TEA FLAVOUR:

DELIVERY AND PERCEPTION

by

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ABSTRACT

A novel method was developed, enabling volatile release from mugs of freshly prepared, hot black tea infusions to be monitored online using atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). Given the number of volatile compounds contributing to the aroma of black tea infusions, and the one-dimensional nature of the APCI-MS technique, it was necessary to determine which ions present on APCI-MS spectra corresponded to which volatile compounds present in tea headspace.

Assignment of APCI ions to specific compounds was achieved by gas chromatography of black tea infusion headspace with simultaneous electron ionization and APCI-MS detectors (GC-EI/APCI-MS). Using this approach, 15 ions were selected for future monitoring, assigned to compounds with varying levels of confidence. Six ions were unequivocally assigned to individual compounds, so monitored and quantified with certainty. In other cases, it was only possible to assign ions to groups of compound, as was the case for isobaric compounds such as heptanal and heptanone (*m/z* 115), or stereoisomers such as *E*-2-heptenal and *Z*-4-heptenal (*m/z* 113). In some cases, although ions could be assigned to compounds, some unknown impurities were also present. The compounds represented by the 15 ions covered a range of physicochemical properties, sensorial significance, formation mechanisms, and odour properties. The analytical system was shown to yield reproducible data, with confidence variation values generally less than 5 %.

The effect of infusion preparation method on volatile release from black tea infusions was determined using this novel method, where three variables; infusion water temperature, infusion concentration, and infusion duration were investigated. Infusion water temperature and concentration were shown to exhibit the greatest effects, the higher the temperature and concentration, the greater the release into the headspace.

The effect of infusion water temperature was shown to be compound dependent, with differences in release partly explained by differences in physicochemical properties affecting extraction out of the leaf matrix into the aqueous phase. It was

suggested that there was very efficient extraction of some compounds such as the more polar, water soluble Strecker aldehydes (2-methyl propanal, 2- and 3-methyl butanal), and less efficient of the more hydrophobic compounds such as β-damascenone and β-ionone. Location of compounds within the leaf matrix, and additional formation of some compounds (e.g. dimethyl sulfide and the Strecker aldehydes) during the infusion process were also thought to play a key role.

The significance of these results for tea consumers was explored based upon the orthonasal aroma discriminability of infusions prepared according to different methods. Utilising a signal detection theory approach, values of d' were obtained for pairs of infusions, prepared using a range of infusion water temperatures and concentrations. Differences in orthonasal aroma caused by differences in preparation method could to a large extent be detected by consumers. Values of d' ranged from 0.08 to 3.26, indicating a range in the magnitude of stimulus differences between different pairs. The ratio of the difference in infusion concentration played a key role in discriminability of samples (e.g. 0.25 vs. 0.5 %w/v d' = 1.34 cf. 1.75 vs. 2.0 %w/v d' = 0.08), although insufficient data were available to be entirely conclusive. In addition, pairs prepared with higher temperature water (90 vs. 100 °C) appeared more discriminable (d' = 1.58) than those prepared with lower temperature water (40 vs. 50 °C, d' = 0.95). These results supported the theory that a d' of 1.0 reflects a 'just noticeable difference' in perception, this value having to be exceeded before differences can be detected.

Given the effect infusion preparation method has on volatile and non-volatile composition of infusions, sensory analysis utilising a quantitative descriptive analysis (QDA) approach was carried out investigating the presence of perceptual interactions between the specific attributes; aroma, bitterness and astringency. Use of a trained panel showed no evidence to support the presence of perceptual interactions between these attributes. This was thought to be due to a combination of factors, including the nature of samples, subjects, and test procedure. Use of consumers revealed a possibility of bitterness suppression caused by black tea aroma, although results were not conclusive.

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I hope that everyone enjoys reading this thesis as much as I have enjoyed writing it.

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1 INTRODUCTION

1.1 RESEARCH OVERVIEW

This thesis explores some key aspects important to black tea flavour, its delivery and perception. The main aim of this study was to explore the volatile component of black tea flavour; how it is affected by infusion preparation method, and how its perception can modify or be modified by other aspects of flavour important to black tea infusions, namely taste and mouthfeel.

The first objective of this research (described in chapter 2), was to develop an appropriate method for studying the release of a selection of important volatile compounds from mugs of freshly prepared, hot, black tea infusions using a realistic system, so as to represent the situation genuine tea consumers would perceive through the orthonasal "sniffing" route. This novel method was utilised in a series of experiments (described in chapter 3), where the objective was to investigate the effect black tea infusion preparation method had on release of these volatile compounds. Chapter 4 explores the relevance of these results in terms of the real-life situation, where the orthonasal discriminability of tea infusions prepared according to different preparation methods was determined using a signal detection theory approach. Perceptual interactions between black tea aroma and key taste and mouthfeel attributes important to overall black tea flavour is the subject of chapter 5, carried out using a series of sensory evaluation tests.

The current chapter introduces some key concepts and background material essential for the understanding of the work carried out in this study. Each subsequent chapter describes a discrete set of experiments, with introduction, materials and methods, results, discussion and future work sections. An overall conclusion, bringing together the results and key findings of all other chapters is presented in chapter 6.

1.2 FLAVOUR

Although the layman often uses the words “taste” and “flavour” interchangeably, flavour as defined by the British Standards Institute is “the complex combination of the olfactory, gustatory and trigeminal sensations perceived during tasting. The flavour may be influenced by tactile, thermal, painful and/or kinaesthetic effects” (BSI, 1992). The following section focuses on the senses of smell and taste, although the sensation of astringency has also been included given its importance in the work to follow.

Tastants and odorants are compounds grouped under the general term “flavour-active molecules”, and whilst “taste” is the sensation induced by tastants, “aroma” (or odour or smell) is the sensation induced by odourants. Some of these terms are used interchangeably, and for the purpose of this thesis the term “aroma” has been used to describe the sensation induced by odourants, be it orthonasally or retronasally. The only exception being in the case of defined words such as “odour threshold” or “odour activity value”.

1.2.1 Olfaction (sense of smell)

The sense of smell occurs when odourants stimulate olfactory receptors in the nasal cavity. This can occur in one of two ways; either orthonasally, when odourants enter the nares during inhalation (or sniffing), or retronasally, during chewing or eating when they travel from the back of the nasopharynx towards the roof of the nasal cavity (Mennella and Beauchamp, 1997).

The olfactory system is reportedly able to distinguish between at least 10,000 different volatiles (Barry et al., 1999), and not surprisingly retronasal olfaction, contributes significantly to the overall perception of flavour (Rozin, 1982). This is well illustrated by the loss of flavour that occurs when olfactory receptors are blocked during a head cold. Whilst threshold concentrations vary, many odourants can be detected at picimolar concentrations (10^{-12} mol/L).

The sensory organ for the sense of smell is the olfactory epithelium, covering an area around 5 cm^2 in the roof of the nasal cavity (Bray et al., 2000). This epithelium contains specialised bipolar receptor cells known as the olfactory receptor neurons, of which around 10^7 are present in humans (Barry et al., 1999).

At the apical region of each neuron is a modified dendrite which ends on the surface in a knob bearing 15-30 long, non-motile cilia embedded in a layer of mucus (Bray et al., 2000). Air-borne volatiles become dissolved in this mucus layer, and as they pass along the olfactory epithelium, bind to specialised receptor cells present on the ciliary membrane. The olfactory structures are illustrated below in figure 1-1.

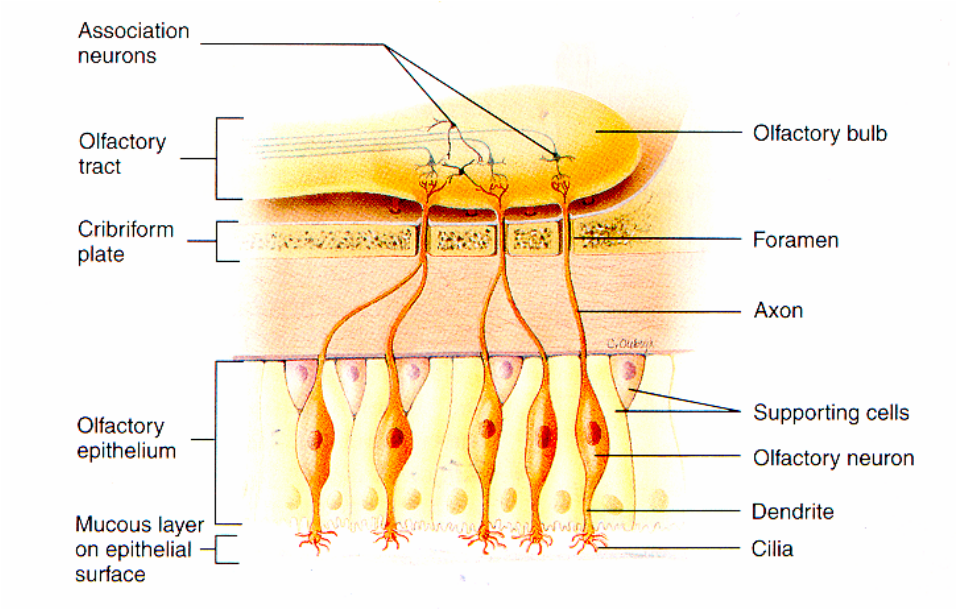


Figure 1-1 - Graphical representation of the olfactory structures - from Seeley et al. (1999)

Binding of an odourant to a receptor cell results in a signal cascade involving one of two second messenger systems; the adenosine 3',5'-cyclic monophosphate (cAMP), and inositol 1,4,5-triphosphate (IP_3) systems. Odourant binding to the cAMP type receptor (the more well known mechanism) causes a membrane bound G-protein to bind to, and activate a membrane-bound adenylate cyclase that catalyses the formation of cAMP from ATP. The cAMP then diffuses and binds to a cyclic nucleotide-gated protein channel, causing it to open, allowing Na^+ into the cell (resulting in depolarisation). The precise role of the IP_3 messenger system is not so well established, although it is thought that on binding to IP_3 type receptors, membrane-bound G-proteins bind to a phospholipase C, catalysing the formation of

IP_3. This causes opening of Ca^{2+} channels and Ca^{2+} activated K^+ channels, again causing depolarisation) (Barry et al., 1999).

The electrical signals generated are transported through axons, which pass in bundles though the foramina of the cribriform plate to the olfactory bulb where they terminate in spherical glomeruli (Bray et al., 2000). The information is passed onto the olfactory cortex of the brain via olfactory tracts where it is finally decoded (Seeley et al., 1999).

Genetic studies by Buck and Axel (1991) suggest the presence of around 1000 different volatile receptor cells, with each olfactory receptor neuron expressing only one type (Axel, 1995). It is also known that an odourant may activate either a single receptor, or many different receptors. Likewise, a receptor may be very specific (only being activated by a few odourants), or recognise a variety (Araneda et al., 2000).

1.2.2 Gustation (sense of taste)

Given that it is the last checkpoint before ingestion of potentially harmful substances, sense of taste is very important for survival and well-being. This is especially true for bitter taste, due to the high bitterness of many toxic plant metabolites (Behrens and Meyerhof, 2006). Current knowledge suggests the presence of five basic tastes; sweet, sour, salty, bitter and umami (Behrens and Meyerhof, 2006).

1.2.2.1 Taste buds

Taste receptor cells (a type of chemoreceptor) lie within specialised onion-shaped structures known as taste buds, which although mostly located on nipple-like protuberances called papillae on the tongue surface, are also found on part of the palate, epiglottis, larynx and pharynx (Behrens and Meyerhof, 2006). There are three types of taste bud-containing papillae (figure 1-2). The fungiform (mushroom-like) papillae are pinkish spots located on the front part of the tongue. Twelve larger circumvillate (wall-like) papillae are located at the back of the tongue, whereas foliate (leaf-like) papillae are found on the sides of the rear of the tongue (Rawson

and Li, 2004). Taste buds are 50-70 µm in diameter (Bray et al., 2000), are located just below the surface epithelium and communicate with the surface via a small opening called the taste pore (Pocock and Richards, 2006) through which finger-like microvilli project (Smith and Margolskee, 2001). The number of taste buds is variable, although in humans, numbers have been shown to vary between 3000 and 10,000 (Guyton and Hall, 1996).

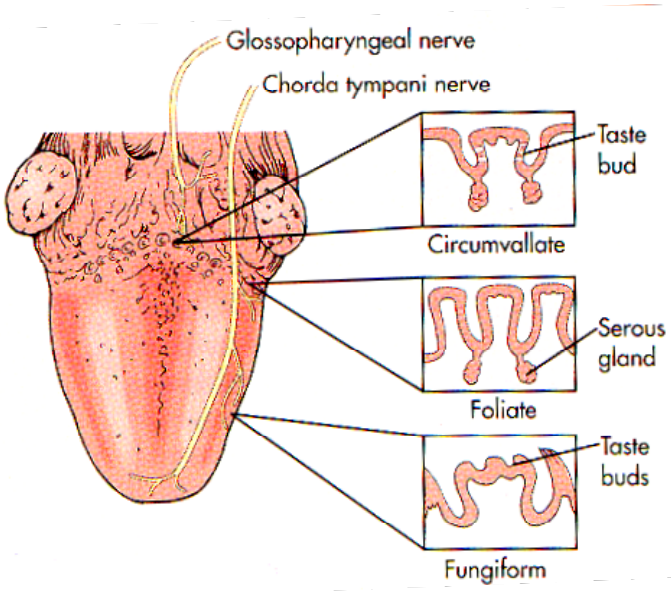


Figure 1-2 - Arrangement of the taste buds on the three types of papillae – from Berne and Levy (2000)

It is worth pointing out the misleading nature of the widely published "tongue map" showing large regional differences in sensitivity across the tongue. In reality, although slight differences in sensitivity do exist, all taste qualities can be elicited from all regions containing taste buds (Smith and Margolskee, 2001).

1.2.2.2 Taste transduction

Taste transduction begins when the stimulus comes into contact with the taste receptor cell. When tastants dissolved in saliva contact the taste cells through the taste pores, they interact either with taste receptors on the surface of the cells, or with ion channels (Rawson and Li, 2004). These interactions cause chemical changes in the taste cells, ultimately resulting in electrical impulses being sent along

either the chorda tympani nerve (anterior tongue), or glossopharyngeal nerve to the brain (Smith and Margolskee, 2001).

Whereas perception of salty and sour tastes involves interaction of taste stimuli with ion channels of the cell membrane, by contrast, perception of sweet, bitter and umami tastes involves membrane receptor proteins coupled to intracellular signalling cascades (Schiffman, 2000, Rosenzweig et al., 1999, Gilbertson et al., 2000). The following paragraph provides a very brief overview of the transduction mechanisms for the basic tastes, salty, sour, sweet and umami (Davies et al., 2001, Smith and Margolskee, 2001), more detailed descriptions can be found elsewhere e.g. Brand (1997). Bitter taste, which is of most relevance to tea, is then described in more detail. In all cases, however cell depolarization leads to an increase in intracellular Ca^{2+} within the taste receptor cell, and release of neurotransmitter (Bray et al., 2000).

The simplest transduction mechanism corresponds to salty taste which does not require any specific membrane receptors. Instead, there are passive Na^+ channels on the microvilli though which Na^+ enters the receptor cell, depolarizing it. The intensity of sourness is dependent upon H^+ concentration. Hydrogen ions act on taste cells in three ways: by directly entering the cell; by blocking K^+ channels on the microvilli; and by binding to and opening channels on the microvilli, allowing other positive ions to the enter the cell which in turn depolarizes the cell. Sweetness depends upon the binding of sugars to specific membrane receptors that are coupled to molecules called “G-proteins”. This binding prompts G-protein subunits to split, activating nearby enzymes. This leads to an elevation in the concentration of the second messenger cyclic AMP (cAMP) reducing K^+ conductance, so producing depolarization. Compounds eliciting the umami taste are also known to bind to G-protein-coupled receptors and to activate second messengers, although the intermediate steps are currently unknown.

Figure 1-3 is a schematic diagram illustrating the transduction mechanisms for the four main basic tastes (sweet, sour, salty and bitter)

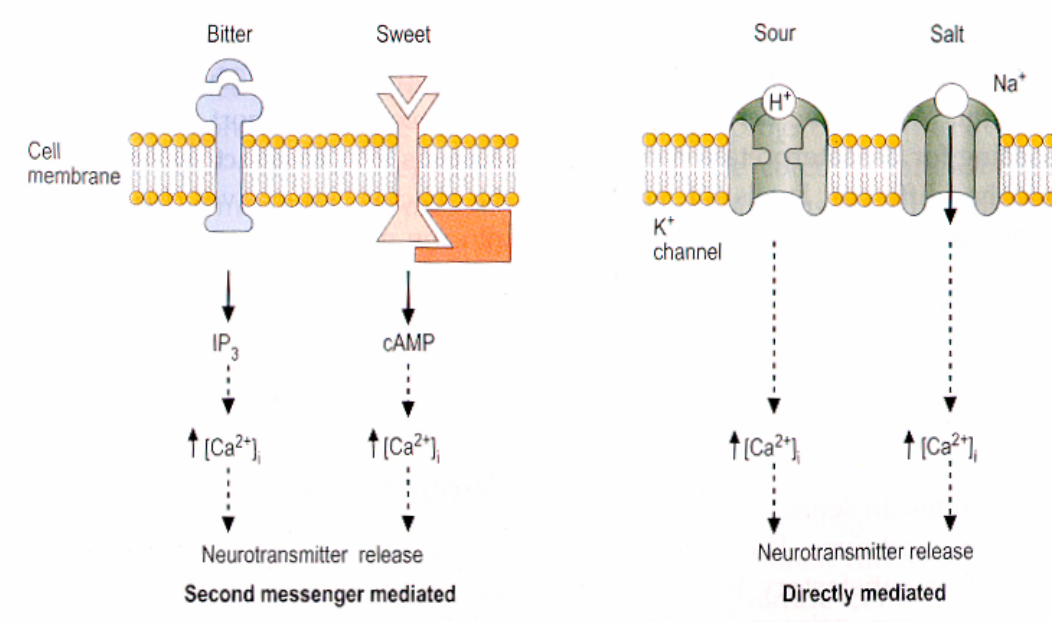


Figure 1-3 - Schematic diagram illustrating the basic taste transduction mechanisms for the four main basic tastes - from Davies et al. (2001)

1.2.2.3 Bitterness

It is believed that sweet and bitter tastes are closely related, and that they may share common transduction pathways (Schiffman, 2000), a view supported by evidence that small changes in structure can convert sweet compounds to bitter ones (and vice versa) (Walters, 1996). There are many different types of bitter compound encompassing a wide range of molecular size and functional groups including amino acids and peptides, esters and lactones, phenols and polyphenols, flavonoids and terpenes, methylxanthines, sulfimides, and organic and inorganic salts (Rouseff, 1990). In light of this structural diversity, the presence of multiple transduction mechanisms is suggested (Brand, 1997, Schiffman, 2000), and it is reported that of the basic tastes, bitter is the most complex and least understood (Drewnowski, 2001).

As with sweet and umami, bitter stimuli also act through G-protein-coupled receptors, with second messengers such as cAMP and inositol triphosphate (IP_3) acting on targets within the cell (Herness and Gilbertson, 1999). It is thought that one pathway involves a G-protein that stimulates enzyme-activated IP_3 (as

illustrated above in figure 1-3). An alternative pathway is thought to involve α-gustducin, that activates the enzyme phosphodiesterase to decrease intracellular cAMP (McLaughlin et al., 1992). As with the other taste mechanisms, the second messengers cause the release of calcium ions from the endoplasmic reticulum, the resulting build-up leading to depolarization and neurotransmitter release. A detailed review of bitter transduction can be found elsewhere (Behrens and Meyerhof, 2006).

Bitterness is generally considered a disagreeable taste, not surprising given that many toxic plant metabolites are bitter, and the corresponding bitter receptor molecules serve an indispensable role as warning sensors (Behrens and Meyerhof, 2006). However, in certain foods (including tea), a certain amount of bitterness is expected and enjoyed (Drewnowski and Gomez-Carneros, 2000). It is well known that the ability to perceive some bitter tastes varies greatly across individuals (Drewnowski, 2001). Studies on bitter sensitivity have focussed on two bitter chemicals; 6-n-propyl-2-thiouracil (PROP), and Phenylthiocarbamide (PTC). It has been observed that a proportion of the population are extremely sensitive to PROP, and are termed “supertasters” (Bartoshuk, 1994). It has also been shown that most PROP tasters give high bitterness ratings to caffeine solutions (Ly and Drewnowski, 2001), and are more sensitive to caffeine aftertaste (Neely and Borg, 1999). On the other hand, caffeine-insensitive respondents are more likely to be PROP non-tasters. It has been shown that supertasters have more fungiform papillae and a higher density of taste buds per papilla (Miller and Reedy, 1990). It has however been cautioned that the perceptual relationship of bitter tastants to PTC and PROP taster status is inconclusive, with several studies on caffeine and quinine showing contrasting correlation results (Schifferstein and Frijters, 1991).

1.2.2.4 Astringency

The concept of astringency has been described as one of the least understood sensory attributes, both by consumers and scientists alike (Bakker, 1998). In its simplest form, it has been described as a dry, rough and puckering feeling in the mouth (Lawless et al., 1996). The American Society for testing and materials (ASTM) committee on the sensory evaluation of materials and products defines astringency as “the complex sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums and tannins” (ASTM, 1989). The chemical definition of astringency is “the ability to precipitate

proteins" (Ishikawa and Noble, 1995). The term astringency is derived from the Latin phrase *"ad stringere"*, meaning "to bind" (Bakker, 1998).

Astringent compounds include polyphenolics (Sarni-Manchado and Cheynier, 1999) organic acids (Lawless et al., 1996), and salts of the multivalent cations (Al, Cr, Zn, Pb, Ca, and B) of which aluminium sulphate (alum) is the most well known (Peleg et al., 1998). Astringency is an important sensory attribute in foods, and examples containing polyphenolics, by far the largest group (and most important for the subject of this thesis), include tea, coffee, wine, apples, cider, berry crops and nuts (Haslam and Lilley, 1988). It has been suggested that, (similar to bitterness), astringency constitutes a form of protection mechanism for plants. Plants produce high levels of (astringent) polyphenolic compounds in certain areas to avoid being eaten (e.g. unripe fruits before seeds are ready) (Breslin et al., 1993).

The senses of touch, pain and temperature are monitored by the trigeminal nerve, whereas the facial, glossopharyngeal and tympani innervate, the taste buds (Clifford, 1997). Historically, astringency has been described as one of the basic tastes (Bartoshuk, 1978), a theory supported by identification of signal transduction involving the chorda tympani nerve in studies involving rodents (Schiffman et al., 1992), and primates (Critchley and Rolls, 1996).

It is however more generally accepted that astringency is in fact a tactile sensation, and whilst debate still exists over the precise mechanisms involved, it is thought to arise from increased friction / reduced lubrication in the mouth transduced by the trigeminal nerve (Green, 1993). Physiological evidence that astringency is a tactile sensation came from the finding that subjects reported a feeling of astringency when tannic acid solution were applied under the upper lip (an area devoid of any taste receptors) (Breslin et al., 1993). The theory is also supported by the fact that there is a failure to adapt on repeated exposure as occurs with taste and smell sensations, instead there being a pattern of partial recovery and build-up in intensity with repeated exposure (Guinard et al., 1986).

Saliva is a diluted aqueous medium whose main function is to form a lubricating layer on the surface of the mucous membranes within the mouth. Proline-rich proteins (PRPs) comprise around 70 % of the proteins present in human saliva (Clifford, 1997, Kaufman and Keller, 1979), and are thought to serve functions of wetting, lubrication and protection of the oral epithelium. It is widely thought that the

perception of astringency is related to the capability of astringent compounds to bind and precipitate these salivary proteins (Bate-Smith, 1973, Peleg et al., 1998). It has been suggested that the binding of salivary proteins to polyphenols may serve to sequester or inactivate polyphenols, so protecting the alimentary tract from their deleterious effect on nutritional uptake (Bacon and Rhodes, 2000). This binding results in a decrease in lubrication and subsequent increase in friction between the mucosal surfaces in the mouth, detected by mechanoreceptors (Breslin et al., 1993, Smith et al., 1996). Support for the theory of delubrication comes from work carried out by Prinz and Lucas (2000) who showed increases in coefficients of friction when tannins were added to saliva, attributed to precipitation of the PRPs. Kallithraka et al. (1998) also provided evidence supporting the role of salivary proteins by showing changes in chromatographic profile of salivary constituents following stimulation with polyphenolic compounds. As well as a decrease in the lubricative properties of saliva, it has also been suggested that the precipitate itself is sensed as particles that increase friction (de Wijk and Prinz, 2006).

Prinz et al. (2006a) suggest that astringents may affect the physical properties of the oral mucosa itself by denaturing proteins in the surface epithelium in a process analogous to that used during leather manufacture (tanning). It has also been suggested that a feeling of roughness in the mouth may be due to the presence of particles in saliva (Tyle, 1993). Aluminium salts such as alum are used in water purification to flocculate suspended particles, aggregating small (and possibly sub-threshold) particles to form larger agglomerations which may give rise to sensations of roughness (and astringency) (Prinz et al., 2006b).

Inevitably, salivary flow rate is important in terms of astringency perception, Fischer et al. (1994), and Ishikawa and Noble (1995), observing a slower onset and slower decay of the astringent sensation in subjects with low salivary flow rates. It has also been shown that subjects with low saliva flow rates evaluated astringency of black tea as significantly higher than high-flow subjects over eight successive sips (Noble, 2002). This is suggested to be due to the greater ability of higher volume of saliva to restore oral lubrication (Noble, 2002). It is thought that the astringent sensation is removed by salivation, which helps clear the mouth of polyphenols and/or provides new proteins to replace the precipitated ones. In contrast to the above results however, Peleg et al. (1999), investigating the astringency of mono- di- and trimers showed that high salivary flow subjects rated the I_{max} of astringency higher than low-flow subjects. The composition of saliva is also thought to be important, with some

workers showing lowered astringency responses amongst individuals with higher overall salivary protein content (Imm and Lawless, 1996). Others however, (Kallithraka et al., 2001, Guinard et al., 1997) found no such correlation, although Kallithraka et al. (2001) did observe significant correlations between the magnitude of specific protein fractions and astringency.

From a perceptual point of view there is little or no consensus as to whether astringency is a single sensation, or a general category made up of multiple sub-qualities (Lee and Lawless, 1991). Lawless et al. (1996) defined astringency as a complex perception arising from a combination of three separate sensations; drying, roughness and puckering. At the extreme level, subjects initially generated a list of 24 separate attributes in a study characterising the astringency of red wine (Gawel et al., 2001). This adds weight to the theory that more than one mechanism may be important. Bertino and Lawless (1993), who carried out a multidimensional scaling approach on mouthfeel attributes observed an overall similarity for the terms; astringent, puckering and drying, and it is likely that many subjects in the study by Gawel et al. (2001) were simply using different terms to describe identical sensations. However, reliable studies of the astringent sensation are made more difficult due to the confusion of bitterness and astringency by untrained observers (Lee and Lawless, 1991). This is at least partly due to the frequent co-occurrence of these two phenomena in nature (e.g. tea, beer), and the difficultly of obtaining pure astringent reference samples. Astringency and bitterness are both very persistent sensations and the intensity and duration of both attributes increase as concentration of polyphenols are raised in model solutions (Robichaud and Noble, 1990)

Correlation between PROP status and astringency perception is currently inconclusive, with some workers observing positive correlations (Pickering et al., 2004), others finding no significant effect (Ishikawa and Noble, 1995).

1.2.3 Taste-aroma interactions

One of the most important factors determining the overall perceived flavour of foods and beverages is the interaction of taste and aroma stimuli. Perceptual confusion between the senses of taste and smell is well known, flavour sensations often

perceived as being located solely in the mouth, rather than correctly as in the mouth and nose (Rozin, 1982). This was well illustrated by Murphy et al. (1977) who presented subjects with solution of ethyl butyrate to sip, with and without noseclips. Whilst subjects assigned intense "taste" ratings in the absence of noseclips, with their nostrils sealed the same subjects reported 80 % of the "taste" to have disappeared.

It is well documented that tastants can influence the perception of odourants and vice versa. Three key findings demonstrate the relationship between the sensations of smell and taste, all of which are closely linked, specifically, i) the attribution of taste qualities to aromas when sniffed, ii) the enhancement (or suppression) of taste intensity induced by aromas, and iii) the reciprocal – enhancement (or suppression) of aroma intensity by taste.

An important question is whether a genuine change in flavour release affects perception, or whether interactions occur at the neural (Rolls, 1997) or cognitive (Calvert et al., 1998) level. Multi-modal neurons which respond to both taste and olfactory inputs have been shown to exist in the brains of monkeys (Rolls and Baylis, 1994); and Rolls (1997) suggested that these multimodal neurons develop from unimodal neurons through learning of appropriate combinations of signals during repeated pairing of particular tastes and aromas. In many cases, the effect of tastants on aroma perception can be explained due to the effect of these solutes on phase partitioning (as discussed in section 1.4.1).

Two similar, yet frequently confused concepts are those of congruency and perceptual similarity. The subtle difference between the two can be seen using an analogy to clothing described by Schifferstein and Verlegh (1996). A pair of jeans is far more similar to a pair of shorts than to a jumper (i.e. are perceptually similar). Yet a pair of jeans goes far better with a jumper as part of a full outfit than does a pair of shorts (i.e. are more congruent). Sometimes making two stimuli more similar can actually make them less congruent. A pair of blue jeans and red jumper is likely go together far better than a pair of red jeans and red jumper.

Congruency describes the compatibility of certain aromas and tastes when combined, and appears to go a long way in explaining the presence of aroma induced taste enhancement, and the reciprocal; taste induced aroma enhancement. Schifferstein and Verlegh (1996) found that taste enhancement only occurred in

congruent combinations. Whilst the addition of strawberry or lemon aroma enhanced the sweetness of sucrose solution, addition of ham aroma did not.

The ability of aromas to enhance the sweetness of sucrose solutions was first reported by Frank and Byram (1988) who observed that addition of strawberry aroma to whipped cream containing sucrose enhanced the perceived sweetness. The fact that the enhancement effect disappeared when subjects were asked to pinch their noses suggested that the effect was not caused by any physical interaction between the odourant and sucrose present in the mouth. It has been widely reported that taste-aroma interactions are both tastant and odorant dependent. Whilst strawberry (Frank and Byram, 1988, Frank et al., 1989, Frank et al., 1993, Clark and Lawless, 1994, Schifferstein and Verlegh, 1996, Stevenson et al., 1999, Frank, 2002), vanilla (Sakai et al., 2001), lemon (Schifferstein and Verlegh, 1996, Frank, 2002), almond (Frank et al., 1993), caramel, maracuja and lychee (Stevenson et al., 1999) have been shown to enhance the sweetness of sucrose solutions, other aromas such as peanut butter (Frank and Byram, 1988, Frank, 2002), ham (Schifferstein and Verlegh, 1996), chocolate and wintergreen (Frank et al., 1993, Frank, 2002) have been shown to have no effect. Maltol, angelica oil and damascone aromas have even been shown to suppress the perceived sweetness of sucrose (Stevenson et al., 1999).

These so called taste-aroma interactions are not limited to sweetness. Whereas strawberry and lemon have been shown to enhance the sweetness of sucrose (Frank and Byram, 1988, Schifferstein and Verlegh, 1996) and sourness of citric acid solutions (Frank, 2002), they have been shown to actually suppress the saltiness of sodium chloride solutions (Shaffer and Frank, 1990). The role of congruency in terms of taste enhancement has been further supported in work by Djordjevic et al. (2004). Strawberry aroma was found to enhance perceived sweetness, but not saltiness, and soy sauce aroma was found in to enhance perceived saltiness, but not sweetness. In a study by Stevenson et al. (1999), caramel aroma was found to enhance the sweetness of sucrose, yet simultaneously suppress the sourness of citric acid. It was noted by Prescott (2004) that addition of sucrose to a solution of citric acid would have similarly resulted in a decrease in its perceived sourness. This suggests that the sweetness of the aroma may be behaving in the same way as genuine sweet taste, even though itself the caramel aroma does not stimulate the sweetness receptors.

As well as aroma induced changes in taste perception, the reverse is also true, with considerable evidence to support tastant induced changes in aroma perception. Addition of sucrose to blueberry and cranberry juice showed little effect on the orthonasal aroma profile, yet produced a significant shift in the retronasally perceived aroma profile, decreasing the intensity of unpleasant notes and increasing the intensity of pleasant notes (von Sydow et al., 1974). It has been reported by Bonnans and Noble (1993) that the intensity of a fruit aroma was enhanced both by an increase in level of sweetener and acid when tasted in solution. Cliff and Noble (1990) reported that whilst increase in sweetness of glucose solutions flavoured with peach essence failed to increase the intensity of fruitiness, the persistence of the retronasal aroma increased. In work by Kuo et al. (1993) it was shown that the addition of acid, or sugar and acid increased the perceived intensity and persistence of citral aroma. A similar finding was observed with vanillin and sugar, where addition of sucrose to a solution of sucrose and vanillin increased the intensity of perceived vanilla flavour. Cayeux and Mercier (2003) investigated the effect of acid on retronasal intensity of certain aromas, observing that in the case of congruent mixtures, intensity was increased in the presence of sour taste. The authors noted however that whatever the congruency of flavours, the aroma appeared to have no effect on either sourness or astringency.

Dalton et al. (2000) showed that olfactory sensitivity to benzaldehyde was increased by the presence of a sub-threshold concentration of (sweet) saccharin in the mouth. As with aroma induced changes in taste perception, congruency appeared to play a key role, the presence of a savoury monosodium glutamate solution not affecting perception of the aroma. This work was also important in that it showed for the first time that taste-aroma interactions were not limited to supra-threshold concentrations, also suggesting direct neural integration of these two modalities are involved in this case. In the other direction, Djordjevic et al. (2003) found that the detection accuracy of sucrose at around threshold level was improved by the addition of an orthonasally presented sweet smelling aroma such as strawberry. Presentation of ham aroma however had no effect.

Prior experience is the main factor behind whether specific taste-aroma combinations are considered congruent or not. Particular aromas become associated with particular tastes due to everyday experiences, a process termed ‘learned association’. Strawberry and vanilla for example tend to be associated with a sweet taste, whereas lemon is frequently also associated with a sour taste.

Stevenson et al. (1995), and Stevenson et al. (1998) studied the effect learned association had on attribution of taste qualities to aromas. In their experiments, two novel aromas (lychee and water chestnut) were repeatedly paired with either sweet or sour tastes in solution in a series of dummy experiments over a period of 5 days. When smelt in isolation, there was a subsequent increase in the smelled sweetness or sourness of the two aromas (compared to prior to the repeated pairing) suggesting that a perceptual relationship had been formed. In a similar study, Prescott (1999) showed that aromas that initially had no impact on sucrose sweetness (water chestnut) or suppressed sucrose sweetness (peanut butter), actually enhanced sweetness of sucrose in solution after a period of repeated exposure. This implies that the association of certain aromas and tastes can be learned. Prescott et al. (2004) has shown that as little as one co-exposure can be sufficient to form an association, subsequently creating an enhancement of sweetness. It is also interesting to note that cultural effects may have a large impact upon congruent combinations, and hence occurrence of specific taste-aroma interactions. Pumpkin aroma is considered sweet in the US due to its association with the sweet tasting pumpkin pie. Elsewhere, the aroma is considered savoury as pumpkin is more frequently associated with savoury dishes.

As well as congruency, the way in which the test is conducted and the cognitive strategy used by subjects has been shown to exert a major effect on enhancement effects. In general, two alternative strategies can be employed by subjects sampling products containing a taste and aroma component; synthetic or analytical. A synthetic approach implies focussing on all sample attributes simultaneously, and results in a merging of the various sensations into an overall flavour. An alternative, analytical approach implies dividing the overall flavour sensation into its sub-sensations, taking and rating each one separately. Whilst consumers generally use a synthetic approach, trained panellists tend to demonstrate a more analytical approach. In fact, one of the key reasons for training panellists is to force them to adopt the analytical approach.

Several studies have been carried out investigating the effect of panel training on the occurrence of taste-aroma interactions. Bingham et al. (1990) found that whilst untrained subjects perceived maltol-sucrose mixtures to be sweeter than sucrose alone, a trained descriptive analysis panel found no such enhancement. This finding is not universal, McBride and Finlay (1990) found no differences in judgements of complex stimuli between experienced and novice subjects.

Linked to training, another possible explanation for aroma-induced taste enhancement is one of confusion, where for example subjects become confused between the genuine sweetness of sucrose and perceived sweetness of strawberry aroma. It was suggested by Murphy et al. (1977) that enhancement could be explained on the basis of taste-aroma confusion by subjects. Stevenson et al. (2001) carried out a study in which the impact of confusion between tastes and smells on aroma induced taste enhancement was explored. Two experimental approaches were employed in an attempt to reduce confusion. One was stimulus pre-exposure in which subjects were given the chance to experience an aromas 'sweetness' in the absence of taste. A second was training in which additionally subjects were explicitly taught that sensations in the mouth may be composed of tastes, aromas, or combinations thereof. Four aromas were used in the study (strawberry, caramel, cherry and sweetness enhancer) and subjects were required to assess sweetness intensity of mixtures using rating and intensity matching procedures. Whilst enhancement of sweetness was observed in both the rating and matching tasks, effects of training and pre-exposure were found to be relatively small.

Subjects from both groups then took part in an additional matching test. Whilst the pre-exposure group received identical instructions to those previously, the trained group were however crucially asked to focus on the training they had received, and rate only the sweetness, ignoring any smell. It was found that trained subjects reported significantly less enhancement with pre-exposed than with non-pre-exposed aromas. The fact that training appeared initially to have no effect on taste enhancement was put down to the fact that the training used in this study was relatively short (2 sessions). It was only when trained subjects were specifically asked to separate out the taste and smell components that a reduction in taste enhancement was observed, and even here this reduction only occurred for those aromas with pre-exposure. In this study at least, it would appear that even with training, subjects were still employing a synthetic (rather than analytical) approach. This finding supports that of van der Klaauw and Frank (1996) who reported that subjects were able to minimise enhancement effects when their attention was specifically directed to specific attributes of the mixture.

Stevenson and Case (2003) carried out a similar study looking at the effect of stimulus pre-exposure, on the attribution of taste terms (sweet and sour) to orthonasally experienced aroma stimuli following a period of association. Pre-

exposure of taste and aroma stimuli in isolation as well as in combination with the tastants was shown to lead to the retardation of acquisition of sweet or sour qualities of the orthonasally presented aromas following a period of conditioning. Training procedures which included subjects being educated about the separate entities of taste and smell appeared to have no effect.

The halo-dumping phenomenon is a measurement artefact caused by the absence of appropriate rating categories. Unable to express a particular sensory quality, subjects tend to dump the attribute intensity onto an alternative scale. It was shown by Frank et al. (1993) that strawberry aroma enhanced the sweetness of sucrose solutions when sweetness was the only attribute rated. When subjects also rated saltiness, sourness, bitterness and fruitiness, the sweetness of sucrose solutions was suppressed. A similar finding was reported by Clark and Lawless (1994), observing that subjects rated the sweetness of strawberry and vanilla flavoured sucrose solutions higher when sweetness was the only attribute rated. Van der Klaauw and Frank (1996) carried out an experiment in which subjects were presented with samples of sucrose and quinine hydrochloride with and without strawberry aroma. It was found that enhancement only occurred when subjects rated only sweetness and bitterness. When fruitiness was added, the enhancement effect disappeared. The fact that enhancement effects were ambiguous when subjects were asked to rate the floralness of solutions very clearly illustrates the importance of ensuring that subjects are fully aware of precisely what they are supposed to be rating. Clearly, some subjects were taking the strawberry aroma to be classified in a floral category, others were not.

Whilst the halo-dumping effect has been shown to inevitably account for a certain amount of enhancement effects, it cannot be entirely responsible. Stevenson et al. (1999) argued that whilst halo-dumping may account for taste enhancement, it would not account for suppression effects. The ability of certain aromas to enhance the sweetness of a sucrose solution, yet decrease the sourness of a citric acid solution cannot be attributed to halo-dumping. The repeated observation that only certain (congruent) aromas enhance taste characteristics also argues against halo-dumping being entirely responsible for taste-aroma interactions.

1.3 TEA

Tea is a fragrant brew prepared from the leaves of the plant *Camellia sinensis,* and depending upon the manufacturing process used can be broadly divided into three main groups; green (unfermented), oolong (partially fermented) and black (fully fermented).

According to Chinese mythology, tea as a beverage was discovered in the year 2737 BC by the Emperor Shen Nung when tea leaves accidentally fell into a pot of boiling water (Harbowy and Balentine, 1997). The worldwide popularity of tea both as a beverage and as a medicinal drink is unquestionable, and is currently the most popular beverage in the world, with an estimated 3 billion cups drunk worldwide every day. Annual consumption of tea lies at 2.84 kg per capita in the UK (Chen, 2002), this corresponding to an approximate average daily consumption of 4.4 (180 mL) cups per person. This popularity is not only due to teas effectiveness at quenching thirst, but also due to its unique sensory properties, stimulating effects, and availability. The effect of tea on human health has been subject to considerable attention for many years, particularly with regards to claims of beneficial effect on cancers and cardiovascular disease (Gardner et al., 2007). Both the volatile and non-volatile components of tea infusion play critical roles in the overall flavour of the beverage, with considerable interactions between the two areas.

1.3.1 Black tea manufacture

The tea manufacturing process consists of a number of stages, described below and summarised schematically in figure 1-4.

The withering stage represents one of the most important stages in tea processing, particularly with regards to aroma development. During the withering stage, the moisture content of leaf is reduced from 75-80 %, down to around 55-70 %; a process usually taking between 12 and 16 hours. This causes concentration of polyphenols in the leaves, and deterioration of leaf structural integrity.

The rolling stage (also known as cutting, crushing and tearing) involves a leaf size reduction, with a degree of cell disruption to allow the exposure of the new surfaces

to air. The process used to macerate the leaf plays an important role in determining the final grade of tea; orthodox and CTC (crush, tear, curl) being the two most commonly employed methods.

Following the rolling stage, the leaf is allowed to contact the surrounding air for a period anywhere between 40 minutes and 3 hours, (depending upon the specific process and temperature used). The fermentation process results in the oxidation of simple polyphenols to more complex polyphenols, extremely important in providing black tea with its characteristic flavour (discussed further in section 1.3.3). The fermenting mass is referred to as “dhool”, and its degree of fermentation largely determines the flavour characteristics of the finished product.

It is extremely important to stop the fermentation process at its optimum point, and this is the role of the drying (also known as firing) stage. The moist fermented leaf particles are exposed to a stream of heated air, reducing the moisture content from 60-72 % down to 2.5-3.5 %, bringing about enzyme inactivation or destruction, necessary for the preservation of the product. This procedure is accompanied by several chemical transformations, responsible for some of teas important flavour characteristics.

Leaf leaving the dryer consists of a mixture of different particle sizes and includes a proportion of stalk and fibre. Sorting equipment consists of stalk and fibre removal apparatus, combined with machinery for separating the different sized tea particles into evenly sized portions. The tea is evaluated for flavour and infusion colour by professional tea tasters who purchase and blend tea based on its sensory characteristics. Tea tasters (like tasters of coffee and whisky) pay very little attention to instrumental analysis of components.

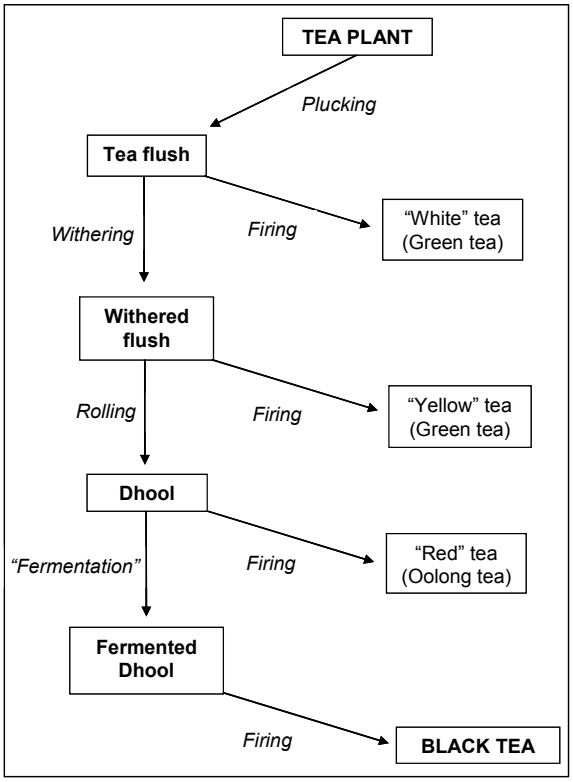


Figure 1-4 - Key stages of the tea manufacturing process - adapted from Harbowy and Balentine (1997)

1.3.2 The volatiles of black tea

Aroma is considered by some to be the most important factor contributing to the quality of tea (Constantinides et al., 1995). Although research into the aroma of tea can be traced back over 160 years, it is only since the 1960's, with the advent of modern techniques that progress on a more scientific basis has been made. The vast majority of this work has made use of gas chromatography, utilising a wide range of sample preparation techniques. Amongst others, these have included conventional steam distillation, used by Yamanishi et al. (1972) investigating the volatile composition of Ceylon tea, and Solvent extraction used by many workers, including Saijo (1967), investigating the volatile composition of black tea essential oil. An alternative technique is that of simultaneous distillation extraction (SDE), utilised by Saijo and Takeo (1973), looking at fresh tea leaves, and Kawakami et al. (1995) looking at volatiles in black tea infusions. Supercritical fluid extraction (SFE) was first used by Vitzthum et al. (1975), who identified 56 constituents of black tea

aroma. Headspace analysis has been widely carried out, both in static and dynamic form: Static headspace analysis was carried out by Heins et al. (1966) in order to identify aroma components from dry tea leaves, whereas dynamic headspace analysis was employed by Clark and Bunch (1997), who used a purge and trap method in order to investigate volatile acids.

The examples selected above, illustrate only a fraction of the work carried out on identification of compounds responsible for black tea aroma, and many hundreds of other examples exist (Constantinides et al., 1995). A recent assessment of all available data has put the total number of volatiles at over 630 (Wang et al., 2002), although it should be remembered that this list comprises those compounds isolated from all types of tea, in fresh leaf, made tea, and tea infusions. It is also important to consider that some of the extraction methods utilised by workers may well have resulted in the development of additional artefact aromas. During harsh techniques such a steam distillation and simultaneous distillation extraction, glycosides can be easily hydrolysed, lactone structures easily opened, and ketones formed as degradation products (Wang et al., 2002).

According to Sanderson and Graham (1973), the aroma compounds in tea can be broadly classified into those biosynthesised in the leaf, and present in the fresh green leaf (primary), and those that are formed during the manufacturing process (secondary). Examples of primary compounds include *n*-hexanol, *Z*-3-hexen-1-ol, *E*-2-hexen-1-ol, linalool (and its oxides), and geraniol (Saijo and Takeo, 1973). The vast majority of volatiles however belong to the second category, formed mainly from glycosides, amino acids, unsaturated fatty acids, and carotenes.

There has been considerable speculation on the mechanism of formation of monoterpene alcohols during the manufacturing process. It was originally thought that linalool was a product of carotene degradation (Sanderson et al., 1971). It was later suggested by Selvendran et al. (1978) that the monoterpene alcohols were produced from oxygenated isoprenoid hydrocarbons. The most recent theory was first suggested by Takeo (1981) who demonstrated that linalool and geraniol were hydrolytic breakdown products of β-D-terpene glycosides. Glycosidic aroma precursors are not limited to monoterpene alcohols, and to date, those (mainly β-primeverosides) of geraniol (Guo et al., 1993), linalool (Guo et al., 1994), methyl salicylate (Moon et al., 1996), linalool oxides I-IV (Moon et al., 1996, Moon et al., 1994) and benzaldehyde (Guo et al., 1998) have all been identified in tea.

Takeo (1981) proposed that the hydrolysis of glycosides by endogenous enzymes is the most plausible mechanism for the formation of alcoholic tea aroma, and that aroma compounds are released during the process of withering, rolling and fermentation (Wang et al., 2000, Wang et al., 2001a, Wang et al., 2001b). It has been suggested by Mizutani et al. (2002) that the β-primeverosidase is localized in cell walls, whilst the aroma precursor primeverosides are separately present in vacuoles.

Another key source of volatile compounds in tea is through conversion of amino acids to their respective Strecker degradation aldehydes, with valine, isoleucine, leucine and phenylalanine converted to 2-methyl propanal, 2-methyl butanal, 3-methyl butanal, and phenylacetaldehyde respectively (Co and Sanderson, 1970). The conversion is catalysed by the strongly oxidising quinines, which are formed by enzymic oxidation of the catechins by polyphenol oxidase during the withering and fermentation stages of manufacture (Saijo and Takeo, 1970). An interaction between amino acids and sugars can also result in the production of furans, pyrroles and pyrazines during the firing stage of manufacture though Maillard reactions (Yamanishi et al., 1989b).

Aldehyde volatile compounds are also known to originate from the oxidative breakdown of fatty acids during processing. Investigations using macerated tea leaves (Hatanaka and Harada, 1973) and isolated chloroplasts (Sekiya et al., 1976) have shown that the enzyme system in chloroplasts catalyses the oxidative splitting of linolenic and linoleic acids to *Z*-3-hexenal and hexanal respectively. The *Z*-3-hexenal is partly reduced to *Z*-3-hexenol by alcohol dehydrogenase, and partly isomerised into *E-2*-hexenal by isomerase, which in turn is reduced to *E*-2-hexenol (Robinson and Owuor, 1992). A similar mechanism has been shown to account for the production of heptanal from palmitoleic acid.

