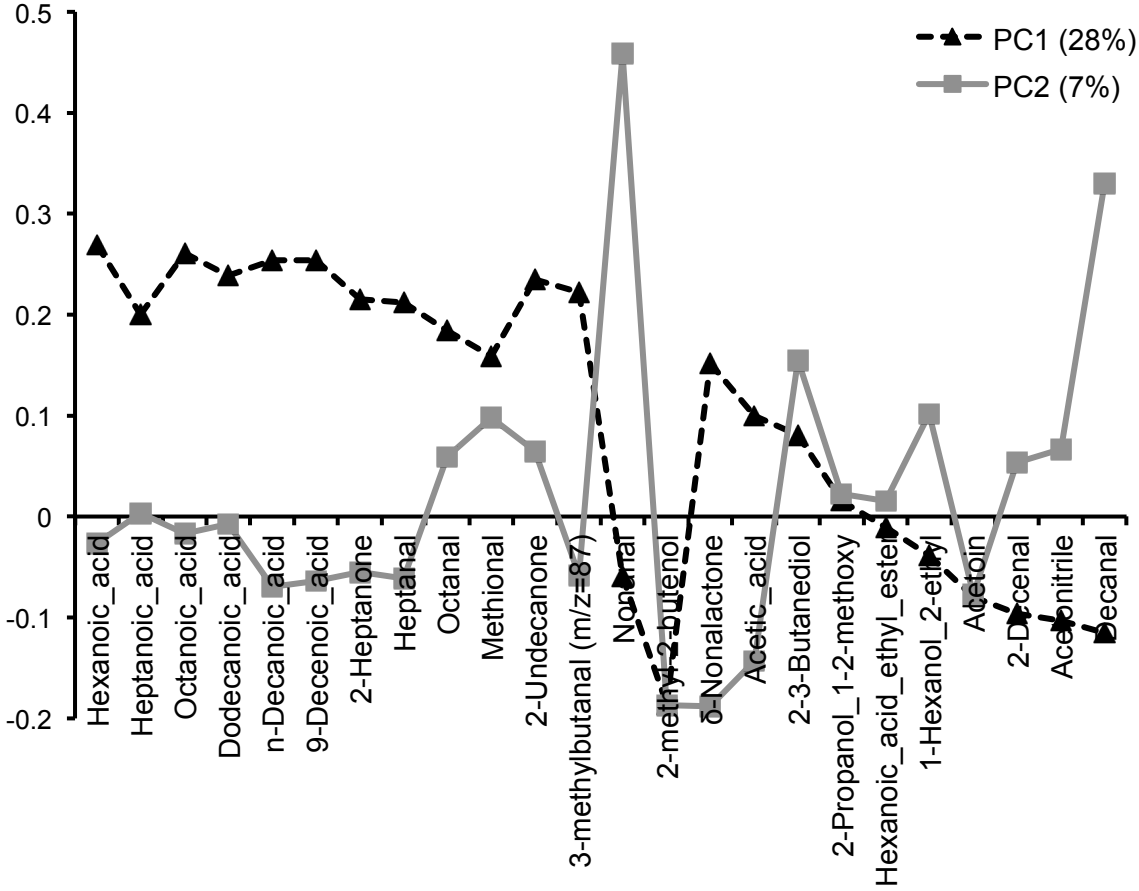

Figure 6.3: PLS-DA loadings for the first two factors of the classification models based on both the GC-MS and APCI-MS data obtained by the headspace analysis of the Cheddar cheeses.

Acetone is the major volatile compound in the headspace of fresh milk which was seen to decrease with ripening due to its reduction to propan-2-ol, likely as a result of adventitious bacteria (Dimos, Urbach, & Miller, 1996). Keen, Walker, & Pederby, (1974) showed that starter culture bacteria produce diacetyl, which is reduced to acetoin, and some starters are also able to reduce the acetoin to 2,3-butanediol, whereas the production of butanone and butan-2-ol from 2,3-butanediol is due to adventitious bacteria. Acetic acid is the major free alkanoic acid in Cheddar. A steady increase in the level of acetic acid over the whole period of maturation was discovered by Urbach (1993) (Dimos, Urbach, & Miller, 1996). This was depicted in Figure 6.2 where acetic acid was correlated to Cheddar of mature grade. However δ-decalactone appeared to play little part in Cheddar flavour. Diacetyl formed from the dehydration of 2,3-butanediol, is a common volatile present in dairy products. While diacetyl showed close association to the mild Cheddar cheese, 2,3-butanediol had a positive correlation with the more matured Cheddar 'ZV' (Fig 6.2).