Formation of aroma compounds from carotenes occurs via oxidative enzymatic reactions during withering and fermentation, and pyrolytic reactions during firing (Sanderson and Graham, 1973). β-ionone is known to be a major degradation product of β-carotene (Tirimanna and Wickremasinghe, 1965), and it is believed that β-damascenone is also derived from the carotenoids (Renold et al., 1974, Ravichandran, 2002).

Several attempts have been made to find a way of assessing the quality of tea based upon the aroma composition as determined by gas chromatographic techniques. The "Wickremasinghe-Yamanishi" ratio (Wickremasinghe et al., 1973, Yamanishi et al., 1978) is based on the sum of peak areas of compounds eluting before linalool to those eluting after. The "Owuor's flavour index" (Owuor et al., 1986b) is the ratio of the sum of peak areas of the sweet /flowery aromas (group I), to the green/grassy aromas (group II). The "Mahanta ratio" (Mahanta et al., 1988) is the ratio of the terpenoids to non-terpenoids, whereas the "Yamanishi-Botheju ratio" (Yamanishi et al., 1989a) is the ratio of peak area of linalool to *E*-2-hexenal. A comparison of these profiling methods was made by Owuor (1992), who observed differences in correlation between to tea tasters evaluations when assessing Kenyan black tea using the different methods.

Despite numerous attempts to identify key compounds responsible for the characteristic aroma of black tea, to date, no single compound, or group of compounds has been identified as being entirely responsible. Whilst possible that a currently undiscovered compound may hold the key to black tea aroma, it is far more likely that the characteristic aroma depends upon a delicate balance of a whole host of trace volatile constituents, as well as several critical compounds.

Despite the large number of volatile compounds, it is clear that not all are contributing to the characteristic aroma, Grosch (2000) estimating that <5 % of volatiles found in foods actually contribute to the aroma. One of the best ways of establishing the importance of a volatile compound to the aroma of a food is to calculate its odour activity value (OAV, ratio of concentration to odour threshold in air) (Grosch, 1993). Only those volatiles giving OAVs >1 will make a significant contribution to the aroma, and it is assumed that the higher the OAV, the greater the contribution of the volatile to the overall aroma (Guth and Grosch, 1999).

Dilution to threshold techniques such as aroma extract dilution analysis (AEDA) are often used as a screening method to identify those volatiles potentially contributing to the aroma of foods prior to calculation of OAVs (Grosch, 1993). An extract obtained from the food is diluted, usually as a series of 1:1 or 1:2 dilutions, and each dilution is analysed by gas chromatography–olfactometry (GC-O). The ratio of the concentration of the volatile in the initial extract to its concentration in the most dilute extract in which the volatile is still detectable by GC-O is expressed as the flavour dilution (FD) factor, the higher the FD factor, the more potent the aroma. The data

obtained from the AEDA technique is not directly related to the food aroma itself because during the GC-O process the compounds are completely volatilized and then evaluated by sniffing. The volatility from the food matrix is likely to differ (due to differences in solubility and/or binding to non-volatiles).

The AEDA technique has been used by several workers in an attempt to identify those compounds in black tea contributing to the unique aroma. Guth and Grosch (1993) applied this technique in order to determine the volatiles responsible for the aroma of Orange Pekoe black tea powders. AEDA of solvent extracts revealed 28 odour-active regions in the chromatograms with FD-factors in the range of 4-512, the highest of which corresponded to linalool, β-damascenone, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone. Linalool was identified as the most potent odourant in the headspace of black tea powder. Also found to be important were 1-octen-3-one, 3-methyl butanal, *Z*-3-hexenal, *E*-2-nonenal, 3-methylnonane-2,4-dione, and *Z*-4-heptenal.

More recently, Schuh and Schieberle (2006) carried out a similar study attempting to characterize the key aroma compounds in the beverage prepared from Darjeeling black tea. These workers found 24 odour-active regions, with FD-factors in the range of 4-128, the highest of which corresponded to vanillin, 2-phenylethanol, (*E,E,Z*)-2,4,6-nonatrienal, and, in agreement with Guth and Grosch (1993), 4-hydroxy-2,5-dimethyl-3(2H)-furanone. Static headspace olfactometry was also carried out to account for any losses of highly volatile compounds which may have occurred during AEDA, revealing 2-methyl propanal, 2- and 3-methyl butanal, hexanal, ethyl 2-methylbutanoate, and 1-octen-3-one as additional odour active compounds. The highest OAV was calculated for linalool, present in the infusion at ~140 times its odour threshold in water, followed by geraniol with an OAV of 45. (*E,E,Z*)-2,4,6-nonatrienal, β-damascenone 3-methylnonane-2,4-dione, 2-methyl propanal, and 2- and 3-methyl butanal all had OAVs between 37 and 41.

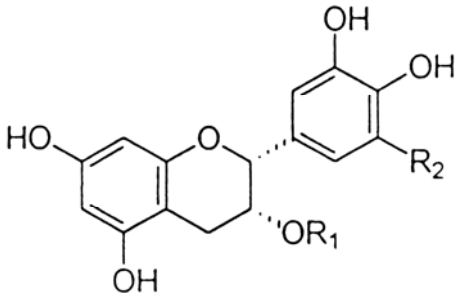
1.3.3 The non-volatiles of black tea

Although the specific aroma of black tea is mainly down to the volatile composition, it is the presence of non-volatiles which generally impart the characteristic colour and taste. Tea contains a wide range of non-volatile constituents including polyphenols, phenolic acids, amino acids, chlorophyll and carotenoids, carbohydrates, organic acids, alkaloids, enzymes, and vitamins and minerals (Chen et al., 2002). One of the most researched, and arguably the most important group is that of the polyphenolic compounds.

The total polyphenol content in tea flush (on a dry weight basis) is in the region of 25-30 %, and has been estimated to account for up to 48.5 % of the solids in a cup of tea (Sanderson et al., 1976). Although the only taste properties associated with tea polyphenolics are astringency and bitterness, they are a central element in determining the overall taste of black tea infusions. Complete infusions containing everything minus the polyphenols were found to have virtually no taste other than a slight bitterness (Sanderson et al., 1976).

1.3.3.1 Catechins

The 2-phenyl benzopyran based phenolic compounds known as flavonoids can be sub-divided into six classes; flavones, flavanones, isoflavones, flavonols, flavanols and anthocyanins. Of these, the flavan-3-ols (catechins) are the predominant form found in fresh tea leaf, usually constituting between 10 and 25 % of the dry matter (Haslam, 2003), and are characterised by di- or tri-hydroxyl group substitution of the B ring, and the meta-5,7-dihydroxy substitution of the A ring (Balentine et al., 1997). Primary catechins include (-)-epicatechin (EC) and (-)-epigallocatechin (EGC), as well as their gallate esters; (-)-epicatechin-3-gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG). In addition, smaller amounts of (+)-catechin (C) and (+)-gallocatechin (GC) are also found (Robertson, 1992). Figure 1-5 shows the structural characteristics of the four main catechins considered to be extremely important for the flavour and colour generation in black tea.



		R_1	R_2
Epicatechin	EC	H	H
Epicatechin gallate	ECG	Gallate	H
Epigallocatechin	EGC	H	OH
Epigallocatechin gallate	EGCG	Gallate	OH

Figure 1-5 - Structural representation of the four most important catechins - from Balentine et al. (1997)

The maceration of fresh tea shoot tips during the rolling stage of manufacture causes extensive disorganisation of the organelles, membranes and cell walls. This enables endogenous enzymes to come into direct contact with vacuolar polyphenolic constituents resulting in enzymatic oxidation and condensation of the various catechins present. Although polyphenol oxidase (PPO), peroxidase (PO) and catalase are all present in fresh tea shoots, it is thought that polyphenol oxidase is the enzyme of prime importance in as far as oxidation of flavan-3-ol substrates is concerned (Davies et al., 1999). Whilst gallocatechins, epigallocatechins and epigallocatechin gallate are the preferred substrate for PPO activity, the enzyme can in fact utilise any catechin as a substrate to produce a range of products. These include the theaflavins and thearubigins, terms first coined by Roberts (1958b), and thought to be responsible for many of the qualities associated with black tea infusions.

Whilst considerable conversion invariably occurs during the fermentation stage of tea manufacture, a proportion of the flavan-3-ols remain unoxidised and are thought to contribute significantly to the character of the resulting beverage. The quantity of theaflavins in a black tea beverage is typically one-third the level of remaining catechins (Balentine et al., 1997).

1.3.3.2 Theaflavins

The theaflavins are characterised by a unique benzotropolone ring system and are formed by the enzymic oxidation and condensation of catechins with di- and tri-hydroxylated β rings. The four major theaflavins found in black tea are formed as a result of the reaction between one dihydroxy flavan-3-ol (EC or ECG) and one trihydroxy flavan-3-ol (EGC or EGCG), the different possibilities of which are shown in figure 1-6.

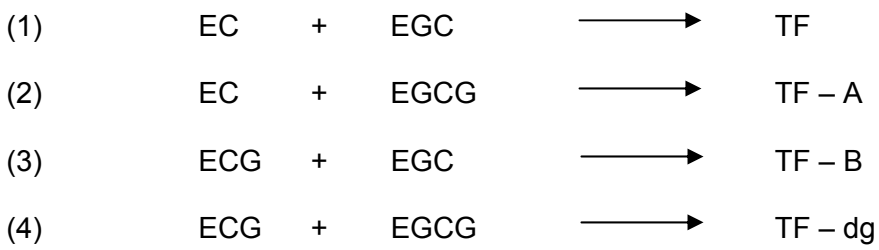
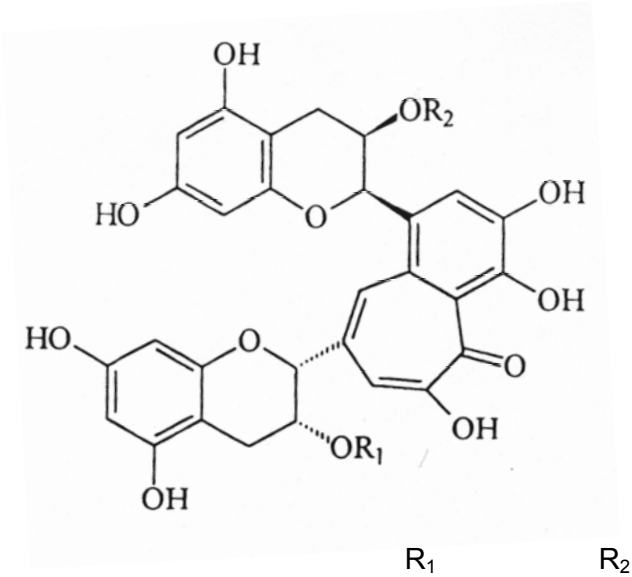


Figure 1-6 - Formation of the major theaflavins in black tea

If neither of the flavan-3-ol substrates are gallated, free theaflavin is formed (1). If the trihydroxy flavan-3-ol is gallated (EGCG), theaflavin-3-gallate (TF-A) will form (2). If the dihydroxy flavan-3-ol is gallated, theaflavin-3'-gallate (TF-B) will form (3). If both flavan-3-ols are gallated, (EGCG and ECG), theaflavin-3,3'-digallate (TF-dg) will form (4).

The structure of the theaflavins was first proposed by Roberts (1962), and has now been confirmed by two independent groups (Mahanta, 1988). Stereoisomers of theaflavin, namely isotheaflavin and neotheaflavin have also been identified in black tea, as have a number of closely related dimers including theaflavic acids and theaflagallins (Davies et al., 1999). Including these minor components, the total number of reported theaflavins to date stands at 11 (Chen et al., 2002). Figure 1-7 shows the chemical structures of the four main types of theaflavin as represented above (Balentine et al., 1997), as well as typical relative proportions present (Coxon et al., 1970).



		R_1	R_2	
Theaflavin	TF	H	H	18 %
Theaflavin-3-gallate	TF3G	Gallate	H	18 %
Theaflavin-3'-gallate	TF3'G	H	Gallate	20 %
Theaflavin-3,3'-digallate	TFDG	Gallate	Gallate	40 %

Figure 1-7 - Structure of the four main theaflavins found in black tea - from Balentine et al. (1997)

Theaflavins contribute 0.3-1.8 % of black tea on a dry weight basis, and between 1.0 and 5.9 % of the solids present in a tea infusion. Due to the presence of the benzotropolone ring system, the theaflavins absorb strongly at a number of wavelengths in the UV and visible regions of the electromagnetic spectrum, contributing significantly to the bright orange/red colour of tea infusions (a characteristic termed "brightness"). It is thought the theaflavins contribute about 30 % of the total colour of tea infusions (Millin and Swaine, 1981). Theaflavins also impart the essential mouth sensations described as "briskness", "freshness" and "aliveness" (Roberts and Smith, 1961). The contribution of the various theaflavins to these characteristics varies somewhat – the digallate for example being six times as brisk as its parent molecule, theaflavin (McDowell and Owuor, 1992).

Much debate exists as to the relative importance of theaflavins in determining the overall quality of black tea infusions, and so price of tea. Early work by Hilton and Ellis (1972) showed a close linear regressive relation between the theaflavin content and brokers valuation of central African black tea. This resulted in much attention being paid to the development of methods to enable the estimation of total theaflavin

levels in tea and so predict quality. The Flavognost method is one such method, and its suitability has been comprehensively reviewed (Reeves et al., 1985, Robertson and Hall, 1989). Work carried out in Kenya however, suggests the composition of the individual theaflavins to be of more importance in predicting the quality of Kenyan black teas than the total content (Owuor et al., 1986a).

1.3.3.3 Thearubigins

Although catechin content has been found to reduce by over 85 % during black tea fermentation, only 10 % of this decrease can be accounted for by the formation of theaflavins and theaflavic acids. The balance of compounds have been clumped together to comprise a complex, heterogeneous, ill-defined group of polymers displaying similar poorly-defined chromatographic behaviour. These compounds are believed to contribute between 10-20 % of black tea dry weight, and 30-60 % of solids in black tea infusions (Roberts, 1962, Wickremasinghe, 1978). Although christened the "thearubigins" by Roberts in 1962, the modern view is that at very best the term thearubigin can be taken as a term to embrace an "assortment of determinate structures from the monomeric to the polymeric, derived by enzymatic oxidation of the flavan-3-ols in fresh green tea leaf" (Haslam, 2003). Despite constituting the largest group of phenolic substances in black tea, (over 75 % of the phenolic flavan-3-ol substrates are thought to ultimately find their way into the thearubigins), they are the least understood of all its components.

Ebullioscopic estimation of the number average molecular weight of various thearubigin fractions gave values in the order of 700 (Roberts, 1962). Further work undertaken by Millin and Rustige (1967) suggested values of up to 40,000, although more recent analysis using size exclusion chromatography has indicated values around the 2000 range (Clifford and Powell, 1996).

It is generally accepted that the orange-brown thearubigins play an essential role in determining the characteristics of black tea infusions, contributing to colour, strength and mouthfeel of the beverage (Roberts and Smith, 1963, Millin et al., 1969). It is believed by some that the thearubigins may contribute up to 35 % of an infusions colour (Mahanta, 1988), although others have suggested that some of the colour traditionally attributed to thearubigins may in fact not be flavonoid in nature at all,

coming instead from compounds such as pheophytins (Sanderson, 1972), or polysaccharides (Millin et al., 1969)

1.3.4 Bitterness and astringency of black tea

Whilst bitterness and astringency are two of the most important sensory attributes in black tea infusions, there is considerable debate, and much conflicting evidence over which compounds are ultimately responsible. Research has been going on for almost 50 years, and even today there is no firm agreement between workers. To add confusion, the vast majority of workers class astringency as a "taste" (rather than as the now more generally accepted tactile sensation). This makes interpretation of literature difficult, especially where workers refer only generally to compounds responsible for the "taste" of tea infusions.

The astringency of black tea infusions has been described as consisting of two components. These have been described as a tangy, sharp and puckery component, sharp little after-effect, and a non-tangy, mouth-drying, mouth coating component with a lingering aftertaste, typical of the astringency of unripe bananas (Sanderson et al., 1976).

Tea tasters employ a bewildering number of terms to describe teas, and amongst others a liquor may be described as being "thick", "thin", "strong", "weak", "brisk", "soft", or "bright" (Millin et al., 1969). Terms such as "strong", "hard" and "harsh" are used by professional tea tasters to describe the intensity and quality of the astringent sensation (Scharbert et al., 2004a), and some workers have suggested that the term "briskness" corresponds to a tangy-type astringency (Sanderson et al., 1976), whilst others (Stahl, 1962) has defined it as "liveliness on the palate".

Early work suggested that it was the theaflavins and thearubigins generated during the tea manufacturing process primarily responsible for the taste characteristics of tea infusions (Roberts, 1958a, Wood and Roberts, 1964). The importance of theaflavins was supported in a study carried out by Millin et al. (1969). In this work, individual catechins were added to black tea infusions in quantities at least as great as those already present. Since a taste panel were unable to detect their presence, it was concluded that these simple monomeric flavanols were of little significance to the taste of tea. However, aqueous solutions of theaflavin isolated from black tea

were reported to exhibit an astringent taste, close to the sensation giving the "briskness" of a tea liquors character. The authors concluded that there was no doubt therefore that theaflavin and other oxidation products of intermediate molecular weight were responsible for the astringency of tea.

This conclusion was however directly contradicted by Ding et al. (1992) who investigated the influence of catechins and theaflavins on the astringency of black tea infusions. These workers found significant correlations between astringency and content of total catechins and individual catechins (except catechin itself), particularly between epigallocatechin-3-gallate and epicatechin-3-gallate. In contrast, no correlations were observed between the astringency of tea infusions and the level of theaflavins, thought to be due to their very low concentrations.

Scharbert et al. (2004b) also evaluated the contribution of theaflavins to the astringent taste of black tea infusions. Whilst it was observed that theaflavin solutions imparted mouth-coating, astringent and long-lasting oral sensations, it was concluded that the theaflavins accounted for less than 0.1 % of the overall astringency of black teas. This conclusion was reached by calculating taste activity values (concentration in tea / taste detection threshold in water), and then relating the taste activity values of the individual theaflavins to the overall tea astringency (This was calculated by determining taste dilution (TD) factors of complete infusions – the TD factor being the dilution at which a certain taste modality can only just be detected).

Recently, Scharbert et al. (2004a) carried out a study investigating the astringency of black tea infusions. These workers reported that it was neither the catechins, theaflavins or thearubigins, but a series of 14 flavon-3-ol glycosides which were the main contributors of astringent "taste" in black tea infusions. This conclusion was reached by applying taste dilution analysis on black tea infusions, a technique based on determination of taste thresholds in serial dilutions of taste-active fractions. The flavon-3-ol glycosides were found to induce a velvety and mouth-coating sensation at very low threshold concentrations, far below those of the catechins or theaflavins. This work was continued by Scharbert and Hofmann (2005) who prepared an artificial taste recombinate of Darjeeling black tea infusion by mixing 51 non-volatile compounds in their natural concentrations. By carrying out taste omission experiments, it was concluded by the authors that nine velvety astringent flavon-3-ol glycosides, two catechins (puckering astringency catechin and astringent and bitter

epigallocatechin-3-gallate), and bitter caffeine were the 12 key taste compounds in Darjeeling black tea infusions (responsible for bitterness and astringency). This conclusion is however in direct contrast to that of McDowell et al. (1995) and Millin et al. (1969), who both report that the flavon-3-ol glycosides play only a minor role in terms of tea taste.

Considerable work has also been carried out investigating differences in bitterness and astringency of individual catechins and theaflavins, again with highly conflicting results. Whilst epicatechin and catechin are chiral isomers, epicatechin has been found to be more bitter and astringent (Thorngate and Noble, 1995, Kallithraka et al., 1997a). Degree of polymerisation has also been shown to affect relative bitterness and astringency, and whilst monomers of flavonoid (and non-flavonoid) phenolics are more bitter than astringent, polymers are reported as being more astringent than bitter (Lea and Arnold, 1978, Robichaud and Noble, 1990). Comprehensive work was carried out by Sanderson et al. (1976), investigating the contribution of the polyphenolic compounds to the taste of black tea infusions., and it was observed that whilst the simple gallated tea flavanols (epicatechin gallate and epigallocatechin gallate), and theaflavins were both bitter and astringent, the non-gallated flavanols (epicatechin, epigallocatechin and catechin) were not astringent, only possessing a bitter taste. The authors reported that this clearly showed the importance of galloyl groups on expression of bitterness and astringency, although it is worth noting that these results conflict with those of other workers e.g. Kallithraka et al. (1997a) who showed that catechin and epicatechin were both bitter and astringent. With regards to the theaflavins, Sanderson et al. (1976) showed that there was a progressive increase in the intensity of astringency (i.e. decrease in threshold level) as the number of galloyl groups per molecule increased; theaflavin was less astringent than the theaflavin monogallates, which in turn were less astringent than the theaflavin digallates. The importance of galloyl groups was proven by treating a whole black tea infusion with a purified preparation of the enzyme tannase. Degallated the black tea infusion had the effect of completely eliminating the tangy portion of astringency.

Yamanishi (1990) reported that the components contributing to the bitterness of tea are catechins, caffeine, saponin and some amino acids. Scharbert and Hofmann (2005) reported that caffeine was quantitatively the predominantly bitter tastant, with concentrations of amino acids too low to be of importance. Tea leaf contains 2.5 to

4.0 % caffeine (dry weight basis), and a typical 180 mL serving of tea contains ~60 mg.

It is well known that interactions between caffeine and polyphenols play a part in moderating both the bitterness of caffeine, and the bitterness and astringency of polyphenols, although again the data is rather fragmented and contradictory. In work carried out by Millin et al. (1969), preparations of phenolics rich in theaflavins were tasted with and without the addition of caffeine. It was observed that addition of caffeine decreased the level of astringency, and some tasters reported an increase in "body" or "thickness". In an apparent contradiction, Scharbert and Hofmann (2005) reported that removal of caffeine led to a reduction in astringency of artificial taste recombinate Darjeeling black tea infusions containing 51 non-volatile compounds in their natural concentrations. Sanderson et al. (1976) also reported that removal of caffeine from tea infusions led to a reduction in the level of tangy astringency, with a shift to the non-tangy type. Whilst these workers reported that decaffeination of tea infusions led to an increase in bitterness, Scharbert and Hofmann (2005) reported the opposite, that removal of caffeine from the taste recombinate led to a decrease in bitterness.

Millin et al. (1969) reported that the samples with added caffeine were only slightly bitter, in direct contrast to the unpleasantly bitter nature of pure caffeine solutions. A similar observation was reported by Sanderson et al. (1976) who reported that the bitterness of caffeine in black tea infusions was only expressed in the absence of polyphenols. These results however appear to contradict those of Scharbert and Hofmann (2005) who report that the bitterness of caffeine solutions was actually increased in the presence of flavon-3-ol glycosides. Additionally, these workers observing that removal of the flavon-3-ols (exhibiting no bitterness of their own) from the artificial taste recombinate led to a decrease in level of bitterness of around 50 %.

Whilst the above appears quite contradictory in nature, it is essential to remember that there are key differences in experimental technique used, and the compounds investigated (i.e. theaflavins, mixture of polyphenolics, flavon-3-ol glycosides). This may well explain differences in interactions observed between workers.

Interactions between polyphenolic compounds and caffeine not only play a role in the taste of black tea infusions, but also in their appearance. As a black tea infusion

cools, a finely divided colloidal precipitate imparts a distinct opacity to the previously clear liquor, and below temperatures of ~60 °C leads to the formation of a visible sediment referred to as “tea cream”. Along with other minor constituents, tea cream has been found to consist essentially of theaflavins and thearubigins in the ratio of approximately 64:17, which compete to form a complex with caffeine (Roberts, 1963). As a result of their lower concentration in the tea infusion, theaflavins are affected by creaming to a greater extent than thearubigins, with up to 62 % of the total precipitated. The term “cream index” is an estimation of the amount of polyphenolic material in the precipitate, providing a measure of the ability of a tea infusion to cream down, a very important cup characteristic considered by tea tasters when purchasing tea from auction. The increasing ratio of thearubigins to theaflavins with increasing fermentation time has been shown to be directly correlated, with a highly significant negative regression between tea tasters’ marks for “briskness” and “cream index”. This correlation strongly supports the view that “briskness” of a tea liquor is at least partly the result of an association of theaflavins with caffeine (Roberts, 1963).

1.4 FLAVOUR RELEASE

1.4.1 Static release

When a mixture of volatiles is partitioned between an aqueous and non-aqueous phase at equilibrium, the quantity of volatiles in each phase is determined by the partition coefficient (K^i) of each of the individual compounds (i), often referred to as the air-water (K_{aw}), or gas-liquid partition coefficient (K_{gl}). The partition coefficient is the ratio of concentration of volatile compound in each of the two phases, as shown in equation 1-1.

$$K^i{}_{gl} = \frac{C^i{}_g}{C^i{}_l} \qquad \textbf{Equation 1-1}$$

Where C^i_g and C^i_l are concentration of the volatile compound (i) in the gas and liquid phases respectively. Partition coefficients can be measured directly using model systems at equilibrium by determining the concentrations in the gas and liquid phases (Chaintreau et al., 1995, Ettre et al., 1993).

The behaviour of volatile compounds in solution (i.e. equilibrium between gas and liquid phases) can be described according to their adherence (or non-adherence) to Henry's law. Henry's law states that "the mass of vapour dissolved in a certain volume of solvent is directly proportional to the partial pressure of the vapour that is in equilibrium with the solution" (Morris, 1968). In other words, the partial pressure of a particular volatile compound is directly proportional to the concentration of that component in solution. Henry's law is an example of a limiting law, only holding true under certain conditions, the most important of which being that the concentration of the volatile compound should be sufficiently low as to be considered "infinitely dilute". Since in food systems, volatile compounds are present at levels ranging from mg/kg to µg/kg, they are considered to obey Henry's law (Taylor, 1998).

In a similar way, Raoult's law defines the behaviour of the solvent, which is obeyed in systems containing solvent and volatile solutes at low concentration with no

interactions between solvent and solute. In solution, solvent molecules behave like a very slightly modified pure liquid, so obey Raoult's law. Since volatile compounds behave entirely differently to their pure state, they obey Henry's law.

The activity coefficient (γ) describes intermolecular interactions between volatiles, solutes, and solvents. These interactions may cause the effective concentration in a solution to differ from the true concentration. However, as long as the volatile is highly diluted in the liquid phase (i.e. obeys Henry's law), the activity coefficient can be assumed to be independent of the volatile concentration in solution, and is equal to a constant value $\gamma^{\infty}{}_i$ known as the infinite dilution activity coefficient.

Partition coefficients can also be determined from fundamental physicochemical parameters, as shown in equation 1-2.

$$K^{i}{}_{gl} = \frac{\gamma^{\infty}{}_i \cdot P^{0}{}_i(T)}{P_T} \cdot \frac{V_l}{V_g} = \frac{H_i}{P_T} \qquad \textbf{Equation 1-2}$$

Where $P^{0}{}_i(T)$ is the vapour pressure for the pure component (i) (Pa) at temperature T. P_T is the total pressure in the gas phase (Pa), and V_l and V_g the molar volumes of the liquid and gas phases respectively (m^3/mol). Assuming an infinitely dilution solution (and an infinitely dilute activity coefficient), the product $\gamma^{\infty}{}_i \; P^{0}{}_i(T)$ is a constant (Henry's law constant, H_i), reflecting the molecules volatility in the medium. The value of $K^{i}{}_{gl}$ in this case therefore depends only on temperature (Reid et al., 1987) .

Simplifying the above equation, Henry's law constants are related to vapour pressure according to equation 1-3.

$$H_i = \gamma^{\infty}{}_i \cdot P^{0}{}_i(T) \qquad \textbf{Equation 1-3}$$

Vapour pressure can be defined as "the pressure exerted by the vapour of a compound at equilibrium with its pure condensed phase". Henry's law constants are therefore a direct measure of volatility, the values of which are directly related to vapour pressure and aqueous solubility.

Henry's law constants can also be expressed as a function of the partial pressure of the volatile compound in the gas phase (p^i) and concentration in the gas phase (C_g) according to equation 1-4.

$$H_i = \frac{p^i}{C^i_g} \qquad \textbf{Equation 1-4}$$

Henry's law constants are large for compounds with a strong tendency to migrate from water to air, and low for compounds with the reverse behaviour. Henry's law constants are temperature dependent since both values of saturated vapour pressure and solubility also are (vapour pressure increases with increasing temperature, whereas solubility of dissolved gases decrease with increasing temperature).

The most reliable Henry's law constant values are those determined by direct measurement using proven experimental procedures, although this type of data is limited and scattered throughout literature. Although compilations are available e.g. Sander (1999), they contain only a fraction of the values obtainable and generally do not provide information regarding validity of the techniques used. A survey of published literature has shown numerous discrepancies, most likely the result of technical problems encountered during measurement (Chaintreau et al., 1995). It is often therefore more appropriate to estimate values using one of several methods available. The bond and group contribution methods are simple structure activity relationship models, originally developed for the calculation of Henry's law constants for a variety of organic compounds (Hine and Mookerjee, 1975). The bond contribution method obtains predictions based upon the presence and relative contribution of chemical bonds within each compound, and has been comprehensively reviewed and adapted over the years (Voutsas et al., 2001, Meylan and Howard, 1991). The group contribution method bases predictions on the relative contribution of functional groups within compounds, where a group is characterised both by the nature of atoms to which it is attached as well as those it contains.

Real food systems differ from simple binary systems held under ideal conditions, and for this reason deviations from the simple laws described above are common. Interaction between volatile compounds and major food components is common, and since only free dissolved molecules exert a vapour pressure (de Roos, 2000),

flavour binding and complex formation can have a significant effect on partitioning behaviour, and ultimately release of volatiles into the headspace (Harrison and Hills, 1997). Examples include interactions with proteins (O'Neill, 1996, Fischer and Widder, 1997) and lipids (de Roos, 1997), and even in cases where physical interactions such as these do not occur, many solute molecules have been shown to influence the vapour pressure of volatile compounds through their effect on the solvent properties of the aqueous phase. (Voilley et al., 1977). Examples include sugars (Nahon et al., 1998, Wientjes, 1968), salts (Kepner et al., 1964), and acids. It is also well known that interaction with non-volatile solutes may cause an increase in vapour pressure (and so headspace concentration) for certain volatile compounds, yet a decrease for others (Nawar, 1971, Wientjes, 1968). Some authors have described the general increase of volatile compounds in the headspace as “salting out”, and a decrease as “salting in”, although these terms originate from work investigating protein solubility and should not be used to describe the general change in volatile headspace concentration upon addition of a solute (Taylor, 1999). In many cases, deviations from ideality can be defined mathematically, although as food systems become increasingly complex, formulating precise definitions becomes considerably more difficult.

Static release data is important and can provide information on the effect of different physicochemical properties on partitioning, and to determine the presence of ideal or non-ideal behaviour in systems. Static systems have been widely used to investigate the behaviour of aroma compounds under equilibrium conditions. Friel et al. (2000) for example looked at static release data when investigating the effect of sucrose concentration on volatile release, whereas (Nawar, 1971) have investigated the effect of sugars, glycerol and acids.

1.4.2 Dynamic release

For the perception of aroma by consumers, volatile compounds present in the food matrix must be released into the gas phase (headspace) and be detected by the olfactory receptors, either orthonasally (via the nostrils), or retronasally (from the mouth cavity via the nasopharynx and lungs) (section 1.2.1).

Two key factors affecting flavour release from foods are phase partitioning and mass transport. Release only takes place when phase equilibria are disturbed, hence non-equilibrium is considered the driving force for mass transport. Under real-life conditions, dynamic factors such as gas flow, sample agitation, and surface area changes disturb the equilibrium headspace concentration, so drive aroma release as the system attempts to re-establish equilibrium.

In terms of aroma release from liquid food systems under dynamic conditions (most relevant to the current study), two key mechanisms have been suggested: convection and diffusion (Darling et al., 1986, Taylor, 2002). These are sometimes referred to as the eddy and molecular (static) diffusion mechanisms respectively (de Roos, 2000).

In the case of the convective mechanism, it is assumed that a constant concentration of volatile exists throughout both the liquid and gas phases. Since volatile compounds can move in the liquid phase (either due to convection currents or agitation), the concentration is uniform throughout. In such a case, mass transfer occurs by diffusion in very thin layers either side of the interface (liquid and gas interfacial layers), with the assumption of an instantaneous equilibrium at the interface itself. This is shown schematically in part (a) of figure 1-8.

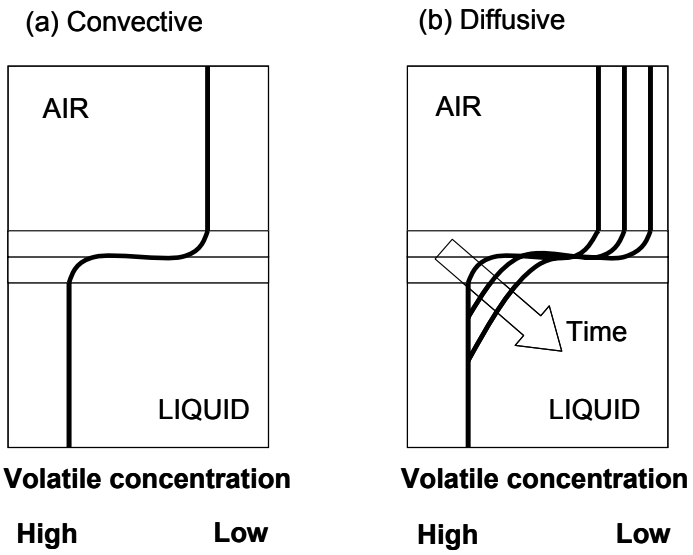


Figure 1-8 - Schematic representation of convective and diffusive mass transport – adapted from Taylor (2002)

In figure 1-8, the lines drawn through the liquid and gas phases represent concentration in each of these phases, with concentration along an imaginary x axis. In the case of the convective mechanism, the vertical line in the liquid phase right up to the boundary layer represents the high, uniform concentration of volatile compound in this phase. There is an instantaneous equilibrium at the interface itself, and a uniform (lower) concentration of volatile compound in the gas phase.

Transport across the interface in this case can be described according to the overall mass transfer coefficient (k) (equation 1-5) (Marin et al., 1999).

$$\frac{1}{k} = \frac{1}{k_g} + \frac{K_{gl}}{k_l}$$ **Equation 1-5**

Where k_l and k_g refer to mass transport through the liquid and gas phases respectively, and K_{gl} the partition coefficient between the gas and liquid phases. Three steps therefore contribute to the overall mass transfer coefficient; i) mass transfer from the bulk liquid phase to the interface, ii) equilibrium at the interface, and finally iii) mass transfer from the interface into the bulk gas phase. In systems where this mechanism is operating, the volatiles at the interface are rapidly replenished.

The diffusive mechanism is more appropriate in cases where one or both of the phases are not well mixed. In this case, the concentration gradient through the liquid phase is initially the same as in the convective mechanism, but as volatiles move into the gas phase, the concentration in the liquid phase decreases (part (b) of figure 1-8). Whilst diffusion over the interfacial layer is still the key mechanism, the distance over which it operates is much larger, and as a result, the rate is much slower.

Compounds with high partition coefficients (~10^{-2}) favour the gas rather than aqueous phase at equilibrium. When the gas phase is disturbed, a lot of molecules are lost, and so have to transfer across the interface as the system tries to maintain equilibrium. In contrast, compounds with low partition coefficients (~10^{-5}) favour the aqueous rather than gas phase at equilibrium, and when the gas phase is disturbed, only a few molecules are lost. As such, only a few molecules need to transfer across the interface in order for the system to maintain equilibrium.

This was shown experimentally by Marin et al. (1999), who used a convective type model to investigate volatile release from aqueous solutions under dynamic dilution headspace conditions. An aqueous phase containing five volatile compounds (chosen to represent a range of air-water partition coefficients and hydrophobicities) was allowed to equilibrate in a closed cell. After reaching equilibrium, the gas phase was diluted by introducing fresh air at a fixed flow rate, and the headspace concentration measured over time using APCI-MS. This system resembled the real-life situation when a sealed container is opened and volatiles are diluted with the surrounding air.

It was observed that the volatile concentration in the headspace varied over time, differing for different compounds. The kinetics of release were shown to be directly related to the partition coefficient, with the lowest change in release observed for the least volatile 2,5-dimethyl pyrazine (K_{aw} = 5.7 x 10^{-5}), and the highest change in release observed for the most volatile dimethyl sulfide (K_{aw} 2.5 x 10^{-2}). A diagram illustrating these two scenarios is shown below in figure 1-9.

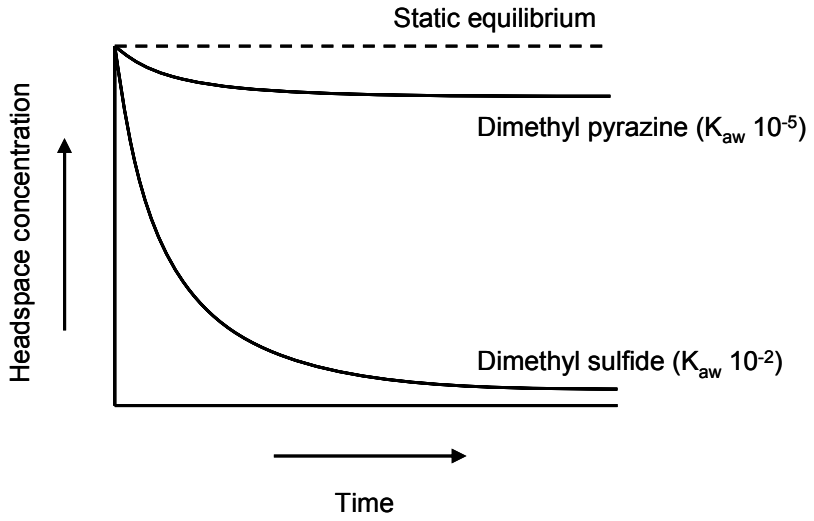


Figure 1-9 - Gas phase stability of volatile compounds under dynamic conditions

In the case of dimethyl sulfide, replenishment was slow, resulting in a rapid fall in the headspace concentration. In contrast, dimethyl pyrazine was rapidly replenished, and as such, the headspace concentration remained fairly stable.

The authors also observed that conditions in the gas phase affected compounds differently, depending upon their value of $K_{aw.}$ In the case of compounds with

K_{aw} >10^{-3} (e.g. dimethyl sulfide), release was determined based upon the partition coefficient alone. In the case of compounds with K_{aw} <10^{-3} (e.g. dimethyl pyrazine), release was determined not only by the partition coefficient, but also on the mass transfer conditions within the gas phase, with the Reynolds number playing a key role in determining the temporal profile of release.

A number of mathematical models have been described over the years in order to predict volatile release from real foods. These have been based either on partitioning e.g. de Roos and Graf (1995)), or on an understanding of the physical processes occurring during the mouth during consumption. Examples include release from boiled sweets (Hills and Harrison, 1995), gels (Harrison and Hills, 1996), and liquids containing aroma-binding macromolecules (Harrison and Hills, 1997). In depth details of these models are not appropriate to the current research, and are provided elsewhere e.g. Taylor (2002) and Overbosch et al. (1991).

1.4.3 Monitoring flavour release

1.4.3.1 Atmospheric pressure chemical ionization-mass spectrometry

Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) is a direct mass spectrometric technique, involving sampling of volatile compounds directly from the gas phase without prior chromatographic separation.

The APCI-MS technique has been successfully applied to many area of work, originally concentrating on analysis of gas mixtures such as the detection of chemical warfare agents in the atmosphere (Ketkar et al., 1991). More recently, a large area of work has involved analysis of the headspace of model systems, foods and beverages, both at equilibrium as in the case of sucrose solutions (Friel et al., 2000), and under dynamic conditions such as ethanolic wine-like solutions (Tsachaki et al., 2005), tomatoes (Boukobza and Taylor, 2002), and aqueous solutions (Marin et al., 1999).

In vivo analysis of volatile release from foods during the eating process is also possible (Taylor, 1996). Typical applications in this area have included study of retronasal release of volatiles from aqueous solutions (Rabe et al., 2004, Hodgson

et al., 2004, Linforth and Taylor, 2000), emulsions (Doyen et al., 2001), biscuits (Brauss et al., 1999), French fries (van Loon et al., 2005), and tomato fruits (Brauss et al., 1998).

The APCI-MS process involves the formation of an initial reactant ion H_3O^+ (as water passes through a corona pin discharge at atmospheric pressure (Raffaelli, 1997), although due to the presence of water clusters, the form $(H_2O)_nH^+$ is often formed. The sample arrives at the ionization region though a fused silica capillary tube by means of a venturi effect created by a high nitrogen gas flow around the end of the silica tube. The volatiles present are ionised by the corona pin discharge (4 kV) before passing into the mass analyzer. A schematic representation of the APCI-MS is shown in figure 1-10.

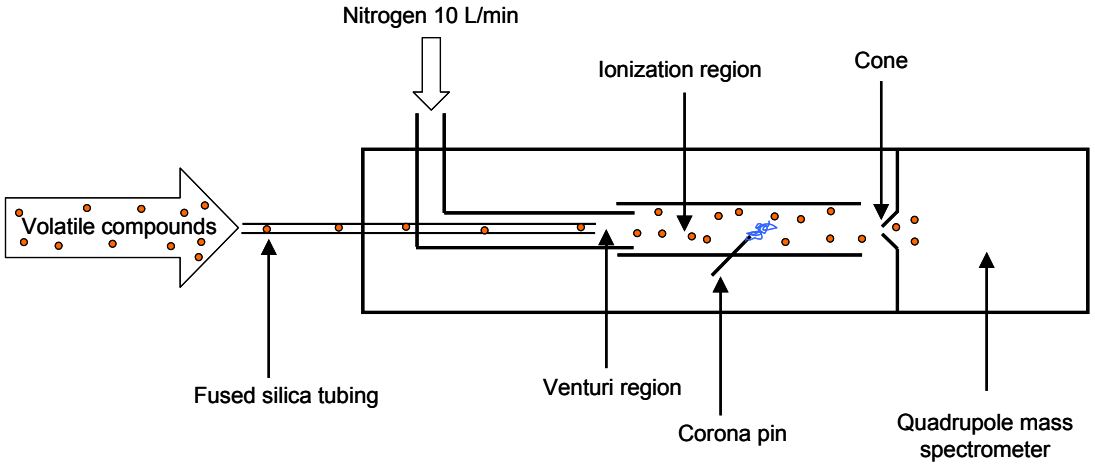


Figure 1-10 - Schematic representation of an atmospheric pressure chemical ionisation-mass spectrometer - adapted from Taylor and Linforth (2000)

The reactant ions (H_3O^+) are able to ionize molecules in the sample, and being a "soft ionization" technique, compound fragmentation is minimal (Taylor et al., 2000). The majority of compounds (R) are therefore converted through protonation (transfer of a proton, H^+) to RH^+ ions (figure 1-11), although other molecules such as alcohols and aldehydes tend to dehydrate forming $[R - H_2O - H]^+$ ions.

$$H_3O^+ + R \longrightarrow RH^+ + H_2O$$

Figure 1-11 - Formation of protonated sample molecules by atmospheric pressure ionization

Only those molecules with proton affinities in excess of H_3O^+ (166.5 kcal/mol) (i.e. most volatile aroma compounds) can accept a proton from H_3O^+, so excluding the major components of air, including N_2, O_2 and CO_2.

The ions formed are sampled into a standard quadrupole mass spectrometer maintained under vacuum where they are detected according to their *m/z*, and displayed as chromatograms in the form of ion intensity against time (or scan number).

The APCI-MS can be run in two modes; fullscan (TIC) and select ion (SIR). As the name suggests, fullscan mode monitors all ions over the range selected. Running the mass spectrometer in select ion mode is advantageous in that it increases sensitivity, although the more ions that are monitored simultaneously, the lower the temporal resolution. Time resolution of APCI-MS is fast, and with the monitoring of five ions simultaneously for 0.01 s each (dwell time), each ion will be sampled every 0.05 s (Taylor et al., 2000).

To a certain extent, compound fragmentation can be controlled by altering the voltage on the sampling cone (cone voltage, V), the voltage applied to the aperture between atmospheric and vacuum pressure regions. In select ion mode it is possible to set up individual cone voltages during the detection of each compound, so ensuring optimal ionization and so fragmentation of different compounds in multi-component mixtures.

It is important to regulate the water content in the APCI-MS source in order to ensure constant ionization, and reproducible data. To overcome issues of water vapour (caused by breath, or analysis of hot beverages for example), a “buffer” nitrogen flow of 10 L/min is used. The sample flow (typically between 5 and 50 mL/min) is therefore insignificant, with water content of the source determined by the water content of the nitrogen “buffer” gas (Taylor et al., 2000).

Providing the analyte concentration is within the linear range of the detector, reliable quantification can usually be achieved over three orders of magnitude (Taylor et al., 2000). The upper limit of detection is determined when all available charge has been utilized by volatiles or the analyser detector is overloaded, and further increase in volatile concentration leads to no further increase in signal. Quantification is carried out by injecting calibration solutions of pure volatile in an inert solvent

(usually hexane or cyclohexane) directly into the nitrogen gas stream. Since the flow rate of the sample can be measured using a flow meter, it is therefore possible to calculate the concentration of volatiles present.

1.4.3.2 Proton transfer reaction-mass spectrometry (PTR-MS)

A similar chemical ionization technique has been developed by Lindinger and co-workers and is known as proton transfer reaction-mass spectrometry (PTR-MS) (Lindinger et al., 1993).

Like APCI-MS, PTR-MS has found a wide range of uses across a variety of areas including environmental, medical and food research. Environmental examples include monitoring of the atmospheric pollutants (Karl et al., 2001) and decaying biomatter (Warneke et al., 1999). Numerous studies have been carried out investigating release of volatiles from foods and beverages such as coffee (Yeretzian et al., 2003) and fruit during ripening (Lindinger et al., 1998a). Examples of *in vivo* monitoring have included analysis of various compounds in garlic breath (Taucher et al., 1996), and acetonitrile and benzene in smokers breath (Jordan et al., 1995). In addition, the PTR-MS has been shown to be an ideal tool for measuring Henry's law constants, particularly their dependence on temperature and matrix (Von Hartungen et al., 2004, Karl et al., 2003).

One of the key differences between the PTR- and APCI-MS techniques is that in the former, generation of H_3O^+ and ionisation of volatile compounds are individually controlled and spatially separated processes. This enables absolute headspace concentrations to be calculated without calibration or use of standards (Lindinger et al., 1998b).

A PTR-MS typically consists of three key features; i) an ion source, ii) a drift tube reaction chamber, and iii) a quadropole mass spectrometer. In the ion source, H_3O^+ ions are produced from pure water vapour within a hollow cathode tube which are then driven by a small electric field into the drift tube. The gas (containing the volatiles to be analysed) is also introduced into the drift tube, and it is here that the proton transfer reaction between the reagent ions and volatile compounds occurs (as shown for APCI-MS in figure 1-11) at a precise temperature and pressure. Protonated volatile compounds drift downstream towards the end of the drift tube

where they are accelerated by an electric field into a quadropole mass spectrometer. An exception to this setup lies in the more recent introduction of PTR-MS based on time-of-flight mass spectrometry, reported to increase sensitivity, especially with regards to complex mixtures (Blake et al., 2004).

Sensitivity for most volatile compounds generally lies in the pptv – ppmv range (Lindinger et al., 1998a), similar to that reported for APCI-MS (Taylor et al., 2000), and a linear response over four orders of magnitude has been reported (Yeretzian et al., 2000, Lindinger et al., 2005). Like APCI-MS, time resolution of the PTR-MS is fast at around 0.2 s (Yeretzian et al., 2000) enabling real-time monitoring of volatile release to take place.

1.4.3.3 Analysis of complex mixtures

In systems containing only two or three volatiles, it is relatively easy to correlate specific ion *m/z* on mass spectra obtained from chemical ionization mass spectrometric methods (e.g. APCI- and PTR-MS) to the presence individual compounds. For example, in a model beverage system containing the two compounds eugenol and ethyl butyrate, two main ions will be present in spectra obtained from headspace or breath analysis. Figure 1-12 shows a typical APCI spectra (TIC) obtained from headspace analysis of solutions of (A) eugenol, (B) ethyl butyrate, and (C) a mixture of the two.

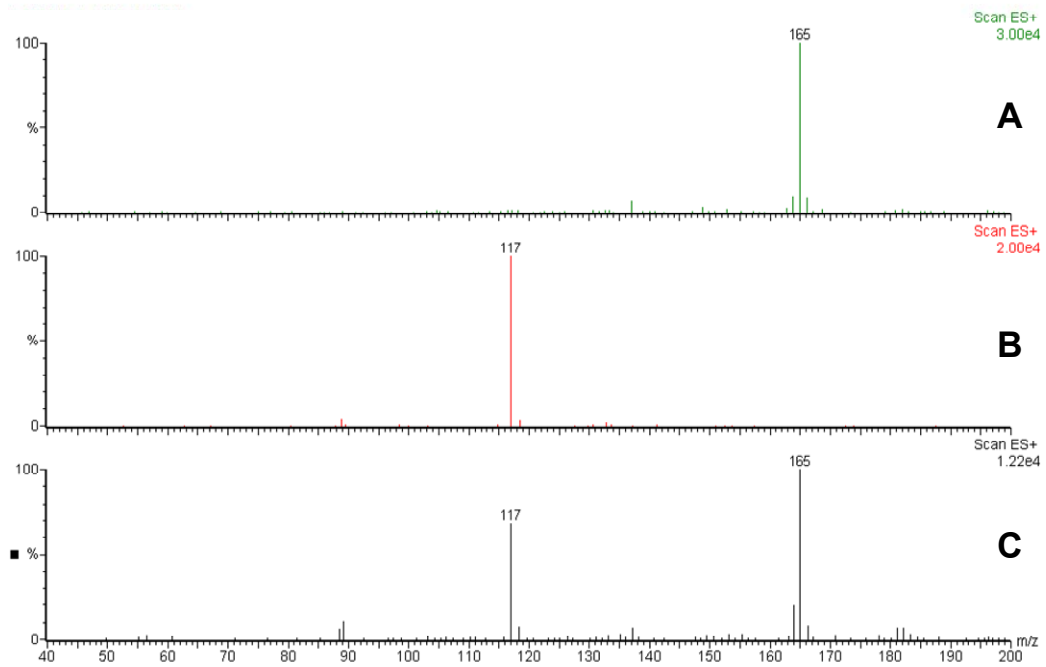


Figure 1-12 - Spectra obtained from (A) eugenol, (B) ethyl butyrate, and (C) a mixture of the two compounds

In this example, ion 165 can be directly related to the presence of eugenol, and ion 117 to the presence of ethyl butyrate. Investigating release of these two compounds can be simply achieved by looking at the intensity of these two distinct ions.

Analysis of complex mixtures such as foods and beverages where many different compounds are present is much more challenging. This difficulty is further complicated in systems containing isobaric compounds (with the same molecular mass) and/or isomeric compounds (stereoisomers; where the atoms are linked in the same order, but differ in their spatial arrangement). As the number of compounds in a mixture increases, so too does the potential for different compounds to produce ions of the same *m/z* on the spectra. This is further complicated by compound fragmentation, which although described as "soft ionization" techniques, can and does occur in both APCI- and PTR-MS analysis. With two or three major fragment ions per compound, it becomes increasingly difficult to find ions that can be uniquely attributed to the presence of individual compounds.

E-2-hexenal for example produces a pronotated ion at *m/z* 99. Many other compounds (e.g. octanone, dodecanol, hexanone) however also fragment to give

ions at this *m/z*. In a mixture containing all four of these compounds, each would contribute to the overall intensity of the ion at *m/z* 99 to an extent dependent upon their individual concentration. It would not be possible to determine the relative contribution of each compound to the overall intensity of the ion at *m/z* 99, and so accurately study release of the compound of interest (*E*-2-hexenal in this case).

Workers have employed a variety of approaches to tackle this problem, and establish which compounds in mixtures contribute to which ions in mass spectra. A commonly used approach involves coupling the APCI- or PTR-MS to a GC (GC-EI/APCI-MS or GC-EI/PTR-MS). Headspace of the complex mixture is usually collected using an offline trapping method (e.g. Tenax trapping) and passed through a GC column where the individual volatiles are separated according to their affinity for each of the phases. Following separation, the flow is split; half is sent to the electron-ionization (EI) detector of the GC, the other half to the chemical-ionization mass spectrometer. This results in the simultaneous production of two chromatograms (one by each of the ionization types). From the chromatograms it is possible to establish which compounds in the complex mixture give rise to which ions in the chemical-ionization mass spectrometer. A schematic of CG-EI/APCI-MS where volatile compounds have been trapped onto a SPME fibre is shown below in figure 1-13.

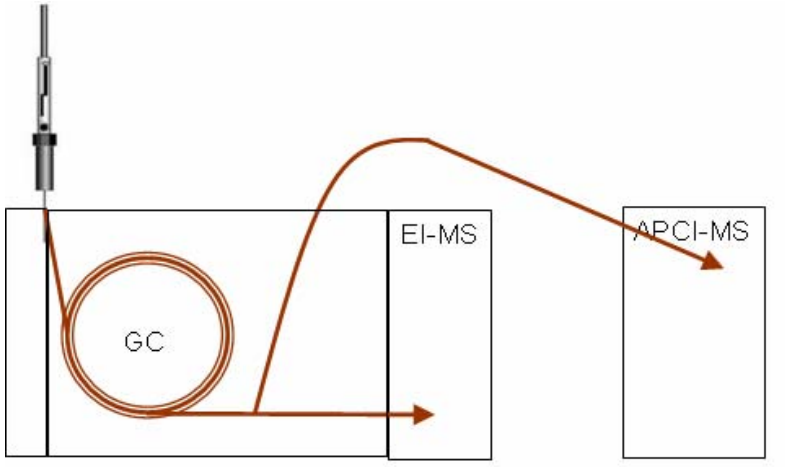


Figure 1-13 - Schematic diagram showing the set up of a GC-EI/APCI-MS system

This GC-coupling approach has been utilized by many workers, with Taylor et al. (2003) for example successfully applying GC-EI/APCI-MS to assign individual ions to volatile compounds released from heated skim milk powder, macerated tomato

and cucumber fruits. The GC-EI/PTR-MS approach has been used by many workers, including Fall et al. (2001) investigating volatiles released from leaves after wounding, and de Gouw et al. (2003) looking at air pollution. More recently, Lindinger et al. (2005) demonstrated the application of PTR-MS coupling with GC-MS using freshly prepared espresso coffee, whilst Mark et al. (2006) studied furan formation in thermally processed foods.

Another way of tackling the problem of identification in complex mixtures is to make use of compound fragmentation. Buhr et al. (2002) analysed 53 flavour compounds using PTR-MS, concluding that differences in fragmentation pattern can provide relevant information on the discrimination of both isobaric and isomeric compounds. In particular, the energy-dependent nature of fragmentation has received much attention, an example of which is illustrated below in figure 1-14 which shows APCI spectra produced following ionization of carvone (*m/z* 150) at three different cone voltages (15, 21 and 27).

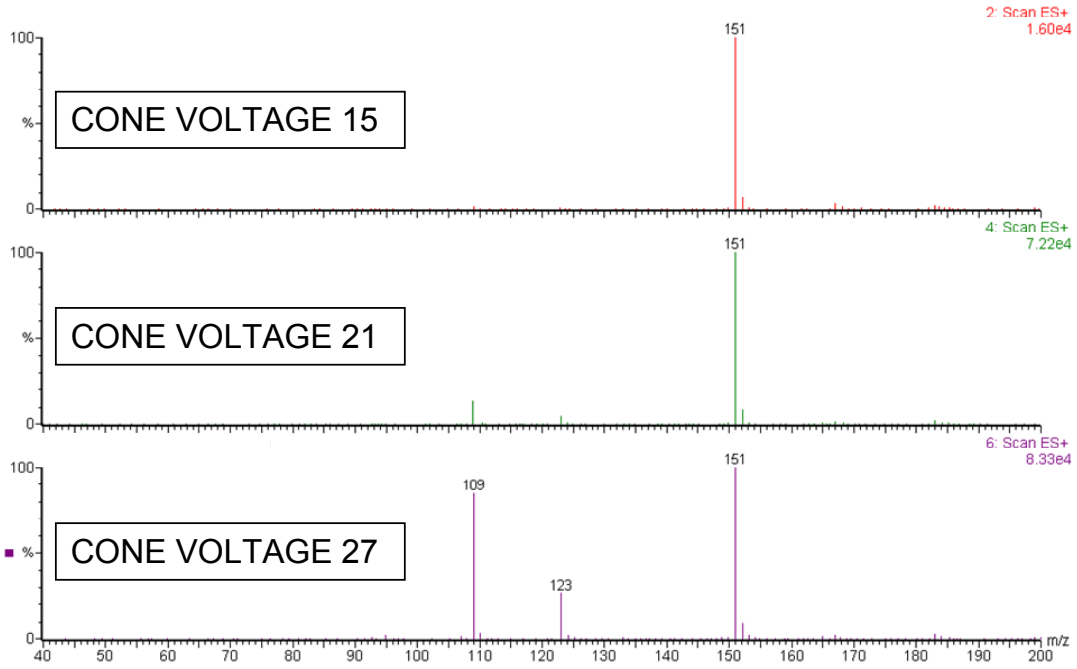


Figure 1-14 - Fragmentation of carvone analysed using APCI-MS at three cone voltages (15, 21 and 27)

Figure 1-14 clearly shows how the ratio of the ions alters as cone voltage, and so ionization energy is increased. With increasing cone voltage, the intensity of ions 109 and 123 increase relative to the intensity of ion 151.

The same is true for PTR-MS, and Lindinger et al. (1998a) describe how altering the fragmentation during PTR-MS analysis by changing the applied voltage in the final region of the drift tube can help differentiate isobaric compounds. Many workers have made use of this characteristic (sometimes termed 'collision-induced break-up') in aiding allocation of ions to individual compounds. Yeretzian et al. (2003) for example, applied this energy-dependent break-up to help link mass spectral peaks observed in PTR-MS to chemical compounds present in the headspace of coffee.

Another approach used by workers attempting to differentiate isobaric and isomeric compounds is the use of different proton transfer reagents. NH_4^+ has been shown as a suitable alternative to the standard H_3O^+ as primary reactant ions (Lindinger et al., 1998a). Work by Yeretzian et al. (2003), has shown several mass peaks disappear or strongly decrease when this alternative ion is used, altering fragmentation patterns of compounds, so offering potential in the identification of compounds from complex spectra. A similar approach has been applied by Wyche et al. (2005), who described an attempt to use chemical ionisation reagents other than proton transfer species inside a PTR-MS instrument. Using NO^+ as an ionization reagent it was demonstrated that for three groups of isobaric aldehyde / ketone compounds, a sufficient shift in the fragmentation occurred, enabling the user to distinguish the two compounds.

A separate approach used for resolution of isobars is the use of an ion trap mass spectrometer with MS/MS capability. Similar studies have been carried out by Prazeller et al. (2003) and Jublot et al. (2005), investigating this technique as a method for distinguishing isobaric compounds using PTR- and APCI-MS respectively. The basic principle involves carrying out a second fragmentation of ions in an ion trap to form further fragment ions, so opening up the possibility to create unique fragment ions which otherwise would not be present from the "soft" ionization process of the APCI- or PTR-MS alone. For some isobaric compounds the additional ionization was found to result in different fragmentation patterns which could then be used in order to aid identification. Both authors reported that the additional fragments still contained common features, and so in mixtures of isobarics, ratios of the ions would be need to be used in order to determine relative contributions.

1.5 SENSORY DISCRIMINATION TESTING

The field of sensory analysis can be broadly divided into two areas; sensory evaluation I (using the human senses as instruments to evaluate food characteristics), and sensory evaluation II (investigating the consumer's ability to discriminate between foods) (O'Mahony and Rousseau, 2002). Discrimination testing is a common prerequisite to more time consuming and costly procedures such as consumer preference testing and descriptive analysis techniques. There is no point in trying to obtain consumer preference information for two samples if they can not be distinguished. Likewise, attempting to describe "how" two samples differ is pointless if it is not first shown that a difference can be detected. Discrimination tests also play a valuable role in their own right, and determining the degree of difference and similarity between samples can be essential in areas such as quality control and competitor assessments.

Discrimination tests can be either directional or non-directional. In the directional test, subjects are directed towards a specific attribute (e.g. sweetness), whereas in the non-directional test, subjects consider all aspects of sample attributes available to them.

The three most commonly used types of discrimination test are the triangle, duo-trio, and paired-comparison methods. Of most relevance to the current study however is the paired-comparison test, and a variant of it, the (non-directional) same-different test. The paired-comparison test is a two product discrimination test in which two products are presented simultaneously, and it is the task of the subject is to identify the sample with the lowest or highest intensity of a pre-stated stimulus (e.g. sweetness). In the same-different test the subject is presented with a pair of products and asked to indicate whether they are they "same" or "different". There is however a lack of appreciation for the complexity of discrimination tests, stemming from the ease and simplicity with which tests are described and implemented by subjects, summed up nicely in a paper by (O'Mahony, 1995) titled "who told you the triangle test was simple?"

1.5.1 Perceptual variance

When a food is repeatedly tasted, the perception of its flavour will vary over tastings. This is known as perceptual variance and there are several sources of this variation, both within the subject, and product. The intensity of a perception at a given moment is therefore not constant, and will vary slightly according to a frequency distribution assumed to be normal. Discrimination testing should only be used to distinguish between confusable stimuli (those with fine differences). This is illustrated schematically in figure 1-15 which shows the frequency distribution of flavour intensity of two samples (A and B) in which (B) has a stronger mean flavour intensity.

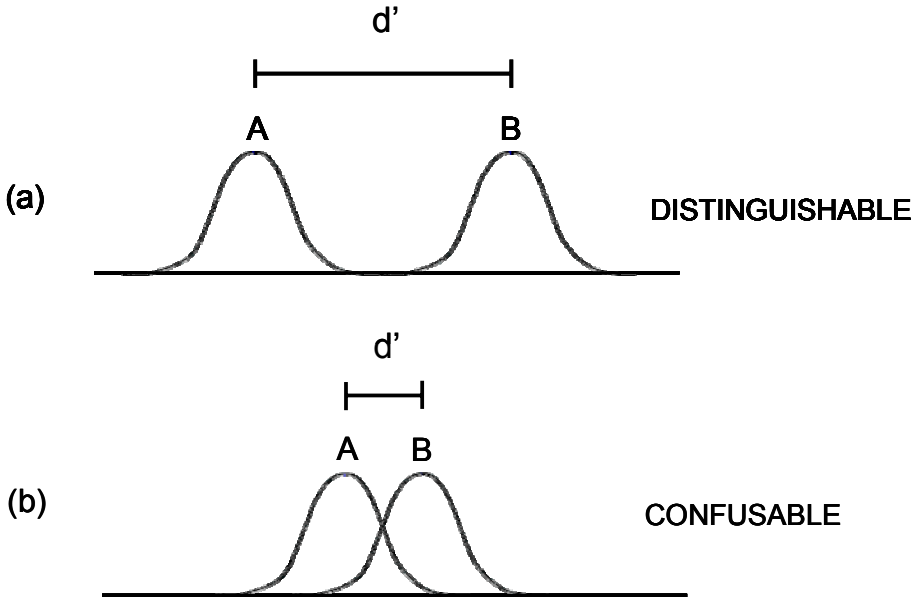


Figure 1-15 - Flavour intensity distributions of easily distinguishable and confusable stimuli

Although in both cases, the mean flavour intensity of (B) is greater than (A), only in the second example (b) are the two stimuli confusable. In the case of (a), the frequency distribution of (B) is so far up the intensity axis that it does not overlap with (A), and as such will never be confused. To carry out a discrimination test on these samples would be a waste of time, effort and money.

The greater the overlap, the more confusable the samples; a simple measure of which is the value of d' which is the distance between the two means, measured in units of standard deviation. Distributions whose means are two standard deviations

apart (d' = 2) will overlap less, and be easier to discriminate that those one standard deviation apart (d' = 1).

Several factors can affect perceptual variance, including sensory fatigue, sequence effects and memory. Sensory fatigue is defined as sensory overload with the senses unable to recover their initial state between the tastings of successive samples. It has been shown that the response to a stimulus is greatly dependent upon the stimulus preceding it. For example, sensory adaptation would render a strong tasting (or smelling) stimulus as not appearing so strong if tasted immediately after another strong tasting stimulus (O'Mahony and Rousseau, 2002). It has also been shown that an increase in stimulus concentration can more easily be detected than a decrease (O'Mahony and Goldstein, 1987). Since a subject's task is to compare the stimulus of several samples, memory also plays a part in determining sensitivity. Having to compare three samples (such as in the triangle test) is more demanding on the memory than having to compare two (as in the same-different test). The effect of memory has been further investigated by Cubero et al. (1995) and Avancini de Almeida et al. (1999), who found that an increase in time delay between tasting citrus flavoured beverages decreased subjects' performance. Factors known to decrease perceptual variance include sample retasting (allowing subjects to restate products before reaching their decision) and repeated exposure (usually in the form of practice sessions).

1.5.2 Response bias

Response bias (criterion variation) is one of the central problems in discrimination testing, coming down to where the subject "draws the line" when making decisions, best illustrated through use of examples. Figure 1-16 shows three cups of tea; N, containing no sugar, X and S and containing two slightly different levels of sugar.

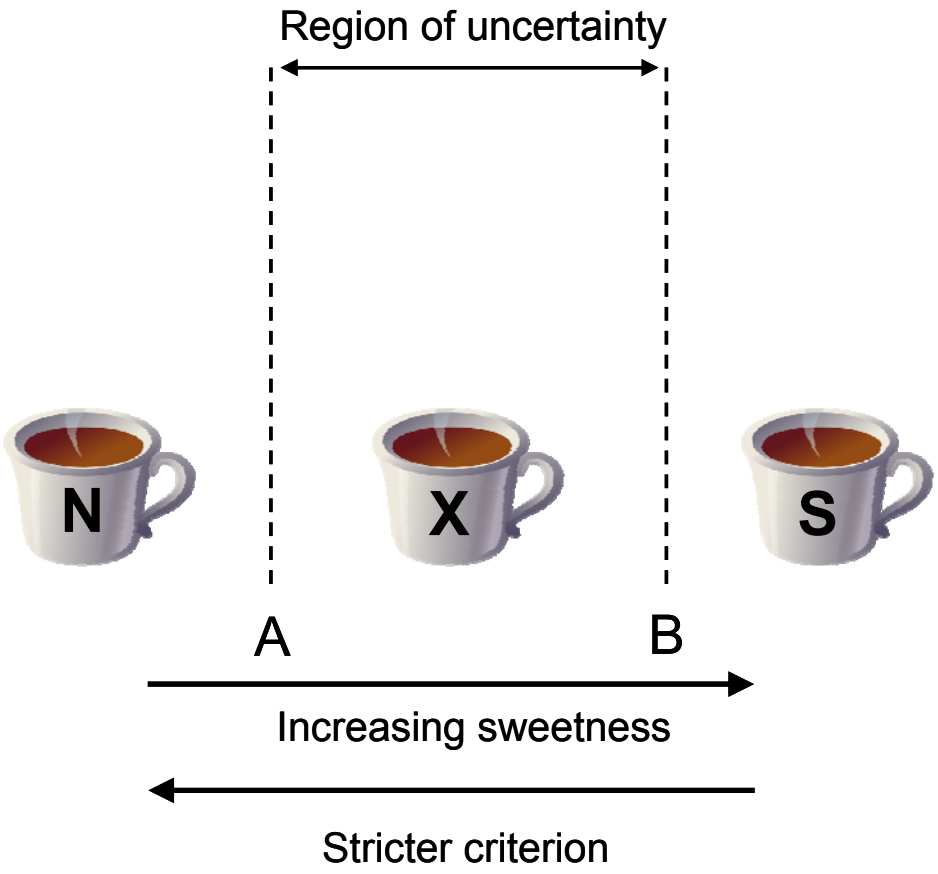


Figure 1-16 - Schematic diagram to illustrate response bias - adapted from O'Mahony (1992)

If a subject were presented with the samples (N) and (S) and asked if sample (S) was sweeter than sample (N), the decision is likely to be easy (comparable to scenario (a) in figure 1-15). If however a subject were presented with samples (X) and (S), and asked if sample (S) was sweeter than sample (X), the decision is likely to be much harder. Whilst sample (S) does indeed contain more sugar, than sample (X), the difference is subtle, sample (X) being said to lie in the "region of uncertainty". The answer the subject provides will depend upon where the subject draws the line between sweet and unsweet. This imaginary line is known as the subject's criterion, and in this example, the point where unsweet ends and sweet begins is known as the β-criterion (Green and Swets, 1966).

If a subject positions his criterion at point (A), he is said to have a strict criterion, and requires a high level of confidence before committing to saying that a sample is sweet. In the above example the subject would report that sample (S) was not

sweeter than sample (X). If the subject positions his criterion at point (B), he is said to have a lax criterion, being happy to commit to saying that a sample is sweeter, and in the above example would report that sample (S) was indeed sweeter than sample (X). Obviously he can draw his line at any point in-between.

A second type of criterion encountered in discrimination testing is the k- (Macmillan and Creelman, 2005) or τ- (Ennis et al., 1988) criterion. This criterion is found in protocols where at least two samples are presented, the subjects task being to indicate whether they are the same or different. Rather than a boundary separating two regions (as shown in figure 1-16), the τ-criterion resembles a sensory yardstick to which the perceived differences between the two intensities are compared. The answer given will depend upon the relative location of the intensities on the distribution at the moment of tasting, and is illustrated schematically in figure 1-17.

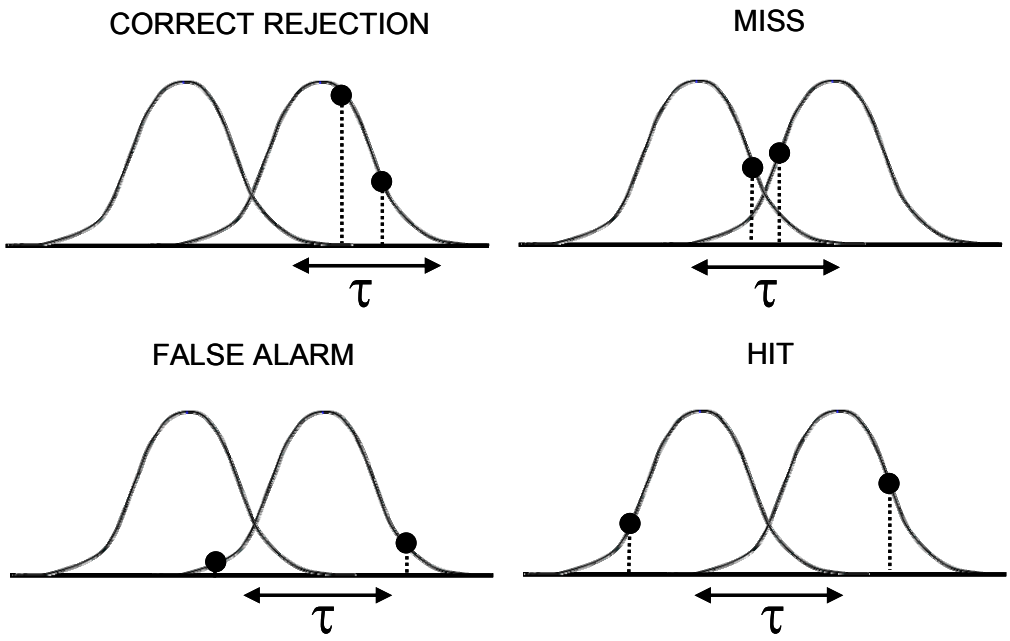


Figure 1-17 - Schematic diagram to illustrate the τ-criterion relevant to the same-different task

Four different outcomes are possible in the same-different task; hit, miss, correct rejection, and false alarm. In the case of a "hit", the two samples presented to the subject are unmatched (i.e. different), and due to the intensity at time of tasting, the distance between the two stimuli exceeds the τ-criterion, and the subject correctly identifies the two samples as "different". In the case of a "miss", although the two samples presented to the subject are still unmatched, due to the intensity at time of

tasting, the distance between the two does not exceed the τ-criterion;, and the subject incorrectly identifies the two samples as "same". In the case of a "false alarm", although the two samples presented to the subject are matched (i.e. the same), the distance between the perceived intensities exceeds the τ-criterion, and so the samples are incorrectly identified as "different". Finally, in the case of a "correct rejection", the two samples presented are the same, and since difference between the perceived intensities is smaller than the τ-criterion, the subject correctly identifies the samples as "same". These four outcomes are shown in figure 1-18.

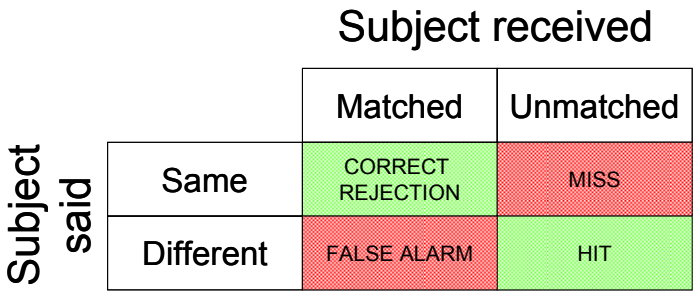


Figure 1-18 - Four possible outcomes in the same-different task

A subject who uses the τ-criterion in the same-different task is said to be using the "differencing strategy", described by Ennis and Ashby (1993), Rousseau et al. (1998) and Rousseau (2001). It is suggested that an alternative strategy (the optimal-, or β-strategy) can also be used in this task (Johnson, 1980, Rousseau, 2001). Rather than using distances, subjects using this strategy attempt to classify samples in sub-areas or categories in order to decide whether they are the same or different. There is however evidence that in the case of flavour based sensory studies, subjects adopt the differencing strategy (Irwin et al., 1993, Rousseau, 2001), the optimal strategy being more relevant to studies of visual stimuli such as work semantic characteristics (Francis and Irwin, 1995, Irwin and Francis, 1995).

Response bias is defined as the tendency of a subject to locate or size his criterion, and is completely independent to a subject's actual sensitivity to the stimulus in question. Since the criterion is a cognitive factor, it can shift positions and size both within and between sessions, so affects a subject's responses. It is therefore essential for any discrimination test to adopt strategies to avoid being susceptible to uncontrolled criterion shifts.

There are generally two ways of handling the problem of response bias. The first is by stabilising the criterion, ensuring that it always falls between two samples. This is achieved in many of the commonly used discrimination tests (e.g. triangle, directional paired comparison, and duo-trio) In the case of the directional paired comparison test, subjects are presented with two samples and asked to indicate the "*x* er" (e.g. sweeter) of the two. These instructions effectively force the criterion to lie between the two samples, and the subject need not worry about how much sweeter the sample has to be in order to be called sweeter.

In other cases, such as the same-different test, the criterion cannot be stabilised in this way. In this case another means of handling response bias is used, making use measures such as d' which are criterion-free (i.e. are not influenced by the actual criterion chosen by subjects).

1.5.3 Signal detection theory

Thustonian modelling is based on ideas first developed by (Thurstone, 1927) and was a precursor to the signal detection theory (Macmillan and Creelman, 2005), developed by psychophysicists' as a theoretical basis for understanding the measurement of human sensitivity. This approach has recently been applied to sensory evaluation, and several reviews exist (Ennis, 1990, Ennis, 1993, O'Mahony et al., 1994, Rousseau, 2001). One of the uses of the signal detection theory is to obtain a measure of a judge's sensitivity to a stimulus, or difference between stimuli that is not affected by criterion variation. Another use is to provide indices as a means of comparing the relative power of different testing procedures which otherwise vary in their sensitivity.

One such indice is d', which can be used for comparing the sensitivity of different test procedures, as well as comparing samples in tests where response bias cannot be controlled. It has been stated by O'Mahony and Rousseau (2002) that a d' of 1 can be considered as a threshold value in psychophysics. Several different procedures are available for calculating values d'. For the forced-choice procedures such as triangle, duo-trio, and directional pair-comparison tests (where the criterion has been stabilised), d' can be obtained directly from tables e.g. Macmillan and Creelman (2005), based upon the proportion of correct responses.

In test procedures where the criterion cannot be stabilised (e.g. same-different test), d' can be obtained through use of receiver operating characteristic (ROC) curves, generated by plotting a subject's proportion of hits and false alarms (figure 1-18) at varying criterion levels. One way of varying the position of the criterion is to offer rewards or punishment for correct or incorrect responses. The promise of a reward for correctly identifying differences would result in the subject adopting a more lax criteria where at even the slightest hint of a difference, the subject would report samples as being different. Likewise, the threat of punishment for incorrectly identifying differences would result in adoption of a stricter criterion; the subject would wait for large definite differences to be present before being prepared to report a difference. Because the proportion of hits and false alarms at various criterions merely provide points on a graph, the actual levels do not matter.

Whilst providing rewards and punishment may be fun, it is not practical or ethical. The subject's criterion can therefore be artificially adjusted by the use of a categorisation procedure, also known as the sureness rating. In this procedure, a subject first decides whether samples are "same" or "different", and then provides a level of confidence associated with that choice. (very sure, quite sure, not at all sure / guess) (Green and Swets, 1966).

The ROC curve provides several unbiased indices of performance, and so allows comparison between other tests. These indices provide a measure of the degree to which one stimulus is discriminable from another, not just whether the difference is significant according to a binomial test (Irwin et al., 1993). The greater the strength of the signal, the more the curve arches up, whereas the smaller the strength of the signal, the less the curve arches up, until d' reaches 0 and the curve is a diagonal line where the proportion of hits and false alarms are equal.

According to O'Mahony (1992), if the assumptions of normality and standard deviation are correct, the ROC curve will be symmetrical along the diagonal, although if not held, the curve will appear unsymmetrical. The proportion of area underneath the ROC curve is defined P(A), and is independent of assumptions of normality and standard deviation. It is therefore often used as an alternative to d', although given the direct relationship between d' and P(A) (Elliot, 1964), most computer packages now calculate values of d' based directly upon values of P(A), thus eliminating the problems of assumption violation.

2 A NOVEL APPROACH TO MONITORING VOLATILE RELEASE FROM FRESHLY PREPARED BLACK TEA INFUSIONS

2.1 INTRODUCTION

As described in section 1.3.2, tea contains in excess of 630 different volatile compounds (Wang et al., 2002). Although this list contains volatiles identified from different types (green, oolong, black etc.) and forms (leaves, powders, infusions etc.) manufactured under different growing and processing conditions, and extracted using a wide variety of techniques, it can nevertheless be concluded that tea infusion headspace comprises a highly complex volatile system.

APCI-MS is an excellent tool for monitoring the volatile release from foods and beverages, and has been used to analyse volatiles in the headspace of food systems, as well as in the breath as samples are consumed (Taylor et al., 2000). As described in section 1.4.3.3 however, the relative "simplicity" of this method is severely compromised in the case of complex mixtures, especially those containing isobaric and isomeric (i.e. stereoisomers) compounds. The headspace of black tea infusions fits well into this category, containing numerous examples of isobarics (e.g. 2-methyl butanal and pentanal), and isomerics (e.g. *E*-2- and *Z*-4-heptenal).

It is for this reason that analysis of volatile release from tea using direct mass spectrometry methods is so difficult without prior work. Figure 2-1 is an example of APCI spectra (TIC, *m/z* 40-250) corresponding to the headspace of a black tea infusion.

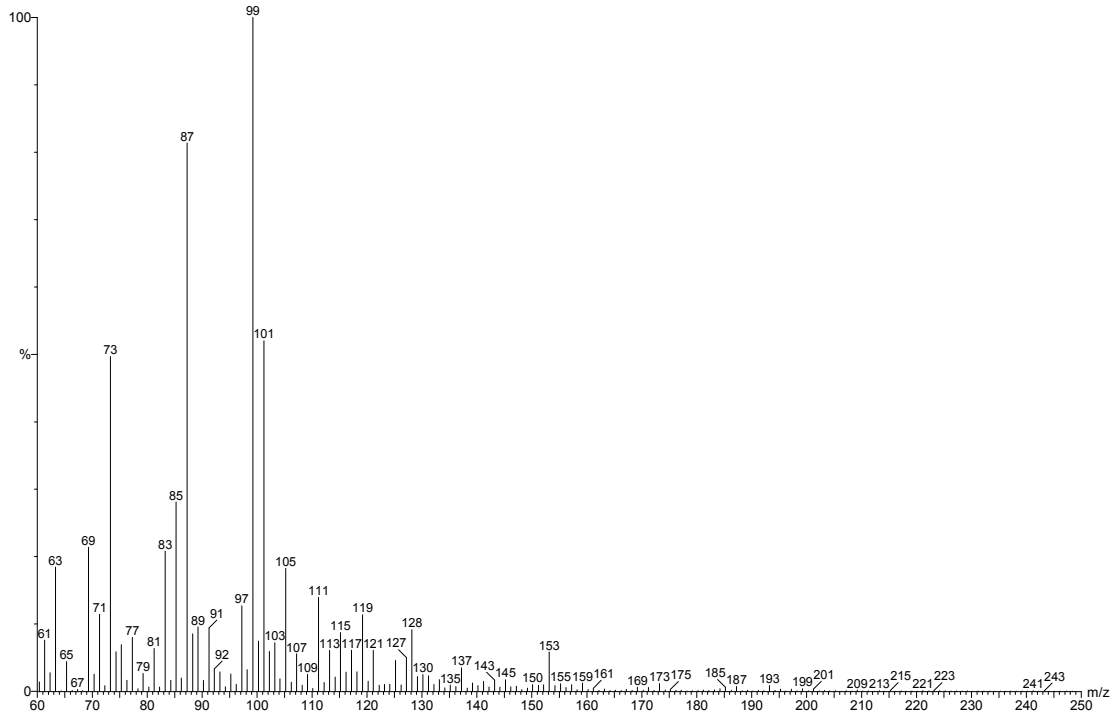


Figure 2-1 – APCI spectra (TIC) of black tea infusion headspace illustrating the many ion masses present

The number of ion masses is large, and without further information on which to base decisions, it is dangerous to make assumptions as to the origins of particular ions. Whilst monitoring ions alone will yield data, the value of this data is limited without knowing which compounds the ions correspond to.

Combined gas chromatography electron impact atmospheric pressure chemical ionisation mass spectrometry (GC-EI/APCI-MS) is one of several techniques which has been used by previous workers in order to tackle this problem (Lindinger et al., 2005, Taylor et al., 2003). This technique can be further enhanced by taking advantage of the fragmentation of compounds, and the fact that fragmentation can be altered by adjusting the cone voltage settings of the APCI-MS.

This chapter describes a GC-EI/APCI-MS approach used in order to assign ions present on APCI-MS spectra to the presence of particular compounds present in freshly infused black tea headspace. The development of a novel protocol for monitoring the volatile release from hot, freshly prepared tea infusions is also described. The aim was to monitor black tea infusion headspace using as realistic a

system as possible, whilst still maintaining a reasonable level of control to enable collection of reproducible data.

The objectives of this chapter were as follows;

- To establish the reproducibility of the standard tea infusion procedure used throughout this research

- To assign ions monitored using APCI-MS to specific volatile compounds present in black tea infusion headspace

- To develop a simple, robust system which could be used to make comparisons between the aroma released from freshly prepared black tea infusions

2.2 MATERIALS AND METHODS

2.2.1 Reproducibility of the standard black tea infusion procedure

A standard tea infusion procedure was developed for use throughout this research, and prior to any experimentation, it was essential to establish an indication of its reproducibility. Since proposed work involved manipulation of infusion water temperature and infusion concentration, it was important to confirm that these factors would not adversely affect reproducibility. Reproducibility was assessed by investigating both total solids and volatile concentration within and between batches of infusions.

2.2.1.1 Standard black tea infusion procedure

In order to avoid confusion between % total solids content of infusions (i.e. g/100mL), and infusion concentration, the latter has been described according to a %w/v basis (a 1 %w/v infusion equates to 1g tea per 100 mL water), and this convention has been followed throughout the thesis. The following section describes the approach used to prepare standard 1 %w/v infusions using boiling water.

All infusions were prepared using bottled mineral water (Highland Spring, Blackford, UK). This water was chosen since it contained an average mineral composition and represented medium water hardness within the UK. Typical analysis at source is as follows (mg/L); calcium (32.0), magnesium (8.0), potassium (0.5), sodium (4.5), bicarbonate (133.0), chloride (5.0), sulfate (7.0), nitrate (<2.0), fluoride (<0.1), iron (<0.01), aluminium (<0.01), total dissolved solids at 180°C (136 mg/L), pH 7.8.

Loose (dry) black tea (3 g, LYL640 blend, Unilever Research, Colworth, UK) was transferred into a pre-heated vacuum insulated flask (Thermos, Rolling Meadows, 500 mL). Pre-heating of vacuum flasks was essential since pouring boiling water onto leaves in a cold or room temperature flask resulted in an immediate reduction in temperature of water. After removing the pre-heat water, freshly boiled mineral water (300 mL) was added to the dry tea, and the flask immediately sealed and

inverted once. After 3 min the flask was inverted five times, and then left for a further minute. At 4 min the tea infusion was inverted once more before the leaves were removed by filtering through double-layered muslin (Moorlands Cheesemakers, Bruton, UK).

Due to the large volumes required for sensory discrimination testing (described in chapter 4) infusions were prepared in larger (1.8 L) vacuum flasks using 950 mL water, although the infusion procedure itself remained unchanged.

2.2.1.2 Total solids analysis

Infusions were prepared according to an adaptation of the standard preparation method described above using water of seven different temperatures (40, 50, 60, 70, 80, 90 and 100 °C), and at eight different concentrations (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 %w/v). In order to accurately control water temperature it was found necessary to measure the volume prior to heating. This was shown to be more reliable that trying to measure an accurate volume post-heating since rapid heat transfer resulted in large differences in temperature.

Three replicate infusions were prepared according to each infusion water temperature and concentration. Infusions were filtered through double-layered muslin into Schott bottles (250 mL), sealed, and cooled rapidly under running water.

From each batch of infusion, three sub-samples (~50 mL) were poured into pre-weighed metal trays, and the accurate mass recorded. Samples were placed in a drying oven (105 °C) and held for 16 hours. Samples were then re-weighed, and by taking into the account the mass of the empty tray and infusion used, total solids content (%) calculated.

2.2.1.3 Gas chromatography-mass spectrometry analysis

An internal standard technique was employed to establish the concentration (ppb) of five selected volatile compounds (hexanal, *E*-2-hexenal, linalool, methyl salicylate, and β-ionone) in the tea infusions prepared using the seven different water temperatures and eight different concentrations as described above in section 2.2.1.2 for total solids analysis.

Calibration curves for each of the five compounds were first created by transferring aliquots (10 mL) of PG granule infusion (total solids content 0.375 %) to 22 mL glass headspace vials. PG granule infusions were chosen in preference to water to more accurately represent the non-volatile composition found in the samples of interest (i.e. LYL640 infusions). It had been previously established that PG granule infusions at the total solids content matching that of a 1 %w/v LYL640 infusion (0.375 %) did not contain any of the compounds of interest so would not interfere with quantification.

The internal standard (carvone, diluted in ethanol) was added (1 µl) to vials, to give a concentration of 80 ppb v/v. A solution containing all five compounds (diluted in ethanol) was prepared so that addition of different levels (0-8 µl) to the vials provided points on a calibration curve. These levels are shown below in table 2-1, and were selected based upon the results of preliminary experiments. Three replicate analyses (i.e. three separate samples) were carried out for each of the concentrations of standard.

Table 2-1 - Concentration of volatile compounds (ppb v/v) used as points on a calibration curve

	Concentrations used to construct calibration curve (ppb v/v)								
Hexanal	0	17	34	51	68	85	102	119	136
***E*-2-hexenal**	0	55	110	165	220	275	330	385	440
Linalool	0	9	18	27	36	45	54	63	72
Methyl salicylate	0	8	16	24	32	40	48	56	64
β-ionone	0	1.7	3.4	5.1	6.8	8.5	10.2	11.9	13.6

Chromatography conditions were selected based upon those routinely used by Unilever Research, where considerable work has previously established the most appropriate parameters for this type of work.

Solid-phase-micro-extraction was carried out using a StableFlex fibre coated with 50/30 µm poly(divinylbenzene) (DVB) / carboxen / poly(dimethylsiloxane) (PDMS), (Supelco, Bellefonte, PA), conditioned as recommended by the manufacturer. Sample vials were transferred to the sample incubation system (65 °C) of a

CombiPal autosampler (CTC analytics, Zwingen, Switzerland). The SPME fibre was exposed to the headspace for 20 min, whilst agitated (500 rpm). Volatile compounds from the SPME fibre were vaporised by injecting into a Trace GC Ultra (Thermo electron corporation, Waltham, MA), fitted with a BP20 column (SGE, Milton Keynes, UK; 25 m x 0.25 mm i.d.; film thickness 0.25 µm). The injector was operated in splitless mode (240 °C, 1 min) with helium as the carrier gas. The oven temperature program was as follows: 45 °C for 2 min, 10 °C/min to 230 °C, and hold for 5 min. The EI-MS (DSQ, Thermo Electron Corporation) was operated in full scan mode over the *m/z* range 40 – 250 (scan time 0.45 s; interscan delay 0.05 s).

Chromatograms were integrated, and a calibration curve constructed for each compound by calculating the ratio of peak area of compound of interest to internal standard, and plotting ratio (y-axis) against compound concentration (x-axis).

From each batch of LYL640 infusion, three samples (10 mL) were transferred into separate 22 mL glass headspace vials, and after adding 1 µl internal standard, were crimp sealed. Following chromatography (using the procedure described above), chromatograms were integrated, and the ratio of compound of interest to internal standard calculated. By referring to the calibration curves previous constructed it was possible to calculate the concentration of compound (ppb) in the aqueous phase.

2.2.1.4 Data analysis

One way ANOVA (p=0.05) (XLSTAT, v. 7.5.2, Addinsoft, New York, NY) was carried out on the total solids and GC-MS data for the nine samples prepared according to each infusion water temperature or concentration. Reproducibility of the infusion procedure was established by comparing the within and between batch variation of total solids and volatile concentration.

2.2.2 Relating APCI-MS spectra to volatile composition of tea infusion headspace

2.2.2.1 Headspace sampling using the solid-phase-micro-extraction (SPME) procedure

Solid-phase-micro-extraction **(**SPME) of the volatiles released from tea infusions was carried out using a 2 cm StableFlex fibre, coated with 50/30 µm DVB / carboxen / PDMS, (Supelco, Bellefonte, PA), conditioned as recommended by the manufacturer. This fibre was chosen since it has been shown to be especially well suited to the analysis of volatile flavour compounds (Doleschall et al., 2003).

Tea infusion (1% w/v, 200 mL) was prepared using 100 °C water according to the standard tea preparation method described above, transferred to a 250 mL Pyrex® bottle (Fisher Scientific, Loughborough, UK) and sealed with a screwcap. The SPME fibre was exposed to the tea infusion headspace through a small hole in the bottle screwcap for 5 min and then transferred to the GC injector. This time period was chosen to capture those volatile compounds released in the first few minutes following the infusion procedure.

2.2.2.2 Gas chromatography-electron impact atmospheric pressure chemical ionization-mass spectrometry (GC-EI /APCI-MS)

A schematic of the GC-EI/APCI-MS setup is shown in figure 1-13, section 1.4.3.3.

Volatile compounds from the SPME fibre were analyzed by GC (Thermo Finnigan 8000, Hemel Hempstead, UK) fitted with a BP5 column (5% Phenyl / 95% Dimethyl Polysiloxane, SGE, Milton Keynes, UK; 30 m x 0.25 mm i.d.; film thickness 1.0 µm). This column was chosen as a compromise between the non-polar BP1, and polar BP20 routinely used for GC flavour analysis. The injector was operated in splitless mode (240 °C; 1 min) with helium as the carrier gas (head pressure 20 psi). The oven temperature program was as follows: 45°C for 2 min, 10 °C/min to 230 °C, and hold for 5 min. The exit of the column was split using an outlet capillary column splitter (SGE), with 0.1 mm i.d. deactivated fused silica tubing (SGE) leading to the

EI-MS source. The EI-MS was operated in full-scan mode over the *m/z* range of 40 – 250 (scan time 0.45 s; interscan delay 0.05 s). The remaining flow was carried through a heated (200 °C) transfer line (0.32 mm i.d. deactivated fused silica tubing; Supelco) into the source of the APCI-MS (Micromass, Manchester, UK) with a corona pin voltage of 4 kV. Compounds entering the source were introduced into the high vacuum region of the mass spectrometer where they were detected according to their *m/z* ratio.

Gas phase APCI-MS was operated in full-scan mode over the *m/z* range of 40 – 250 (scan time 0.45 s; interscan delay 0.05 s). The chromatographic separations of tea headspace were carried out in duplicate at six different cone voltages (12, 15, 18, 21, 24, and 27V), thus altering the fragmentation patterns of the individual volatiles.

Eluting compounds were identified using retention time and mass spectral library matching (NIST 1998, Ringoes, NJ) against authentic standards. It was accepted that not all compounds could be positively identified.

2.2.3 Analysis of volatiles released from tea infusion headspace

Figures 2-2 and 2-3 show a schematic diagram and photograph of the apparatus developed in order to analyse the headspace of freshly infused black tea.

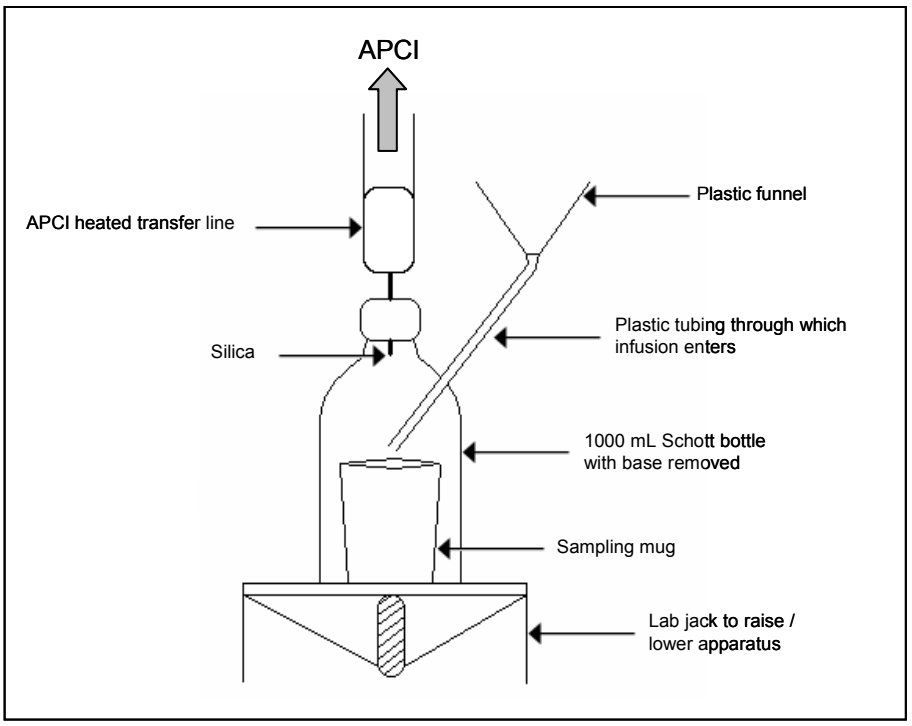


Figure 2-2 – Schematic diagram of tea infusion headspace sampling apparatus

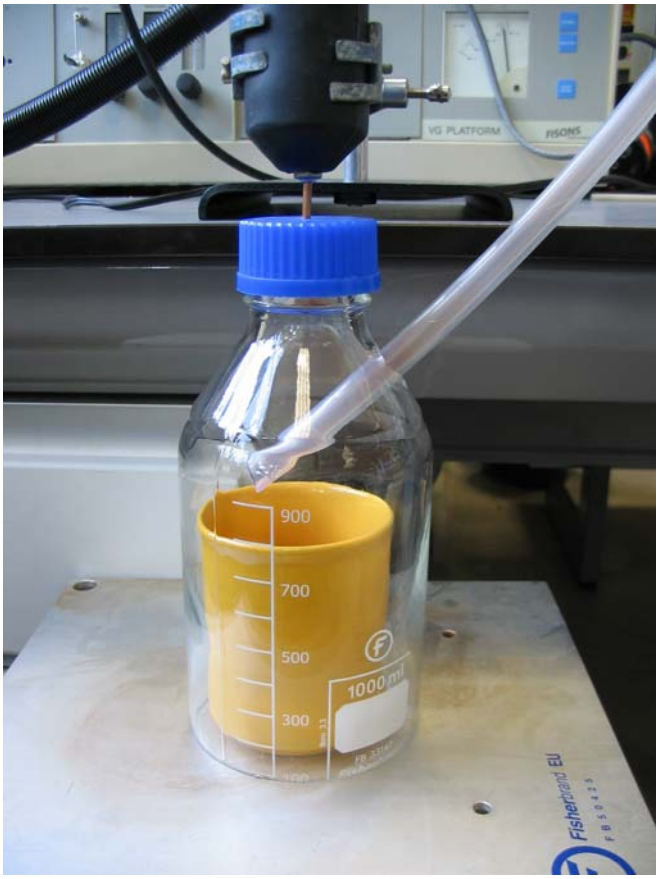


Figure 2-3 – Photograph of tea infusion headspace sampling apparatus

The tea infusion was poured through a double layer of muslin into a polypropylene funnel connected to plastic tubing (20 cm, 5 mm i.d.). The tea infusion flowed along the tubing, through a hole in the neck of a borosilicate glass bottle with the base removed (1 L, Fisher Scientific, Loughborough, UK), and into a mug. (The muslin layer resulted in separation of the tea leaves from the supernatant if appropriate, and also prevented a vortex from forming, enabling a more reproducible transfer of infusion). The APCI-MS probe entered through a 1 mm diam. hole in a polypropylene screw top. Headspace were drawn at 25 mL/min into the ionization source through deactivated fused silica tubing (1 m x 0.53 mm i.d.) surrounded by a heated (160 °C) transfer line to prevent condensation of water or volatiles.

The scan file was set to simultaneously monitor the intensity of selected ions at selected cone voltage settings using a dwell time of 0.1 s and interscan delay of 0.02 s for 5 min. (This later had to be reduced to 4 minutes due to performance issues whereby the APCI-MS was unable to cope with the amount of steam present).

The APCI system was calibrated by injecting (using a syringe pump) known concentrations of volatile compound in hexane or cyclohexane solutions at 1.5 µL/min into the heated make up gas entering the APCI-MS (10 L/min) and measuring peak height. From the calibration curve, the quantity in any sample could be calculated by taking into account the flow rate.

2.2.3.1 Data processing

Raw data were processed using in-house software to smooth the data (moving five point average) and to extract a data point every 3 s. The data points were expressed as the intensity of the ion count (I_{max}) or as the cumulative counts (cumulative ion count; equivalent to area under the curve) during the sampling period.

2.2.3.2 Reproducibility of the headspace sampling technique

Reproducibility of the headspace sampling technique was determined by carrying out 25 consecutive analyses of 1 %w/v tea infusions prepared according to the

standard procedure (described above). Values of cumulative ion count and I_{max} were determined for each of the ions of interest, and the corresponding % coefficient of variation (%CV= SD x 100/mean) values calculated.

2.3 RESULTS AND DISCUSSION

2.3.1 Reproducibility of the standard black tea infusion procedure

The results presented in this chapter indicate only reproducibility of total solids and volatile concentration of infusions prepared using different water temperatures and concentrations. The actual effect these modifications in preparation method had on the total solids and volatile concentration of infusions is reserved for chapter 3 where it is more relevant.