6.3.2 Prediction of Cheddar Cheese Maturity

PLS2 regression model was performed to predict the age of Cheddar cheese from the instrumental variables, i.e. the headspace volatiles and physical variables that are the measurements of % salt and colour. Salts of calcium and phosphorus are released, which influence the consistency of the cheese and help to increase the firmness of the curd. Colour of the cheese is to a great extent determined by the colour of the

milk fat and undergoes seasonal variations. These are measured to observe if changes in these physiochemical properties vary in cheese during maturation. It seemed that the spectrophotometric colour measurements (L, a, b) and % salt showed insignificant correlation to the PLS-factors since they gave low PLS loadings. Therefore physical measurements proved not important in predicting age of Cheddar cheese in our study. Furthermore, food colours have been commonly added in many of the industrial varieties of Cheddar cheeses, which make the measurement less distinguishing. Use of GC-MS and APCI-MS variables alone was essential for the age prediction ability of the Cheddar cheese data. Hence, PLS1 regression model was built to interpret and predict the age of Cheddar using headspace data from GC-MS and APCI-MS. PLS combines the properties of multiple linear regression and PCA to make linear combinations in the dependent matrix. By means of these regression models, relationships between aroma compounds and age/ maturity of Cheddar cheese were established. Cross-validation of order one was used in order to optimize the use of a small set of measurements. Parameters used to evaluate the models prediction ability were: root mean square error prediction (RMSEP) and coefficient of determination (R^2) for the derived model between actual and predicted Y-variables (Capone, S., Tufariello, M., Francioso, L., Montagna, G., Casino, F., Leone, A., Siciliano, P., 2013). The RMSEP and R^2 values for the prediction model were 3.94 and 0.85 respectively.

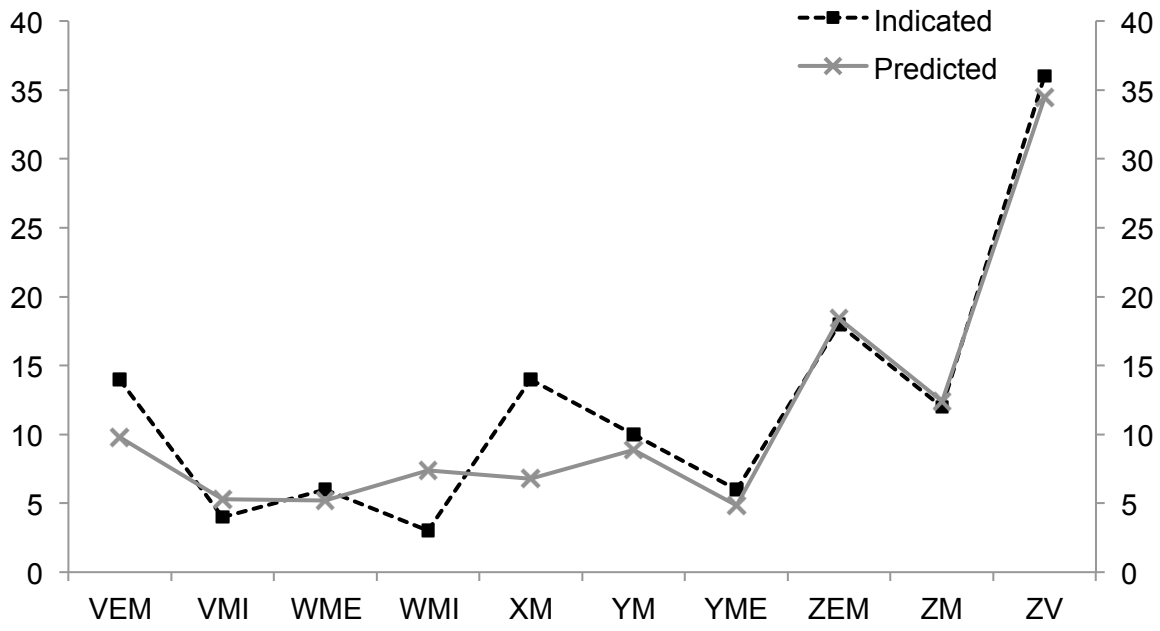

Figure 6.4 Comparison of the age of 10 Cheddar cheeses ('predicted' set) given by the manufacturer (labeled as *'indicated'*) and predictive values from regression model (labeled as *'predicted'*)

The estimates of Cheddar cheese age supplied by the manufacturers, compared to those predicted by the PLS model for the 10 prediction samples used, were shown in Figure 6.4. As illustrated in Table 6.2, the age of the Cheddar was 67 % correctly predicted. Despite this, the predictive power of the herein constructed model was reasonably satisfactory, especially when a lot of parameters, like the time between the grading of the Cheddar cheese to the analysis of them, the processing protocols and storage conditions, were not known.