Tables 2-2 and 2-3 summarise the results of the ANOVAs carried out on the total solids and volatile concentration data for infusions prepared according to the different infusion water temperature and concentrations respectively. Only those cases where the significance level (p) was <0.05 have been included. In these cases, a statistically significant batch-to-batch variation in concentration was evident.

Table 2-2 - Significance level (p) associated with infusion water temperature (40-100 °C) for concentration of five volatile compounds and total solids content of infusions where p<0.05

	Infusion water temperature (°C)						
	40	**50**	**60**	**70**	**80**	**90**	**100**
Hexanal		0.021			0.042		
***E*-2-hexenal**				0.016	0.004	<0.001	
Linalool				0.047		0.014	
Methyl salicylate					0.022		
β-ionone		0.004		<0.001		0.038	
Total solids				0.001		0.041	

Table 2-3 - Significance level (p) associated with infusion concentration (0.25-2.0% w/v) for concentration of five volatile compounds and total solids content of infusions where p<0.05

	Infusion concentration (%w/v)							
	0.25	**0.5**	**0.75**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**
Hexanal							0.017	
***E*-2-hexenal**				0.020				
Linalool	0.039				0.001			0.002
Methyl salicylate	0.021			0.040	0.006			0.009
β-ionone				0.001	0.001			
Total solids		0.007			0.020	0.014		0.005

Tables 2-2 and 2-3 show that in the majority of cases, a significant difference in the concentration of at least one volatile compound (or total solids content) was present. In no cases was a significant difference in the concentration of all five volatiles and total solids present between batches of a particular infusion. Only in the case of infusions prepared using 40, 60 and 100 °C water, and those prepared at a concentration of 0.75 %w/v was no significant batch-to-batch variation apparent.

2.3.2 Relating APCI-MS spectra to volatile composition of tea infusion headspace

The GC-EI/APCI-MS procedure provided a means of linking the ions observed in APCI spectra to the presence of specific compounds in the headspace of freshly infused tea. This procedure resulted in two chromatograms (for each analysis at a particular APCI-MS cone voltage). Figure 2-4 shows typical chromatograms obtained from simultaneous gas chromatography analysis of tea infusion headspace using both EI-and APCI-MS detectors. The lower chromatogram corresponds to analysis at cone voltage 18 using APCI-MS. The upper chromatogram is the corresponding EI-MS trace, although since detected by EI, is independent of the cone voltage setting of the APCI-MS. Since the peak height of linalool (12.81) on the (upper) EI trace is considerably larger than other peaks on a relative basis

compared to the (lower) APCI trace, in order to illustrate similarities between the smaller peaks, the y-axis of the upper trace has been magnified.

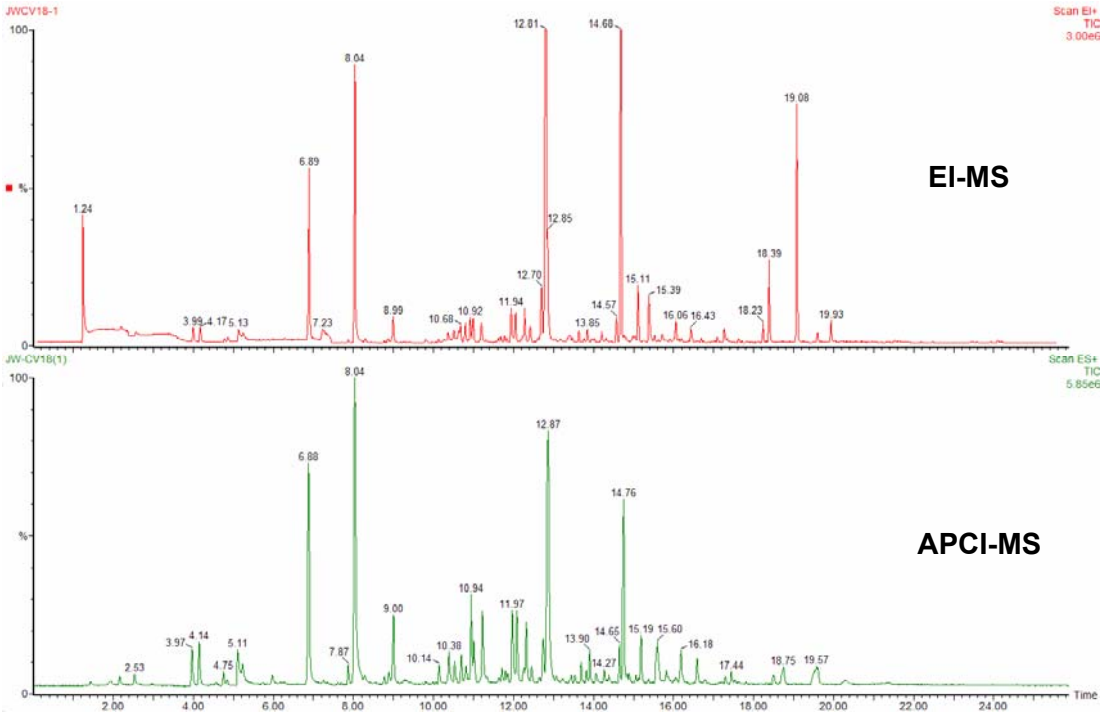


Figure 2-4 – Traces obtained from simultaneous gas chromatography analysis of tea infusion headspace, using both EI-MS and APCI detectors

It can be seen from figure 2-4 that generally, the two chromatograms are very similar, both in terms of compounds detected and their relative intensities. This observation has been reported by previous workers using the technique (Lindinger et al., 2005, Turner et al., 2002).

A couple of exceptions can however be seen. A peak at 1.24 on the EI trace is not present on the APCI trace. This peak corresponds to carbon dioxide, and is not present on the APCI trace as it is not ionised by proton transfer under these conditions. Another difference is obvious towards the right hand side of the chromatogram where the elution of higher molecular mass compounds occurs. The peaks at 18.39 and 19.08 on the EI-MS trace (referring to an unidentified compound and β-ionone respectively) are still present on the lower chromatogram although their peak shape is poorly defined (18.75 and 19.57). This is likely a result of the distance (~3 meters) between the two machines over which volatiles exiting the GC column had to travel prior to entering the APCI mass detector. Although a heated transfer line at 200 °C (maximum operable temperature) was used in order to

prevent condensation of volatiles, some condensation and peak broadening likely occurred.

By carrying out analysis at six different cone voltages, the effect cone voltage had on fragmentation of the identified compounds in tea infusion headspace could be determined. Figure 2-5 clearly illustrates the importance cone voltage had on the assignment of ions to the presence of particular compounds. At cone voltage 15, the ion at *m/z* 121 can be almost entirely attributed to the presence of the protonated molecular ion of phenylacetaldehyde (11.98 min on the APCI-MS trace, MW 120). At cone voltage 27 however, methyl salicylate (MW 152) also produces an ion with *m/z* 121 due to the loss of methanol (14.76 min on the APCI-MS trace).

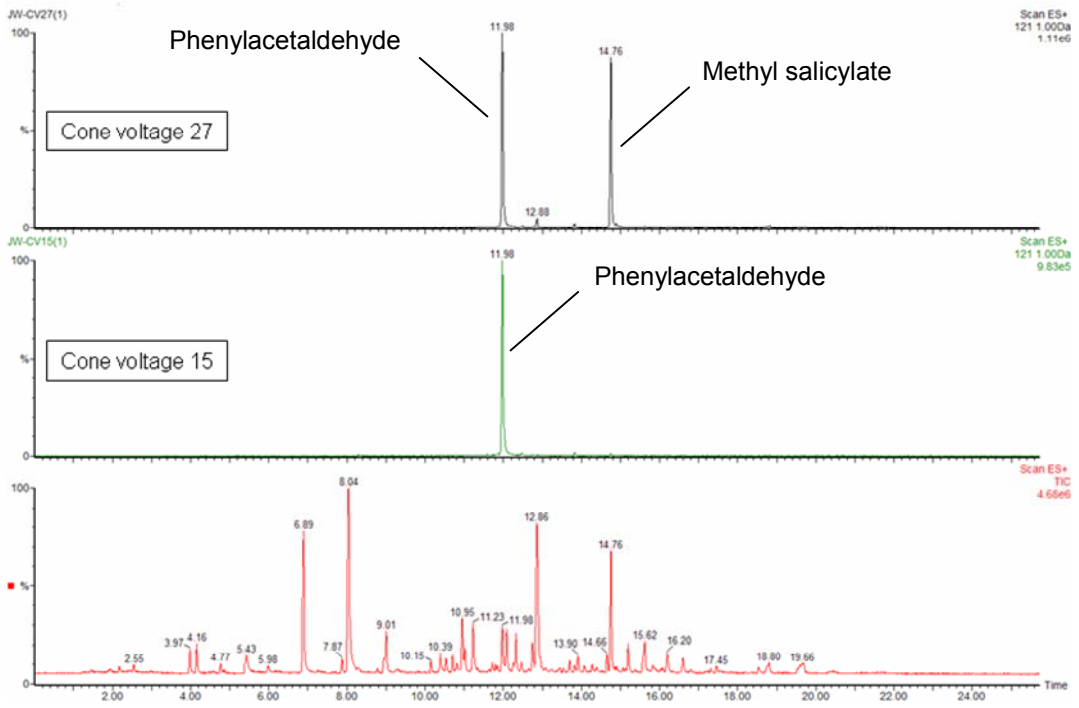


Figure 2-5 – Manipulation of cone voltage to aid ion allocation

Monitoring ion 121 at a cone voltage of 27 is therefore undesirable since two separate compounds in tea infusion headspace contribute to the total intensity. Monitoring this ion at cone voltage 15 however leads to the unequivocal detection of phenylacetaldehyde in this system.

Inspection of the other compounds, identified by retention times and the EI-MS library, showed three different levels of confidence in assigning ions to the presence

of specific compounds in tea headspace. Figure 2-6 shows ion chromatograms obtained from the APCI detector following the GC-EI/APCI-MS procedure.

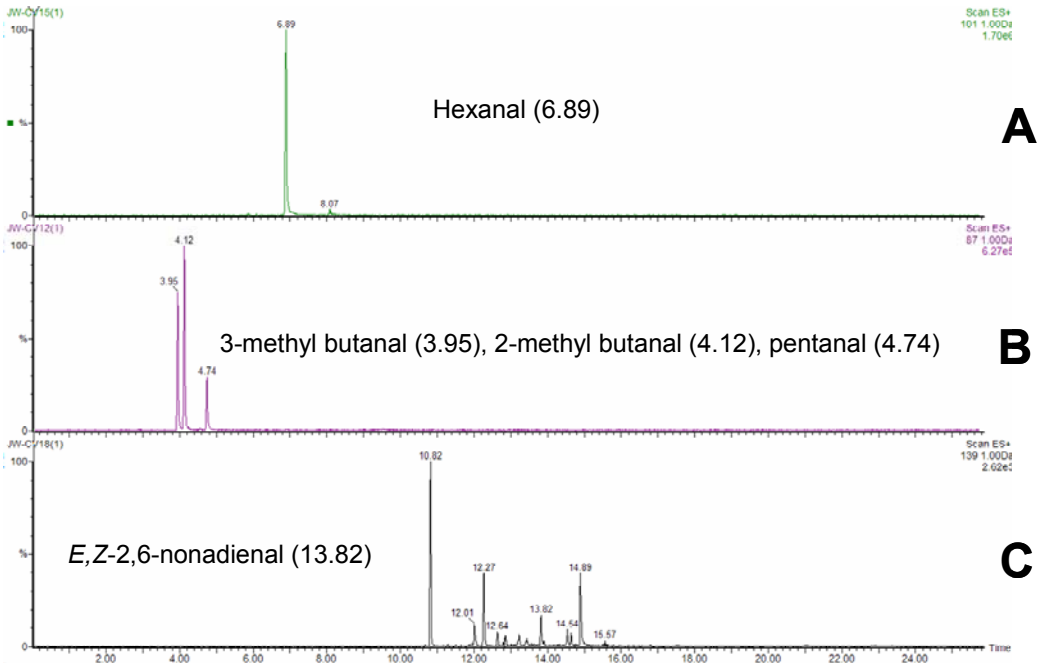


Figure 2-6 – Ion traces showing differences in confidence of assignments

Trace A clearly shows that the ion at *m/z* 101 is almost entirely due to hexanal (retention time 6.89 min), with a very minor contribution from an unidentified compound at retention time 8.07 min. Trace B shows that ions with *m/z* 87 are found at three different retention times, corresponding to 2- and 3-methyl butanal and pentanal. Trace C is the ion chromatogram at *m/z* 139 which contains over twelve peaks including *E,Z*-2,6-nonadienal, known to be an important aroma in tea. It is not however possible to monitor *E,Z*-2,6-nonadienal with any degree of certainty due to the contributions from the other eleven compounds also significantly contributing to this ion mass. This is one of the limitations of the technique, and although fragmentation has been manipulated in order to identify ions which can be related to single (or small groups) of compound, the sheer number of compounds present in tea headspace has meant that this has not always been possible.

Examination of all ion chromatograms led to the selection of 15 ions that represented individual compounds or groups of compounds, and are shown in table 2-4. In the case of six ions, a compound contributed over 90 % of the signal at that *m/z.* These compounds could therefore be monitored and quantified with a degree

of certainty. For two ion masses, *m/z* 111 and 113, the compounds involved were stereoisomers (heptadienal and heptenal respectively) while for three ions, *m/z* 87, 109 and 115, the compounds were isobaric (2- and 3-methyl butanal, pentanal; 2-octenal, 6-methyl-5-hepten-2-one; heptanone and heptanal respectively). For the remaining four ions, some key volatile compounds from tea were present but were associated with some unknown compounds.

Table 2-4 also indicates the confidence associated with these allocations, and has been achieved by looking at the contribution of peak area of known peaks to total peak area for an ion of interest. In cases such as the ion at *m/z* 101 at CV15, 98 % of the peak area came from the peak at 6.89 (hexanal), with just 2 % from the unidentified peak at 8.07. In other cases, it has not been possible to assert so much confidence to a particular assignment. In the case of ion at *m/z* 153, 66 % of the overall ion intensity on the GC-EI/APCI-MS trace could be attributed to the presence of methyl salicylate, with a further two unidentified compounds contributing 25 % of the total peak area.

Only those peak areas contributing ≥ 5 % of total peak area have been included in this table (hence contributions do not always tally to 100 %). Two replicate GC-EI/APCI-MS chromatograms for each ion / cone voltage combination were used to determine percentage peak area contribution.

It is essential at this point to define future terminology to describe the compounds released from tea infusions. Rather than referring to "the compounds represented by ion *x*", a decision has been made to use the name of the genuine compounds. In cases where an ion has been allocated to more than one (positively identified) compound, the compound contributing the greatest % peak area will be referred to (i.e. *E*-2-octenal in the case of the ion at *m/z* 109, heptanal in the case of the ion at *m/z* 115, and β-damascenone in the case of the ion at *m/z* 191). The stereoisomers represented by the ions at *m/z* 111 and 113 will be termed the "heptadienals" and "heptenals" respectively. The compounds represented by the ion at *m/z* 87 will be referred to as the "methyl butanals" (although it is appreciated that pentanal is also present). This terminology has been applied purely to aid readability, and in future discussion it must be remembered that in several cases ions represented more than one compound.

Table 2-4 - "Marker" ions chosen to monitor volatile release from tea infusions

Ion (*m/z*)	Cone voltage (V)	Compounds monitored (molecular weight)	% of total peak area
63	18	dimethyl sulfide (62)	100%
73	18	2 methyl propanal (72)	96%
87	15	2 methyl butanal (86), 3 methyl butanal (86), pentanal (86)	51% 33% 17%
99	18	*E*-2-hexenal (98) unidentified compound	91% 8%
101	15	hexanal (100)	98%
107	21	benzaldehyde (106)	95%
109	21	*E*-2-octenal (126), 6-methyl-5-hepten-2-one (126)	63% 22%
111	21	*E,E*-2,4-heptadienal (110), *E,Z*-2,4-heptadienal (110) † unidentified compound	49% 43% 6%
113	15	*E*-2-heptenal (112), *Z*-4-heptenal (112) unidentified compound	49% 28% 13%
115	15	heptanal (114), heptanone (114)	79% 19%
121	15	phenylacetaldehyde (120)	98%
137	15	linalool (154), unidentified monoterpene	78% 15%
153	21	methyl salicylate (152), unidentified compound unidentified compound	66% 13% 12%
191	15	β-damascenone (190), β-ionone (192) unidentified compound unidentified compound	55% 7% 16% 10%
193	18	β-ionone (192) unidentified compound	87% 12%

† - tentatively assigned

2.3.3 Analysis of volatiles released from tea infusion headspace

The system shown in figures 2-2 and 2-3 in section 2.2.3 has the capability of monitoring selected volatile compounds released from hot, freshly prepared black tea infusions. The system is a compromise between one which tries to represent a realistic system, and one which attempts to maintain a reasonable level of control. The real-life situation of volatile release from mugs of tea infusions is uncontrolled, particularly with regards to the impact of air movement on the transfer and distribution of volatiles, driven partly by the thermal effects from the hot cup as well as natural convection currents present in the atmosphere. Surrounding the mug by a glass bottle has the effect of stabilizing the air movement, preventing the random air currents from interfering with the release and transport of volatiles into the mass spectrometer. Positioning the APCI-MS sample inlet directly above mugs of hot infusion was shown to be completely inadequate in terms of generation of consistent data.

Figure 2-7 (overleaf) illustrates this visually, showing three replicate analyses of fresh tea infusion headspace carried out (A) using the novel system where currents are protected from the atmosphere, and (B) holding the sampling inlet directly above a mug of hot tea. For illustrative purposes only two ions (*m/z* 87 and 99) are shown, although all 15 ions were monitored simultaneously.

2.3.3.1 Reproducibility of the headspace sampling technique

The reproducibility of the cumulative ion count and I_{max} for each of the 15 monitored ions obtained from analysis of 25 replicate tea infusions was determined. Both cumulative ion count and I_{max} values yielded acceptable %CV values for all ions (most <5 %) indicating a very reproducible infusion and analytical technique. Irrespective of the data form, the highest values of %CV (about 8 %) were obtained for ion *m/z* 191, which corresponded mainly to β-damascenone. The headspace concentration and signal intensity for this ion was lower than the other compounds, and the lower signal:noise ratio for this compound probably accounts for the greater variation.

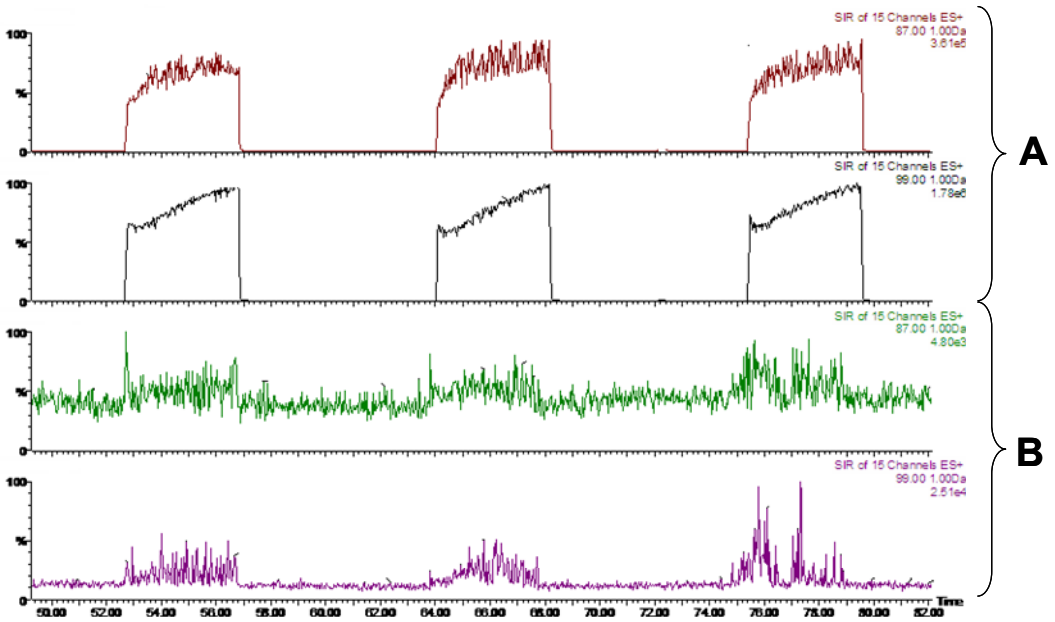


Figure 2-7 – APCI-MS chromatograms showing (A) sampling above a mug of tea using the newly developed sampling apparatus, and (B) directly above a mug of tea with no atmospheric protection

2.3.3.2 Relevance of monitored compounds

Calibration of the APCI-MS using authentic standards enabled approximate headspace concentrations of the monitored compounds to be established and these are displayed in table 2-5 along with published odour threshold values, obtained from a compilation database of experimentally determined values (van Gemert, 2003) . In cases where two or more compounds were assigned to an individual ion mass, calibration was carried out using only one of the compounds. As such, the concentrations obtained are expressed in terms of "equivalents" for that particular compound. The ion at *m/z* 87 for example corresponded to 2- and 3-methyl butanal, and pentanal, although since calibration was carried out using 3-methyl butanal, values of concentration are expressed in terms of 3-methyl butanal equivalents. An asterisk has been used where necessary to highlight the compound used for calibration purposes. Table 2-5 also shows the odour descriptors for the compounds represented by the 15 ions, obtained from various literature sources.

In order to obtain consistent values for all compounds, the concentration recorded refers to the point at which the signal plateaued following the initial increase from baseline level. This is in view of the slight accumulation of volatile compounds in the headspace due to the closed nature of the sampling apparatus, and taking the maximum (or average) intensity would have yielded misleading data.

Table 2-5 - Approximate concentration of monitored compounds in tea headspace, comparison with published values, and odour descriptors

Ion (*m/z*)	Compound(s)	A †	B †	Odour descriptor ‡
63	dimethyl sulfide	3.32	0.0003 - 20.6	Sulfury, marine [c]
73	2-methyl propanal	4.50	0.015 - 0.41	Pungent, fruit, chocolate [a]
87	2-methyl butanal 3-methyl butanal * Pentanal	5.76	0.01 - 0.1 0.0016 - 0.004 n/a	Cocoa, fresh, fruity [a] Fruity, peach, malty [a] n/a
99	*E*-2-hexenal	2.78	0.034 - 1.8	Herbal, green [a]
101	hexanal	2.46	0.02 - 0.33	Fatty, green [a]
107	benzaldehyde	0.07	<0.01 - 3400	Almond [a]
109	*E*-2-octenal * 6-methyl-5-hepten-2-one	0.30	0.009 - 0.25 0.0063 - 2.5	Fresh, cucumber [c] n/a
111	*E,E*-2,4-heptadienal * (*E,Z*-2,4-heptadienal)	0.30	0.057 n/a	Fatty [b] n/a
113	*E*-2-heptenal * *Z*-4-heptenal	0.11	0.034 - 2.8 0.0034 - 0.040	Grassy, green [c] Fatty, fishy [a]
115	heptanal heptanone *	0.10	0.006 - 9.5 0.045 - 3.3	Green, floral [c] n/a
121	phenylacetaldehyde	0.72	0.0006 - 0.0017	Hyacinth, rose [a]
137	linalool	0.54	0.0004 - 6	Lily of the valley [a]
153	methyl salicylate	0.53	0.002 - 119	Sweet, phenolic [a]
191	β-damascenone * β-ionone	0.006	0.000002 - 0.05 0.00007 - 11500	Fruity [b] Cedarwood, violet [a]
193	β-ionone	0.04	0.00007 - 11500	Cedarwood, violet [a]

* - compound used for calibration purposes

† - where (A) = headspace concentration (mg/m^3), and (B) = published odour threshold range (mg/m^3)

‡ - Odour descriptors from [a] Bauer et al. (2001), [b] Schuh and Schieberle (2006), [c] Prost et al. (2004)

n/a – information not available

The very high variability of odour threshold values is due to the wide variations in techniques used by different workers, and the fact that some values refer to detection, whereas others refer to recognition thresholds (not stated in many cases). Published values for β-ionone for example range from 0.00007 to 11500 mg/m^3. Differences are also apparent between the isomeric forms of compounds, where for the heptenals, values are quoted at 0.14 – 2.4 mg/m^3 for the *E*-2- isomer, and 0.0034 mg/m^3 for the *Z*-4- isomer. For all monitored compounds, calculated concentrations are generally above the range of values of odour threshold in air. It is also very important to appreciate the concept of synergism between different volatile compounds. Ito and Kubota (2005) for example observed that sub-threshold aroma constituents play an essential role in the characteristic aroma of Jasmine tea.

Table 2-6 provides a compilation of several key physicochemical parameters of the compounds represented by the 15 ions. Those shown are values of K_{aw}, Henry's law constant (HLC), LogP, and water solubility. Values were all obtained from EPI suite^{TM} software (US Environmental Protection Agency) using the Henrywin v1.90, and Wskowwin v1.41 programs. Where possible, experimentally determined values are provided, although estimates (italicised) are presented where unavailable. Henry's Law constants shown are averages of those determined using the bond and group contribution methods (section 1.4.1). Values of K_{aw} have been obtained from these by multiplying by 1000 and dividing by 24.46 (1 mole of gas occupies 24.46 litres). Water solubility data has been calculated based upon values of logP using a method discussed by (Meylan et al., 1996). All values correspond to those determined (or estimated) at the standard temperature of 25 °C and 1 atm.

Table 2-6- Compilation of physicochemical parameters for volatile compounds monitored in tea headspace

Compound	K_{aw}	HLC (m^3/mole)	LogP	Water solubility (mg/L)
Dimethyl sulfide	6.58×10^{-2}	1.61×10^{-3}	*0.92*	*22000*
2-methyl propanal	7.36×10^{-3}	1.80×10^{-4}	*0.74*	*89000*
2-methyl butanal	*8.63×10^{-3}*	*2.11×10^{-4}*	*1.23*	11230
3-methyl butanal	1.66×10^{-2}	4.05×10^{-4}	*1.23*	11230
Pentanal	6.01×10^{-3}	1.47×10^{-4}	*1.31*	*11700*
E-2-hexenal	2.00×10^{-3}	4.89×10^{-5}	*1.58*	5261
Hexanal	8.71×10^{-3}	2.13×10^{-4}	1.78	3527
Benzaldehyde	1.09×10^{-3}	2.67×10^{-5}	1.48	*6570*
E-2-octenal	3.00×10^{-3}	7.34×10^{-5}	*2.57*	612.7
6-methyl 5-hepten-2-one	*5.40×10^{-3}*	*1.32×10^{-4}*	*2.06*	1651
E,E-2,4-heptadienal	*2.21×10^{-3}*	*5.41×10^{-5}*	*1.86*	2805
E-2- heptenal	*3.99×10^{-3}*	*9.77×10^{-5}*	*2.07*	1810
Z-4-heptenal	*6.83×10^{-3}*	*1.67×10^{-4}*	*2.07*	1810
2-heptanone	6.91×10^{-3}	1.69×10^{-4}	1.98	*4300*
Heptanal	1.10×10^{-2}	2.70×10^{-4}	*2.29*	*1250*
Phenylacetaldehyde	*2.24×10^{-4}*	*5.48×10^{-6}*	1.78	3026
Linalool	8.79×10^{-4}	2.15×10^{-5}	2.97	*1590*
Methyl salicylate	4.01×10^{-3}	9.81×10^{-5}	2.55	1875
β-damascenone	*3.33×10^{-3}*	*8.15×10^{-5}*	*4.21*	12.48
β-*ionone*	7.40×10^{-3}	1.81×10^{-4}	*3.84*	25.16

It is clear from table 2-6, that the compounds represented by the 15 monitored ions possess a wide range of different physicochemical properties. Phenylacetaldehyde and linalool possess the lowest values of K_{aw} at 2.24×10^{-4} and 8.79×10^{-4} respectively. Correspondingly, values of Henry's law constant for these compounds are the lowest at 5.48×10^{-6} and 2.15×10^{-5} m^3/mole respectively. At the other extreme, dimethyl sulfide possesses the highest values of K_{aw} and Henry's law constant at 6.58×10^{-2} and 1.61×10^{-3} m^3/mole respectively.

These differences will inevitably result in differences in temporal profile of release from infusions under dynamic conditions as demonstrated by Marin et al. (1999), and described in section 1.4.2. Compounds with high values of K_{aw} (~10^{-2}) will favour the gas rather than aqueous phase at equilibrium, and when the gas phase is disturbed (i.e. under non-equilibrium conditions), produce unstable gas phase concentrations. Those compounds with lower values of K_{aw} will produce more stable gas phase concentrations under dynamic conditions. These differences will be discussed in more detail in the following chapter.

Table 2-6 also shows that the compounds represented by the 15 ions exhibit a wide range hydrophobicity and water solubility values. These range from the least hydrophobic, most water soluble 2-methyl propanal (LogP and water solubility 0.74 and 89000 mg/L respectively), to the most hydrophobic, least water soluble β-damascenone (LogP and water solubility 4.21 and 12.48 mg/L respectively). These differences in physicochemical properties have key implications on extraction of volatile components out of the leaf matrix during the infusion process, and subsequent release into the gas phase, and will be discussed in more detail in the following chapter.

It is clear from tables 2-5 and 2-6 that the compounds represented by the 15 ions possess a wide range of different odour descriptors and physicochemical properties. They are also formed according to a variety of different mechanisms in the leaf and during manufacture as will be discussed later. The contribution that many of these compounds play towards the overall tea aroma profile has also been reported by previous workers.

Guth and Grosch (1993) used the concept of AEDA to identify the compounds responsible for the aroma of black tea leaf (orange pekoe). Amongst the 28 odour active peaks were the compounds *Z*-4-heptenal, β-damascenone, linalool, phenylacetaldehyde, hexanal, 3-methyl butanal and 2-methyl propanal. The same technique was applied to retorted black tea drinks manufactured from Darjeeling tea (Masuda and Kumazawa, 2000), where high flavour dilution factors were observed for linalool, β-damascenone, methyl salicylate, phenylacetaldehyde and 3-methyl butanal, amongst others. Also relevant, is recent work carried out by Schuh and Schieberle (2006) looking to identify the key aroma compounds in the Darjeeling black tea beverage. Amongst the important compounds identified through the AEDA method were *Z*-4-heptenal, *E,E*-2,4-heptadienal, *E*-2-hexenal, linalool,

phenylacetaldehyde, β-damascenone, β-ionone, 2-methyl propanal, and 2- and 3-methyl butanal. These workers went on to calculate odour activity values (OAVs: ratio of concentration to odour threshold) for the compounds, confirming the importance of compounds such as geraniol, β-damascenone, 2- and 3-methyl butanal, and 2-methyl propanal. Further details can be found in section 1.3.2.

It is therefore clear that a large proportion of the compounds monitored using the approach developed in this study are of sensory significance. Whilst the sensory significance of others (e.g. heptanal and heptanone) is unconfirmed, monitoring is still of value in terms of investigating the behaviour of different classes of compound. Whilst dimethyl sulfide is not reported to contribute significantly to the aroma of freshly infused tea, it is well known to be of major importance in terms of off-notes generated during ageing and stewing.

2.4 OVERALL DISCUSSION

Based on the results obtained in this study, it cannot be concluded that the standard infusion procedure is completely reproducible, with the volatile composition and total solids content of infusions likely to differ slightly between batches. The fact that there does not appear to be any pattern suggests that the two variables, infusion water temperature and concentration do not adversely affect the reproducibility of the infusion procedure. The infusion procedure was however not entirely irreproducible, post-hoc testing revealing that in the majority of cases, of the three batches, only one batch differed to the other two, and never in the concentration of all five volatile compounds monitored. These results were not unexpected, given that the infusion procedure involves a lot of variables that are difficult to control. Whilst mass of tea, volume and temperature of water were carefully controlled, it is inevitable that differences still existed. Given that tea is a natural product, it is also possible that variations were brought about by differences in composition of the made tea. This experiment has therefore highlighted the need for careful precautions to be taken in future work in order to prevent batch-to-batch variation from biasing results.

The following section discusses the approach taken in the current study to allocate the ions observed on APCI spectra to volatile compounds present in tea headspace, and the novel method developed to monitor the release of these compounds from freshly prepared black tea infusions.

The tendency of isobaric and isomeric compounds to produce unique fragmentation patterns has been described by Buhr et al. (2002). These workers analysed pure solutions of 53 flavour compounds, observing that in many cases differences in fragmentation pattern could easily be used to distinguish between isobaric and isomeric compounds. Whilst true, this finding is of limited use in terms of complex mixtures where it is often not known i) which compounds are present in the first place, and ii) how much each are contributing to the intensity of each ion at a particular *m/z*. Similarly, Yeretzian et al. (2003) investigated the energy-dependent break-up patterns of 40 pure compounds observing that clear differences in fragmentation pattern could be used in order to differentiate isobaric compounds. The authors admit that applying this data to complex mixtures such as coffee

headspace would be rather difficult to exploit since it would first require exhaustive databases of energy-dependent break-up patterns of pure compounds.

The approach of coupling GC with multiple detectors described in this chapter has previously been applied successfully by many workers e.g. (Taylor et al., 2003, Fall et al., 2001, de Gouw et al., 2003, Mark et al., 2006, Lindinger et al., 2005), and has been described in section 1.4.3.3. The degree of success at assigning ions to individual compounds has been found to vary depending upon the complexity of the particular sample under investigation. Taylor et al. (2003) concluded for example that ion 97 could at best be used to monitor "sugar degradation products" in the headspace of heated skim milk powder. In studies of urban air samples, (Warneke et al., 2003, de Gouw et al., 2003) it was found that benzaldehyde, three xylene isomers, and ethylbenzene all contributed to the ion at *m/z* 107. Since all of these compounds have common sources in car exhaust fumes, and have similar atmospheric reactivity, the authors concluded that PTR-MS measurements of this ion were still worthwhile. A recent paper by Lindinger et al. (2005) claims the "unambiguous identification of volatile organic compounds by proton-transfer reaction mass spectrometry coupled with GC-MS". Despite the apparent suggestion made in the title, the authors admit that in several cases the overall intensity of PTR-MS ion signals are superpositions of ions originating from several different compounds. These previous findings are therefore in agreement with the current study, where it was determined with confidence that some, but not all ions could be unambiguously assigned to individual compounds

Whilst used in isolation, the GC-EI/APCI-MS approach would have still provided useful information, utilizing energy-dependent compound fragmentation by altering the cone voltage resulted in a significant increase in its ability to allocate ions to individual compounds. Only by altering the cone voltage was it possible to select an ion (*m/z* 121) which could be used to unambiguously monitor phenylacetaldehyde for example, as was shown in figure 2-5. The allocation of many other ions to compounds was also greatly improved by manipulation of cone voltage.

Although the approach described in the current chapter has been successful in allocating specific ions on APCI spectra to volatile compounds in tea headspace, accuracy of assignments were nevertheless not perfect. Of the 15 ions monitored, only six corresponded to individual compounds, the remaining nine corresponding to groups of two or more isobaric or isomeric compound. One suggested means by

which the current technique could be improved is to further develop the GC-EI/APCI-MS procedure. Whilst fragmentation patterns were manipulated by altering cone voltage, it may also be possible to further manipulate fragmentation by using different proton transfer reagents. The use of alternative proton transfer reagents (such as NH_4^+) have been employed successfully by several workers using PTR-MS e.g. Yeretzian et al. (2003) investigating the headspace of coffee, and there is no reason why a similar approach could not be applied to APCI-MS. It may be that use of an alternative reagent (such as NH_3^+ or NO^+) would yield additional (or reduced) fragments, enabling the unambiguous assignment of additional volatiles in black tea aroma. It must be considered that this approach would add complication to the analysis of black tea in real-time using the current setup, with each analysis having to be carried out several times (using different reagents), or using some kind of mechanism enabling very rapid switching throughout the analysis period.

Another possibility would be to make use of an ion trap, as used by several workers to successfully differentiate isobaric compounds (Prazeller et al., 2003, Jublot et al., 2005, Warneke et al., 2004). Whilst this method differs somewhat from the approach described in this chapter, it still makes use of compounds' tendency to fragment differently under different conditions in the same way as altering the reagent or ionization energy. As with the suggestion of using an alternative reagent however, this would require changes to be made to the APCI-MS setup, namely its linkage to an ion-trap mass spectrometer during the analysis of volatile release from freshly infused tea infusions, adding unnecessary complication to the specifically simple technique.

Several authors have made meticulous attempts to accurately relate all ions observed in spectra to sample composition. Yeretzian et al. (2003) for example has utilised a variety of techniques to assign the ion masses observed on PTR-MS spectra to volatile compounds in the headspace of coffee. As well as investigating the energy-dependent fragmentation of compounds, and varying H_3O^+ and NH_4^+ as primary reactant ions, they also used a technique in order to establish whether or not multiple compounds were contributing to a given ion mass based on a method usually used to calculate Henry's law constants.

In the current work, an attempt was made to estimate the relative contribution of particular compounds to individual ions, so establishing some form of confidence

over the allocations. This was achieved by calculating total peak area on the APCI trace for each ion of interest, and then calculating the relative contribution of each compound to this by taking the area of the individual peaks as a percentage of the total area. It must be emphasised that this method has its limitations, and therefore only provides approximate information regarding relative contribution. Although SPME fibres trap volatiles in amounts relative to their concentration in the headspace (up until saturation point), not all classes of compound are trapped with equal efficiency (Wardencki et al., 2004). The peak area of compounds on the APCI trace is therefore inevitably partly a function of trapping efficiency of the fibre, and whilst attempts were made to minimise this by using a DVB / carboxen / PDMS fibre specifically designed to adsorb a wide range of volatile classes, this effect can not be eliminated completely. A similar comment was made by Lindinger et al. (2005) who found Tenax traps to have variable trapping efficiencies depending upon the physical properties of molecules. These workers do state however that they remained a good compromise given the high humidity of the sample (hot coffee in this case).

Using of a selection of different SPME fibres of differing polarity is one suggestion for minimising the problem of variable trapping efficiency. A possible alternative would be the use of large volume headspace injection since the headspace directly injected into the GC (and subsequently sent to the detectors) would then accurately resemble headspace being drawn into the APCI (or nose) during real-time monitoring. This method was not employed in the current study due to the unavailability of a machine fitted with this sampling capability in proximity to the APCI-MS, although this could be investigated in the future.

The technique of GC-EI/APCI-MS employed in the current study utilised the trapping of volatiles using SPME from black tea infusions prepared as according to the standard procedure (i.e. 1 %w/v, 100 °C). This had important implications in terms of the range of samples which could subsequently be analysed using the APCI-MS procedure developed. It would, for example have been inappropriate to apply the same compound allocation data to the analysis of green tea infusions, the volatile profile of which is known to differ markedly to black tea (Kumazawa and Masuda, 2002). Even if restricted to investigating different types of black tea (e.g. Assam, Darjeeling, Ceylon), it would be necessary to carry out a new GC-EI/APCI-MS procedure before analysis of each type. The presence of a single compound may

mean the difference between an unequivocal assignment, and one in which two compounds contribute to a particular ion mass.

It is also worth noting that all trappings took place over the same time period (i.e. for 5 min following the infusion procedure). Employing a similar approach to investigate aroma released from coffee infusions, Lindinger et al. (2005) used Tenax traps to collect volatiles from the headspace in discrete 120 s time windows. This was designed in order to account for changes in relative concentrations of volatiles in the headspace over time and is valid given the fact that in some cases two or more volatiles contributing to a single ion mass may have differing physicochemical properties, resulting in very different release profiles over the subsequent period of monitoring. Subsequent analysis of coffee headspace using PTR-MS was carried out using the same discrete time windows. Whilst this approach was not utilised in the current study, neither was the objective of the research to investigate the change in release profile over time. This point leads directly onto the next stage of the discussion, which is the method developed in order to monitor release of volatile compounds from hot tea infusions.

Whilst much analysis of food aroma has been carried out using static (equilibrium) headspace approaches, this provides an unrealistic picture of volatile release experienced in real-life. The objective of the current study was to develop a simple method for rapidly analysing volatiles released from freshly prepared, hot, black tea infusions in a mug system as genuine consumers would experience, enabling the effect of certain parameters such as the infusion preparation method to be investigated.

Dutta et al. (2003) used a metal oxide sensor based electronic nose (containing four sensors) in order to discriminate between five samples of tea manufactured under different processing conditions. In this work, tea leaves (10 mg) were added to 200 mL boiling water in a sample vessel with a plastic tube connected to the sensor chamber. These authors used procedures such as principal component analysis to model the data, and were able to successfully classify future tea samples based upon the sensor responses. Whilst this technique was rapid, and made use of freshly infused tea samples, the method lacked the specificity of that used in the current study, namely the ability to monitor and quantify specific volatile compounds.

It is however acknowledged that the adopted approach in the current study is not without its limitations, one of which is the relatively closed system into which infusions were poured during analysis, found to be necessary in order to obtain reproducible data. Since this had a direct impact upon release profiles of the volatiles monitored over the 5 minute sampling period due to accumulation in the headspace above the infusions, release profiles of (such as those shown in figure 2-7 section 2.3.3.1) do not therefore represent release with time (i.e. temporal profiles), and no attempt has been made to describe them as such. In reality, differences in the physicochemical properties of the compounds such as K_{aw} and Henry's law constant (table 2-6) will inevitably result in differences in temporal profile of release from infusions under dynamic conditions as demonstrated by Marin et al. (1999), and described in section 1.4.2. Compounds with high values of K_{aw} (~10^{-2}) such as dimethyl sulfide (K_{aw} 6.58 x 10^{-2}) will favour the gas rather than aqueous phase at equilibrium, and when the gas phase is disturbed (i.e. under non-equilibrium conditions), produce unstable gas phase concentrations. As such the headspace concentration of this compound would be expected to peak and then fall rapidly as shown in figure 1-9, section 1.4.2. Those compounds with lower values of K_{aw} (e.g. phenylacetaldehyde K_{aw} 2.24 x 10^{-4} would be expected to produce more stable gas phase concentrations under dynamic conditions showing a more gradual decrease in concentration over time.

Whilst the ideal approach would be to monitor release of volatile compounds from directly above a free-standing mug of tea (as described in section 2.3.3.1), this was found to be inappropriate due to acquisition of completely irreproducible data caused by random atmospheric air movements. A compromise dynamic approach has been used by several workers where the headspace of the sample under investigation is continually flushed or purged with an inert gas stream. Such studies have been successfully used to study release of aroma compounds in model systems such as aqueous (Marin et al., 1999), and ethanolic solutions (Tsachaki et al., 2005), and emulsions (Doyen et al., 2001). Model systems such as these allow sample conditions to be carefully controlled, and volatile compounds to be added at concentrations suitable to obtain a good detector response. This dynamic approach has also been used in the case of real food systems, including tomatoes (Boukobza et al., 2001), Maillard reaction products (Turner et al., 2002), and coffee infusions (Lindinger et al., 2005).

Boukobza et al. (2001) investigated release of selected volatile compounds from tomato fruits under dynamic conditions in an attempt to simulate, in a controlled environment, what is likely to occur during consumption. The approach involved sampling the headspace from above tomatoes (subsequently macerated) in an adapted blender, designed to protect the sample from the atmosphere. Air at a high flow rate (170 mL/min) was continually flushed through the system, and a portion (11.5 mL/min) sampled into the APCI-MS (the excess released into the atmosphere). Analysis of APCI traces enabled volatile compounds to be classified according to their release profiles into those showing rapid release immediately after maceration, and those released more gradually. Samples in this case were of ambient temperature, and levels of volatiles present were much greater than those found in tea infusions. Hexanal and methyl butanals for example were reportedly present in the headspace at concentrations of up to 80 and 110 mg/m^3 respectively. This compares to concentrations of around 2 and 6 mg/m^3 observed for the same compounds in the current study (table 2-5)

Also utilising a dynamic approach were Turner et al. (2002) who monitored release of volatile compounds from skimmed milk during thermal processing. Although samples were heated (70–120 °C), the headspace was not moisture-laden as is the case for hot tea infusions. As was the case of tomatoes described above, headspace concentrations of volatile compounds were very high, it being necessary to dilute samples just to keep chromatograms on the scale (Taylor 2007, personal communication).

Lindinger et al. (2005) employed PTR-MS to monitor release of selected volatile compounds from freshly prepared espresso coffee in a closed glass vessel, purging the headspace with a continuous flow of air at 200 mL/min. After exiting, 14 mL/min of the sample gas was continually introduced into the PTR-MS. Whilst hot beverages were used in this case, problems of humidity were controlled by further diluting the outlet gas with dry air prior to introduction into the PTR-MS. These workers also used a magnetic stirrer in order to ensure an efficient and reproducible transfer of volatile compounds into the headspace.

Adopting a similar approach in the current study would have enabled the temporal profile of selected volatile compounds to be monitored. Due to the very low concentration of volatile compounds present in tea headspace, purging samples with a high gas flow rate was shown to generate very inconsistent data due to the

diluting effect this had. As described in section 2.3.3.1, analysis of some compounds such as β-damascenone were already near their limit of detection (based on its signal:noise ratio).

The approach described in this chapter has however clearly achieved the objectives it set out to, with the development of a simple approach in which a selection of key volatile compounds could be monitored in a very reproducible nature (as shown by the low %CV values in table 2-4). The system was realistic in nature, allowing the study of release from genuine mugs of freshly prepared hot tea (as shown in figure 2-3). Analyses were rapid, with potential to be carried out every 10 minutes (4 min infusion + 5 min analysis). This compares to a typical GC analysis which would take around 50 minutes. Whilst not showing temporal profile information, values of cumulative ion count used in the current study do provide an indication of the "overall" release, enabling direct comparisons to be made between different infusion preparation methods (discussed in the following chapter).

2.5 FUTURE WORK

This chapter has described a method used to successfully assign compounds to 15 ions present on APCI-MS spectra of freshly prepared black tea infusion headspace, and described an approach to monitor the release of these selected compounds from freshly, prepared black tea infusions in a realistic system in a reproducible manner.

Future work should be carried out in order to improve the allocation of ions to compounds, aiding the differentiation of isobaric compounds. Use of alternative proton-transfer reagents such as NH_4^+ or NO^+, or incorporating an ion trap, are two key potential techniques worth exploring further.

Another area of future work lies in developing the headspace sampling system to further enhance its realism. Whilst the current approach achieved all of its set objectives, the system could be modified to further represent the real-life system experienced by consumers in terms of dynamic release of volatiles. One approach involves continually purging the system with an inert gas stream, and has been used successfully in the case of several foods and beverages. An added benefit of such a technique is that it would allow the temporal profile of release of volatile compounds to be explored.

There are many potential uses for the system described in this chapter, and the following chapter describes one such application, where effect of infusion preparation method on the volatile release from black tea infusions has been investigated.

3 EFFECT OF PREPARATION METHOD ON VOLATILE RELEASE FROM BLACK TEA INFUSIONS

3.1 INTRODUCTION

Worldwide consumer observations and questionnaire studies on tea preparation habits have shown wide variations both between countries, and between individuals within countries (Astill et al., 2001). In Western countries, infusion time is generally short (less than 3 minutes). In India, Pakistan, and some Middle Eastern countries, the drink is prepared by boiling black tea leaves in a pan for several minutes. In Turkey, infusions are often prepared with brew times of around 30 minutes (Tascioglu and Kok, 1998), whereas in Far Eastern countries, it is common practice to brew the same leaves three or more times (Hicks et al., 1996).

Spiro and co-workers have studied the kinetics of the tea infusion procedure in depth, focussing on the rate of extraction of non-volatile tea components such as caffeine and mineral ions from both black and green teas. Investigations have focussed on the effect of leaf size, manufacturing method (Jaganyi and Price, 1999, Price and Spiro, 1985), composition of the aqueous extracting medium (Spiro and Price, 1987), and infusion water temperature (Spiro et al., 1992).

Health benefits of tea are generally attributed to the antioxidant properties of the major flavonoid components; catechins, theaflavins, bisflavonols and theaflavic acids (Rice-Evans, 1999). A large proportion of studies on the effect of infusion preparation method have therefore been health orientated, with a range of studies investigating the polyphenols (Lakenbrink et al., 2000, Astill et al., 2001) and their corresponding antioxidant activity (Liebert et al., 1999, Langley-Evans, 2000).

Negative health implications of tea have also been cause for concern, and several workers have investigated the effects that preparation method has on levels of naturally occurring methylxanthines, including caffeine, theobromine and theophylline (Hicks et al., 1996, Stavric et al., 1988, Shishikura and Khokhar, 2005).

The transfer of certain pollutants from tea leaf to brew during the infusion process has been widely investigated, the main areas being heavy metals (Tascioglu and Kok, 1998), pesticides (Jaggi et al., 2001, Nagayama, 1996, Wan et al., 1991) and polycyclic aromatic hydrocarbons (Lin et al., 2005, Lin et al., 2006).

It is important to determine the effect preparation method has on the content of these compounds in order to formulate advice on daily consumption levels. Published dietary tables may under or over-estimate actual levels, depending upon the specific infusion preparation method used. Dietary estimates are essential in epidemiological and intervention studies, and clinical research. Fujiki et al. (1992) has suggested drinking ten cups of green tea per day as a form of cancer prevention. The preparation method is not stated; the Western bag method would result in three times as much as the Asian method.

In comparison, little work has been carried out to specifically investigate the effect of infusion preparation method on aroma release from the complete beverage. It is, however, reported that the main factor determining the taste and aroma of the tea is the infusion preparation method (Sharma et al., 2005). Whilst the non-volatiles are generally responsible for the characteristic taste, it is the volatiles that contribute to the aroma, and hence the overall flavour of the beverage.

The following chapter utilises the approach and apparatus developed and described in Chapter 2 to study the factors most likely to vary within the continental European setting, namely infusion water temperature, concentration, and duration. These are factors which may be chosen due to consumer preference, but are just as likely to be varied unintentionally in the domestic environment.

The objectives of this chapter were as follows:

- Investigate how infusion water temperature, concentration and duration affect the volatile release from black tea infusions using the APCI-MS approach developed and described in chapter 2

- Investigate differences in the behaviour of different volatile compounds, and apply these findings to the relevant release mechanisms

3.2 MATERIALS AND METHODS

All analyses of tea infusion headspace were carried out using the procedure previously described in section 2.2.3. The 15 marker ions were monitored over a 4 or 5 min period, corresponding to selected volatile compounds found in tea infusion headspace.

3.2.1 Effect of infusion water temperature on volatile release

A series of experiments were carried out in order to investigate the effect infusion water temperature had on volatile release from tea infusions, and to decouple the effects of extraction of volatiles from tea leaf and release of volatiles into the headspace.

3.2.1.1 Immediate analysis of tea infusions (experiment 1)

Infusions were prepared according to a modified version of the standard infusion procedure described in section 2.2.1.1. Infusions were prepared using water of seven different temperatures (40, 50, 60, 70, 80, 90 and 100 °C), from a kettle fitted with a thermocouple. Five replicate infusions were prepared using water of each temperature, and analysed in randomised order. As soon as infusion was complete, infusions were poured into the mug via the sampling apparatus connected to the APCI-MS (described in section 2.2.3, shown in Figure 2-2 and Figure 2-3) and release measured for 5 min.

3.2.1.2 Analysis of tea infusions following 30 minutes incubation at 60 °C (experiment 2)

The procedure described in the previous section enabled the overall effect of infusion water temperature on the volatile release from tea infusions to be determined. That is, the effect a consumer would experience via the orthonasal route were they to prepare infusions using different water temperatures. Whilst the temperature of infusion water has been intentionally varied, so too has the

temperature of the resulting infusion within the mug. In order to decouple the effect of temperature on extraction of volatile compounds from the leaf matrix and partition between the aqueous and gas phases, a second experiment was carried out.

Infusions were prepared using the same seven water temperatures as above. Following the infusion procedure, infusions were filtered through a double layer of muslin into 250 mL Schott bottles (Fisher Scientific, Loughborough, UK), and sealed immediately. Bottles were held in a water bath set at 60 °C for 30 min. The purpose of this procedure was to ensure that all infusions were of equal temperature during the analysis step, irrespective of the temperature of water originally used. Preliminary experiments showed that 30 min was the minimum time necessary in order for all infusions to equilibrate at this temperature. Although holding at a higher temperature would have enabled a shorter incubation period, this was avoided due to the potential of chemical reactions occurring, potentially altering the volatile composition of infusions.

3.2.1.3 Investigating the relative importance of infusion and incubation temperature (experiment 3)

In order to investigate the relative importance of infusion and incubation temperature on volatile release, a third experiment was conducted. Infusions were prepared as previously described using water at 40, 70 and 100 °C. As soon as infusion was complete, infusions were filtered and poured into 250 mL Schott bottles and sealed. Bottles of infusion were held in water baths set at 40, 60 and 80 °C for 30 min before being poured into the mug via the sampling apparatus. Infusion water temperatures were chosen to represent a range (i.e. low, medium, and high) based upon those used in the previous two experiments. Incubation temperatures were chosen to represent temperatures of infusion in a mug had they been prepared using these three infusion temperatures. The maximum incubation temperature was limited to 80 °C due to the increasing risk of chemical reactions occurring due to prolonged storage at higher temperatures, potentially significantly altering the volatile composition. This experiment conformed to a full-factorial design; infusions were made using each water temperature and were incubated at each incubation temperature, resulting in nine possible combinations (five replicates of each).

3.2.2 Effect of infusion concentration on volatile release

Effect of infusion concentration (ratio of tea leaves to water) was studied using a modified version of the standard infusion procedure described in section 2.2.1.1. Infusions were prepared at nine different concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 %w/v), five replicates of each, and analysed in random order.

3.2.3 Effect of infusion duration on volatile release

Infusions were prepared according to the standard procedure using boiling water (section 2.2.1.1), but were allowed to infuse for varying durations from 0 to 8 min in 1 min intervals. These durations were chosen to cover a range around the standard 4 min infusion. In the case of the 0 min infusion, water (boiling) was added to the loose leaves and the flask lid sealed. The flask was inverted once, before the infusion was filtered and poured into the sampling apparatus. For all other duration infusions, flasks were inverted immediately upon addition of water, and immediately prior to filtration. A decision was made to eliminate the five inversions usually taking place at 3 min. This was to eliminate any bias which may occur since otherwise relatively more mixing would have occurred in the shorter duration infusions.

3.2.4 GC-MS and total solids analysis

The aqueous phase concentration of five volatile compounds (hexanal, *E*-2-hexenal, linalool, methyl salicylate, and β-ionone) was determined for infusions prepared using the same infusion water temperatures and concentrations as described above for the APCI-MS experiments. Three batches of each infusion were prepared, and three sub-samples from within each were analysed. Full details of the experimental protocol can be found in section 2.2.1.3.

Total solids analysis was also carried out in order to establish the solids content of infusions prepared using the same infusion water temperatures and concentrations, and is described in section 2.2.1.2. In addition, total solids analysis was carried out for infusions prepared using each of the infusion durations from 0 – 8 min. Again,

three batches of infusion were prepared, and three sub-samples from within were analysed.

3.2.5 Statistical analysis

The effect of infusion water temperature (experiments 1 and 2) and concentration (investigated using APCI-MS) were analysed using one-way-ANOVA (p=0.05) (SPSS for Windows, v11.0, SPSS Inc, Chicago, IL). Data were manually checked for normal distribution, and post-hoc testing was carried out using Tukey's honestly significant (Tukey's HSD) test. In order to investigate the effect of infusion water and incubation temperature (experiment 3), data were analysed using two-way ANOVA (p=0.05).

Principal Components Analysis (PCA) was also applied to the data from experiment 3 in order to provide a visual representation of the relative influence of infusion and incubation temperatures on compounds. The software used for PCA was Matlab v7.1 (The MathWorks, Inc, Natick, MA) in combination with the PLS toolbox v3.5.2 (Eigenvector Research, Inc., Wenatchee, WA).

GC-MS and total solids data were analysed using one-way ANOVA in order to determine the effect of infusion water temperature, concentration (and duration in the case of total solids analysis) on the concentration of selected volatiles and non-volatiles in solution.

3.3 RESULTS

Due to the large amount of data collected, only a summary of data is shown here illustrating key features. All data is however provided in appendices 1 - 5.

3.3.1 Effect of infusion water temperature on volatile release

3.3.1.1 Immediate analysis of tea infusions (experiment 1)

For all 15 ions, ANOVA indicated that release increased as infusion temperature increased ($p=0.05$). Post-hoc testing revealed that for each temperature there was a significant difference in release for most of the compounds. For 2-methyl propanal, *E*-2-octenal, heptanal and β-damascenone, release at the lower or upper end of the temperature range used was not significant due to higher variation. Although all compounds showed a significant effect of infusion water temperature on release, the relative effect varied. This is illustrated in figure 3-1 which shows the effect of infusion water temperature on the release of the methyl butanals, and β-damascenone (ions 87 and 191), which represent the extremes. In order to enable direct comparisons to be made, maximum release has been normalised to 100 %.

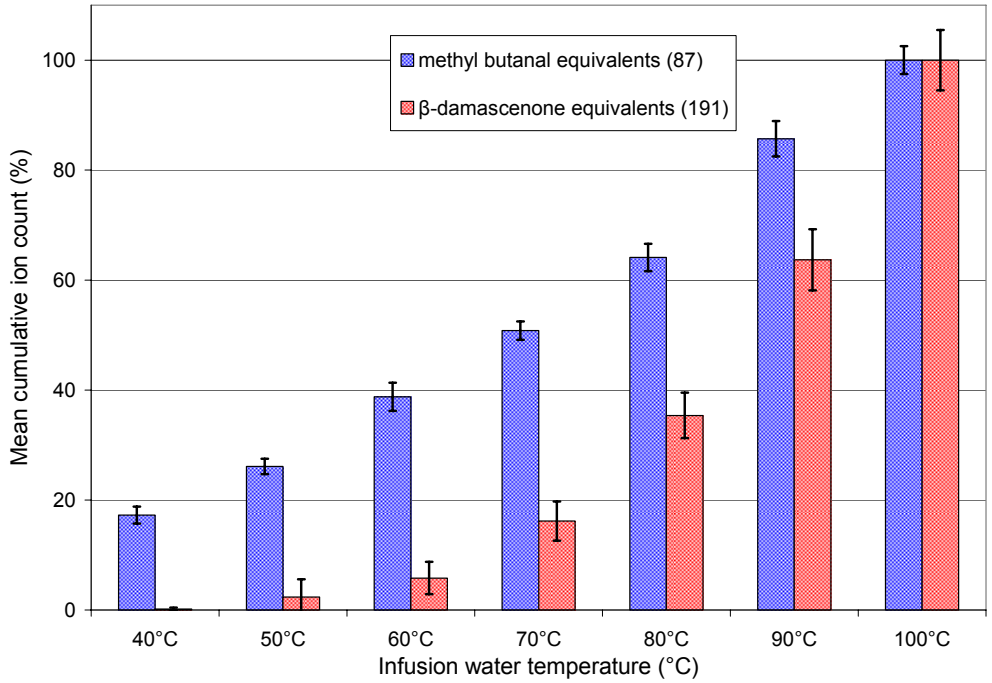


Figure 3-1 - Overall effect of infusion water temperature on release of different compounds from tea infusion headspace (error bars show standard deviations)

Figure 3-1 shows a clear difference in release between the two groups of compound. For the aldehyde compounds represented by *m/z* 87, temperature had a linear effect on release (a similar pattern was seen for 2-methyl propanal, *m/z* 73), whereas for β-damascenone and the other high molecular weight compounds, the relationship is more an exponential function with increased release at higher temperatures.

Whilst it is clear that temperature has a substantial effect, from this data alone, it is not clear whether preparing tea using hotter water resulted in greater extraction of volatile compounds from the dry tea leaf during the infusion process, or whether release levels observed were solely a function of the final temperature of the infusion within the mug.

3.3.1.2 Analysis of tea infusions following 30 minutes incubation at 60 °C (experiment 2)

ANOVA indicated that for all 15 ions monitored, increase in infusion water temperature led to an increase in volatile release. Relative differences in release behaviour between the compounds appeared more marked when all infusions were analysed at a constant temperature with a greater variation in extreme profiles. Since the APCI-MS was calibrated using authentic standards, it was possible to align the data obtained from the two experiments and is shown for *E*-2-hexenal in Figure 3-2 where maximum release across both experiments has been normalised to a value of 100 %.

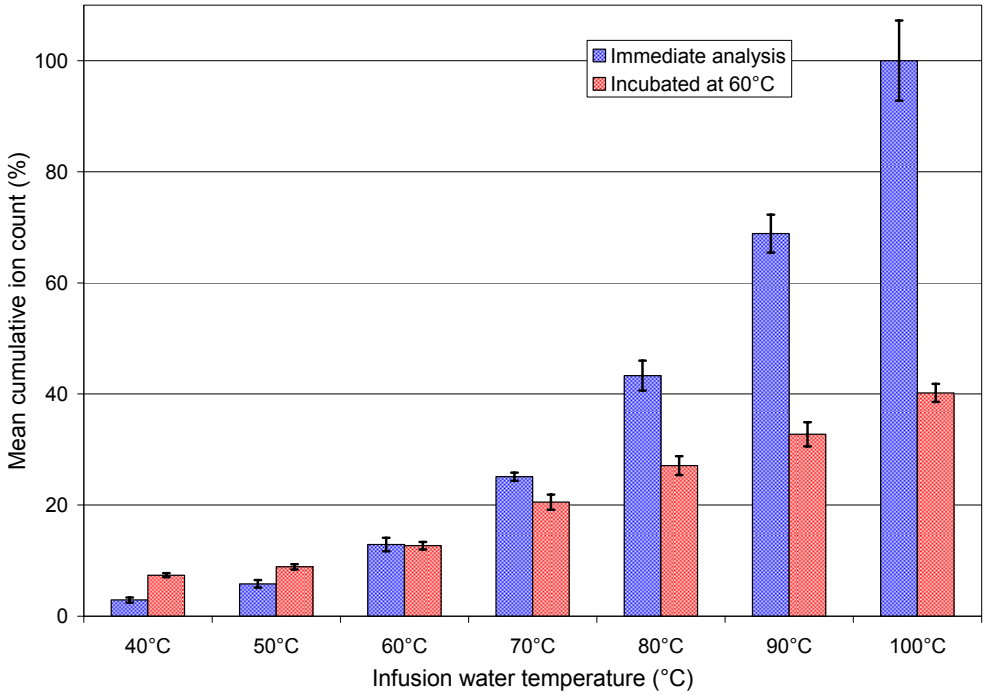


Figure 3-2 - Effect of infusion water temperature on the release of *E*-2-hexenal when analysed immediately, and after incubation at 60°C (error bars show standard deviations)

It can be seen from figure 3-2 that release levels are similar for both sets of infusions prepared using 60 °C water. This is not surprising, given that both infusions were ~60 °C in the mug at time of analysis. This similarity in release indicates that the incubation step had no effect on release, and was shown to be the case for all 15 ions.

Whilst infusion water temperature still has a clear effect on release of *E*-2-hexenal when temperature within the mug is kept constant, absolute levels of release tended to be lower (for infusions >60 °C). Release of *E*-2-hexenal from the incubated infusion following infusion using 100 °C water was just 40 % of that released from that which was immediately analysed. This clearly illustrates the effect that the temperature of the infusion within the mug is having on the release of the release of *E*-2-hexenal. The same behaviour was evident for all other ions to a greater or less extent.

3.3.1.3 Investigating the relative importance of infusion and incubation temperature (experiment 3)

General linear model univariate analysis (2-way ANOVA) confirmed that both infusion and incubation temperatures had significant (p=0.05) effects on release of all monitored compounds from tea infusions. Post-hoc testing revealed that for all volatile compounds, each incubation temperature had a significant effect on release, compared to each other incubation temperature. For of all compounds (except benzaldehyde), each infusion temperature had a significant effect on release, compared to each other infusion temperature. In the case of benzaldehyde, there was a significant difference in release using 40 °C water to both other temperatures, although there was no significant difference in release between 70 and 100 °C.

Figure 3-3 shows an example of the data obtained for four compounds (dimethyl sulfide, the methyl butanals, benzaldehyde and β-ionone), which have been chosen to illustrate differences in the relative importance of the infusion and incubation temperature. For each of the four compounds, charts have been plotted to show the effects of infusion and incubation temperatures.

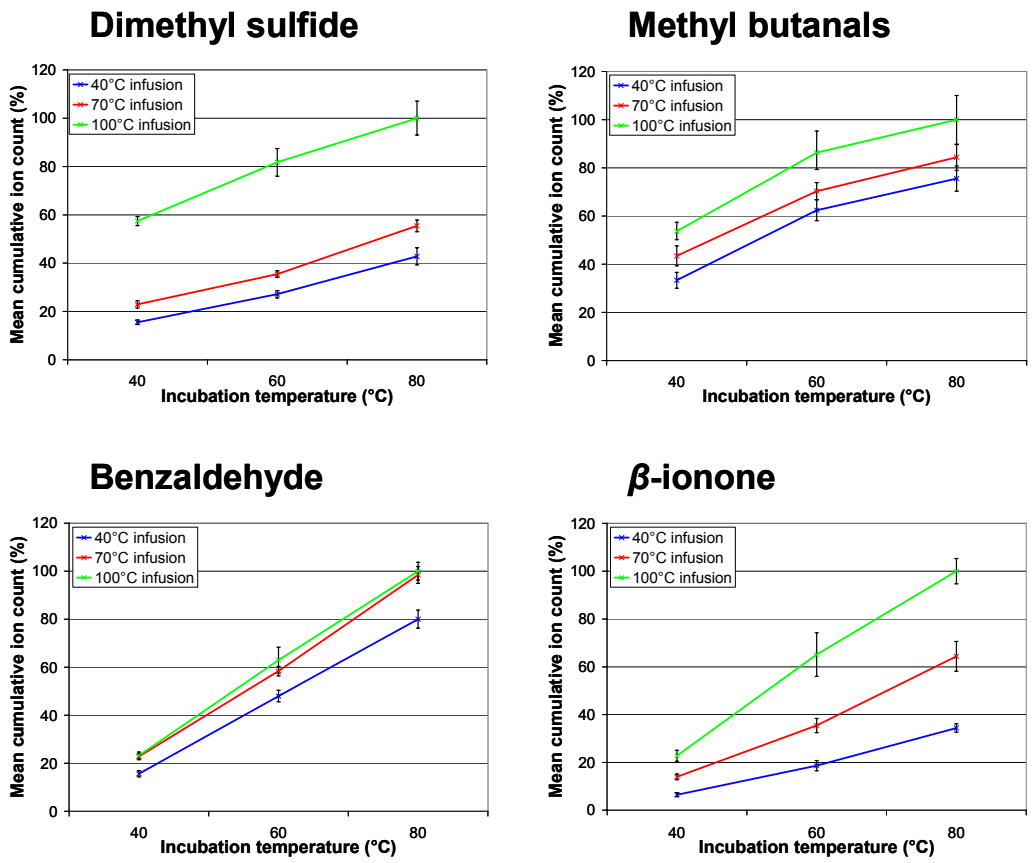


Figure 3-3 - Relative effects of infusion and incubation temperature for dimethyl sulfide, the methyl butanals, benzaldehyde, and β-ionone (error bars show standard deviations)

It can be seen from figure 3-3 that a range of behaviours are exhibited by the different compounds. In the case of dimethyl sulfide, there appeared to be a relatively small effect of increasing infusion water temperature from 40 to 70 °C (at all three incubation temperatures). The relative difference in release observed between 70 and 100 °C was however substantially greater.

The methyl butanals and benzaldehyde appeared to show a much smaller effect of increasing infusion water temperature than other compounds. This is particularly true for benzaldehyde where there is almost no effect of infusion water temperature from 70 to 100 °C (irrespective of incubation temperature).

In the case of dimethyl sulfide, benzaldehyde and β-ionone there appeared to be a fairly consistent increase in release as a function of increasing incubation temperature (irrespective of the infusion water temperature). For the methyl

butanals, the increase in incubation temperature from 60 to 80 °C led to a relatively smaller increase in release, compared to from 40 to 60 °C. This was also true for some of the other aldehyde compounds (2-methyl propanal and hexanal).

Differences in behaviour can also be illustrated by use of response surface curves. The same four compounds are represented in figure 3-4, (created in Design Expert v6.0.2, Stat-Ease, Minneapolis, MN). Although the curves show a profile, it must be remembered that they have been created using the distinct three values of incubation and infusion temperature used in the original experiment, and as such there is some degree of uncertainty regarding the exact profiles.

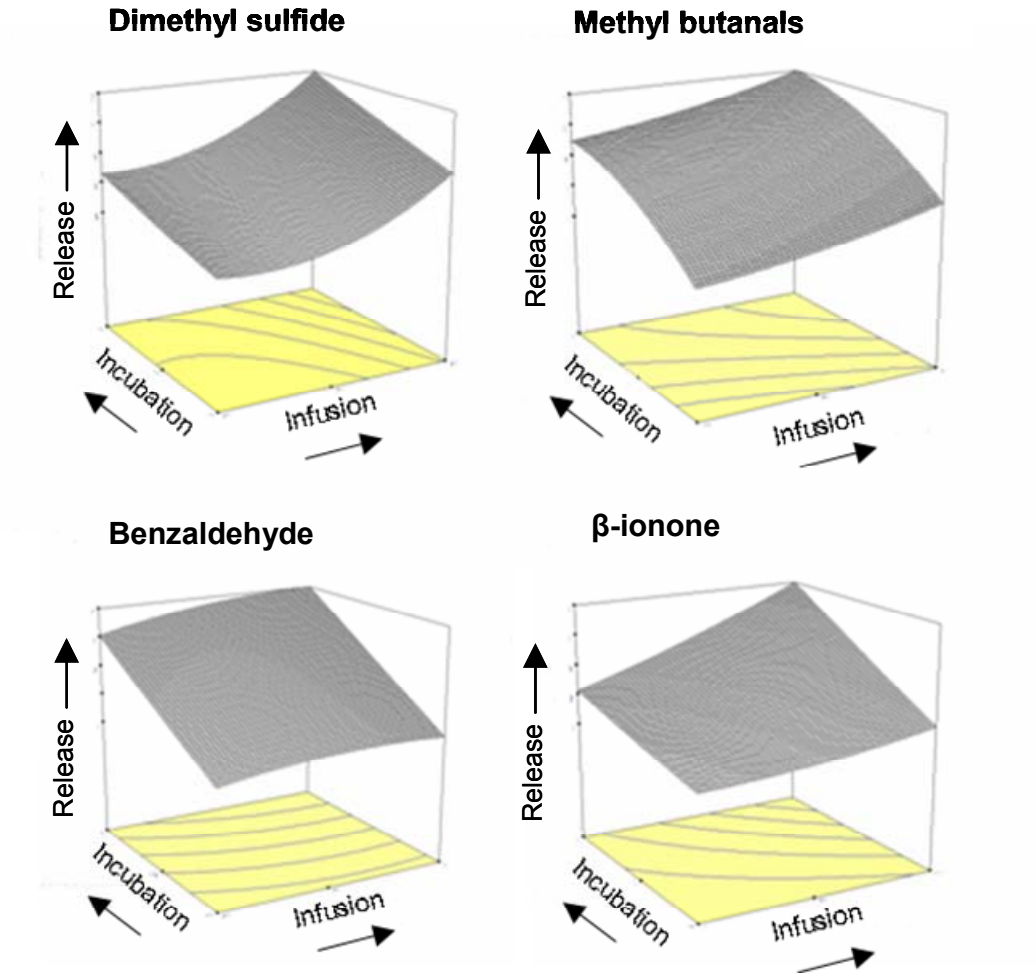


Figure 3-4 - Response surface curves for dimethyl sulfide, the methyl butanals, benzaldehyde and β-ionone illustrating differences in the relative influence of infusion and incubation temperatures

Prior to Principal Components Analysis, the data were auto-scaled, i.e. for each value, the average for the respective ion was subtracted and divided by its standard deviation. This resulted in a dataset where for each ion the average over all samples was 0 and variance equaled 1. This pre-processing ensured that each ion had the same amount of influence on the subsequent data analysis, irrespective of its absolute concentration or variation over different samples. This was a valid choice since it was appropriate to investigate the relative effect of infusion and incubation temperature for each ion, ignoring actual differences in concentration.

The PCA on the auto-scaled data revealed that 97 % of the total variance present in the dataset could be explained with only two Principal Components (PC's) as shown in the biplot (figure 3-5).

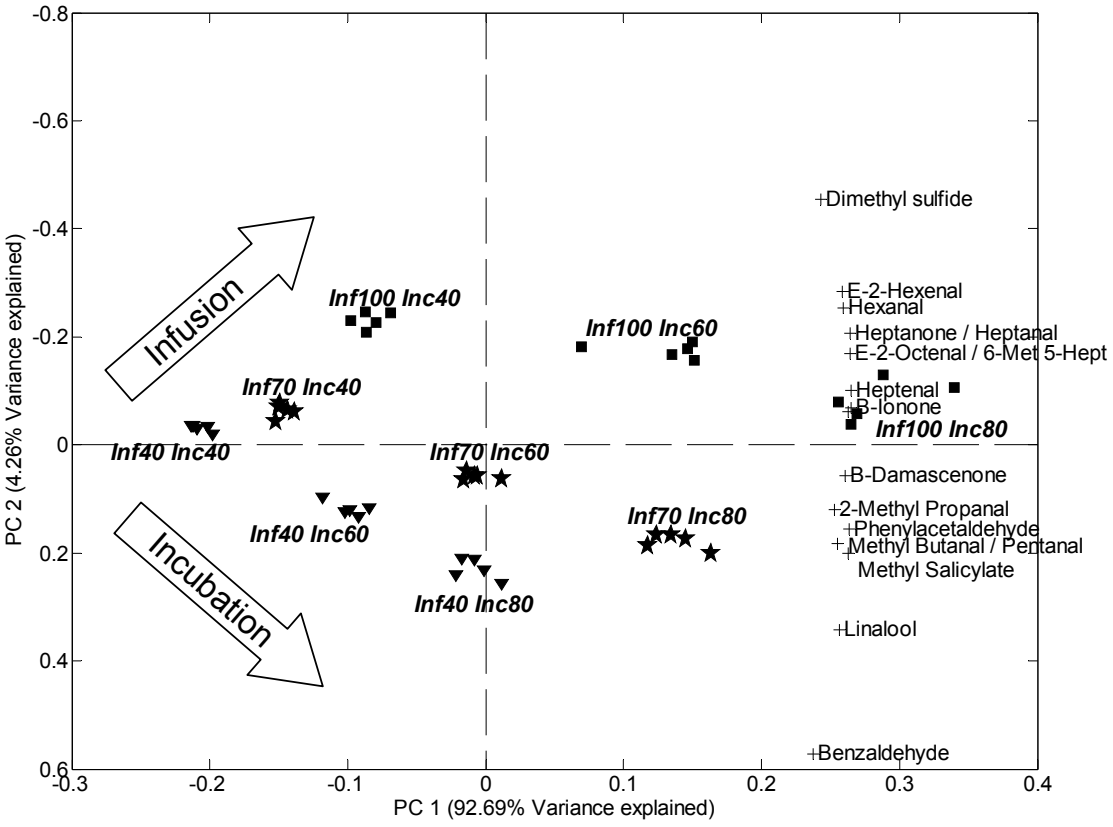


Figure 3-5- Biplot from PCA analysis of infusion and incubation data from tea samples infused at 40, 70 and 100 °C, and then incubated at 40, 60 or 80 °C

Figure 3-5 shows the effects of infusion and incubation temperatures as diagonal trend lines. The three clusters of data for a constant incubation temperature of 40 °C show that increasing the infusion temperature from 40 °C (▼) through 70 °C (★)

to 100 °C (■) shows a linear change in release. The same linear pattern can be observed for incubation temperatures of 60 and 80 °C although there is now a clear incubation effect, working in the opposite direction to infusion. The arrows on the plot show the direction of the effects of infusion temperature and incubation temperature on volatile release.

The individual volatile compounds are also included in the biplot, their position governed by the relative effect of infusion or incubation on their release. Dimethyl sulfide is the compound most affected by infusion temperature while benzaldehyde is the compound most affected by incubation (on a relative basis). Compounds lying near the zero axis of PC2 are equally affected by both effects.

3.3.2 Effect of infusion concentration on volatile release

For all 15 monitored ions, infusion concentration had a positive correlation with volatiles released; the greater the concentration, the higher the level of release. Figure 3-6 shows the effect of infusion concentration on release of *E*-2-hexenal, the same trends being seen for all 15 ions (appendix 4).

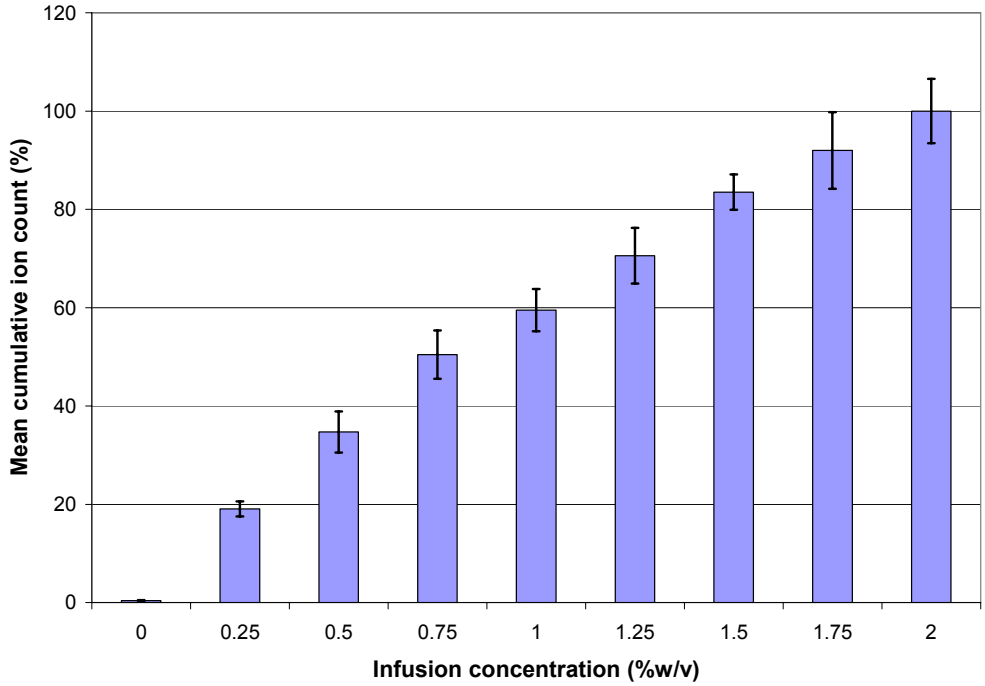


Figure 3-6 - Effect of infusion concentration on release of *E*-2-hexenal (error bars show standard deviations)

One-way ANOVA confirmed that the effect was significant (p=0.05) for all monitored compounds. Results of post-hoc testing varied between ions, although in no case was release significantly different at all concentrations.

3.3.3 Effect of infusion duration on volatile release

For all 15 ions monitored, a significant difference (p=0.05) in release was observed between infusions of 0 min duration, and infusions of all other durations (1-8 min). Of the 15 ions, only two showed significant differences in release between any of the other infusion durations (i.e. between 1-8 min). These corresponded to dimethyl sulfide, and β-damascenone. Figure 3-7 illustrates the release of these compounds showing the three alternative scenarios (heptenal gives an example where infusion between 1 and 8 min does not lead to any statistically significant difference in release). In order to enable suitable comparisons to be made, maximum release for each compound has been normalised to 100 %.

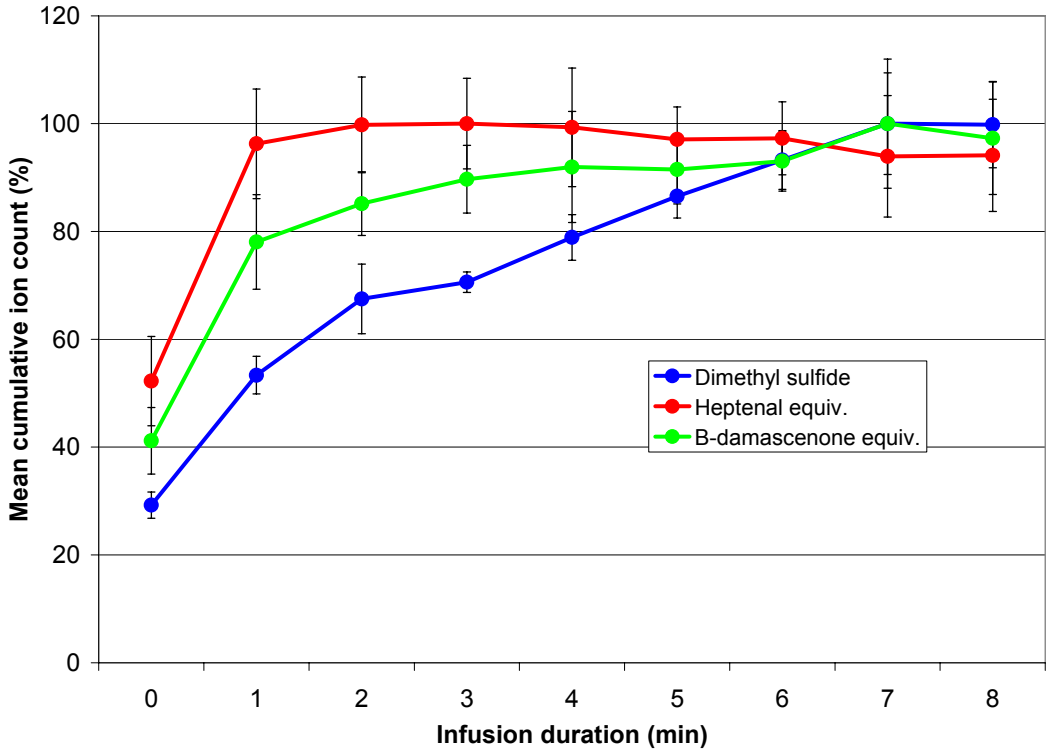


Figure 3-7 - the effect of infusion duration on release of dimethyl sulfide, the heptenals, and β-damascenone (error bars show standard deviations)

The red line illustrates the behaviour of the heptenals (similar profiles to this compound were evident for 13 of the 15 ions monitored). Whilst there were differences between infusions of duration 0 and 1 min, the release was not significantly different to that for all other infusion durations.

Release of β-damascenone increased with infusion duration (green line). The release at 1 min was significantly different to release following all other infusion durations, although between 2 and 8 min, levels of release were not significantly different from one another. A very similar release profile was observed for β-ionone, although in this case release between 1 min and the longer duration was not statistically significantly.

Dimethyl sulfide release (blue line) increased the most with increasing infusion duration. As infusion duration was increased, there appeared to be a steady increase in the amount of this compound released.

3.3.4 GC-MS analysis

3.3.4.1 Effect of infusion water temperature

ANOVA showed that infusion water temperature had a significant ($p=0.05$) effect on the concentration of all five volatile compounds studied, with an increase in concentration with increasing infusion water temperature.

Figure 3-8 shows the effect infusion water temperature had on the concentration of the five selected volatile compounds. Values of mean and standard deviation (error bars) are derived from the total of all three batches and three replicate analyses.

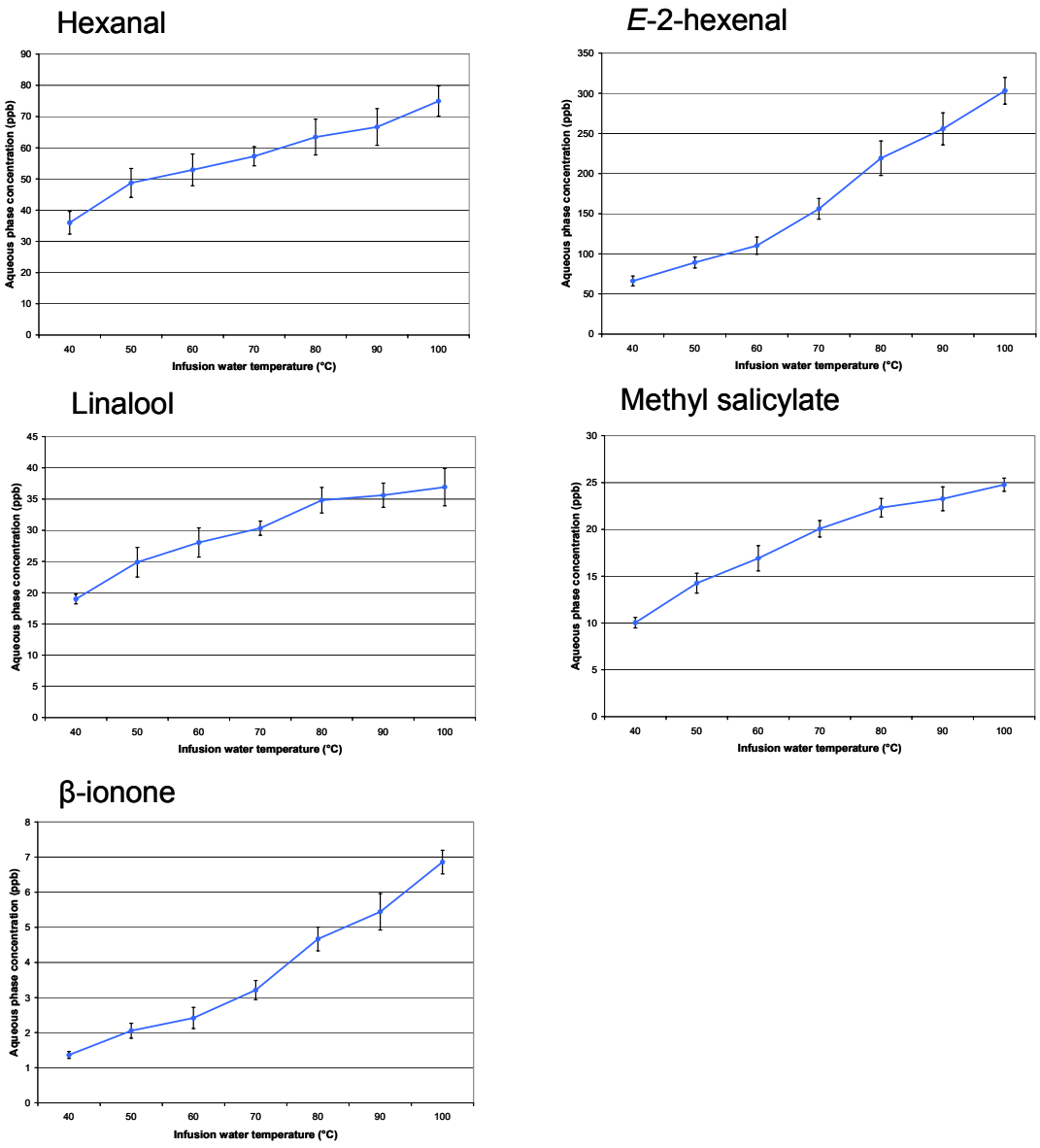


Figure 3-8- Effect of infusion water temperature on the concentration of five volatile compounds

3.3.4.2 Effect of infusion concentration

ANOVA showed that infusion concentration had a significant (p=0.05) effect on the concentration of all five volatile compounds studied, with an increase in concentration with increasing infusion concentration. This is shown in figure 3-9.

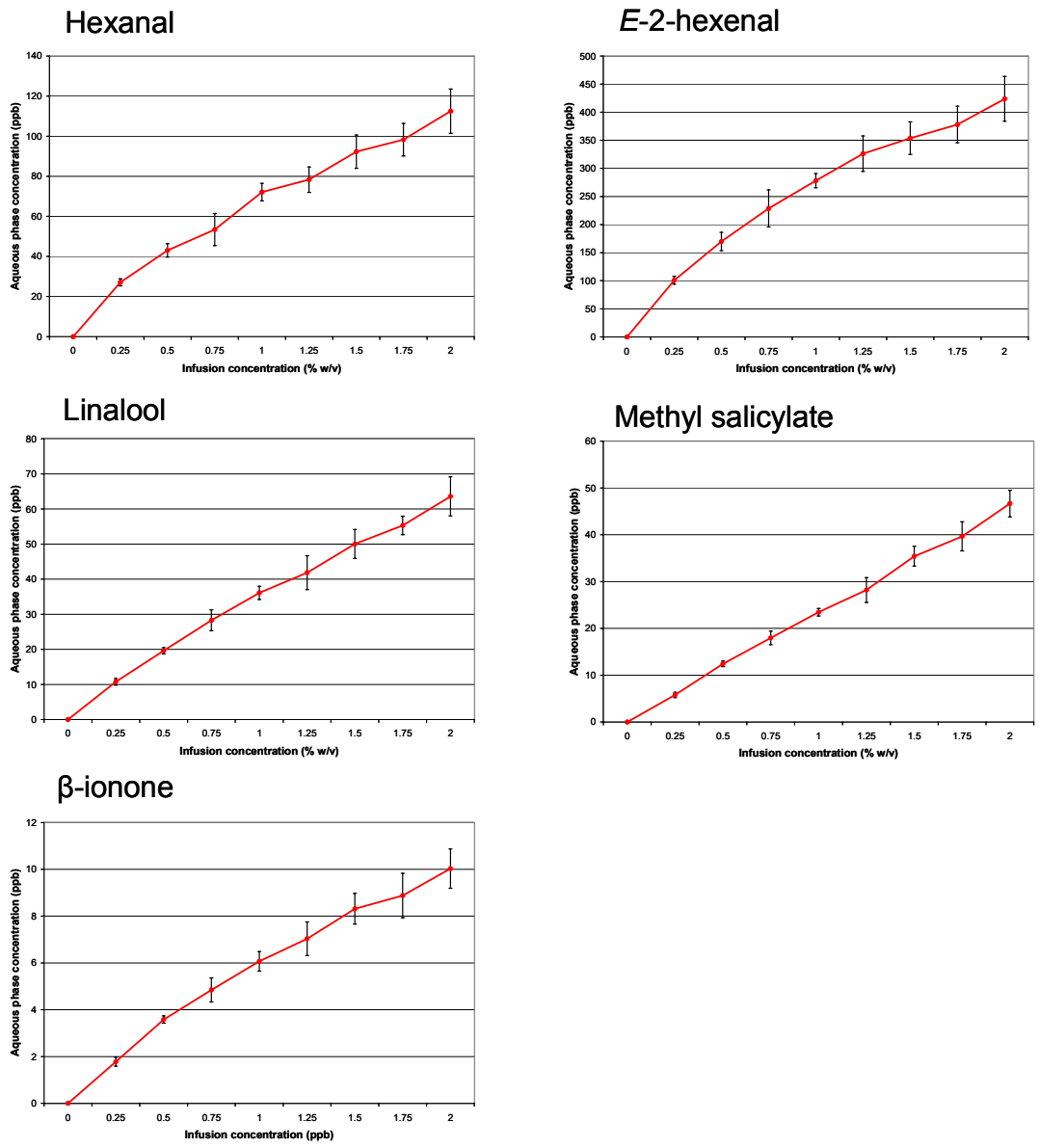


Figure 3-9 - Effect of infusion concentration on the concentration of five volatile compounds (error bars show standard deviations)

Post-hoc testing revealed that for all five compounds, in most cases each infusion water temperature and concentration resulted in a significant difference in the concentration to all others.

3.3.5 Total solids content

Figures 3-10 to 3-12 show the effect of infusion water temperature, concentration and duration on total solids content of tea infusions. As with GC-MS data, values of mean and standard deviation (error bars) are derived from a total of all three batches and three replicate analyses. Values of % total solids refer to g/100 mL infusion.

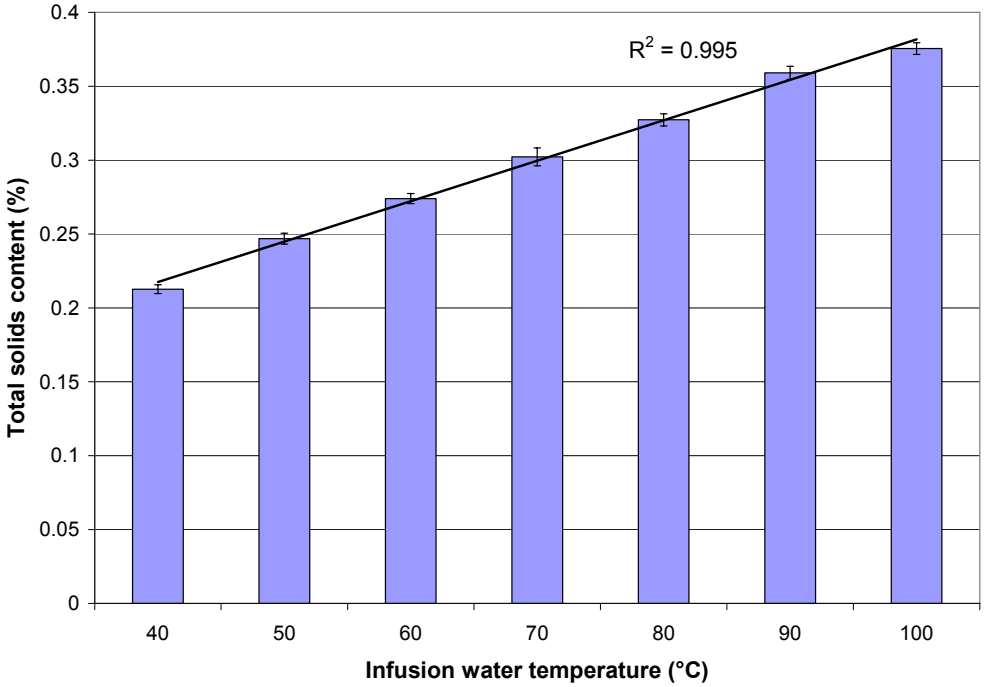


Figure 3-10 - Effect of infusion water temperature on total solids content (error bars show standard deviations)

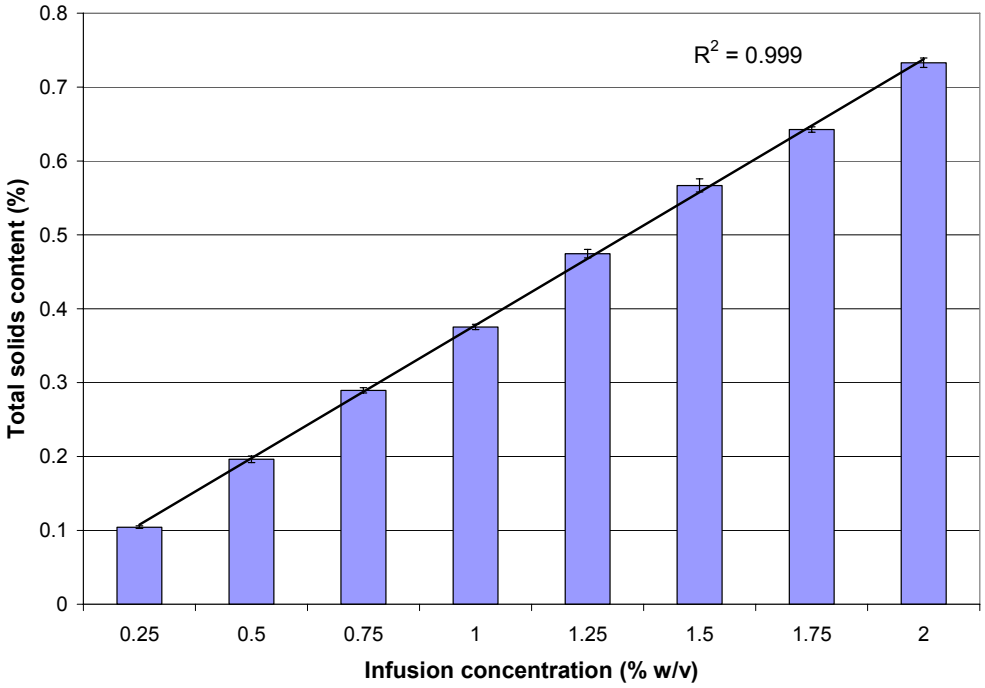


Figure 3-11- Effect of infusion concentration on total solids content (error bars show standard deviations)

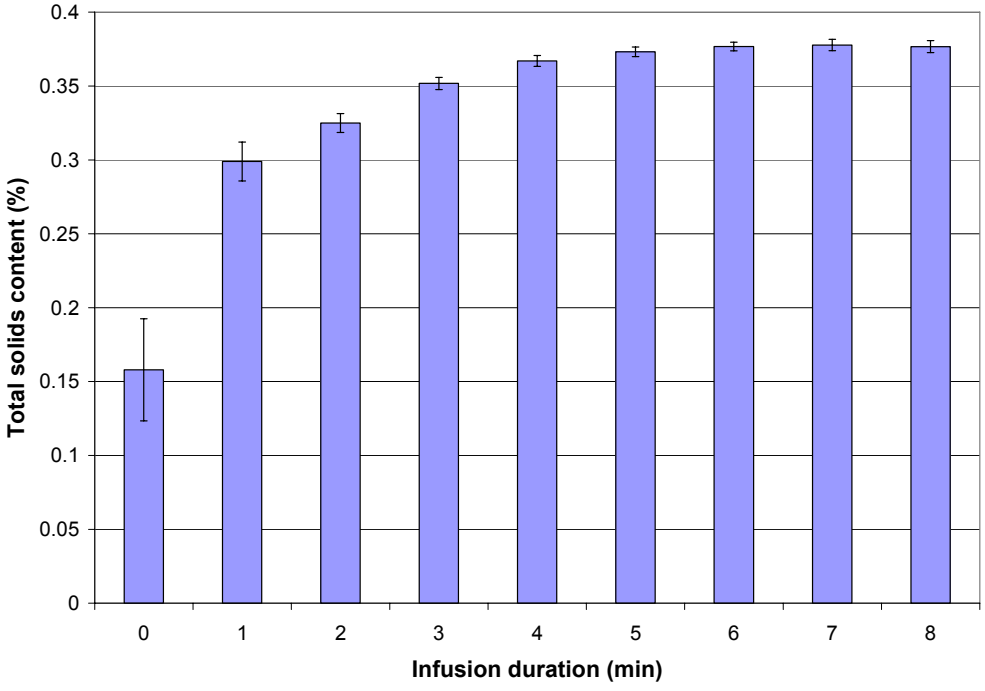


Figure 3-12- Effect of infusion duration on total solids content

It can be seen from figures 3-10 and 3-11 that there is a clear effect of both infusion water temperature and concentration on total solids content of the infusions. For both factors there was a linear relationship on solids content, with R^2 values of 0.99. For infusion temperature, total solids content ranged between 0.21 % (40 °C) and 0.38 % (100 °C) whereas, for infusion concentration, the total solids content varied from 0.10 % (0.25 %w/v) to 0.73% (2.0 %w/v). A 1% w/v infusion prepared with 40 °C water therefore contained approximately the same percentage total solids as a 0.5 %w/v solids infusion (i.e. half strength) prepared using 100 °C water. Infusion concentrations had a greater effect than infusion water temperatures as the change observed from 40 to 100 °C was equivalent to the changes seen when concentration changed from 0.5 to 1 %w/v.

In terms of infusion duration (figure 3-12) there was a large, initial increase in the amount of solids extracted between 0 and 1 min which continued to increase at a slower rate until it plateaued after 5 min. There were no significant differences in total solids content of infusions brewed between 5 and 8 min.

3.4 DISCUSSION

Two key mechanisms affect the concentration of volatile compounds in the headspace above mugs of fresh black tea infusions (illustrated schematically in figure 3-13). The first (a) takes place during the tea infusion process, and involves transfer of volatile compounds from the tea leaves into aqueous solution. The second (b) involves transfer of the volatile compounds (present in aqueous solution) into the headspace via the interfacial layers of the liquid and gas phases.