Table 6.2: Results of PLS1 predictive model (based on 3 PCs accounting for 92% of Y) applied for the prediction of Cheddar cheese by means of age

	Correct prediction	Incorrect prediction	% Correct prediction
Prediction samples			
Mild (MI)	1	1	50
Medium (ME)	2	0	100
Mature (M)	1	1	50
Extra mature (EM)	1	1	50
Vintage (V)	1	0	100
Total	**6**	**3**	**67**

6.4 Conclusion

GC-MS and APCI-MS headspace analysis were effective techniques for determining aroma compounds relevant to Cheddar cheese. Characterization of Cheddar cheese maturity based on headspace measurements using GC-MS and/ or APCI-MS combined with chemometric treatment of data was shown to be effective when presented within a PCA format. With the PLS-DA chemometric approach, it was possible to classify and predict the age of the Cheddar cheeses on the basis of their headspace analysis. The PLS model generated was robust enough to accurately predict ~ 70 % of the Cheddar cheeses using the aroma compounds from the headspace data alone. This further established the applicability of multivariate statistical technique as a tool to monitor the quality of foodstuff. Results

could be improved by analysing more Cheddar cheese samples with GC-MS and APCI-MS in order to train the model and more accuracy in the prediction.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

Aroma release from food is influenced by two different principles: a static distribution of the aroma into the different phases of a product: solid matrix, hydrophilic or hydrophobic liquid phase and the gas phase. This behaviour is controlled by the partition coefficients of the molecules. The second principle is a dynamic factor, controlled by the mass transfer through a matrix and interfaces. Both factors influence the perception of the food (Grab & Gfeller, 2000).

Direct mass spectrometry techniques like APCI-MS have been developed to monitor the concentrations of known volatile compounds in air. While the kinetics of volatile release can be readily measured and compared from solid or liquid systems of varying formulation or under different conditions using APCI-MS, the capacity to monitor the kinetics of volatile release into the headspace systematically in model systems can also provide a valuable tool in helping to elucidate the most important factors controlling volatile release. The applications therefore range from aroma release in foods to characterizing aroma fingerprints for quality control. Understanding matrix parameters that influence the release of aroma compounds from foods provide essential information to control their delivery in food products and allow effective use of these materials (Schober & Peterson, 2004).

APCI-MS was the primary technique used in this research to measure real-time aroma partitioning and release by static headspace and dynamic headspace approaches, as this method had been previously

shown to be reliable and reproducible (Taylor 1998). Aroma -matrix interactions affect in vitro and in vivo flavour delivery, hence quality of foods and consumer overall acceptance. The type of interaction depends on the physicochemical properties of the aroma compounds and the food matrix.

An increase in the concentration of fructose above 25 % (w/ w) was shown to significantly ($p < 0.05$) increase the release of ethyl butyrate, ethyl acetate and benzaldehyde into the headspace above the aqueous solutions. The 'salting out' occurrence was observed since the fructose and FOS polymers interacted with water molecules, decreasing the ratio of free to bound water, therefore increasing the concentration of aroma compounds in the solutions. This resulted in the displacement of these aroma compounds into the headspace, so as to maintain equilibrium in the static environment. Hence, competing effects of intermolecular attractions among the fructose, aroma molecules and water made it difficult to formulate a general theory for sugar-aroma interactions. In the dynamic system, property of interface was very much affected by K_{aw}, as aroma with higher K_{aw} showed less stability, which explained the most rapid depletion of ethyl butyrate (with highest K_{aw}) into the headspace. Mass transfer of aroma compounds through matrices decreased with increasing concentration of fructose/ FOS. The persistence of benzaldehyde in the headspace seen in the dynamic environment could be due to surface concentration of the aroma volatile. Movement of benzaldehyde compounds through the sugar solutions was impeded because of the bulky aromatic ring structures. These

molecules thus remained on the surface, resisting headspace dilution.