It is important to emphasise that APCI-MS monitored volatile compounds in the gas phase, and so although preparation conditions inevitably affected both mechanisms, only the overall effect was monitored.

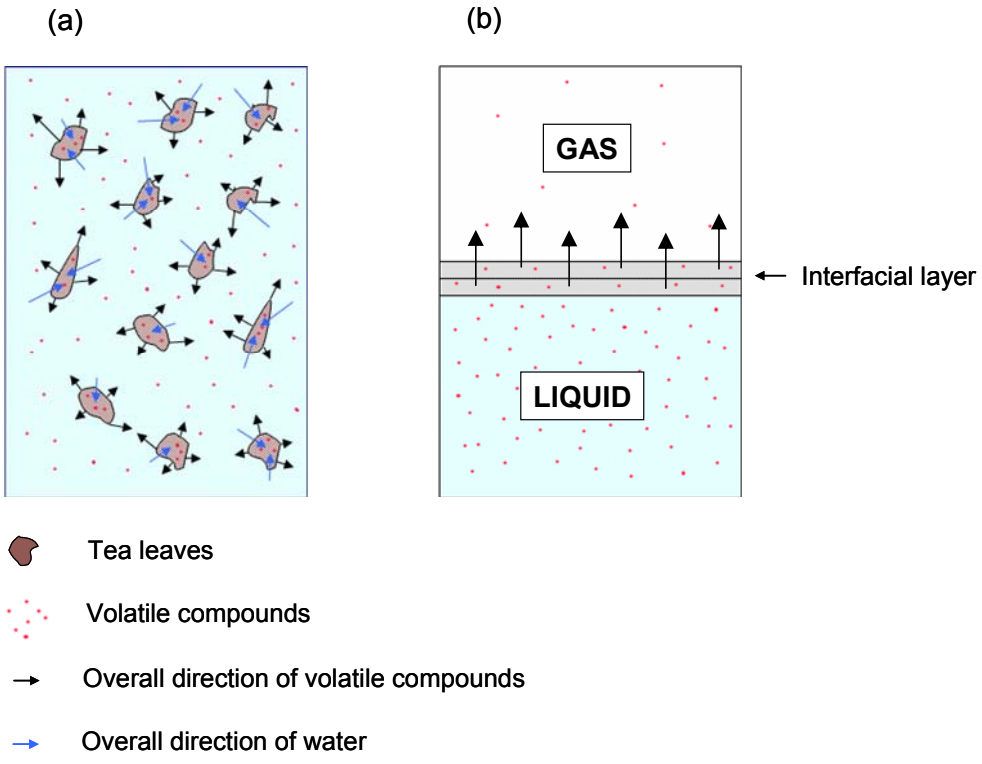


Figure 3-13 - Two key mechanisms contributing to overall volatile release from tea infusions

Considerable work has been carried out by Spiro and co-workers investigating transfer of soluble constituents out of the leaf matrix during the infusion process (mechanism (a) in Figure 3-13). Whilst the purpose of the current study was to

study volatile compounds, this work is still relevant as it is likely that the mechanisms involved in both cases are similar.

Immersion of loose tea in hot water results in rapid water absorption, leading to swelling of the leaf, and increase in water content from ~5 to 75 % (Spiro and Price, 1987). This inward flow of water has been shown to retard the outward diffusion of solubles such as caffeine (Spiro, 1997b). It has been shown that the rate of infusion of caffeine (and other solubles) is fastest at the beginning of infusion, slowing down until a plateau value is reached at equilibrium, that the concentration at equilibrium is always higher in the water-filled solids than in the beverage itself (Spiro, 1997a), and the partition coefficient (*K*) describes the relative distribution of tea solubles between the swollen leaf and water (Spiro and Siddique, 1981). The rate at which this overall process takes place is known as the rate constant of infusion, and for the extraction of soluble constituents can be divided into three distinct parts; i) diffusion through the leaf, ii) transfer across the leaf / water interface, and iii) diffusion away through the Nernst layer (Spiro and Jago, 1982). It has been suggested that the rate determining step of the tea infusion process is slow diffusion through the tea leaf matrix, with the diffusion coefficients of most solubles within the leaf around 100 times smaller than in pure water at the same temperature (Spiro, 1997b).

Considerable work has also been carried out investigating the second mechanism (mechanism (b) in figure 3-13) concerning transport of volatile compounds from the aqueous phase into the headspace. The two main mass transfer mechanisms (convective and diffusive) have been previously described in section 1.4.2, although due to the nature of the samples (i.e. hot tea infusions) it is suggested that the convective mechanism is of most relevance to the current study. The volatile compounds in the aqueous phase are likely to be uniformly distributed due to eddy currents created by convection, exaggerated by the elevated temperatures of the samples. It is assumed that an instantaneous equilibrium exists at the air-water interface, with transport across the interface occurring by diffusion, mainly governed by physicochemical properties such as partition coefficient, vapour pressure, and Henry's law constant, all of which are closely linked. The following discussion attempts to describe the results of this study in terms of the mechanisms described above.

In terms of investigating the effect of infusion water temperature on the aroma of tea infusions from a consumer perspective, the results of the first experiment are of

most relevance. These show the "overall" effect of infusion water temperature on release of different volatile compounds into the headspace where they could be detected via the orthonasal "sniffing" route. Infusion water temperature was shown to exert a significant effect on overall release of all monitored compounds with clear compound-dependent differences evident. For example, it was shown (figure 3-1 section 3.3.1.1) that for the methyl butanals, 90 °C infusion water resulted in release of approximately 85 % of the level released using 100 °C water. This is in comparison to just 65 % the level of β-damascenone. Differences in release between these two groups of compound were even more pronounced at lower temperatures. At 40 °C, release was 17 % for the methyl butanals, but only 0.2 % for β-damascenone.

Whilst these results illustrated the real-life situation, they did not immediately identify the mechanisms involved. Temperature affected many of the parameters involved in the extraction and partition behaviour e.g. vapour pressure, Henry's law constants, and the gas-liquid partition coefficients (K_{aw}). The temperature-dependence of gas-liquid partition coefficients has been previously reported by Kolb et al. (1992), namely that the log-transformed gas-liquid partition coefficient is linearly related to temperature. Solubility of dissolved volatile compounds (in solution) decreases with increasing temperature, again resulting in an increase in values of Henry's law constants. These basic physical laws therefore help explain why hotter infusion water resulted in greater release into the headspace, irrespective of the actual concentrations present in the aqueous phase (i.e. irrespective of mechanism (a) in figure 3-13).

In order to examine this theory further and to test whether it could help explain the different behaviours seen in figure 3-1, values of vapour pressure at different temperatures for the monitored compounds were estimated using the Mpbpwin v1.41 program within the EPI suite™ software (US Environmental Protection Agency).

Figures 3-14 and 3-15 compare the overall effect of infusion water temperature on release of the methyl butanals and pentanal, and β-ionone along with values of predicted vapour pressure. EPI suite uses several different methods to estimate the vapour pressure of compounds; values plotted are mean values of those obtained using the Antoine (Lyman et al., 1990) and modified grain (Lyman, 1985) methods. Since experimentally determined boiling point values (used to calculate vapour

pressure) were unavailable for β-damascenone (compound shown in figure 3-1), it was substituted with β-ionone. Since values of vapour pressure differed slightly for the methyl butanals and pentanal (i.e. ion 87), an average value of the three compounds at the highest temperature was taken, other values then plotted as a proportion of this.

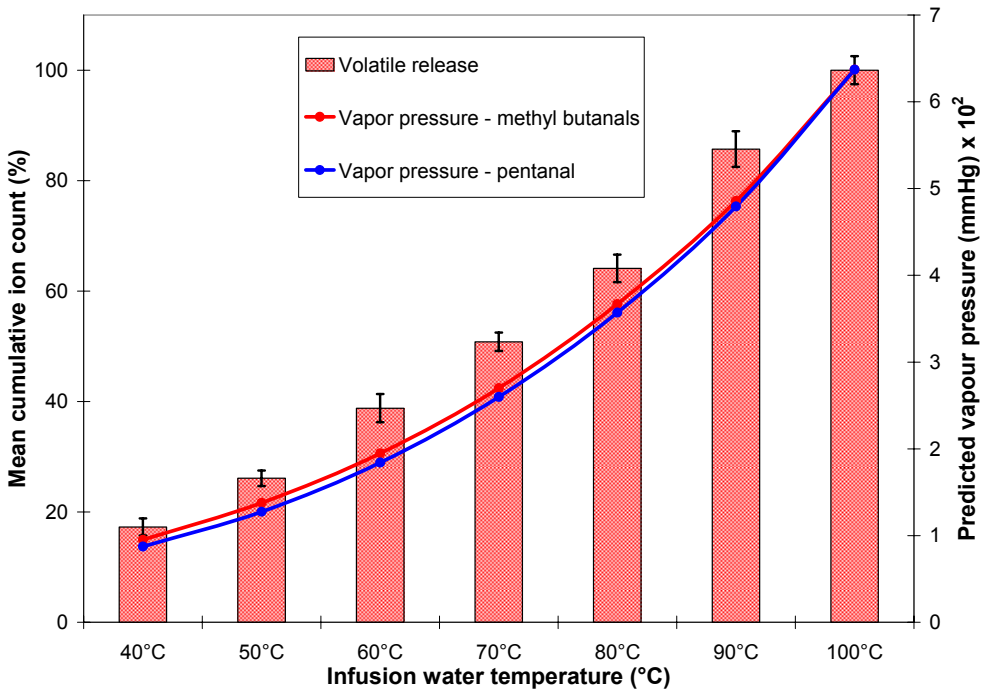


Figure 3-14- Effect of infusion water temperature on release of the methyl butanals and pentanal from tea infusions along with values of predicted vapour pressure (error bars show standard deviations)

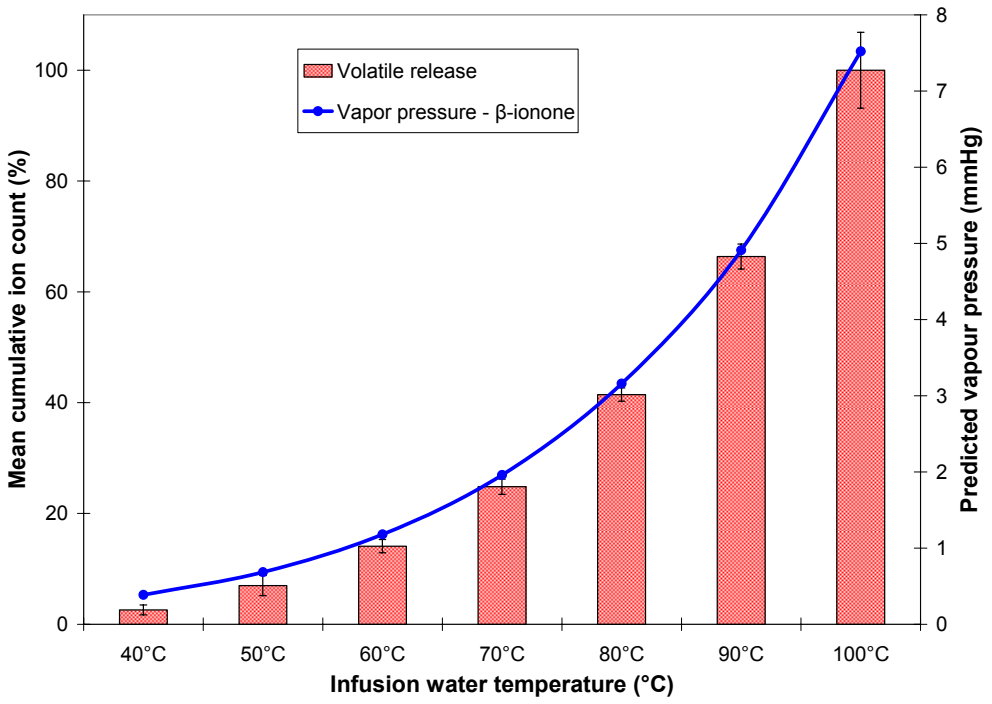


Figure 3-15- Effect of infusion water temperature on the release of β-ionone from tea infusions along with values of predicted vapour pressure (error bars show standard deviations)

In the above two examples, predicted vapour pressure correlated well with volatile release as a function of increasing infusion water temperature. The reasonably linear increase in release of the methyl butanals corresponds to a fairly linear increase in vapour pressure with temperature for these compounds. The exponential release of β-ionone with increasing temperature also correlates well with vapour pressure. This correlation was true for the majority of volatile compounds studied, suggesting that the increase in release observed as a function of increasing infusion water temperature was at least partly attributable to effect of temperature on compound volatility (i.e. the effect of temperature on mechanism (b) in figure 3-13).

A particularly poor correlation between release and vapour pressure was observed for dimethyl sulfide, (figure 3-16). The increase in release of this compound with increasing infusion water temperature appearing to be greater than can be explained by an increase in vapour pressure alone.

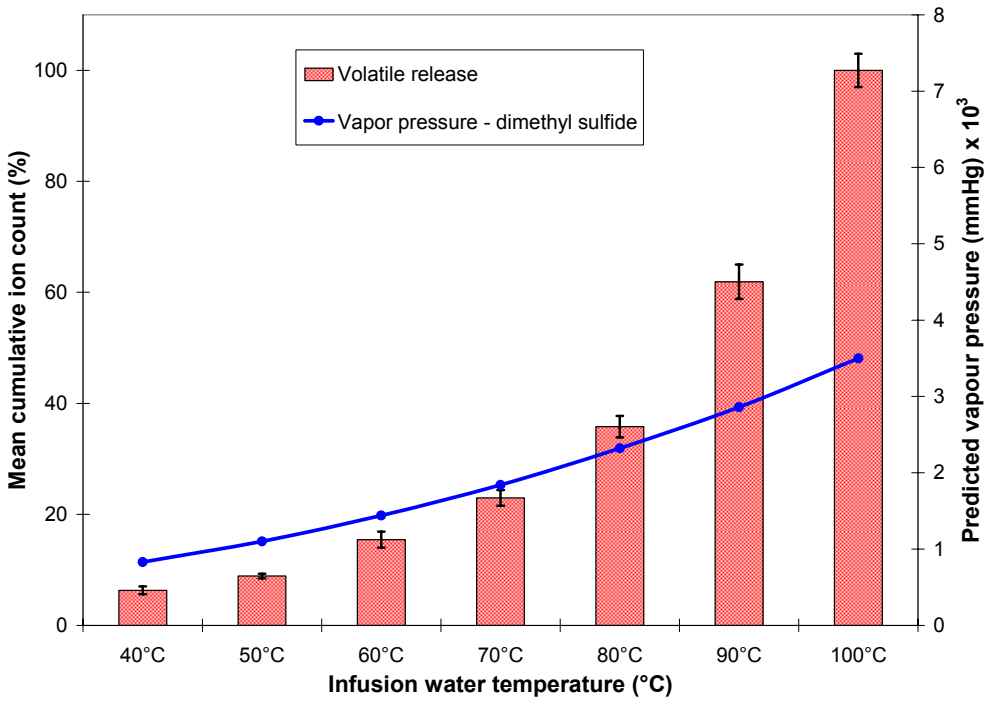


Figure 3-16 - Effect of infusion water temperature on the release of dimethyl sulfide along with values of predicted vapour pressure (error bars show standard deviations)

There are several explanations for this discrepancy, one being that estimation of vapour pressure using a software package makes assumptions about the ideality of solutions, ignoring interactions with non-volatile components which are well known to affect phase partitioning of volatile compounds (van Ruth and Roozen, 2002, Harrison and Hills, 1997). From literature evidence e.g. Natarajan et al. (1962), and total solids analysis carried out in the current study, infusion water temperature has been show to have a significant effect on the non-volatile content of infusions, increasing from 0.21 % for 40 °C to 0.38 % for 100 °C (1 %w/v) infusions. The polyphenols are estimated to account for up to 48.5 % of the solids in a cup of tea, (Sanderson et al., 1976), and Aronson and Ebeler (2004), investigating the effect of polyphenolic compounds on the headspace volatility of flavours observed that these can exert a significant effect - the volatility of benzaldehyde for example showing a decrease in the presence of polyphenolic compounds. Jung and Ebeler (2003) observed that the volatility of hexanal was decreased by ~20 % in a solution of catechin (10 g/L), whilst that of 2-heptanone was increased by ~15 %.

It is also important to remember that values of predicted vapour pressure obtained using software packages are indeed only estimates. Previous workers have found discrepancies between experimentally determined values and those obtained using mathematical models, due to errors in terms of properties such as polarity used to calculate values (Espinosa Diaz et al., 1999).

With particular regards to dimethyl sulfide, another potential reason for the discrepancy may be due to the generation of significant amounts of this compound during the infusion process itself; this is the subject of later discussion.

The most likely reason for any discrepancies however is the fact that plotted values of volatile release correspond to the overall effect of infusion water temperature (i.e. the combined temperature-dependent effect of both mechanisms (a) and (b) in figure 3-13). Whereas values of vapour pressure correspond to those of pure compounds at different temperatures, values of release from tea originate from dilute solutions in which the concentration (as suggested by experiments 2 and 3, and subsequently confirmed by GC-MS) also changes.

Although results from the first experiment clearly illustrated that temperature of infusion water had a significant effect on volatile release, it did not conclude whether this was due to the effect of temperature on extraction of volatiles out of the leaf (mechanism (a), figure 3-13), or whether it was an indirect effect caused either by effect of the temperature on vapour pressure, or shifts in partitioning caused by changes in non-volatile composition (mechanism (b)). Additional experiments using APCI-MS and GC-MS were therefore necessary to decouple the effect of infusion water temperature on extraction of volatiles from the leaf during infusion, and partitioning between the aqueous and gas phase in the final infusion.

The fact that infusion water temperature had an effect on volatile release even when all infusions were at the standard temperature of 60 °C during analysis (experiment 2) confirmed that the effect of infusion water temperature on release observed in experiment 1, was not entirely attributable to differences in temperature of infusion within the mug during headspace sampling. This agrees with data of Schuh and Schieberle (2006) who infused tea with water at 20 and 95 °C. Although only three compounds were studied (linalool, geraniol, and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone), extraction from the leaf to the aqueous infusion was shown to be very temperature dependent.

This clearly provides one explanation why values of vapour pressure did not correlate perfectly with release for all compounds. The fact that volatile release was lower in the second experiment (an example shown for *E*-2-hexenal in figure 3-2, section 3.3.1.2), supports the theory that observed differences in release were at least partly attributed to the effect of temperature on Henry's law constants, and so air-water partition coefficients of the compounds. This was subsequently confirmed in experiment 3, which showed that, for all monitored compounds, temperature of the infusion at time of analysis (incubation temperature) had a significant effect on release, irrespective of the actual temperature of infusion water used (infusion temperature). Results of experiment 3 also showed clear differences in the effect of infusion and incubation temperature on release of the different volatile compounds and is the subject of future discussion.

Whilst analysing all infusions at identical temperatures (60 °C in experiment 2; 40, 60 and 80 °C in experiment 3) confirmed that the overall effect of infusion water temperature on release was not entirely due to differences in partitioning of volatiles between the aqueous and gas phases, it still did not provide conclusive evidence that infusion water temperature affected extraction of volatile compounds out of the leaf matrix. Given the effect of infusion water temperature on non-volatile content of infusions, a possibility remained that increases in observed volatile release were simply caused by matrix effects (i.e. shifts in the partition coefficients of compounds with increasing non-volatile concentration). The same possibility also applied to experiments investigating infusion concentration and duration, where in both cases an increase in volatile release corresponded to an increase in total solids content of the infusions. Increasing infusion concentration from 0.25 to 2.0 %w/v for example resulted in a seven-fold increase in total solids content from 0.10 to 0.73 %.

GC-MS data was crucial in confirming that differences in infusion water temperature and concentration genuinely had an effect on extraction of volatile compounds out of the leaf matrix into the aqueous infusion. For the five volatile compounds for which GC-MS data was obtained, concentration in the aqueous phase (ppb) increased as a function of increasing infusion water temperature and concentration. The results were in very good agreement with the APCI-MS headspace data, an example of which is shown below. Figure 3-17 shows the relative amount of *E*-2-hexenal in the headspace of infusions prepared using the seven infusion water temperatures as determined using APCI-MS, overlaid with the concentration (ppb) obtained using

GC-MS. (APCI-MS data corresponds to that obtained in experiment 2 where all infusions were incubated at 60 °C prior to analysis).

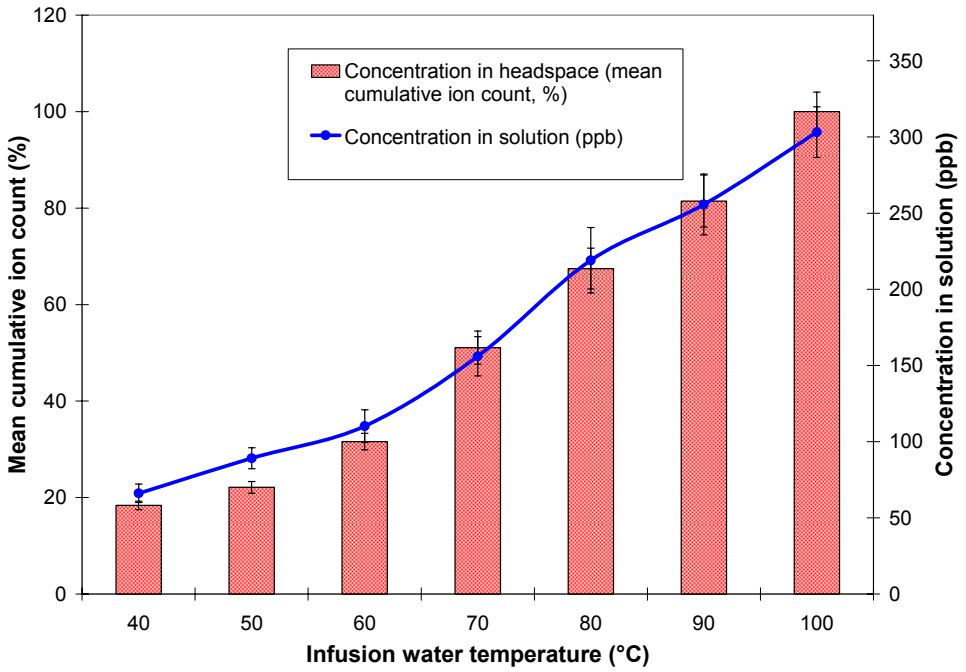


Figure 3-17- Effect of infusion water temperature on concentration of *E*-2-hexenal in the aqueous phase and released into infusion headspace as a function of infusion water temperature (error bars show standard deviations)

The similarity of these plots is striking, and was observed for all five compounds both in terms of infusion water temperature and concentration. Whilst the effect of the non-volatile composition on volatile release cannot be entirely ruled out, it can be said with some certainty that both infusion water temperature and concentration had significant effects on extraction of the volatile components out of the tea leaf into the aqueous phase. Although GC-MS was only carried out on five compounds, it is a reasonable assumption that the trend is indicative of all of those compounds monitored using APCI-MS. It is also reasonable to assume that the same was true of the experiment investigating infusion duration where complementary GC-MS data were not available. The extent to which the non-volatile content of infusions affects volatile release could be investigated further by preparing a number of aqueous solutions containing various concentrations of the compounds constituting the non-volatile portion of infusions. By adding specific amounts of volatile compound(s) and measuring the release by APCI-MS using a static equilibrium approach, the effect of

non-volatiles on aroma release (i.e. partitioning between phases) could be determined.

It is clear that there are distinct differences in release behaviour between the volatile compounds studied in terms of all three preparation method variables investigated. These compound-dependent differences were most apparent in the case of infusion water temperature where, in all three experiments, distinct differences in behaviour of the volatiles were evident. This is particularly well illustrated in the PCA plot (figure 3-5, section 3.3.1.3) which shows clear differences in the relative effect of incubation and infusion temperature for the compounds monitored.

Figures 3-18 and 3-19 show extremes of release behaviour observed in the experiments investigating infusion water temperature and concentration respectively. The compounds displayed in each case have been chosen to illustrate different behaviours, based on their release profiles over the temperature and concentration ranges studied (graphs for infusion water temperature refer to those infusions incubated at 60 °C prior to analysis i.e. experiment 2).

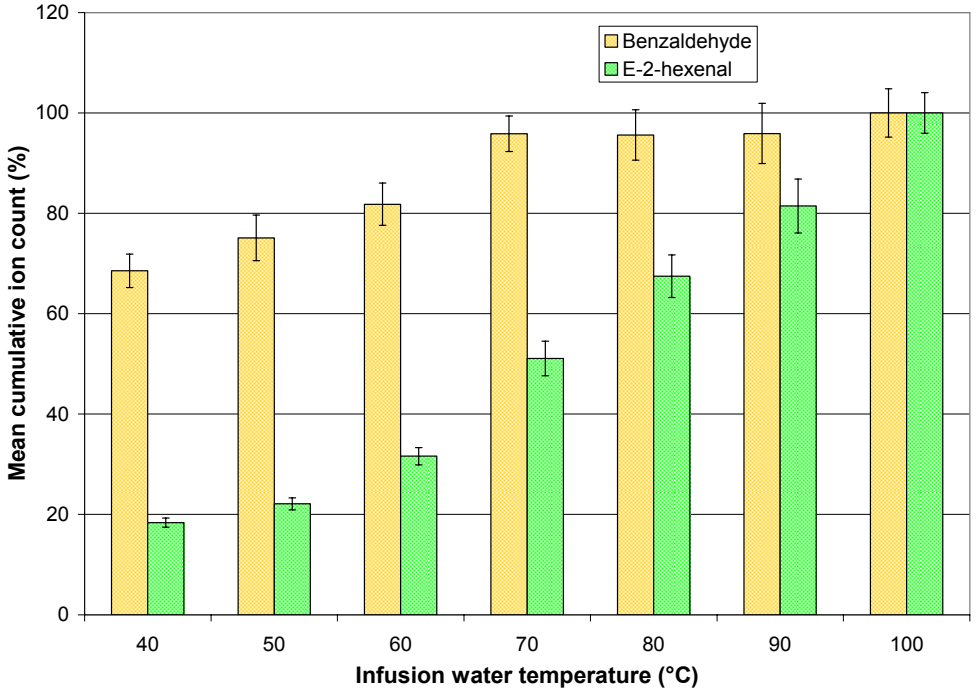


Figure 3-18 - Effect of infusion water temperature on the release of benzaldehyde and *E*-2-hexenal – (incubated at 60°C) (error bars show standard deviations)

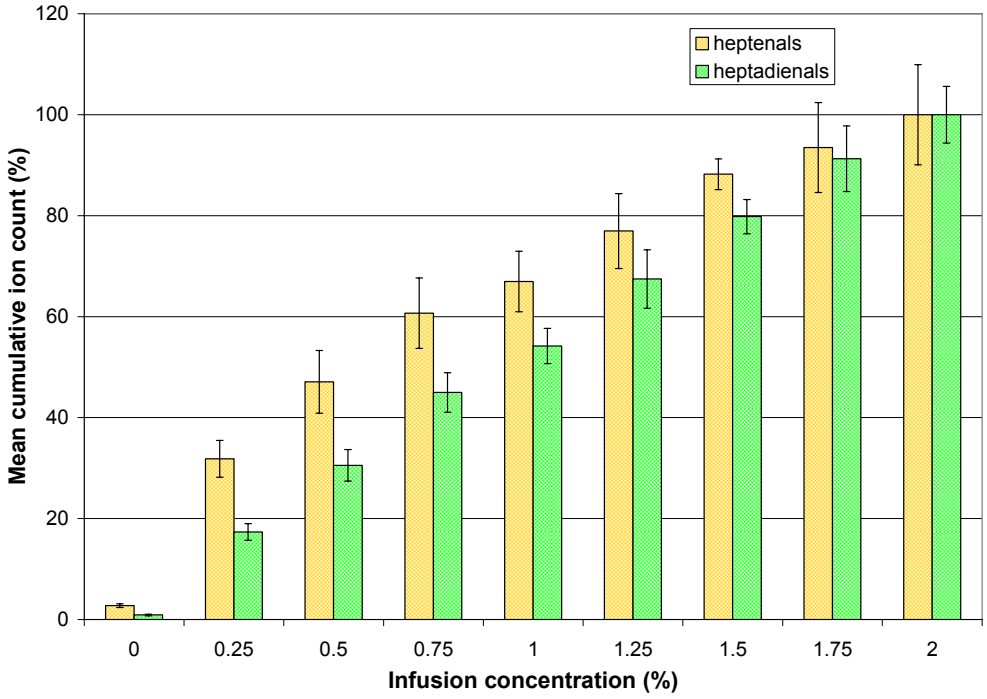


Figure 3-19 - Effect of infusion concentration on release of the heptenal and heptadienals (error bars show standard deviations)

Figure 3-18 shows distinct differences in the effect of infusion water temperature on release of *E*-2-hexenal and benzaldehyde. Whilst there is an almost exponential increase in *E*-2-hexenal release as temperature of infusion water is increased, increase in release of benzaldehyde is more gradual. Release of benzaldehyde from infusions prepared using 70, 80, 90 and 100 °C water was not statistically significantly different between samples. (This was experimentally proven not to be a simple result of the APCI-MS reaching its saturation detection limit for this compound).

In contrast, in the case of all volatiles monitored, there was an approximate linear increase in release as infusion concentration increased from 0 to 2 %w/v. Although the heptenals and heptadienals have been chosen to illustrate differences in behaviour, it can be seen from Figure 3-19 that the effect of concentration on their release follows a similar pattern.

The fact that all compounds were affected by infusion concentration to a similar extent suggests that the increase in release into the headspace with increasing infusion concentration was a direct function of concentration in the aqueous phase,

largely unaffected by the compounds' physical properties. This suggests that compound-dependent differences in release as a function of infusion water temperature were likely due to differences in extraction efficiency of the compounds from the loose tea leaves into the aqueous phase (i.e. mechanism (a) in figure 3-13).

The following section discusses potential reasons for differences in behaviour between compounds, focussing on the effect of infusion water temperature on concentration within the aqueous phase, thought to be largely related to extraction efficiency of compounds.

Whilst relatively little work has been carried out investigating extraction of volatile compounds from tea leaves during the infusion process, considerably more has been carried out on the non-volatile compounds, particularly caffeine and the polyphenols. Whilst it is inevitable that extraction mechanisms will differ slightly, this field of research does provide some suggestions to help explain differences in behaviour of the volatile compounds investigated in the current study.

The rate at which constituents are extracted from the leaf plays a key role in determining the concentration in the aqueous phase, and in the case of volatile constituents, the amount that are available to partition between the liquid and gas phase (i.e. the amount released into the headspace). It has been widely reported that a large proportion of the non-volatile components are extracted from the leaves within the first few minutes of infusion. It has been observed that over 80 % of the total caffeine (Hodgson et al., 1999), and 60 % of the total catechins (Shishikura and Khokhar, 2005) are extracted within the first 30 seconds. Likewise, Langley-Evans (2000) reported that 90 % of the maximum antioxidant activity (associated with the polyphenolic compounds) was present after an infusion of 2 minutes duration. These results support data from the current study, where over 80 % of the total solids content present in infusions of 8 minutes duration were released from leaves after just one minute.

Differences in the extraction efficiency of different non-volatile constituents are widely reported. Hodgson et al. (1999) and Astill et al. (2001) both report that caffeine is extracted from black tea leaf faster than the polyphenols. Similar findings have been shown by Price and Spiro (1985), who observed that under typical infusion conditions, caffeine always infused faster than theobromine, which in turn

infused faster than the polyphenols. These differences have generally been attributed to differences in physicochemical properties of the compounds, Astill et al. (2001) for example suggesting the fast rate of caffeine extraction to be a direct consequence of compound size and so diffusion efficiency; the smaller caffeine molecules diffusing faster than the large polyphenolics. Differences in rate of extraction are not limited to different classes of compounds, Price and Spitzer (1994) observing that the two ungallated flavanols (EC and EGC) infused at a faster rate than the gallated forms (ECG and EGCG) irrespective of the temperature of infusion water used.

Considerable work has also been carried out on non-volatiles investigating effect of infusion water temperature on extraction. Natarajan et al. (1962) investigated the effect of infusion water temperature (40, 60, and 80 °C) on extraction of total soluble solids, tannins, caffeine, total sugars, and nitrogen. In all cases, an increase in infusion water temperature was shown to result in greater efficiency of extraction. These results therefore agree with those of the current study, where infusion water temperature was shown to have a direct effect on the total amount of soluble solids extracted. Similar results were observed by Spiro et al. (1992), and Jaganyi and Price (1999) who studied caffeine extraction, observing an increasing diffusion coefficient and so faster rate of extraction with increasing temperature. Interestingly, Spiro and Siddique (1981) found caffeine extraction was unaffected by infusion water temperature between 79.5 and 94 °C, although they did report a difference in extraction of the theaflavins and thearubigins. The effect of infusion water temperature on extraction of non-volatile constituents has been attributed to a range of factors, Jaganyi and Price (1999) for example identifying faster penetrative ability, less adsorption on the leaf matrix, and a dissolution of caffeine complexes with other solutes (i.e. the polyphenols) which might have otherwise associated to form bulky, slow moving entities. In addition, Spiro and Siddique (1981) suggested the possibility that some compounds such as the thearubigins were present in inaccessible sites within the leaf structure, so only becoming fully extractable using water at higher temperatures.

It is important at this point to recognise similarities between the work carried out on non-volatiles, and the volatiles investigated in the current study. As with the non-volatile components, it was observed that rate of extraction was very rapid in the first minute of infusion, soon plateauing off. With the exception of dimethyl sulfide and β-damascenone, release from infusions prepared under standard conditions

(i.e. 1 % w/v, 100 °C) was not significantly different for infusion durations between 1 and 8 min. Extraction of volatile compounds also appeared to be affected by infusion water temperature in a similar way to the non-volatiles, with clear temperature-dependent extraction, and differences in behaviour between compounds. This suggests the possibility that similar explanations may apply to the extraction of volatile compounds during the infusion process as those discussed above for the non-volatiles.

Whilst the location of volatile compounds in tea leaves has not yet been elucidated, it is known that they are formed in a variety of ways, both in the green leaf, and during processing (section 1.3.2). The monoterpene alcohols (linalool and geraniol) (Takeo, 1981), methyl salicylate (Moon et al., 1996), linalool oxides (Moon et al., 1994) and benzaldehyde (Guo et al., 1998) for example are all known to be formed to a large extent during withering and fermentation due to hydrolytic breakdown of the corresponding glycosides. The Strecker aldehydes (2-methyl propanal, 2- and 3-methyl butanal, and phenylacetaldehyde) are formed due to Strecker degradation reactions during the withering and manufacturing processes (Saijo and Takeo, 1970). Oxidative breakdown of fatty acids is another major source of volatile compounds, with hexanal and *E*-2-hexenal formed from linoleic and linolenic acids respectively.

With such a range of formation mechanisms, it is unlikely that all volatile compounds are present in the same form, and in the same location within the leaf matrix. Some may be present deep within the leaf structure, possibly bound to non-volatile constituents, extracted with great difficulty, whilst others may be present towards the outside in easily accessible areas. Although unlikely (due to the firing process) it is nonetheless possible that some volatile compounds are present on the outside of the tea leaves, as has been suggested for some non-volatile constituents (Long, 1979). It would make sense (as suggested by Spiro and Siddique (1981) in the case of thearubigins) that the more inaccessible volatile compounds would require hotter water in order to become extractable, potentially explaining the exponential-type behaviour seen by some compounds such as *E*-2-hexenal. In contrast, easily accessible compounds would require much less energy in order to be extracted, with high amounts extracted even at the lower infusion water temperatures. It may be that 2-methyl propanal, the methyl butanals, and benzaldehyde belong to this class of compound since infusion water was shown to have a much smaller effect on extraction efficiency.

Whilst the majority of compounds appeared to be extracted to their maximum possible extent within the first minute (under the infusion conditions used), the level of β-damascenone released from infusions of 1 min duration was significantly lower than that from infusions of 2 min duration. This suggests a lower extraction efficiency for this compound, with a slower transfer from the leaf matrix into the aqueous phase. A similar behaviour was observed for β-ionone, although in this case differences in release were not statistically significant. It is also interesting to note that of the compounds monitored, percentage of maximum release of β-damascenone and β-ionone following the 0 min infusion (i.e. water added to tea leaves, inverted, then immediately filtered) was amongst the lowest for all compounds.

One explanation for this may be differences in the location of these compounds within the leaf matrix, leading to a more tortuous and so slower extraction. β-damascenone is derived from the carotenoids (Renold et al., 1974), whilst β-ionone is known to be a major degradation product of β-carotene (Tirimanna and Wickremasinghe, 1965). Whilst the location of these compounds is unknown, due to similarities in formation mechanism it may be that they are present in similar places.

It is also important to consider differences in the physicochemical properties of the volatile compounds which may affect their ease of extraction, especially those of water solubility and hydrophobicity (LogP) which are closely related, as previously described by Chiou et al. (1977). Of particular relevance to the current study is work carried out investigating transfer of pesticide residues and pollutants from tea leaves into the aqueous phase during the infusion process. Wan et al. (1991) reported that extraction of pesticides during the infusion process can be regarded as a reversible equilibrium process between adsorption and dissolution; residues present in the tea leaves can be dissolved in the water, and those in water can be adsorbed to the leaf – until equilibrium is reached. Lin et al. (2005) observed extraction efficiency of polycyclic aromatic hydrocarbons to be inversely proportion to LogP; the higher the LogP, the lower the extraction efficiency. Similar findings have been observed in the case of pesticides, with water insoluble pesticides (e.g. organochlorines and synthetic pyrethroids) showing negligible transfer into infusion compared to the water soluble organophosphates. Nagayama (1996) suggested that pesticides with high LogP values enter the leaf strongly, combining with it, and not moving with infusion water during the infusion process.

Whilst the chemistry of pesticides is different to that of aroma compounds, the same explanations may well provide some explanation for differences in behaviour of the compounds in the current study. Table 2-6, section 2.3.3.2 shows values of LogP varied widely from the relatively polar methyl propanal (LogP 0.74), to the more non-polar β-damascenone (4.21). Similarity, water solubility ranged from 89000 mg/L for 2-methyl propanal, to 12.48 mg/L for β-damascenone.

To a certain extent, differences in hydrophobicity of the volatile compounds does appear to explain differences in their behaviour. Of the compounds monitored, β-damascenone and β-ionone have the highest values of LogP (4.21 and 3.84 respectively), and lowest values of water solubility (12.48 and 25.16 mg/L respectively). The fact that these two compounds appear to be extracted less efficiently from the tea leaf matrix (observed in the experiment investigating infusion duration) therefore makes logical sense. In contrast, compounds such as 2-methyl propanal, the methyl butanals, and pentanal have amongst the lowest values of LogP (0.74 – 1.31) and highest values of water solubility (11230 – 89000) and would be expected to be extracted much more easily. The effect of infusion water temperature in experiments 2 and 3 of the current study appear to support this theory, where temperature had relatively little effect on release. Given the ease of their extraction, a large proportion of these compounds are likely to be extracted with 40 °C water (around 70 % of that of 100 °C water), increases in temperature only resulting in little further increase in extraction.

From table 2-6 it can be seen that dimethyl sulfide also has a relatively low value of LogP (0.92) and high water solubility (22000 mg/L), and on this basis would be expected to be extracted with similar efficiency to 2-methyl propanal, the methyl butanals and pentanal as discussed above. This was not the case since this compound was affected by infusion water temperature to a far greater extent, specifically, release following infusion using 100 °C water was considerable greater than that following infusion with 90 °C water. Results of experiment 2 showed that release of this compound from 100 °C infusions was 52 % greater than 90 °C infusions, compared to 15 and 17 % for 2-methyl propanal and the methyl butanals respectively. One explanation is that dimethyl sulfide (despite its high water solubility, and low hydrophobicity) is poorly extracted from the leaf matrix using cool water due to its location; requiring boiling water for efficient extraction. Whilst the precise mechanism of formation of dimethyl sulfide in tea leaf is unknown, it is

known to be present in both green leaf and manufactured tea (Natarajan et al., 1962), and likely formed by a different mechanism to the aldehyde compounds. This could account for differences in location within the leaf matrix, and so differences in ease of extraction.

However, the most likely explanation for the results observed for dimethyl sulfide is not related to either of the mechanisms illustrated in figure 3-13. Instead, it is likely that the unique effect infusion water temperature has on this compound is related to a third mechanism; additional generation during the infusion (or incubation) procedure. It has been shown that release of dimethyl sulfide increased linearly with increasing infusion duration (figure 3-7, section 3.3.3). Whilst it is possible that this compound was so inefficiently extracted that it was still being extracted after 8 minutes, evidence supports the theory of additional generation. Of most relevance is an experiment carried out as part of a separate area of work (section 4.2) where the effect of storing fresh (filtered) infusions (1 %w/v, 100 °C) in vacuum flasks was determined. It was shown that there was a large increase in dimethyl sulfide release with increased storage, and even after just 10 minutes storage release of this compound was 135 % greater than the fresh equivalent, increasing to 250 % after 20 minutes. Another experiment showed release of dimethyl sulfide from infusions prepared using 100 °C water to be unaffected by storage in sealed Schott bottles in a 60 °C water bath for 30 minutes, suggesting that generation of this compound occurred during the infusion procedure when hot (i.e. >60 °C) water was used. This probably accounts for the position of dimethyl sulfide on the PCA plot (figure 3-5) which appears to show outlier behaviour. As already suggested, it is also likely to be a major reason for the poor correlation of overall release to vapour pressure (figure 3-16), where specifically, it appeared that at infusion water temperatures above around 80 °C, release was greater than could be explained by increases in vapour pressure alone.

Whilst the limited effect of infusion water temperature on extraction of 2-methyl propanal, the methyl butanals and pentanal may be the result of the relatively high water solubility and low hydrophobicity of these compounds, it is also possible that additional generation during infusion or incubation provides some explanation for the behaviour. It has been suggested by Finot et al. (1967) (referenced by Co and Sanderson (1970)) that amino acids can also give rise to volatile aldehydes through interaction with black-tea solids through Strecker degradation reactions under conditions existing during the normal brewing of a cup of tea, a theory also

suggested by Schuh and Schieberle (2006). Whilst these compounds were also shown to be formed during storage in vacuum flasks (section 4.2), increases were considerably lower than those of dimethyl sulfide, release of compounds represented by ions at *m/z* 73 and 87 from infusions stored for 10 minutes being 10 and 13 % greater than their fresh equivalents respectively. As with dimethyl sulfide, storage of 100 °C infusions in water baths at 60 °C for 30 minutes had no effect on release of these compounds. Release from infusions prepared using 40 °C water however was significantly increased following incubation at 60 °C for 30 minutes, with release of 2-methyl propanal and the methyl butanals being 13 and 9 % greater than that from fresh infusions respectively. The fact that release of these compounds from infusions prepared using 60 °C was not significantly different between experiments 1 and 2 (as was shown in the example of *E*-2-hexenal in Figure 3-2) shows that generation only occurred in infusions prepared using cooler (i.e. <60 °C water) water, at this temperature of incubation. This may explain, or at least contribute to the relatively little effect that infusion water appeared to have on release of these compounds from tea infusions observed in experiments 2 and 3. The additional generation in low (i.e. <60 °C), relative to high temperature infusions counteracted any effect infusion water temperature may have had on extraction efficiency. Release of dimethyl sulfide from 40 °C infusions stored in 60 °C water baths also increased, with a 130 % increase relative to the fresh sample. The fact that dimethyl sulfide release appeared to be affected by infusion water temperature to a much greater extent than 2-methyl propanal and the methyl butanals is most likely due to much greater extent of generation of the former in high temperature infusions during the infusion process itself.

Of particular relevance to the current study is recent work carried out by Schuh and Schieberle (2006). As part of a wider study, these workers investigated the extraction efficiency of a range of volatile compounds from loose tea leaves into the aqueous infusion. The authors found that the volatiles studied could be classified into two main groups; those in which the concentration appeared to increase as a result of the infusion process, and those in which the concentration remained constant, or decreased. Of the twenty-two compounds investigated, ten matched those studied in the current work. Of these, *E*-2-hexenal, 2-methyl propanal, 2- and 3-methyl butanal and phenylacetaldehyde had high ratios (all greater than 11) indicating significantly greater concentration in the infusion relative to the loose leaves. At the other end of the spectrum, β-ionone had a ratio of 0.7 suggesting

inefficient extraction into the infusion. *Z*-4-heptenal, β-damascenone, and linalool had ratios between 1.1 and 1.8 indicating similar concentrations in both.
The additional generation of volatile compounds during the tea infusion procedure was suggested by the workers as a reason for the greater concentration of certain volatiles in the tea infusion compared to the leaf. In the case of geraniol (ratio of 32), the authors suggested that this was due to the presence of yet unknown precursors of geraniol which might be released by hydrolytic processes during the infusion procedure. They claim that concentration increases could not have been due to the much simpler explanation of incomplete extraction of the compound during extraction from the tea leaves since the extracted leaves were checked to be odourless by sniffing. Whether simply sniffing the tea leaves (previously extracted using methylene chloride and diethyl ether) is a valid approach to take is questionable. It certainly seems a plausible explanation that the solvents used were unable to fully extract the volatiles from the leaves and it would be very interesting to see whether adding hot water to the so called "odourless" tea leaves results in a perceivable aroma.

Another interesting study has been carried out by Xian et al., (2006), who investigated extraction of components during the tea infusion process using ultrasonic cleaning baths. It was observed that extraction efficiency of the polyphenols, caffeine and amino acids was enhanced through use of ultrasonic baths, whilst that of protein and pectin was inhibited. Particularly interesting, was the effect this procedure had on extraction of volatile compounds, and whilst linalool and geraniol were found to be present at higher quantities in conventionally prepared infusions, *Z*-3-hexanol, benzyl alcohol, 2-phenylethanol, methyl salicylate and nerolidol were all more efficiently extracted by the ultrasonic procedure.

Given the effect the ultrasonic procedure likely had on penetration of water into the leaf structure, it is likely that the procedure resulted in a more efficient extraction of those volatile compounds normally extracted from the leaf matrix with most difficulty, such as those present in inaccessible places. It would be very interesting to utilise a similar technique using the infusions from the current study, infusing two samples of tea simultaneously, one under normal conditions, the other in a sonic water bath. In theory, the ultrasonic extraction would be expected to have limited effect on extraction of those compounds such as 2-methyl propanal, the methyl butanals, and benzaldehyde as these already appear to be extracted with ease. Extraction efficiency of compounds such as β-damascenone and β-ionone however is likely to

be enhanced through use of ultrasonic extraction, since these compounds appear to be extracted with the least efficiency, hypothesised to be at least partly due to their hydrophobicity and location within the leaf matrix. The results of this experiment may help to further explain the differences in behaviour of the volatile compounds seen in the current study, particularly with regards to infusion water temperature.

It is important at this point to return to the results of the experiment investigating infusion duration. The fact that infusion water temperature appears to affect extraction of volatile compounds to a large extent, yet infusion duration appears to have very little effect appears contradictory. It might be expected that differences would be seen between compounds depending upon ease of extraction from the leaf matrix into solution. Whilst this does appear to be the case for β-damascenone, no statistically significant differences were observed for the other compounds (other than dimethyl sulfide). Release of *E*-2-hexenal appeared to be affected by infusion water temperature to a greater extent than these compounds, with only 18 % of the amount released with 100 °C water when using 40 °C water, compared to 38 and 29 % for β-damascenone and β-ionone respectively (shown in experiment 2). The most plausible explanation for this lies in the conditions used in the experiment investigating infusion duration, namely the use of 100 °C water. Irrespective of the rate of extraction, maximum possible extraction of volatiles had generally occurred within the first minute or two of infusion.

It is however worth noting differences in the percentage of maximum release from the 0 min infusions (i.e. those in which the boiling water was added to the tea leaves, then filtered immediately). It is particularly interesting that in the case of those compounds least affected by infusion water temperature (i.e. 2-methyl propanal, the methyl butanals and benzaldehyde), a large proportion of the maximum release occurred as a result of this extremely short (matter of seconds) infusion procedure. Levels of release of these compounds were 72, 68 and 70 % of the maximum respectively. In contrast, those compounds shown to be least affected by infusion water temperature, showed much lower percentage of maximum extraction after this 0 min infusion duration. In order to effectively investigate differences in behaviour between volatile compounds, it would therefore be necessary to use much smaller increments in infusion duration. It is necessary however to consider the practicalities of such an experiment where such short infusion durations would be very difficult to accurately control.

As described in the previous chapter, due to the nature of the headspace sampling system, it has been inappropriate to draw any conclusions regarding differences in temporal profile of release between the different compounds. However, it has previously been suggested that large differences in temporal profile will exist, mainly as a result of differences in the value of K_{aw} of the compounds (as shown in table 2-6). It is also possible that infusion water temperature will affect the temporal profiles of the compounds in different ways. It has already been demonstrated that vapour pressure increase with temperature is compound-dependent, and as such so too will be increases in K_{aw} and Henry's law constant. Also relevant are the findings made by Marin et al. (1999) that conditions in the gas phase affect different compounds in different ways. These workers observed that for compounds with K_{aw} $>10^{-3}$ the temporal profile was based upon K_{aw} alone, whilst for compounds with K_{aw} $<10^{-3}$ conditions in the gas phase (i.e. Reynolds number) played a part in determining the temporal profile. Whilst impossible to accurately measure in the system used in the current study, it is likely that conditions in the gas phase did differ depending upon the temperature of infusion in the mug at time of analysis (due to convection currents caused by differences in steam released). These differences in gas phase conditions may well have affected some compounds (e.g. dimethyl sulfide, 3-methyl butanal, and heptanal, K_{aw} 10^{-2}) to a lesser extent than others (e.g. phenylacetaldehyde and linalool, K_{aw} 10^{-4}) in experiment 1.

Even if a dynamic headspace sampling approach such as that used by Lindinger et al. (2005) (looking at coffee infusion headspace) had been used in the current study, it would still not have been appropriate to make any firm conclusions regarding differences in temporal profiles between the different compounds based upon their physicochemical properties. The main reason for this is the fact that in many cases several compounds collectively contributed to the intensity of ions at particular *m/z* values (table 2-4, section 2.3.2). Although in some cases (e.g. heptanal and heptanone represented by the ion at *m/z* 115), the physicochemical properties of the compounds varied only slightly, in many cases, other unknown compounds contributed to ion intensity, the physicochemical properties of which were also unknown. Although online techniques, such as APCI-MS and PTR-MS are excellent choices for rapid, convenient monitoring of volatile release for foods and beverages, they are not designed for accurate quantification of specific compounds. If this were the aim, accurate (but time consuming) techniques such as GC-MS would be more appropriate).

3.5 FUTURE WORK

It has been shown that the preparation method plays an important role in determining the volatiles released from black tea infusions. This is especially the case in terms of infusion water temperature where differences in the behaviour of different compounds has been observed.

The APCI-MS approach utilised in this chapter has proved to be an excellent tool for identifying differences in aroma release from infusions as a function of infusion preparation method, and has shown clear compound-dependent effects. In order to investigate there results further it is necessary to use alternative techniques such as gas chromatography. A key area of future work would involve accurate quantification of volatile compounds in the aqueous infusion during the time course of the infusion process itself. This would enable the rate of extraction from the leaf matrix of the different volatile compounds to be calculated, and determine how preparation method affects extraction rate of different compounds. A study of extraction rate of different volatile compounds, and the effect infusion preparation method has on this would complement the considerable work that has been carried out on non-volatile components in recent years.

This study has focussed on three of the many variables encountered in the typical tea infusion process. Future work should be carried out to further investigate the effect of preparation method on volatile release from black tea infusions. Proposed areas of study include the use of tea bags, agitation, addition of milk and sugar, extended brewing, and the practice of multiple infusions from the same leaves. It would also be interesting to determine whether there are any interactive effects as a function of preparation method. For example - does infusion water temperature affect volatile release in the same way in low concentration, as in high concentration infusions? With sufficient data, it would then be possible to predict the aroma profile released from an infusion prepared according to a particular infusion procedure (e.g. use of 70 °C water, 1.25 %w/v, 6 min duration, with milk and sugar). This information would benefit the commercial sector as it would be possible to then optimise tea infusion preparation procedures in order to always obtain the optimal aroma.

Whilst all three variables studied were shown to have significant effects on volatile release, the relevance of this to real-life tea consumers cannot be determined from this data alone. The following chapter addresses this issue, where the orthonasal discriminability of freshly prepared black tea infusions prepared according to different preparation methods (infusion water temperature and concentration) has been investigated.

4 EFFECT OF INFUSION PREPARATION METHOD ON BLACK TEA AROMA DISCRIMINATION

4.1 INTRODUCTION

APCI-MS data has shown preparation method to have a significant effect on aroma release from black tea infusions (chapter 3). The aim of this chapter was to explore the significance of these findings based upon the orthonasal aroma discriminability of infusions prepared according to different methods, namely infusion concentration and water temperature.

Choice of discrimination test is an area of much debate within the sensory community, and their use within the field of sensory evaluation is described in section 1.5. APCI-MS work showed that release of selected volatile compounds increased with increasing infusion water temperature and concentration, although given the fact that the precise nature of the difference between samples was unknown (and that it was possible that some compounds showed different behaviours), it was necessary to choose a test where the nature and direction of perceived difference was unspecified (i.e. a non-directional test). A two sample test was also desirable, enabling subjects to smell and compare samples with minimal interval, minimising any effect of temperature or temporal profile on aroma release. Since discrimination testing is reliant upon memory (i.e. comparing samples) a two-sample test was less memory demanding. It also enabled subjects to be presented with realistic volumes of freshly prepared infusion, mimicking the real-life situation.

The same-different test is a non-directional two sample test, particularly appropriate where samples are limited, or unstable. This test is well suited for use with naïve subjects due to the very simple instructions, namely whether the two presented samples are the “same” or “different”. It has been used across a range of food products, including orange drinks (Irwin and Hautus, 1993), raspberry beverages (Stillman and Irwin, 1995), citrus beverages (Cubero et al., 1995), yoghurts (Rousseau et al., 1998), and mustard (Rousseau et al., 1999). There are two main variants of this test; in the first, one pair of stimuli (either the same or different) are presented to subjects (Delwiche and O'Mahony, 1996). The alternative version

involves two successive tests whereby (unknowingly) judges are provided with two pairs, one of which is the same and the other different (Rousseau et al., 1998). Used in isolation however, both versions of this test suffer from response bias (section 1.5.2). This is due to the nature of the human response, namely that some individuals are risk takers and will respond "different" from minimal perceptual evidence whereas others are more conservative and require a stronger perceptual response. In the case of the differencing strategy, the response criterion is a measure of how far apart two samples have to be before an individual is confident enough to say they are different and is discussed further in section 1.5.2.

As described in section 1.5.3, one way of negating the effect of response bias is through the use of signal detection theory, more specifically the calculation of criterion-free measurements such as d'. This approach has been used in the current study, where the sureness rating response has been used to provide points on a ROC curve.

The objectives of this chapter were as follows;

- Develop an appropriate protocol for determining the discriminability of black tea infusions prepared according to different methods

- Confirm the robustness of the chosen method, particularly that volatile release was unaffected by any aspects of the procedure

- Investigate whether consumers could detect differences between the orthonasal aroma of infusions prepared according to different methods

- Apply the signal detection theory to the data in order to negate the effect of response bias and determine degree of difference between samples

4.2 PRELIMINARY EXPERIMENTS

To maximise efficiency, several subjects were required to carry out the test simultaneously, and therefore having some form of storage capability for the prepared tea infusions was essential. This way infusions could be prepared in advance of a session and stored, to be used within a specified time-window. The colour of the infusions varied significantly as a result of differences in preparation method. This is not surprising given that infusion colour is dependent upon non-volatiles present, the composition of which are known to vary widely as a function of preparation method e.g. Astill et al. (2001). Preliminary experimentation showed that addition of 200 µL dye to 200 mL infusion, coupled with red lighting, was sufficient to mask differences between extremes of colour. As with colour, differences in temperature of infusions are easily detectable (even through sniffing), and so where infusions were prepared using water of different temperatures; they were all brought to a common temperature (60 °C) before assessment through incubation in water baths.

Prior to carrying out discrimination tests, extensive preliminary work was therefore required to determine an appropriate preparation protocol for the tea infusion samples. It was important that factors, such as addition of food dye, storage in vacuum flasks, and incubation in water baths, did not provide a source of variation in the data confounded with that from important experimental variables under investigation. Failure to identify systematic errors would have seriously compromised the validity of sensory data obtained.

APCI-MS was used in order to investigate the effect of storage in vacuum flasks and incubation in water baths on release of volatile compounds represented by the 15 ions described in chapter 2. In both sets of experiments, analysis was carried out using a static equilibrium approach where, following appropriate treatment, infusions were rapidly cooled under running water and allowed to equilibrate for 30 minutes at room temperature in sealed Schott bottles prior to headspace analysis.

Infusions (1 %w/v) were prepared according to the standard procedure described in section 2.2.1.1, and stored in vacuum flasks for varying durations of 0, 10, 20, 30 and 40 min. APCI-MS analysis revealed that for most of the monitored compounds, storage duration had no effect on release. There was a slight increase in release of

2-methyl propanal and the methyl butanals with increasing duration of storage, the samples stored for 40 min released around 35 and 43 % more of these compounds respectively compared to the fresh sample. There was a large increase in dimethyl sulfide release with storage, the sample stored for 20 min released around 250 % more than the fresh sample, this increasing to 480 % more after storage for 40 min .

Infusions (1 %w/v, 40, 70 and 100 °C) were prepared according to the standard infusion procedure and held in water baths (60 °C) for either 30 or 50 min. This simulated a 30 min incubation (necessary to equilibrate samples at 60 °C), and a standard 30 min incubation plus an additional 20 min storage in a vacuum flask respectively. Fresh infusions (i.e. no incubation or storage) were also analysed for comparison purposes. In the case of infusions prepared using all three temperatures of water, APCI-MS analysis revealed that holding duration (i.e. 30 or 50 min) had no significant effect on release of any of the volatile compounds monitored. It was shown however that in the case of infusions prepared using 40 °C water, storage of samples stored at 60 °C for 30 min led to a significantly greater release of dimethyl sulfide, 2-methyl propanal, and the methyl butanals compared to the fresh infusions. Storage for 30 min resulted in an increase in release of these compounds by 130, 13 and 9 % respectively (compared to the fresh infusion). Whilst this difference was unimportant in the context of the current sensory work (all subjects received samples incubated for a minimum of 30 min), it clearly helps explain the effect infusion water temperature had on release of 2-methyl propanal and the methyl butanals as observed in the previous chapter.

GC analysis confirmed that no additional compounds were present in the headspace of infusions containing added food dye at the concentrations used. APCI-MS analysis was also carried out in order to confirm that addition of food dye did not alter the partitioning of volatiles between the liquid and gas phases.

Based on these results, controlling colour and temperature differences by use of food dye and water baths was concluded to be acceptable. Since storage of infusions in vacuum flasks (even for as little as 10 min) was shown to significantly affect release of certain volatiles it required further investigation.

The storage of infusions in vacuum flasks was further investigated by use of a same-different discrimination test. Subjects were presented with two samples (fresh vs. stored for 20 min), asked to smell them, and decide whether they were the

"same" or "different". This test confirmed that even though differences in the volatile composition of infusions were present, naïve assessors (consumers) were unable to detect these. It was therefore concluded that storage of infusions for a maximum of 20 min prior to presentation to subjects was acceptable.

4.3 MATERIALS AND METHODS

Two completely separate discrimination tests were performed; one investigating the effect of infusion concentration, the other investigating the effect of infusion water temperature on discrimination between samples.

4.3.1 Subjects

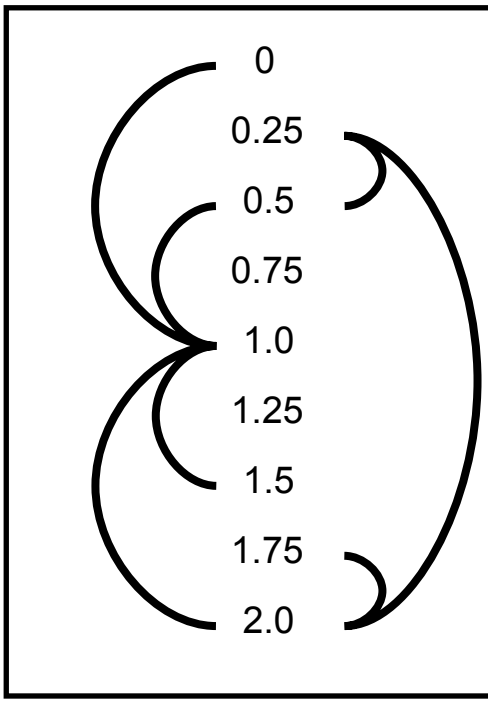
For each experiment, 120 regular black tea consumers were recruited on the basis of availability, each subject attending a single session. Whist not carrying out a "consumer test", it made logical sense to use tea consumers so that results would represent the real-life situation as far as possible.

4.3.2 Samples

4.3.2.1 Effect of infusion concentration

Infusions were prepared according to a modified version of the standard tea infusion procedure (section 2.2.1.1), the mass of loose tea varying according to the desired infusion concentration. Following the standard 4 min infusion procedure, infusions were filtered through double-layer muslin and stored in pre-heated vacuum flasks (1 L) for a maximum of 20 minutes.

Ideally, one comparison test would have been carried out per session, the result of which directing the comparisons used for the next test. For example, if subjects had been unable to detect a difference between a 0.25 and 2.0 % w/v infusion (ratio 1:8) it would have been reasonable to assume that they would also have been unable to detect a difference between a 0.25 and 0.5 % (ratio 1:2) or 1.75 and 2.0 % (ratio: 1:1.1) infusion. This approach is not logistically feasible, and as such, a range of comparisons were carried out in each session. Seven pairs were considered to be the maximum number which could be incorporated into a single session without causing fatigue or boredom. The selected pairs are shown in figure 4-1.



Pair		
1	0.0 %w/v	1.0 %w/v
2	0.5 %w/v	1.0 %w/v
3	1.0 %w/v	1.5 %w/v
4	1.0 %w/v	2.0 %w/v
5	0.25 %w/v	2.0 %w/v
6	0.25 %w/v	0.5 %w/v
7	1.75 %w/v	2.0 %w/v

Figure 4-1 - Seven comparison pairs investigating infusion concentration (% w/v as prepared)

In three pairs (2, 4, and 6), although absolute differences in concentration varied, the relative difference in the two concentrations represented a doubling (i.e. 100 % increase relative to the original, or ratio 1:2). Pairs 6 and 7 were chosen to represent small absolute differences in concentration at either end of the concentration range (although the relative difference in concentration differed in each case). Pair 3 was specifically chosen since the relative difference in concentration between samples was a 50 % increase (ratio 1:1.5), enabling direct comparison to pair 2 with the same absolute difference in concentration, but relative difference in concentration of 100 % increase.

It is important to note that differences in concentration stated above (absolute and relative) were based upon the concentration of infusions as prepared (i.e. % w/v tea leaves to water), and not directly comparable to total solids content of the infusions themselves. For example, although relative differences in concentration (as prepared) between samples in pairs 6, 2 and 4 were identical (100 % increase, ratio 1:2 in all cases), the relative difference in total solids content of samples within these three pairs differed slightly, with % increases relative to the original of 89, 91 and 96 % respectively.

Table 4-1 illustrates this information more clearly. For each pair, the absolute difference in concentration between samples is shown. This information is provided both for the concentration as prepared, (displayed in g/100 mL), and for the total solids content of the resulting infusions (g/100 mL). The relative difference in concentration (prepared and total solids) is also shown. In the case of the prepared concentration this is displayed as the ratio of the concentration of the two samples. In the case of total solids content, it is more appropriate (to aid future discussion) to display relative increases as % increases of the original (i.e. lowest concentration sample). Since it was shown (section 3.3.2) that infusion concentration affected release of compounds represented by all 15 ions to a similar extent, absolute and relative differences in volatile release (average of the 15 ions) are also shown (displayed in arbitrary units, and % increase respectively). This data does however need to be interpreted with a degree of caution since it is not guaranteed that all compounds contributing to the overall aroma behaved in the same way as those monitored using APCI-MS.

Table 4-1 - Absolute and relative difference in concentration and total solids content of prepared infusions and complete infusions, and in average volatile release from infusions within the seven pairs

	Pair						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Absolute difference in leaf tea concentration (g/100 mL) †	1.0	0.5	0.5	1.0	1.75	0.25	0.25
Ratio of leaf tea concentration (1 : *x*) †	n/a *	1:2	1:1.5	1:2	1:8	1:2	1:1.1
Absolute difference in total solids content of infusions (g/100 mL)	0.36	0.17	0.19	0.35	0.62	0.09	0.09
Relative difference in total solids content of infusions (% increase)	n/a *	91	51	96	605	89	14
Absolute difference in average volatile release (arbitrary units)	61	23	21	38	77	16	8
Relative difference in average volatile release (% increase)	n/a *	58	34	62	338	72	9

***** - it is not appropriate to calculate relative differences for pair 1 (0 vs. 1.0 %)

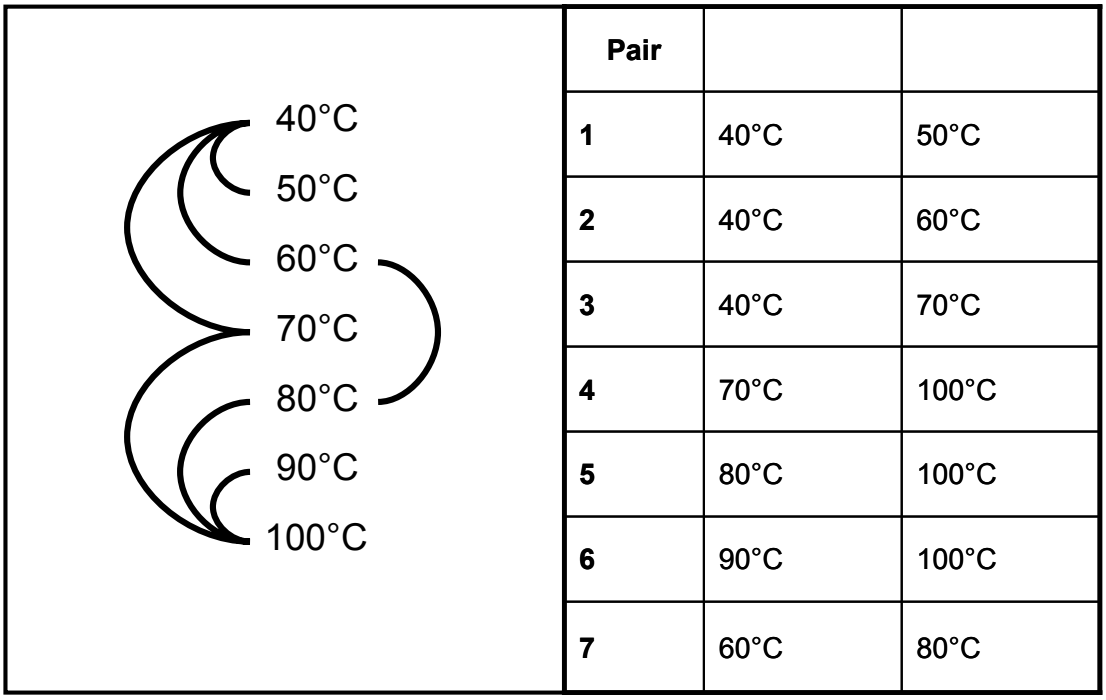
† - leaf tea concentration refers to concentration as prepared

4.3.2.2 Effect of infusion water temperature

Infusion water of the required temperature was pre-measured and held in 1 L vacuum flasks (which maintained temperature within the timeframe used). Preliminary experiments determined the temperature of water required to ensure that temperature at time of preparation was correct (e.g. 93 °C water required for the

90 °C infusion). An exception lay with the 100 °C infusions which were prepared using freshly boiled water.

Infusions (1 %w/v) were prepared according to a modified version of the standard tea infusion procedure (section 2.2.1.1). Prior to infusion, vacuum flasks (1.8 L) were pre-heated using water of approximately the required temperature. At time of preparation, pre-heat water was removed, tea added, and water of the required temperature added. Following the standard 4 min infusion procedure, infusions were filtered though double-layer muslin into Schott bottles (1 L) and held in a water bath (60 °C) for 30 minutes. This time was shown (via preliminary work) to be necessary for all infusions to reach a common temperature, although inversion of bottles every 5 minutes was necessary in order to ensure efficient heat transfer. To limit unnecessary transfer of infusions (and any subsequent loss of volatile compounds), storage (for a maximum of 20 minutes) was carried out in the same bottles in the water bath (as opposed to in vacuum flasks). As with infusion concentration (section 4.3.2.1), seven pairs were selected for this investigation, infusions prepared using water temperatures ranging from 40 to 100 °C, as shown in figure 4-2.



Pair		
1	40°C	50°C
2	40°C	60°C
3	40°C	70°C
4	70°C	100°C
5	80°C	100°C
6	90°C	100°C
7	60°C	80°C

Figure 4-2 - Seven comparison pairs investigating infusion water temperature

Infusion water temperature differences of 10, 20 and 30 °C were investigated at both the low and high range previously investigated using APCI-MS (chapter 3). A mid-range pair was also investigated (60 vs. 80 °C). It is important to remember that temperature in °C constitutes an interval scale (40 °C water is not half as hot as 80 °C water).

Table 4-2 shows the absolute and relative differences in infusion water temperature between the samples of the seven pairs, displayed in Kelvin (K), and as a ratio respectively (values have been derived from the temperature in Kelvin since this represents an absolute, rather than interval scale). As in table 4-1 (effect of concentration), absolute and relative differences in total solids content of the complete infusions is also displayed (displayed in g/100 mL and % increase respectively). Since APCI-MS data (section 3.3.1) very clearly showed that infusion water temperature caused wide variations in behaviour of the compounds represented by the 15 monitored ions, taking an average was inappropriate in this case.

Table 4-2 - Absolute and relative difference in infusion water temperature and total solids content of infusions for the seven pairs

	Pair						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Absolute difference in temperature (K) *	10	20	30	30	20	10	20
Ratio of infusion water temperature (1 : x)	1.03	1.06	1.10	1.09	1.06	1.03	1.06
Absolute difference in total solids content of infusions (g/100 mL)	0.03	0.06	0.09	0.07	0.05	0.02	0.05
Relative difference in total solids content of infusions (% increase)	16	29	42	25	15	5	19

* - refers to infusion water temperature as prepared

4.3.3 Sensory procedure

The basic principle of the same-different test is for subjects to receive two samples simultaneously and decide whether they think they are the "same" or "different". Half of the subjects receive a matched pair (i.e. two identical samples); the other half an unmatched pair (i.e. two different samples). Four sample combinations are therefore possible (AA, BB, AB and BA), equally divided across subjects.

Two samples of tea infusion (200 mL) were presented to subjects in white porcelain bowls (109 mm int. diameter, 60 mm depth), (Teacraft Ltd., Bedford) as recommended by BS6008:1980 - *Method for preparation of a liquor of tea for use in sensory tests* (BSI, 1999). In all cases, infusions were poured up to a line marked on the inside of the bowl.

Black food dye (200 µL) (Supercook, Leeds) was added to the bowls prior to pouring the infusion in order to mask any visual differences. It was shown that the water movements created due to pouring were sufficient to ensure complete mixing of the dye. Bowls were individually labelled using randomly assigned three-digit codes. Presentation to subjects was made two minutes after pouring – both to limit the level of steam released from the infusions, and to reflect more accurately the APCI-MS measurements whereby volatile release were monitored for a period of 4 or 5 minutes after pouring.

Assessment took place in partitioned sensory evaluation booths under red lighting. Subjects were instructed to smell each sample in turn from left to right, and state whether they thought the two samples were the "same" or "different". Prior to entering the booths, subjects were informed that they would either be presented with two "same" samples, or two "different" samples of tea. Subjects were instructed to state how sure they were of their decision, based on three available options of "sure", "quite sure?" and "not at all sure??". Subjects were forced to wait a minimum of 2 minutes in-between successive pairs in order to minimise any effects of fatigue.

Sessions were designed to ensure that the combination and order of sample pairs (same or different) were balanced across subjects as far as possible. Presentation order of individual samples within the pairs (i.e. AB, BA) was also balanced. This was important, partly due to the possibility that subjects' ability to discriminate may

have improved with practice, yet partly because discriminative ability may decrease with fatigue. Due to logistical reasons it was only possible to balance across groups of four people. Each of the four subjects present in a particular session therefore received the same pairs of samples in the same order because once a batch of infusion had been opened, it was necessary to pour immediately. Continually opening and resealing infusions during the session may have affected the aroma profile and/or temperature, biasing results.

The two samples presented to subjects were always taken from different batches of infusion (irrespective of whether they were "matched" or "unmatched" pairs). This decision was made in view of the infusion reproducibility data reported in section 2.3.1, and the possibility that slight differences in aroma profile existed solely as a function of batch-to-batch variation. Had subjects been presented with samples from the same batch when presented with "matched" pairs, and inevitably different batches when presented with "unmatched" pairs, it is possible that statistical differences may have been reported on the basis of batch-to-batch variation, as opposed to infusion preparation method. This procedure minimised the risk of a type I error occurring (α-risk), and so the risk of falsely rejecting the null hypothesis (i.e. concluding that samples could be discriminated when in fact they could not).

4.3.4 Data analysis

4.3.4.1 Chi-square test

The Chi-square test was used to determine whether or not subjects could discriminate between infusions prepared under different conditions (i.e. infusion concentration and water temperature).

Results were tabulated according to the samples tested and responses given. Table 4-3 gives an example of such a table, the columns indicating the samples tested, the rows indicating how they were identified by the subjects.

Table 4-3 - Example of data obtained in the two discrimination tests

		Subjects received:		
		Matched pair AA or BB	**Unmatched pair AB or BA**	**TOTAL**
Subjects said:	**Same**	35 (27)	19 (27)	**54**
	Different	25 (33)	41 (33)	**66**
	TOTAL	**60**	**60**	***120***

Values in brackets indicate the "expected" number of responses, calculated by multiplying the total number of respondents saying same or different, by the total number receiving a matched or unmatched pair, then dividing by the total number of responses (Meilgaard et al., 1999). For example, in terms of the number of subjects expected to say "same" when presented with a matched pair, E = (54 x 60) / 120. E therefore equals 27.

The χ^2 analysis is used to compare the placebo effect (35 / 25) with the treatment effect (19 / 41), calculated as follows;

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Where *O* is the observed number, and *E* is the expected number in each of the four boxes. For the above example;

$$\chi^2 = \frac{(35-27)^2}{27} + \frac{(19-27)^2}{27} + \frac{(25-33)^2}{33} + \frac{(41-33)^2}{33}$$

$\chi^2 = 8.62$

The null hypothesis is that there is no significant difference perceived by subjects between the two samples. The alternative hypothesis is that there is a significant difference perceived between the two samples. The null hypothesis is rejected if χ^2 is greater than the critical value with one degree of freedom found in statistical tables.

With α=0.05, 1 df, the critical value of χ^2 is 3.84, and so in the above example the null hypothesis is rejected and it be concluded that subjects could discriminate between samples.

4.3.4.2 Calculation of d', P(A) and generation of ROC curves

Values of d' and P(A) for each discrimination pair were calculated using specialist ROC fitting software for single-interval rating procedures obtained from the internet (Hautus, 1991). This software can be used to model ROC curves based on either the differencing or optimal strategies as described in section 1.5.3 (Irwin and Hautus, 1997). Based upon previous evidence suggesting the use of the differencing strategy within the field of sensory evaluation (Irwin and Hautus, 1993), a decision was made to use the differencing strategy in this area of work. Using this model, the final estimate of d' (and the criteria) is obtained by systematically adjusting its value (and those of the criteria) to minimize the normalized squared distance between each data point and the ROC curve; that is, chi-square (Hautus, 2007, personal communication).

4.4 RESULTS

4.4.1 Effect of infusion concentration

A full breakdown of results obtained in both discrimination tests can be found in appendices 6 and 7. Table 4-4 shows values of χ^2 obtained for each of the comparisons, along with values of d' and P(A) determined by generation of ROC curves. Pairs are listed in order of increasing d'.

Table 4-4 - Values of χ^2, d' and P(A) obtained for the seven infusion concentration pairs

Pair	Description	$\chi^2_{1,0.05}$	d'	P(A)	
7	**1.75 vs. 2.0 %w/v**	0.13	0.08	0.50	**Increasing d'** ↓
2	**0.5 vs. 1.0 %w/v**	4.06 *	1.34	0.62	
6	**0.25 vs. 0.5 %w/v**	8.62 *	1.34	0.62	
3	**1.0 vs. 1.5 %w/v**	4.06 *	1.50	0.65	
4	**1.0 vs. 2.0 %w/v**	11.42 *	1.81	0.70	
5	**0.25 vs. 2.0 %w/v**	23.63 *	2.61	0.83	
1	**0 vs. 1.0 %w/v**	40.34 *	3.26	0.90	

* - significant difference between pairs i.e. critical values of χ^2 (3.84) exceeded ($p<0.05$)

Table 4-4 shows that significant differences existed for six of the seven pairs ($p<0.05$). Only in the case of pair 7 (1.75 vs. 2.0 %w/v) were subjects unable to detect a difference between the two samples. Values of d' ranged from 0.08 to 3.26 indicating a range of discriminability between the pairs. It is interesting to note that d' and P(A) values of 1.34 and 0.62 respectively were calculated for both pairs 2 and 6 (0.5 vs. 1.0 %w/v, and 0.25 vs. 0.5 %w/v), whereas higher values of d' and P(A) (1.81 and 0.70) were calculated for pair 4 (1.0 vs. 2.0 %w/v), even though the ratio of concentration (as prepared) between samples was the same for all three pairs

(i.e. 1:2). Another point of interest is the fact that pair 3 (1.0 vs. 1.5 %) gave higher values of d' and P(A) than both pairs 2 and 6, even thought the relative difference in (as prepared) concentration between samples was greater in the latter two pairs (i.e. ratios of 1:1.5 and 1:2 respectively).

Figure 4-3 shows ROC curves obtained for the seven pairs combined on a single plot. Individual ROC curves (showing the five criterion levels) are shown in appendix 8.

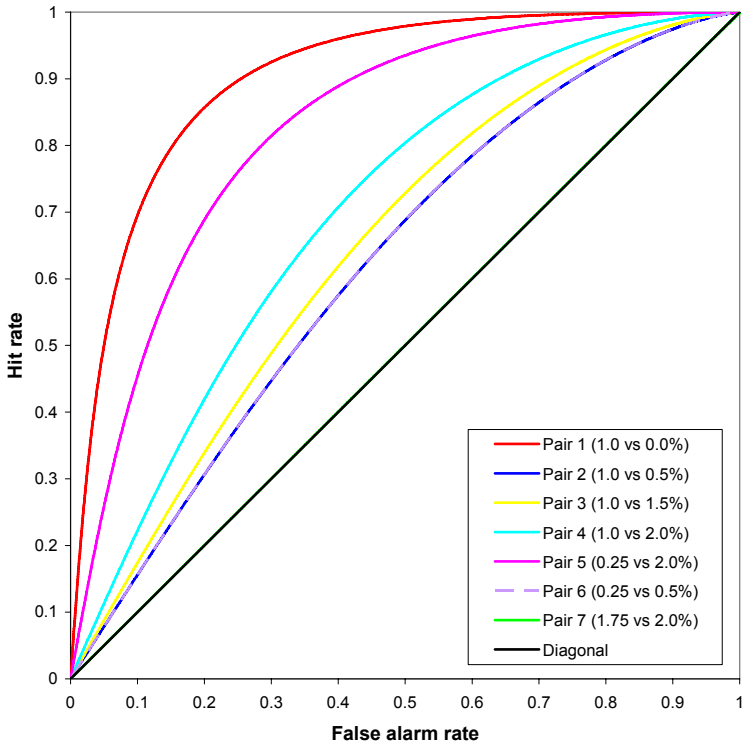


Figure 4-3 - ROC curves (differencing strategy) obtained for the seven infusion concentration pairs

The diagonal line (black) indicates the scenario where proportion of hits = proportion of false alarms (d'=0 and P(A)=0.50, indicating that subjects are unable to discriminate). As described in section 1.5.3, the curvature of individual curves represents degree of discriminability; the greater the curvature, the higher the value of d', and the greater the area under the curve, P(A). ROC curves for pair 2 (0.5 vs. 1.0 %w/v) and pair 6 (0.25 vs. 0.5 %w/v) are identical, and in the above figure are represented by a hatched line. The green ROC curve for pair 7 (1.75 vs. 2.0 %w/v) lies underneath the diagonal, and so is difficult to distinguish.

4.4.2 Effect of infusion water temperature

Table 4-5 shows the values of χ^2 obtained for each of the seven comparisons along with values of d' and P(A) determined by use of ROC curves as described above.

Table 4-5 - Values of χ^2, d' and P(A) obtained for the seven infusion water temperature pairs

Pair	Description	$\chi^2_{1,0.05}$	d'	P(A)	
1	**40 vs. 50 °C**	2.14	0.95	0.57	Increasing d' ↓
2	**40 vs. 60 °C**	2.14	0.99	0.57	
7	**60 vs. 80 °C**	2.72	1.16	0.60	
6	**90 vs. 100 °C**	6.53 *	1.58	0.66	
3	**40 vs. 70 °C**	15.42 *	1.86	0.71	
5	**80 vs. 100 °C**	16.81 *	1.93	0.72	
4	**70 vs. 100 °C**	18.80 *	2.27	0.78	

* - significant difference between pairs i.e. critical values of χ^2 (3.84) exceeded ($p<0.05$)

Table 4-5 shows that significant differences existed for four of the seven pairs, with values of d' ranging from 0.95 to 2.27, again indicating a wide range of discriminability between the pairs. It is interesting to note that d' and P(A) values were significantly smaller for pairs prepared using cooler infusion water. Samples within pairs 2, 7 and 5 were all prepared using water with a temperature difference of 20 °C (40 vs. 60 °C, 60 vs. 80 °C, and 80 vs. 100 °C). Values of d' for these pairs increased in the order 0.99, 1.16, 1.93 for these samples, indicating greater degree of discriminability at higher temperatures. The same was also true for samples with 10 and 30 °C differences in infusion water temperatures (i.e. pairs 1 and 6, and 3 and 4 respectively).

Figure 4-4 shows ROC curves obtained for the seven pairs combined on a single plot. Individual ROC curves (showing the five criterion levels) are shown in appendix 9.

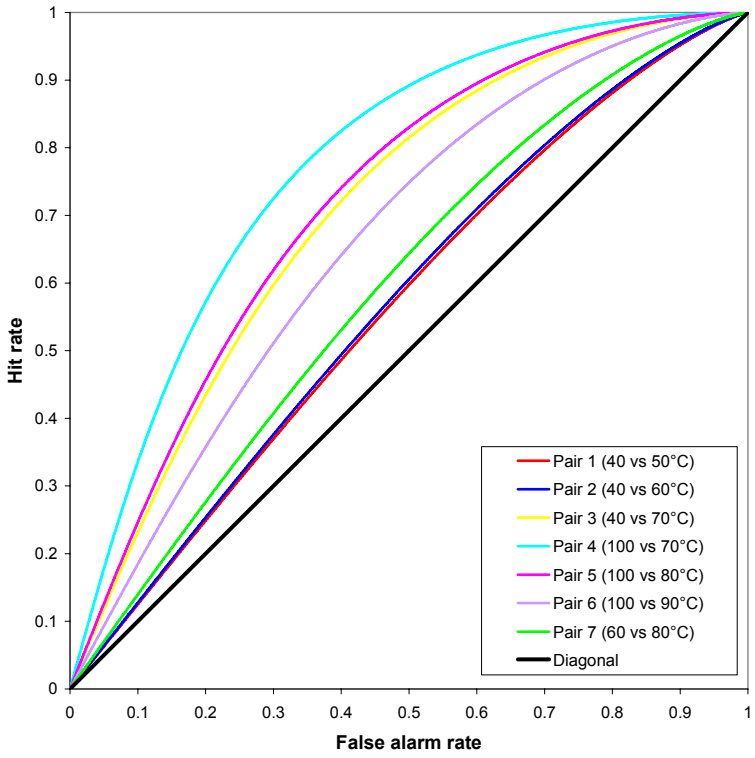


Figure 4-4 - ROC curves (differencing strategy) obtained for the seven infusion water temperature pairs

4.5 DISCUSSION

The following discussion explores the results obtained in this study, paying particular attention to reasons for differences in discriminability of samples within the pairs for both concentration and infusion water temperature experiments. The effect of absolute and relative differences, both in infusion concentration and temperature is explored, as is average volatile release in the experiment investigating infusion concentration.

As stated in section 1.5.3, a d' value of 1.0 can be considered as a threshold value in psychophysics and has to be exceeded in order for differences to be detected (O'Mahony and Rousseau, 2002, Kuesten, 2001). This appears to apply reasonably well to the data shown in tables 4-4 and 4-5. In the infusion concentration discrimination test, the only sample where the null hypothesis was not rejected (i.e. subjects could not discriminate) had a d' value of 0.08 and corresponding P(A) of 0.5. The second highest value of d' of 1.34 applied to two pairs, in which cases the null hypothesis was rejected and it was concluded that subjects were able to discriminate. In the infusion water temperature discrimination test, pairs 1 and 2 had d' values of 0.95 and 0.99 respectively (both values being below 1.0). In neither case, was the null hypothesis rejected and it was concluded that subjects were not able to discriminate between samples. Whilst in pair 7 (60 vs. 80 °C) the critical value of χ^2 was not exceeded, and the samples were not confirmed as being discriminable, the d' value was still very close to 1 (1.16).

In the infusion concentration discrimination test, the only pair not discriminated was pair 7 (1.75 vs. 2.0 %w/v). There are several possible explanations for this which will now be discussed. It can be seen from table 4-1 that of the seven pairs, the relative difference in concentration between the two samples of this pair was smallest (both for the as prepared concentration and total solids content). The absolute and relative difference in average volatile release of samples within this pair were also the smallest.

It is interesting to note that whilst the absolute difference in concentration (as prepared, and total solids) was identical for both pairs 6 and 7 (0.25 vs. 0.5 %w/v and 1.75 vs. 2.0 %w/v) (table 4-1), only in the latter case were subjects unable to discriminate between the samples, the χ^2 value of pair 6 being 8.62 (i.e. greater than

the critical value of 3.84), with a corresponding d' value of 1.34. The key difference between these two pairs lies in the relative difference in concentration of the two samples. Whilst the 2.0 %w/v infusion was prepared using 14% more loose tea than that of the 1.75 %w/v infusion, the 0.5 %w/v infusion was prepared using 100 % more loose tea than the 0.25 %w/v infusion (i.e. ratios of 1:1.1 and 1:2 respectively). Relative difference in average volatile release was also considerably greater between the samples of pair 6, with a 72 % increase, compared to just 9 % for the samples of pair 7.

The difference in discriminability between these two pairs can be explained by referring to Weber's law. This law states that the size of the difference threshold (just noticeable difference) grows in size proportionally as the size of the stimulus increases (Coren et al., 2004). To put this into context, an example of a similar situation may be encountered if asking subjects whether they can detect the difference in the overall light intensity from candles. If a darkened room were lit by two candles, the increase in light intensity as the result of addition of an extra hundred candles would almost certainly be perceivable. If a darkened room already contained a million candles, the addition of a hundred more is unlikely to make any perceivable difference to the overall light intensity.

Sensory fatigue is defined as a sensory overload, with the senses unable to recover their initial state between the tasting of successive samples, and is a factor known to increase perceptual variance and subsequently decrease d' (Rousseau et al., 1999). Of the samples encountered, those of pair 7 are likely the most fatiguing due to both samples within the pair having high aroma intensity, and may therefore provide an additional explanation for the least discriminable nature of these samples. It is possible that some subjects allowed insufficient time to elapse between smelling the two samples within this pair, subsequently suffering from the effects of fatigue. Alternatively, other subjects may have allowed extra time between the two samples in an attempt to allow their senses to recover. This increase in interstimulus delay may however have had an effect on memory, with subjects less able to recall the characteristics of the first sample after the extended delay, so adding to confusion. An extended interstimulus delay is also likely to exaggerate any differences in the aroma of samples caused by differences in temporal profile of volatiles. Looking at the raw data however (appendix 6) the number of "misses" (subjects incorrectly saying "same" when presented with an unmatched pair) was highest in pair 7 out of

all pairs. This would suggest that exaggeration of temperature profile differences caused by an extended interstimulus delay did not exert a major effect.

The non-discriminability of pair 7 is likely a result of several contributing factors discussed above. Most important is the fact that the samples had the lowest relative difference in concentration (both as prepared and total solids), and exhibited the lowest absolute and relative difference in average volatile release. The higher stimulus intensity possibly also played a part, in terms of increasing the perceptual variance due to fatigue and memory effects.

In pairs 2, 4 and 6, the ratio of concentration (as prepared) between the two presented samples was identical (1:2), each representing a doubling of concentration (i.e. 0.5 vs. 1.0 %w/v, 1.0 vs. 2.0 %w/v and 0.25 vs. 0.5 %w/v respectively). It can be seen in table 4-4 however that the values of d' for three pairs were not identical, with pair 4 (1.0 vs. 2.0 %w/v) giving a higher value of d' and P(A) (1.81 and 0.70) than the other two pairs (1.34 and 0.62 in both cases) indicating that subjects found it easier to discriminate between the samples of pair 4. Looking at the average increase in aroma release for all 15 monitored ions using APCI-MS (table 4-1) it can be seen that that relative increase in release of volatile compounds does not vary significantly between the samples in pairs 2 and 4 (0.5 vs. 1.0 %w/v, and 1.0% vs. 2.0 %w/v), with increases of 58 and 62 % respectively. In the case of pair 6, (0.25 vs. 0.5 % w/v), there was a 72 % increase in average volatile release between the two samples. This difference does not however explain why the 1.0 vs. 2.0 %w/v pair gave a higher value of d' since from this data it would be expected that pair 6 would in fact be the easiest to discriminate, having the largest relative difference in volatile release.

A likely explanation for this apparent anomaly is that pair 4 (1.0 vs. 2.0 %w/v) had a higher absolute difference in level of volatile release than the other two pairs due to the largest absolute difference in concentration (38 compared to 23 and 16 arbitrary units respectively). Whilst subjects are likely to use relative differences as the basis for their decision strategy, absolute difference in release may also contribute to a certain extent. It should also be remembered that whilst the relative difference in concentration (as prepared) between the samples was the same for all three pairs, slight differences were evident in terms of the absolute differences in concentration of the infusions (total solids content). Of the three pairs, the relative difference in concentration based upon total solids content was greatest between the samples in

pair 4 (1.0 vs. 2.0 %w/v) with an increase of 96 %. For pairs 2 and 6 (0.5 vs. 1.0 %w/v and 0.25 vs. 0.5 %w/v), the relative differences were lower, at 91 and 89 % respectively. This could potentially explain differences in the discriminability of the pairs, since the pair with the greatest relative difference in concentration did yield the highest d' value.

It is also interesting to note that the value of d' obtained was higher for pair 3 (1.0 vs. 1.5 %w/v), than for pair 2 (0.5 vs. 1.0 %w/v) at 1.50 and 1.34 respectively. Although absolute differences in concentration (as prepared and total solids) and volatile release were similar between these two pairs, relative differences were greater between the samples in pair 2. It is therefore surprising to find a higher value of d' for pair 3 where a smaller relative difference existed contradicting the theory that it is relative (as opposed to absolute) differences that are more important in determining discriminability.

It is however important to appreciate that in the current study, only one value of d' and P(A) was calculated for each pair-wise comparison. Whilst 120 subjects were used in each experiment, this constitutes only one replicate and may well account for the reason why some of the d' values appeared slightly contradictory in nature. The sureness rating response has a large impact on calculated values of d', with a minor difference in proportion of subjects saying 'sure' or 'not sure' having an effect on d' values. It is not possible to calculate confidence intervals (i.e. variation) associated with each value of d', and is possible that small differences such as those described above are not in fact statistically significantly different. Ideally several replicate studies would be carried out for each pairwise comparison enabling several values of d' to be obtained for each pair. It should however be emphasised that carrying out replicates in this way is not normal practice for this type of test given the extremely labour intensive nature of the experimental procedure.

In the discrimination test investigating infusion water temperature, differences in orthonasal aroma were detected between samples of four of the seven pairs. No significant difference ($p<0.05$) existed between the samples of pairs 1, 2 and 7, corresponding to 40 vs. 50 °C, 40 vs. 60 °C and 60 vs. 80 °C respectively. It is interesting to note that whilst no differences were observed in the case of pairs 1 and 2, they were identified in the cases of pairs 5 and 6, even though absolute differences in infusion water temperature between samples were the same in both cases (10 or 20 °C). Temperature in Kelvin (K) constitutes a ratio scale (because an

absolute zero value exists) and as such was used to calculate the relative differences in temperature as a ratio shown in table 4-2.

In the case of pair 6 (90 vs. 100 °C), the ratio of water temperature was 1:1.03. This compares to a ratio of 1:1.06 for pairs 7 and 2 (60 vs. 80 °C and 40 vs. 60 °C) which both had absolute differences of 20 °C. Neither the absolute or relative differences in infusion water temperature however appear to correspond well to the discriminability of these samples since in theory it would be expected that the more discriminable samples would be those prepared using the largest relative difference in infusion water temperature. The relative difference in total solids content also does not correspond well to the discriminability of these samples. The infusion prepared using 100°C water contained 5 % more solids than that prepared using 90 °C water and the two samples could be discriminated. The infusions prepared using 40 and 50 °C, and 40 and 60 °C water contained 16 and 29 % more total solids than each other respectively yet could not be discriminated.

Unlike the case with infusion concentration, it has not been possible to draw any conclusions from APCI-MS data with regards to the explanation of the infusion water temperature discrimination test results. Compounds represented by the 15 monitored ions responded to infusion water temperature to varying extents, showing quite different release profiles (see for example figure 3-4 in section 3.3.1), and averaging data for the 15 monitored ions (as done for concentration) is therefore inappropriate. Aroma perceived by participating subjects originated from all released volatiles (above their odour threshold), the relative impact of each depending largely upon its odour activity value (OAV). A potent volatile present only at a trace level (e.g. β-damascenone) will be of greater sensorial significance than a volatile with negligible aroma present at much higher concentration. According to Schuh and Schieberle (2006) for example, the OAV of linalool in black tea infusion aroma is 140, compared to <1 for *E*-2-hexenal. To make any reasonable inferences from APCI-MS data, profiles for all volatile compounds released from the infusion would be required along with OAV's (indicating their relative impact).

For some compounds monitored by APCI-MS, the relative difference in release occurring between 40 and 50 °C was considerably smaller than between 90 and 100 °C, the increase in release of dimethyl sulfide for example was 3.3 %, compared to 52.4 % respectively. In the case of other compounds (e.g. the heptadienals) the difference in release was greater between 40 and 50 °C than between 90 and

100 °C (26.3 and 5.2 % increases respectively). It is possible that the presence of compounds exhibiting similar behaviour to that of dimethyl sulfide (i.e. greater relative differences in release at higher temperatures) could explain why subjects were able to discriminate between the 90 and 100 °C samples of pair 6 yet not the samples of pairs 7 and 2. It is however unlikely that dimethyl sulfide itself was the cause, a theory based upon results of the storage trial whereby subjects were unable to distinguish between fresh and infusions stored for vacuum flasks for 20 minutes (despite the latter being shown to release 250 % more dimethyl sulfide).

It is also important to note that in order to respond "different", a subject need only detect that a difference exists between samples. Whilst likely that perceivable differences resulted from differences in release of a number of compounds, it is possible that just one or two key compounds were responsible for differences in perception. Masuda and Kumazawa (2000) reported linalool and β-damascenone to be the two most odour-active constituents in the tea beverage, a finding supported by recent work of Schuh and Schieberle (2006) who investigated odour activity values of selected volatiles in Darjeeling black tea infusions. APCI-MS data (chapter 3) showed that release of these two compounds were significantly affected by both infusion concentration and water temperature and it is quite possible that a select few key compounds such as these were responsible for the differences perceived (or not perceived) by subjects.

Schuh and Schieberle (2006) recently claim to have identified *E,E,Z*-2,4,6-nonatrienal as a very important contributor of Darjeeling black tea aroma. Whilst Darjeeling tea was not used in the current study, it is quite possible that compounds such as this (i.e. not monitored using APCI-MS) played a key role in determining the discriminability of samples. For example, a hydrophobic compound very poorly extracted without the use of boiling water may have resulted in a characteristic aroma note only being present in the infusions prepared using 100 °C water. This could account for the fact that subjects easily discriminated between infusions prepared with 90 vs. 100 °C water, yet struggled when presented with 40 vs. 60 °C and 60 vs. 80 °C samples, even though the absolute and relative difference in temperature and concentration were greater in the latter two cases.

In comparing the results of the two discrimination test sessions (concentration and infusion water temperature), it is important to remember that all samples presented in the sessions investigating infusion water temperature were 60 °C at time of

evaluation. As shown previously in section 3.3.1, temperature of the infusion at time of analysis had a significant effect on release of volatiles, believed to be a direct effect of temperature on physicochemical parameters such as the Henry's Law constant. Release of *E*-2-hexenal for example from an infusion prepared using 100°C water, then incubated at 60°C for 30 minutes was only 40 % that of the immediately analysed equivalent (figure 3-2). The 1.0 %w/v infusions (prepared using 100 °C water) used in the concentration discrimination tests were identical to the 100 °C infusions (with an as prepared concentration of 1 %w/v) used in the water temperature tests. In terms of the samples presented to subjects, and considering the effect of the incubation procedure, release of *E*-2-hexenal from the 100 °C infusions in the infusion water experiment would therefore only have been around 40 % that from the 1 %w/v infusions in the infusion concentration experiment. With the exception of the 0.0 and 0.25 %w/v infusions, *E*-2-hexenal release was therefore greater from infusions used in the concentration discrimination tests, as were absolute differences in release between samples (compared to those in the temperature study). Whilst behaviour of the compounds represented by the other 14 ions did differ slightly, in all cases release levels were significantly lower in the infusions incubated at 60 °C prior to analysis.

This finding supports the theory that differences in relative (rather than absolute) release to a large extent determine whether or not subjects can discriminate. Subjects were able to discriminate between the 90 and 100 °C infusions, yet not the 1.75 and 2.0 %w/v infusions. Using *E*-2-hexenal as an example, on an absolute basis, difference in release of this compound between infusions prepared using 90 and 100 °C water was around half that of between the 1.75 and 2.0 %w/v infusions. Release from the 100 °C infusion was however around 23 % greater than that of the 90 °C infusion. This compares to around 9 % by which release from the 2.0 %w/v infusion was greater than that of the 1.75 %w/v infusion. As mentioned previously however, it is dangerous to make any more concrete conclusions with regards to volatile release due to the large differences in behaviour observed by the different volatiles as a function of infusion water temperature.

Since APCI-MS analysis showed that (of the compounds monitored by the 15 ions), all volatile compounds were affected by infusion concentration in roughly the same way (i.e. linear increase in release with increasing concentration), in theory, infusions prepared at different concentrations should have smelled identical in terms of aroma quality, differing only in the overall intensity (this theory assumes that all

compounds exceeded their threshold values, which in the lower concentration infusions may not have been the case). In contrast, APCI-MS analysis showed volatile compounds were affected by infusion water temperature to different extents, thus having a direct impact on the aroma profile and quality of the different infusions. The infusions prepared using higher infusion water temperatures not only smelled stronger, but also smelled different due to a shift in the relative release of different compounds. It may have been that subjects found it easier to discriminate between samples differing in the aroma profile, rather than just differing in aroma intensity.