^{1}H-NMR was used to investigate the molecular mobility of water in the simple aroma-solvent-fructose model system. The T_1 relaxation times and diffusion coefficients provided preliminary clues to the interactions between water, aroma and the biopolymer. From the NMR results, binding of aroma compounds to the simple sugars fructose did not seem probable, since their T_1 values and diffusion constants were different, implying that interaction between them would be minimal, and the motions of the sugar and aroma compounds were independent of each other.

Van Ruth et al (2000) confirmed the impact of matrix composition and mastication on aroma release for aroma compounds. Real time determination and monitoring of the aroma compound concentrations in the oral and nasal cavities could also be carried out using APCI-MS, although the in vivo behaviour of hydrophilic compounds in the frozen dessert consumed were not easily detected with this technique due to the intermolecular interactions that existed between the aroma and the matrix. Proteins and aldehydes are known to form non-covalent interaction via reversible physical adsorption. Flavour compounds bind to protein only when binding sites are available; that is, if the sites are not engaged in protein-protein or other interactions. covalent bonds (Wang, Xie, Ren, Yang, Xu, & Chen, 2011). A proportion of food aldehyde thus becomes unavailable for release during consumption. During mastication of frozen desert, the presence of mucins in saliva (which are heavily glycosylated proteins) and alpha-amylases was

shown to result in a reduction in the release of these aldehydes.

Technical optimization for measuring in-nose/ in-mouth volatiles was studied. The signal to noise ratio of the hydrophilic aldehyde volatile was subsequently improved by at least five-fold when the length and temperature of the transfer line, which appeared to have the greatest impact on the response signal, were optimised. Shortening the transfer line to 10 cm and heating it to 150 °C increased the airflow rate to 400 min/ ml. The detection limits of breath signals of the aldehyde in water was then more easily determined at 200 ppm for in-mouth and 600 ppm for in-nose.

Capability of APCI-MS as a diagnostic tool for establishing the provenance of apple juice and the age of cheese was investigated. The development and validation of mathematical models of flavour release with greater predictive power was shown to be greatly facilitated using APCI-MS, which could enable reductions in the resources required for product development.

Profiling or fingerprint matching methods often offer powerful tools for forensic applications. The complex nature of chemical profiles provides a specific fingerprint for food fraud prevention if reproducible. APCI-MS could be used for the monitoring of food authenticity with the validation using GC-MS; it also proved effective as a novel tool for the classification (based on geographical and botanical origin) of a foods volatile compliment, using a real food (clarified apple juice) with broad commercial diversity as an exemplar. When combined with multivariate statistical technique such as PLS-DA chemometric data pretreatment,

the models generated based on headspace data, were robust enough to reliably discriminate apple juices prepared from Braeburn, Golden Delicious, Granny Smith, Jazz and Pink Lady varieties. Furthermore, discrimination of the geographical provenance of monovarietal-clarified apples from Chile, New Zealand and South Africa achieved 94 % accuracy.

An extension to evaluate the soft modelling approach based on chemometric analyses of the chromatographic profiles of real food with broad commercial diversity was using chromatographic techniques, such as GCMS and direct injection APCI-MS, to identify and characterise the aroma volatiles of commercial Cheddar cheese volatiles during different stages of ripening. With the PLS1 chemometric approach, it was possible to classify and predict the age of the Cheddar cheeses on the basis of their headspace data. This might present an opportunity whereby expensive and time-consuming sensory panels or cheese graders could be supplemented or replaced.

In conclusion, the extensive application of the APCI-MS headspace analysis in characterisation of different food systems, and the monitoring of flavour delivery in vitro and in vivo under both static and dynamic conditions, not only demonstrated the complexity of aroma-matrix interaction but also the potential of this technique as a rapid non-destructive analytical tool for quality control.

Future work will involve the measurement of water diffusion directly and evaluating diffusion coefficients of ethyl butyrate over a range of fructose concentrations and in the presence of FOS to gain better

understanding of solvent-matrix-aroma interactions. Mathematical models of flavour release can be more thoroughly studied using optimised APCI-MS measurements, with more panels consuming a variety of natural food; to enable manufacturers to appreciate the performance and release of the food flavours in their new products. Predictive models derived for apple juices and Cheddar cheeses could be further validated with produce of different seasons to constitute higher repeatability and reproducibility of results before routine use in the food industry could become a reality.

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