Of those subjects who participated in both tests, several commented that they had found the trial investigating infusion water temperature easier; they claimed that this was due to less steam being released from infusions, making it easier to concentrate on the aroma. In order to carry out an unbiased comparison between the effects of infusion water temperature and concentration, it would be necessary to present samples of equivalent temperature (i.e. 60 °C). It would also be necessary to either randomise all samples, or use different subjects in each experiment in order to prevent the effect of learning from influencing results.

O'Mahony and Rousseau (2002) have shown that training has the effect of increasing subjects' sensitivity with regards to discriminating using the same-different test, and in an experiment investigating ice cream discrimination, it was observed that a consumers d' of 1 corresponded to an experts d' of 2. Whilst training subjects is likely to have increased values of d' and made samples more discriminable, the aim of the current study was to determine whether genuine tea consumers (i.e. not trained) could discriminate.

An observation worth mentioning is the high proportion of subjects who reported samples as "different" when in fact they were the same. On average (over the 14 pairs provided), 43 % of subjects responded "different" when presented with matched pairs (i.e. false alarms). Of those false alarms 46 % rated "sure" that the two samples were different (appendices 6 and 7). One possible explanation for the high proportion of false alarms is the expectation of participating subjects. It has been noted by Coren et al. (2004) in work related to signal detection, that if a signal is "expected" in every trial, the subject may respond to even the slightest stimulus. This point is reiterated by Stone and Sidel (2004) who say that "almost all subjects have an expectation about product differences, that is, they expect the products to be different". Instructions to subjects in the current study were explicit in that

samples were just as likely to be the same as they were to be different, and not to assume that they were different. Even so, it was clear that many subjects entered the booths with the preconception that the samples were going to be different as several subjects emerged disappointed that for many pairs they had been unable to detect a difference and so had resigned themselves to responding "same". In truth, many of these subjects had genuinely been presented with matched pairs, and so the "same" response would in fact have been classed as a correct response (correct rejection). It is recognised that presenting seven pairs in a single session may have contributed to the high proportion of false alarms. Whilst subjects seemed prepared to accept that some pairs were the same, some found it hard to believe that all seven pairs could possibly be the same. Some subjects stated that they started off by responding "same" to the pairs, but upon reaching the last few begun thinking that surely some should be different, and so responded accordingly. This tendency was accounted for by the careful balancing of experimental design but clearly illustrates how thought behaviour can influence the results of discrimination tests where subjects use predictions in a bid to get "correct" answers.

In addition, the possibility exists that the two matched samples were genuinely different in terms of their aroma release, and that these subtle differences were detected by subjects. It has been shown (section 2.3.1) that despite careful protocols, batch-to-batch variation can sometimes result in slight differences in volatile release. As discussed previously, a decision was made to always present subjects with samples from different batches of infusion, irrespective of whether they were matched or unmatched pairs. This was done in an attempt to prevent incorrect conclusions that samples could be discriminated due to the unmatched pairs being identified as "different" solely as a result of them coming from different batches. In effect, the batch-to-batch variation of infusions became inherent in the background noise.

An experiment carried out to investigate this theory further showed that the proportion of subjects saying "different" when presented with identical infusions presented from different batches was almost identical to the proportion saying "different" when presented with identical infusions from the same batch. This suggests that the high false alarm rate was unlikely due to variation between batches.

Assuming that aroma composition of infusions was identical at time of pouring, it is however still possible that differences were present in the aroma profile experienced at the time of sniffing. This is due to the unpredictable release of volatile compounds in the open-environment, and it is evident from looking at the patterns of steam released from the bowls of hot infusion that the patterns were highly random. This is also illustrated in figure 2-7, section 2.3.3 which show APCI-MS release profiles of *E*-2-hexenal and the methyl butanals obtained from directly above a mug of tea. It is also possible that the temporal profile of volatile release from infusions could account for differences in aroma at time of sniffing. In work investigating the temporal release of volatile compounds from coffee infusions, it was shown that different volatiles were released at different rates (Lindinger et al., 2005). Whilst it has not been possible to prove this is also the case with tea, based on the differences in physicochemical properties of those compounds monitored using APCI-MS (table 2-6), it is highly likely. Both infusions within each pair were poured at time=0, and after 2 minutes standing were presented to subjects. Since subjects were unable to smell both infusions simultaneously, depending upon the interstimulus delay they allowed could potentially have perceived differences in aroma due to differences in the temporal profile.

Presenting all seven pairs within a single session may also have influenced the responses of subjects with regards to the decision strategy used. In both experiments a range of differences existed, some very obvious (e.g. 0.25 vs. 2.0 %w/v), and some very subtle (e.g. 1.75 vs. 2.0 %w/v). Some subjects receiving a clearly different pair first may have made the conscious decision that this magnitude of difference represented a “different” sample. Whilst they may have been able to detect some of the smaller differences present in other pairs, based on the experience of the pair with the large difference may have felt that the difference in the other pair was too small in comparison to be called “different” (i.e. a contrast error). In other words, being presented with a pair exhibiting a large difference first would have set their τ-criterion too large. This does illustrate a limitation of the technique used in this study, and whilst the only way to eradicate this would have been to use different subjects for each discrimination test, the balanced experimental design does much to compensate for the contrast effect

4.6 FUTURE WORK

This chapter has shown that to a large extent, differences in volatile release caused by variations in preparation method can be detected by consumers via the orthonasal route.

Whilst the current study has focussed on the orthonasal aroma discriminability, this is only one of many factors known to be affected by infusion preparation method. It is well known that different infusion preparation procedures also alter the non-volatile content (Astill et al., 2001, Natarajan et al., 1962), and are likely to result in perceivable taste and mouthfeel differences being present. Investigating the discriminability of infusions based on these attributes would be a potential area of future work.

Although it has been shown that in many cases consumers can distinguish between infusions, it has not been possible to fully determine the reasons for this. APCI-MS analysis (chapter 3) has shown significant differences in volatile release from infusions prepared according to different preparation methods. Since the volatile compounds monitored using APCI-MS covered a range known to be of high sensorial significance, it is certainly likely that these played a role in the discriminability of samples. Results of discrimination tests alone however cannot conclude that it is these monitored compounds which were responsible for the perceived differences.

With further work it would be possible to provide some firmer answers to this question. Descriptive analysis techniques such as quantitative descriptive analysis (QDA) using a trained panel would be an appropriate means of quantifying the differences in aroma between samples. Observing that one sample had a more intense “floral” aroma note for example would suggest that it was floral compounds such as phenylacetaldehyde, linalool and geraniol (amongst others) which were responsible for the perceived differences in aroma. Observing that one sample had a more intense cabbage note would suggest that it was a sulphur compound such as dimethyl sulfide responsible for perceived differences.

This study has clearly shown that based on orthonasal aroma, infusions prepared according to different methods are discriminable from one another. This is an

important discovery, but covers only half of the story. It is essential to determine consumer preference for infusions prepared according to these different preparation methods. Whist this research has shown for example that consumers can distinguish between infusions prepared using 90 and 100 °C, it has not shown which is the most desirable. This information is necessary in order make full use of the results, and should be carried out using a program of consumer preference testing.

Black tea infusion preparation method has been shown to have a significant effect on volatile release, which has (to a large extent) been shown to be detectable by genuine tea consumers via the orthonasal route. The following chapter describes a series of experiments designed to explore the effect differing aroma composition of black tea infusions may have on the perception of taste and mouthfeel characteristics.

5 INVESTIGATING PERCEPTUAL INTERACTIONS BETWEEN VOLATILE AND NON-VOLATILE COMPONENTS OF BLACK TEA INFUSIONS

5.1 INTRODUCTION

The overall aim of this chapter was to investigate the presence of perceptual interactions occurring between volatile and non-volatile components of black tea infusions. For the purpose of this research, the taste attribute of bitterness, tactile sensation of astringency, and specific aroma notes important to overall black tea flavour were focussed upon.

The non-volatile composition of black tea, particularly the polyphenolic compounds (including catechins, theaflavins and thearubigins) plays a major role in the overall sensory characteristics experienced by consumers. Whilst much debate still exists, it is widely accepted that these compounds, along with others such as caffeine and amino acids contribute to the characteristic bitterness and astringency of tea infusions. In turn they reflect the quality attributes judged by professional tea testers, and amongst others affect infusion “briskness”, “freshness”, “aliveness”, “fullness” and “richness” (McDowell and Owuor, 1992).

The overall aroma profile of black tea infusions depends upon a fine balance of hundreds of different volatile compounds contributing to varying extents depending mainly upon their concentration and odour threshold. The infusion preparation method has been shown to have a significant effect on the release of volatiles (chapter 3), and in the case of infusion water temperature significantly affects the aroma profile. These subtle differences in aroma can to a large extent be detected by consumers via the orthonasal route (chapter 4).

It is well known that physicochemical interactions can occur between the many constituents of tea infusions, and these can have a direct effect on their sensory attributes. Physical interactions between caffeine and polyphenols for example are known to have a marked effect, not only on the relative bitterness and astringency of

tea infusions, but also on the type of astringency. Removal of bitter caffeine for example has been shown to actually result in an increase in bitterness, and shift in the type of astringency from tangy to non-tangy as a result of changes in complex formation (Sanderson et al., 1976)

Research on the multi-modal perception of flavour, and taste-aroma interactions in particular is widespread. Whilst considerable work has been carried out investigating taste-aroma interactions involving sweet and sour tastes (section 1.2.3), little in comparison is known about bitter-aroma interactions. Whilst studies have shown the impact of basic taste components such as sweetness on perception of astringency, there is little information on the impact of aroma on astringency perception. Relatively little work has been carried out investigating perceptual interactions involving the aroma of black tea infusions, particularly with regards to bitterness and astringency. Given the high economic importance of tea, and the fact that bitterness, astringency and aroma are three key attributes affecting tea quality, investigating perceptual interactions is of importance.

This chapter aims to address this gap in knowledge, describing a series of three experiments designed to investigate the presence of perceptual interactions which may occur during the consumption of black tea infusions. Whilst it is generally accepted that astringency is a tactile sensation (Breslin et al., 1993), for the sake of clarity, aroma-bitter, and aroma-astringent interactions discussed in this chapter will be all referred to under the general heading of taste-aroma interactions.

This work has utilised the solvent assisted flavour evaporation (SAFE) technique developed by Engel et al. (1999). Unlike many conventional separation techniques (e.g. freeze drying, solvent extraction), this technique is relatively mild in nature and has been used to separate freshly prepared black tea infusions into their constituent volatile and non-volatile fractions which have subsequently been used as raw materials for the basis of sensory evaluation. The volatile fraction contains all of the volatile compounds (responsible for the characteristic black tea aroma) and water present in the original infusion. The non-volatile fraction contains all of the non-volatile compounds (responsible for the characteristic taste and mouthfeel of black tea infusions), and can be reconstituted with water to create infusions containing the characteristic taste, but lacking the aroma of black tea.

The study of perceptual interactions in tea infusions was divided into the three key experiments. The first two utilised the non-volatile fraction of the complete tea infusion, using a trained panel. The third experiment made use of the volatile fraction, utilising naïve tea consumers.

In the first experiment different levels of volatile compounds were added to reconstituted non-volatile black tea infusions to determine whether the nature or level of the aroma note had any effect on perceived bitterness or astringency intensity. The second experiment followed a similar approach whereby different levels of hexanal and caffeine were added to reconstituted non-volatile infusions to determine whether the level of hexanal (green note) had any effect on the perception of bitterness intensity; or the reciprocal – whether the level of caffeine had any effect on the perception of green aroma note intensity. In the third experiment, caffeine was added to solutions of distilled water and volatile SAFE extract to determine whether tea volatiles had any effect upon bitterness perception intensity.

Based on the findings of previous workers, it was hypothesised that the some taste-aroma interactions would be observed in the first study, although the direction (enhancement or suppression) was unknown and expected to be concentration and aroma-note specific. An enhancement of bitterness caused by the presence of the green note was expected in the second study, based on the findings of previous workers (Caporale et al., 2004). In the third study, it was expected that the presence of a complete tea aroma would enhance the bitterness of caffeine solutions, based upon results of previous workers (Scharbert and Hofmann, 2005).

The objectives of this chapter were as follows:

- Investigate how the presence and concentration of specific aroma notes affects perception of bitterness and astringency of black tea infusions

- Investigate how the green aroma note affects bitterness perception, and how bitterness affects green aroma perception in black tea infusions

- Investigate how the presence of the complete aroma profile of black tea affects bitterness perception of caffeine solutions

5.2 SOLVENT ASSISTED FLAVOUR EVAPORATION (SAFE)

The following section relates specifically to the SAFE procedure used to create the volatile and non-volatile infusions utilised during forthcoming sensory evaluation sessions. It is separated from the main section of this chapter to aid reading.

5.2.1 Materials and methods

Tea infusions (500 mL, 2 %w/v) were prepared according to the standard procedure (section 2.2.1.1), transferred to Scott bottles, sealed and cooled rapidly under running water. Given the lengthy distillation procedure, it was desirable to use as high a concentration of infusion as possible, although the formation of tea cream (polyphenol-caffeine complexes), (section 1.3.4) upon cooling in infusions of concentrations greater than ~2 %w/v meant that this was the highest feasible concentration. Preliminary work showed problems with the resolubilisation of this cream thus altering the non-volatile composition in reconstituted infusions.

Figure 5-1 shows a diagram of the SAFE apparatus consisting of a dropping funnel, a cooling trap, and a central head bearing two “legs”, to which distillation flasks were attached. The outlet of the dropping funnel led to the bottom of the left “leg”, the vapour inlets to the head, with the inlet to the trap mounted on the sides of each “leg”. The head and two “legs” were thermostated with water (40 °C), ensuring a constant temperature during distillation, preventing volatile condensation.

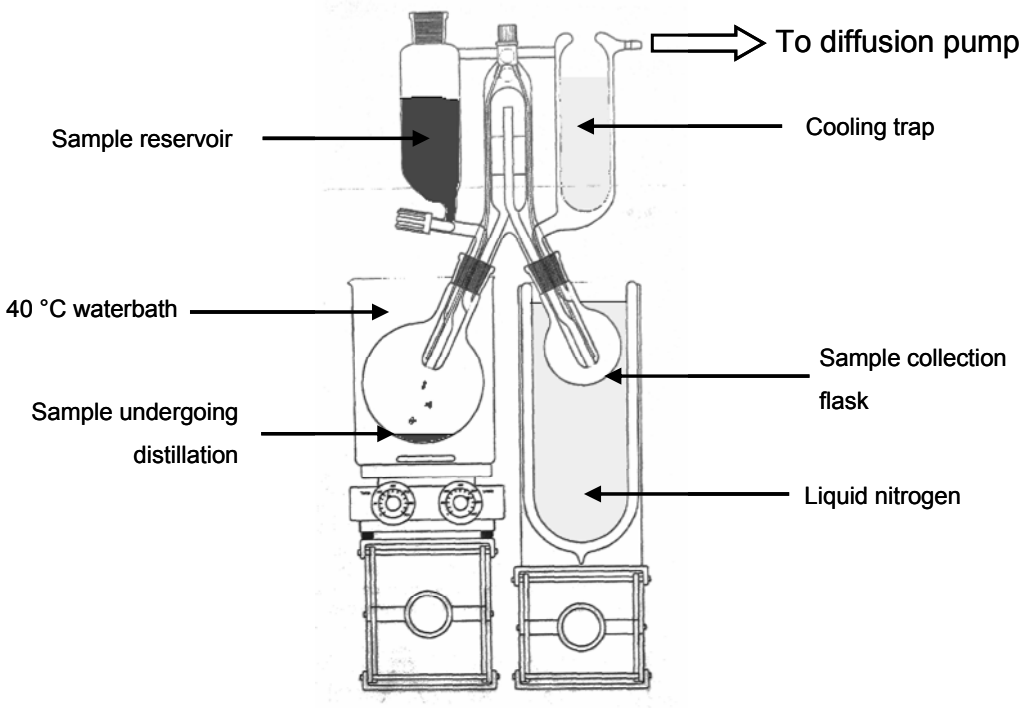


Figure 5-1 – Diagram of SAFE apparatus - adapted from Engel et al. (1999)

The distillation flask was immersed in a water bath (40 °C), and high vacuum (10^{-3} Pa) was applied to the apparatus by a diffusion pump (Leybold, Cologne, Germany). With the stopcock of the dropping funnel closed, liquid nitrogen was poured into the trap and bucket surrounding the sample collection flask.

The distillation procedure was started by allowing aliquots (~5 mL) of tea infusion to pass from the sample reservoir into the left vessel. Under the high vacuum, the vapour containing volatiles and solvent were transferred into the distillation head. Condensation of volatiles, and solvent (water) occurred in the sample collection flask maintained in liquid nitrogen. Liquid nitrogen levels were constantly topped up, and infusion was continually dropped to maintain an extraction volume of ~5 mL. This batch extraction procedure was repeated many times to build up a sufficient quantity of sample for sensory evaluation purposes.

Given variation in the efficiency of the SAFE process, it was necessary to combine the individual extracts of both the volatile and non-volatile fractions to create a single uniform batch of each. In order to determine how much water was required to reconstitute the non-volatile extract, total solids content was first determined.

The volatile composition of the two extracts was compared to that of the fresh infusion using SPME GC-MS. In order to make a fair comparison it was essential to compare like with like. A 2 %w/v LYL640 infusion was therefore prepared and a small amount stored in a sealed Schott bottle in a refrigerator. Following the SAFE procedure using the remaining infusion, the total solids content of the aqueous non-volatile extract was established and re-constituted with boiling water (Highland Spring) in order to create an infusion of identical total solids content to that of the 2 %w/v infusion (i.e. 0.733 %), as determined previously (section 3.3.5).

SPME GC-MS was carried out on the three samples (complete infusion, reconstituted non-volatile SAFE infusion, and volatile SAFE extract), along with a mineral water blank. GC parameters used were identical to those used previously, described in section 2.2.1.3.

5.2.2 Results

Figure 5-2 shows chromatograms obtained for the three samples of infusion. The y-axis has been normalised so that all three chromatograms utilise the same scale.

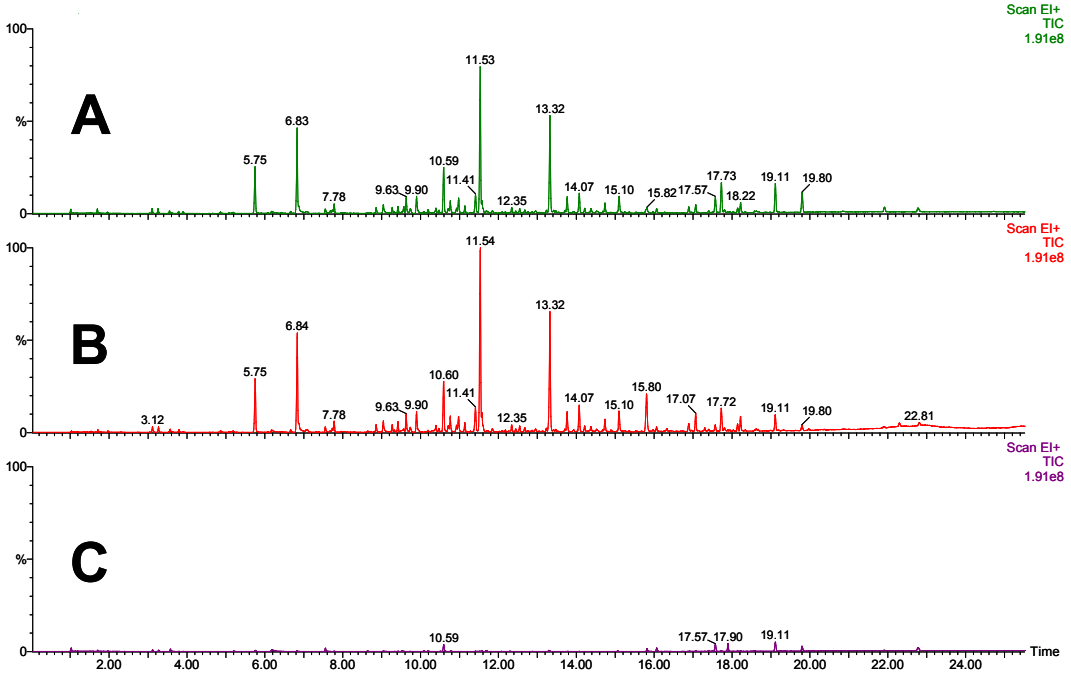


Figure 5-2 - Chromatograms of a complete LYL640 infusion (A), volatile SAFE extract (B), and reconstituted non-volatile SAFE infusion (C)

Figure 5-2 shows that qualitatively, the volatile portion of SAFE extract was almost identical to that of the complete infusion. All peaks present in the original infusion were present in the extract, and relative proportions similar between the two. Absolute peak areas did differ slightly between the two samples, although since no internal standard was included, it is not valid to draw any conclusions from this. Some small differences are present between the two chromatograms, most notably the presence of a peak at 15.80, in the volatile extract which is completely absent in the original infusion. This peak exhibits exactly the same spectra as that of β-ionone and has tentatively identified as an isomer (α- or γ-ionone). Since authentic standards were unavailable at time of analysis, this was not possible to confirm.

A large difference was evident between the complete infusion and the volatile extract compared to the reconstituted non-volatile infusion, specifically the complete absence of a large number of peaks. For example, peak areas of hexanal, *E*-2-hexenal and Linalool (5.75, 6.84 and 11.54) decreased in terms of absolute area by 97, 99 and 100 % respectively. The reconstituted non-volatile infusion did however contain several volatile compounds present in the complete infusion. A peak at 10.59 corresponding to phenylacetaldehyde was still present, although its absolute peak area was only 15 % that of the complete infusion. The same were true for 2 and 3-methyl butanal, and whilst present at lower levels (29 and 39 % respectively) were clearly still present in the reconstituted non-volatile infusion. Of the three peaks evident towards the end of the chromatogram, those at 17.57 and 19.11 were present in the water blank, whereas that at 19.11 remained unidentified.

5.2.3 Discussion

The high efficiency of the SAFE procedure observed supports findings of previous workers. Engel et al. (1999) investigated the yields obtained by distilling a solvent solution of seven volatile compounds in a model system using the SAFE procedure, results indicating 100 % recovery for five of the seven.

On the whole, the aroma composition of the volatile SAFE extract was very similar to that of a complete infusion as can be seen by the almost identical nature of

chromatograms (A) and (B) in Figure 5-2, and considered to be highly suitable for the proposed sensory analysis.

The large number of peaks completely absent in the reconstituted non-volatile infusion indicated complete extraction and transfer to the volatile extract. Some clearly identifiable peaks were still present, and it is likely that these remaining compounds were responsible for the residual aroma detected in the reconstituted non-volatile infusion. Whilst levels appear to be lower than in the complete infusion, the complete absence of a significant number of other volatile compounds is likely to have made the aroma of these remaining compounds more noticeable.

One possibility for the presence of these residual compounds is inefficient extraction, although given the high volatility of the methyl butanals for example this seems unlikely. A more likely reason for their presence is regeneration upon reconstitution using hot water since 2- and 3-methyl butanal and phenylacetaldehyde are formed from the amino acids isoleucine, leucine and phenylalanine respectively via Strecker degradation reactions (section 3.4). It would therefore never be possible to completely eliminate them from the reconstituted non-volatile infusion - as soon as the non-volatile extract is reconstituted with hot water (essential for the resolubilisation of non-volatile material), they would be reformed.

Whilst the reconstituted non-volatile infusions did have an aroma, it was weak and not tea-like. The infusion was therefore also considered suitable for the objectives of the current research. In future work, it may be possible to prevent the Strecker degradation reactions from taking place, perhaps by chemical means, and this should be explored further.

5.3 MATERIALS AND METHODS

Ethical approval for these experiments was granted by the University of Nottingham ethics committee.

5.3.1 Experiment 1 - effect of aroma note level on perception of bitterness and astringency

5.3.1.1 Panel screening

Subjects (23) from the University of Nottingham external panel (aged 40 – 67, four male) were screened for suitability and introduced to six potential aroma notes to be used in the study. Discriminative ability was determined by presenting subjects with solutions of water and tea containing individual volatile compounds at four different concentrations. Subjects were provided with a reference of the particular volatile compound in water then instructed to rank samples in order of increasing intensity. Ranking was also carried out on bitter and astringent solutions in order to assess discriminative ability for these attributes. Subjects who found the samples excessively unpleasant were eliminated from the pool.

Based on the results of this initial screening, 12 subjects were selected for extensive training prior to taking part in the first two experiments (two male, aged 40-67).

5.3.1.2 Training samples

Six individual aroma notes were developed which could be added to reconstituted non-volatile SAFE infusions in varying amounts to manipulate the aroma profile, enhancing the intensity of certain notes. The compounds used and target notes are shown below in Table 5-1. Wherever possible, compounds were chosen based upon their natural occurrence in black tea. Stock solutions of each volatile compound were prepared in ethanol at such concentration that very small amounts could be added (up to 400 µL/L) to the infusions.

Table 5-1 - Target aroma notes and volatile compounds added to infusions

Target aroma note	Volatile compound(s) added
Cabbage	dimethyl sulfide
Floral	linalool / geraniol (50:50)
Fruity	hexyl acetate
Green	hexanal
Medicinal	methyl salicylate
Woody	damascenone

Green, medicinal, floral and woody are terms commonly used within the tea industry to describe the characteristic flavour of black tea infusions. Whilst not usually noticeable in fresh tea, cabbage is a term used to describe stewed or aged infusions. Fruity was chosen in order to represent the emerging market for flavoured black teas.

Due to a very limited supply of aqueous non-volatile SAFE extract, panel training was carried out using PG granule infusions (total solids content 0.34 %). PG granules are readily available, and manufactured using a freeze drying process. This process results in a significant loss in volatile compounds, and the resulting infusions have only a slight aroma. GC-MS analysis confirmed that PG granule infusions contained a very similar aroma composition to that of the reconstituted non-volatile SAFE infusions, confirming their suitability as training material.

5.3.1.3 Training procedure

Panel training was over a period of four months conducted in 16 three-hour sessions

Stage 1 – generation and agreement of descriptors

During the first session, subjects were introduced to the research project and provided with a brief outline of the quantitative descriptive analysis approach (Stone, 1992). Each subject was presented with a tray containing seven pots of PG granule infusion, six of which possessed a different aroma note at an easily detectable level.

In individual sensory booths, subjects were instructed to record all descriptors they felt described the flavour and mouthfeel attributes of each of the samples.

A total of 105 different descriptors were generated from this exercise, although through discussion it was clear that different subjects had used different words to describe the same thing or the same word to describe different things.

In subsequent sessions, this pool of descriptors was rationalised to provide a list of attributes to describe the flavour of the samples, including the bitter taste and astringent sensation. Whilst it would have been possible to have simply presented subjects with solutions of the volatile compounds to be used in the study, it is better practice to involve a panel in discussion of this sort. This ensures that all subjects are happy with the descriptors used and understand them clearly. Table 5-2 shows the target aroma notes and the agreed descriptors. In order to avoid confusion, future reference to aroma notes in this chapter will be made using the target aroma note terminology (i.e. first column).

Table 5-2 - The six key aroma notes and agreed descriptors

Target aroma note	Agreed descriptor (flavour)
Cabbage	Cabbage / garlic
Floral	Floral
Fruity	Pear drop
Green	Nutty / grassy
Medicinal	Antiseptic
Woody	Pruney / tobacco

As well as the six aroma descriptors listed in table 5-2, subjects chose the terms “bitterness” and “astringency” to describe the bitter taste and astringent sensation associated with the tea infusions.

Since many bitter substances are also astringent and vice versa, these two attributes are commonly confused. This was evident when the panel were initially asked as a group to describe bitterness and astringency. Several subjects stated

that “bitter is very drying in the mouth”, or that “astringency is very bitter”. It was therefore essential to provide adequate training to ensure that subjects were absolutely clear of the difference between these two attributes and could rate them independently. Subjects eventually all agreed that bitterness was the taste associated with caffeine and quinine solutions, detected at the sides and back of the mouth, whereas astringency was the drying sensation associated with alum solution and green banana skin, most easily detectable at the sides and roof of the mouth.

A standard tasting protocol was developed to ensure that all subjects consumed samples and rated attributes in the same way. It was unanimously agreed that consuming samples directly from medicine pots was the best way to ensure that a consistent quantity of each sample was taken. 10 mL per pot was chosen as a suitable volume; large enough to get a reasonable feel for the attributes, yet not so large as to be fatiguing after a session of samples. The developing sensation of astringency with time is a well documented phenomenon (section 1.2.2.4), and for this reason it was necessary for all subjects to rate the astringent sensation at the same time after consumption. Samples were placed in the mouth and the sides and mouth were coated for 2 to 3 seconds. The samples were swallowed, and the aroma note and bitterness levels rated. Astringency intensity was rated 10 seconds after swallowing (determined using a timer).

Stage 2 – recognising varying intensity of defined attributes

A considerable portion of training time was devoted to training subjects to recognise and score the eight attributes at varying intensities. For the six aroma notes, this was initially carried out using aqueous solutions in water around a training table. Subjects were presented with three solutions containing a particular aroma note at different levels and asked to rank them in order of increasing intensity. Results were discussed as a group and retasting carried out wherever subjects gave inconsistent answers.

Subjects were then presented with four samples of PG granule infusion, each containing a different level of the aroma note, instructed to drink each of the samples and rank them in order of increasing intensity. Again, results were discussed as a group, and retasting carried out whenever incorrect rankings were given.

Training of bitterness and astringency was initially carried out using aqueous quinine hydrochloride and alum solutions respectively. Subjects ranked solutions containing various levels of the added compounds around the table as a group, and individually in the booths with subsequent discussion of results.

Stage 3 – rating attributes on a line scale

During training, it was necessary to introduce, and familiarise subjects with the two extremes of attribute intensity to be encountered during the forthcoming experiment through use of reference samples.

Providing reference samples for the aroma notes was easy, since the highest and lowest concentrations of the volatile compounds to be used in the experiment had previously been determined. Providing suitable reference samples to anchor the scale for the bitterness and astringency attributes was more difficult. These reference samples needed to encompass the range of intensity subjects were likely to perceive as a result of the differing aroma note levels (assuming a range of perceived intensities did indeed exist). It was not possible to use experimental samples to illustrate the scale extremes, since in theory levels of bitterness and astringency should have been identical for all samples. Reference samples were therefore created using aqueous solutions of caffeine (bitter) and alum (astringent). The range of the two reference samples (lowest and highest) was chosen to enable all samples to be scored within the scale, yet to ensure that the whole scale was used. Determining suitable reference sample concentrations was therefore a trial and error process, requiring input from the panel. Low and high reference samples for caffeine were chosen as 0.8 g/L and 1.5 g/L, and for alum; 0.8 g/L and 1.2 g/L.

Subjects were presented with the reference samples and asked to provide word anchors for each end of the scale. It was important to ensure that all subjects agreed on and understood these word anchors. Figure 5-3 shows an example of the type of line scale used, along with the word anchors chosen by the panel. Whilst this example only shows nutty / grassy flavour (green note), the anchors “none” and “high” were chosen for all six aroma notes. Scores provided by subjects were converted to numerical values (0-10) from left to right along the scale axis.

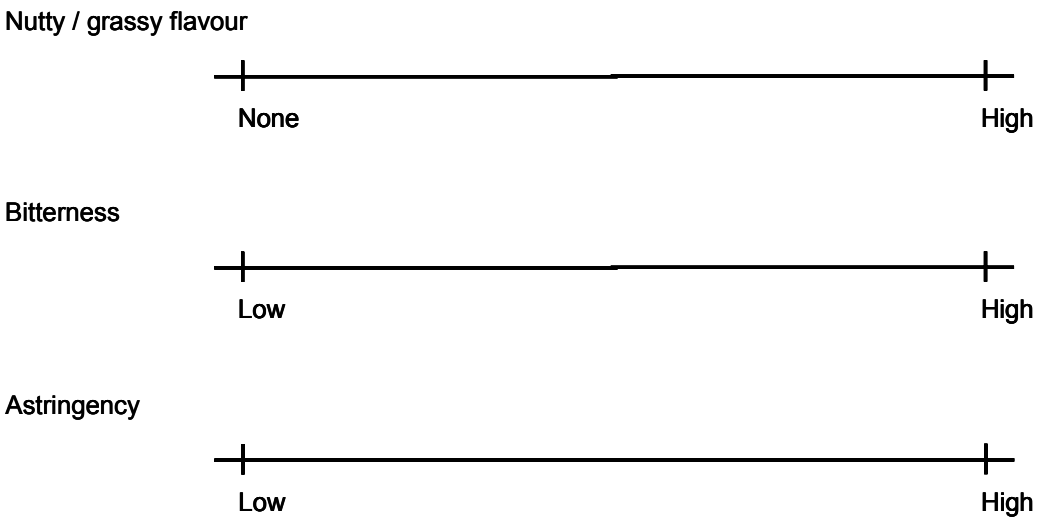


Figure 5-3 - Scales and word anchors for the chosen attributes

Rank-rating is a training procedure commonly used to familiarise subjects with different intensities of attributes and rating them on a line scale. Subjects were presented with five samples of PG granule infusions containing differing levels of a specific aroma note. They were instructed to rank samples in order of increasing intensity, and then rate these intensities on line scales using the scale anchors previously agreed upon. Since the results of these sessions were discussed as a group, it was necessary to vary the levels of added aroma notes to prevent subjects from detecting any patterns. The rank-rating procedure was also used to familiarise subjects with rating the intensity of bitterness and astringency, using aqueous solutions of caffeine and alum respectively.

5.3.1.4 Test subjects

Ten of the twelve trained subjects took part in the first experiment (two male, aged 43-67), two withdrawing due to illness.

5.3.1.5 Test samples

Whilst training had been carried out using all six aroma notes, due to limited availability of the non-volatile SAFE extract, only four were selected for use in

experiment 1. These were cabbage, fruity, green and medicinal, chosen partly based upon panel performance in terms of rating ability and partly due to relative importance and interest in terms of tea aroma research.

Preliminary experiments confirmed that a reconstituted non-volatile SAFE infusion with a total solids content of 0.52 % resembled the bitterness and astringency of the PG granule infusions used during training. Five 500 mL batches of reconstituted non-volatile SAFE infusion were prepared by adding boiling mineral water (468 mL, Highland Spring) to non-volatile SAFE extract (31.67 g) contained in Schott bottles. The five batches were then combined, so ensuring that the non-volatile composition of all samples within the experiment was consistent. It also acted to minimise any differences in composition between replicate samples prepared on different days. Reconstituted non-volatile infusions were rapidly cooled under cold running water, and 120 mL aliquots were divided between 16 Schott bottles (100 mL).

Four levels of each aroma note were chosen so as to represent very subtle differences in flavour intensity (i.e. none, low, medium and high) (table 5-3), and aroma stock solution added to the non-volatile SAFE infusions in varying amounts (0, 20, 40, and 80 µl). The low level could only just (with practice) be distinguished from the base infusion (i.e. none), and the high level was noticeable, yet not far outside the realms of true black tea flavour. Whilst effort was taken to ensure that intensities of each note were closely matched (i.e. equi-intense), it was not possible to confirm that this was definitely the case. Preliminary experiments using noseclips showed that the volatile compounds used produced no taste sensations at the concentrations used.

Table 5-3 - Concentration of volatiles added to samples of reconstituted non-volatile SAFE infusions

	None	Low	Medium	High
Cabbage	0 ppm	5 ppm	10 ppm	20 ppm
Fruity	0 ppm	20 ppm	40 ppm	80 ppm
Green	0 ppm	6 ppm	12 ppm	24 ppm
Medicinal	0 ppm	0.7 ppm	1.4 ppm	2.4 ppm

Samples (10 mL) were poured into medicine pots, coded using random three-digit numbers and sealed immediately.

5.3.1.6 Test procedure

Prior to commencement of experiment 1, subjects completed four replicate practice sessions. The procedure used was exactly the same as that described below, with the exception that PG granule infusions were used in place of the valuable reconstituted non-volatile SAFE infusions.

Subjects were presented with the low and high reference samples for bitterness and astringency in the training room and reminded that these represented the far left and right extremes of the line scale. Sessions were carried out in individual sensory booths under northern daylight conditions. For each aroma note a reference sample of reconstituted non-volatile SAFE infusion containing the highest aroma level was first presented to anchor the scale. Samples corresponding to the four aroma notes were studied on separate visits to the booth, and a Latin square design used to ensure that order of presentation, both of the aroma notes, and levels within each aroma note were balanced across subjects.

All instructions were provided on a computer screen, and forced breaks used to ensure that subjects allowed sufficient time between samples (2 minutes) for palate cleansing using Honeydew melon, milk chocolate and crackers provided. Whilst these are not standard palate cleaners, work at Unilever research has shown them to be very effective in removing the lingering bitterness and astringency associated with black tea infusions. Samples were presented one at a time and subjects instructed to consume the whole sample and rate the intensity of the three attributes (bitterness, astringency and aroma note). A 20 minute break was applied between each set of aroma note samples. This was essential in order to minimise fatigue, especially given the lingering effect and accumulation of bitterness and astringency. Between the second and third aroma note, subjects re-visited the training room where they were again presented with the low and high reference samples for bitterness and astringency.

In order to confirm that subjects were able to rate intensity bitterness and astringency within the tight region provided by the reference samples, a separate

session was carried out where subjects were presented with five aqueous samples of caffeine and alum at a range of concentrations between those used in the low and high bitter and astringency reference samples (three replicates of each). Subjects were instructed to rate these samples for bitterness and astringency intensity respectively.

5.3.2 Experiment 2 – effect of hexanal and caffeine level on perception of green aroma and bitterness

This experiment explored the possibility of reciprocal interactions between bitterness and aroma perception. The initial intention had been to manipulate the concentration of the reconstituted non-volatile SAFE infusion in order to provide different levels of bitterness and astringency. However, due to the residual aroma this was not possible, as with increasing concentration of the infusion there was an obvious increase in aroma which would have biased results. It was decided instead to use a weak reconstituted non-volatile infusion, adding different levels of bitter and astringent compounds to manipulate intensity of the two attributes.

Manipulation of astringency however proved problematic, mainly due to the necessity of adding something which could be safely consumed by subjects. Whilst alum was used as a reference sample to provide astringency anchors, it was expectorated in this case. Additionally, alum is an aluminium salt, and whilst is suitable as a general astringency reference sample (due to an absence of any other taste or aroma) does not exhibit the same kind of astringency found in black tea infusions. Addition of food-grade compounds thought to be responsible for the astringency of black tea (e.g. epigallocatechin gallate) was also found unsatisfactory due to their intense associated bitter and aroma attributes.

It was therefore decided that this second experiment would investigate only bitter-aroma interactions. The investigation of astringent interactions is an area of future research which can be carried out when suitable means of enhancing this attribute become available. Due to limited non-volatile SAFE extract availability only the green aroma note was studied in this second experiment. Green was chosen, partly based on its known importance as a key aroma note in black tea infusions, and partly due to the presence of literature evidence suggesting that it may enhance

bitterness perception in certain food systems such as olive oils (Caporale et al., 2004).

5.3.2.1 Training procedure

With the exception of providing different reference samples, it was not necessary to provide a separate period of training for this second experiment. Details of training are therefore as described previously in section 5.3.1.3.

Reference samples anchoring the ends of the scale for green and bitter attributes were created using aqueous solutions of hexanal and caffeine respectively. It was necessary to create solutions of hexanal in water (as opposed to tea infusion) since it was unknown whether the addition of caffeine would enhance or suppress bitterness. Concentrations of the references were prepared slightly above the maximum concentration added to the tea samples in order to provide a small amount of leeway, ensuring that subjects' attribute scores were not off the end of the scale. Low and high reference samples for caffeine were chosen as 0.3 g/L and 1.5 g/L; hexanal as 0 ppm and 30 ppm.

5.3.2.2 Test subjects

Eleven of the twelve trained subjects took part in the second experiment (two male, aged 43-67).

5.3.2.3 Test samples

The experiment conformed to a full-factorial design using four levels of caffeine (0 g/L, 0.3 g/L. 0.6 g/L, 1.2 g/L) and four levels of hexanal (0 ppm, 6 ppm, 12 ppm, 24 ppm), resulting in a total of 16 samples of varying bitter and green note flavour levels.

Evidence exists to show that caffeine can affect release of volatile compounds through formation of caffeine-flavour complexes resulting in increased solubilisation and decrease in headspace concentrations (King and Solms, 1982). APCI-MS

analysis of the samples was therefore carried out to confirm that at the concentrations used, caffeine had no effect on hexanal release.

Reconstituted non-volatile SAFE infusions were prepared in a similar way to previously described (section 5.3.1.5). A weaker infusion with a total solids content of 0.1 % was used so that upon addition of caffeine, differences in bitterness intensity could clearly be detected, yet still be within the realms of reality. As in experiment 1, five individual infusions were prepared and combined. Caffeine and stock hexanal solution were added to each of the samples to produce the above concentrations. It should be noted that whilst four samples contained caffeine concentrations of 0 g/L, background bitterness from the reconstituted non-volatile SAFE infusion was still present. After thorough mixing, 10 mL aliquots were poured into coded medicine pots and sealed immediately.

5.3.2.4 Test procedure

Subjects were presented with one sample at a time and asked to rate the intensity of the green flavour and bitterness by marking line scales. A forced 2 minute break was applied between samples to allow sufficient time for palate cleansing. A 20 minute break was applied after every four samples in order to minimise any effects of sensory fatigue and/or bitterness carryover. Subjects were familiarised with all reference samples in between the second and third visits to the booth.

5.3.3 Data analysis for experiments 1 and 2

Data were analysed using two- and three-way ANOVA with interactions (XLSTAT, v. 7.5.2, Addinsoft, New York, NY) to determine whether interactions between the aroma notes and bitterness (and astringency) were present. Where subject-sample interactions were not present, analyses were rerun omitting the interaction term as is common practice in sensory data analysis (O'Mahony, 1986). Tukey's HSD tests were carried out as appropriate in order to identify those samples and/or subjects where significant differences occurred.

In experiment 1, the aroma note flavour, bitterness, or astringency score were classed as dependent variables. Level of aroma note addition and subject were

both classed as fixed factors. In experiment 2, green flavour and bitterness were classed as dependent variables. Level of hexanal addition, caffeine addition, and subject were all classed as fixed factors.

5.3.4 Experiment 3 - investigating interactions between black tea aroma and bitterness using tea consumers

5.3.4.1 Subjects

64 naïve subjects (tea consumers), recruited on the basis of availability were invited to take part in a series of directional paired comparison tests where they would be presented with two samples and asked to indicate the most bitter of the two.

5.3.4.2 Samples

Solutions of caffeine were prepared by dissolving a mass of caffeine in a volume of either distilled water (Highland Spring) or volatile SAFE extract. Distilled Highland Spring Mineral was used in this experiment to match the water present within the volatile SAFE extract which had undergone a distillation procedure. Caffeine, water and volatile SAFE extract were all measured using a balance (accurate to three decimal places), and four separate batches of each sample were prepared and randomly assigned to subjects. This was to prevent any perceived differences in bitterness being detected due to genuine differences in caffeine concentration between samples. Levels of caffeine were selected to represent bitterness at low (0.3 g/L), medium (0.6 g/L), and high (1.2 g/L) levels within the range associated with tea infusions of varying strengths. A control pair was also included, where no caffeine was added to either the water or volatile SAFE extract.

A preliminary experiment using 30 tea consumers was carried out under controlled conditions, confirming that consumers were unable to perceive any difference in bitterness between the water and volatile SAFE extract (containing no added caffeine). Subjects wore noseclips during the test to ensure that differences in the aroma had no effect on bitterness perception.

5.3.4.3 Sensory procedure

Subjects were presented with two samples simultaneously (20 mL of each) in medicine cups, coded using three digit numbers. Each pair consisted of a sample of water and a sample of volatile SAFE extract, both containing the same concentration of caffeine. Subjects were instructed to drink each sample in turn and indicate which they thought was the most bitter of the two using a forced-choice approach. Subjects were instructed to cleanse their palate thoroughly between samples using melon, cracker and water. Sessions took place in individual sensory booths under northern daylight conditions. All four pairs (consisting of the four levels of caffeine) were presented within a single session with a forced 5 minute break between each pair. Presentation order was balanced to ensure that order within each pair was balanced, as was the order of the four pairs.

Prior to entering the booths, subjects were provided with clear instructions and a bitter reference sample to ensure that they were clear on the attribute they were instructed to look at. It was made very clear that the samples differed in other attributes (i.e. aroma), yet it was specifically "bitterness" they were being asked to focus on. This was to prevent (as far as possible) subjects from confusing attributes, and basing their decision on other attributes.

5.3.4.4 Data analysis

Results were analysed using a two-sided test approach (i.e. neither answer was necessarily "correct"). The number of agreeing responses for each sample within a pair were calculated and the sample with the highest frequency was compared to published critical values in a table for 64 subjects and $\alpha=0.05$ using published tables (BSI, 2006).

5.4 RESULTS

5.4.1 Experiment 1 - effect of aroma note level on perception of bitterness and astringency

Table 5-4 provides an overall summary of the results obtained in experiment 1. Mean scores (across all reps and subjects) for each of the samples are shown, along with values of standard deviation in brackets. For clarity, the four levels of each aroma note are identified as none, low, medium and high. A mean score for the four samples within each aroma note is also shown.

Separate analyses were carried out for each of the four aroma notes under investigation. It was not appropriate to make comparisons of bitterness and astringency scores between the four aroma notes, since it had not been possible to ensure that levels within each note were equivalent (i.e. a low level of cabbage note was not necessarily equivalent in intensity to a low level of fruity note). It was however possible to make a comparison between the four samples with no added aroma as these samples were all identical in terms of composition (this was also carried out using two-way ANOVA).

Table 5-5 summarises the results of the ANOVA's carried out on the data obtained from the four aroma notes.

Table 5-4 - Summary of mean attribute scores (0-10) and associated standard deviations

		Aroma note	Bitterness	Astringency
Cabbage	**None**	0.25 (0.80)	8.24 (2.53)	7.08 (3.39)
	Low	3.95 (3.05)	8.74 (1.88)	6.69 (3.59)
	Medium	6.13 (2.83)	8.06 (2.42)	6.85 (3.13)
	High	6.98 (3.04)	7.23 (2.93)	6.29 (3.57)
	Total	*4.33 (3.67)*	*8.07 (2.50)*	*6.73 (3.39)*
Fruity	**None**	0.15 (0.34)	7.65 (3.11)	6.50 (3.47)
	Low	5.09 (2.80)	8.06 (2.16)	7.27 (3.20)
	Medium	7.61 (2.30)	8.25 (2.52)	6.83 (2.81)
	High	8.79 (1.67)	7.50 (2.47)	6.68 (3.41)
	Total	5.41 (3.88)	7.86 (2.57)	6.82 (3.20)
Green	**None**	1.01 (1.99)	7.93 (2.56)	6.15 (3.36)
	Low	5.93 (2.60)	7.53 (3.00)	6.43 (3.61)
	Medium	7.25 (2.28)	7.74 (2.48)	6.94 (3.04)
	High	9.24 (1.25)	8.30 (2.41)	7.44 (2.88)
	Total	5.86 (3.68)	7.88 (2.61)	6.74 (3.23)
Medicinal	**None**	0.14 (2.41)	6.67 (3.29)	5.82 (3.65)
	Low	3.19 (2.51)	7.37 (2.79)	5.96 (3.25)
	Medium	5.26 (2.93)	7.19 (3.25)	6.94 (3.61)
	High	8.17 (2.26)	7.17 (2.72)	6.50 (3.49)
	Total	4.19 (3.68)	7.10 (3.00)	6.31 (3.49)

Table 5-5 - Significance level (p) associated with subject and aroma note level for each of the tea samples calculated during 2-way ANOVA

	Attribute	Aroma note level	Subject
Cabbage	**Cabbage / garlic flavour**	**<0.001***	**0.023***
	Bitterness	0.099	**0.021***
	Astringency	0.653	**<0.001***
Fruity	**Pear drop flavour †**	**<0.001***	**<0.001***
	Bitterness	0.548	**<0.001***
	Astringency	0.458	**<0.001***
Green	**Nutty / grassy flavour**	**<0.001***	**0.011***
	Bitterness	0.623	**<0.001***
	Astringency	0.152	**<0.001***
Medicinal	**Antiseptic flavour**	**<0.001***	0.548
	Bitterness	0.780	**<0.001***
	Astringency	0.325	**<0.001***

***** = significant effect at p=0.05

† = ANOVA included significant subject-sample interaction term

Unsurprisingly, the 2-way ANOVA showed that for all four aroma notes, aroma note level had a significant effect on the perceived intensity of the aroma note flavour, post-hoc testing revealed that for fruity, green and medicinal, each aroma note level was significantly different to all others (p=0.05). In the case of cabbage, the medium and high levels were not significantly different to one another, but all other levels were.

Of most relevance to the current study is the result that for all four aroma notes, the aroma note level had no significant effect on either the bitterness or astringency score.

Significant differences were present between the subjects for all three attributes for three of the four aroma notes. In terms of the aroma note attribute, post-hoc testing revealed that this was generally due to inconsistencies in scoring between two or three subjects. In terms of bitterness and astringency however, it is clear that subjects made use of different portions of the scale when rating these two attributes (discussed later). In the case of the fruity aroma note there was a significant subject-sample interaction term. Subject-sample interactions are undesirable since they imply that different subjects are ranking samples in different orders. These interactions may be due to a lack of attribute understanding, poor homogeneity of samples, or human error (either in sample presentation or recording of results).

The four samples with no added aroma were all identical in terms of composition, and as expected, scores of bitterness and astringency were not significantly different from one other. There was however a significant difference in the aroma note scores given, with a significantly higher score given to the green flavour associated with the green aroma note sample compared to the corresponding scores for the other three notes.

5.4.2 Experiment 2 – effect of hexanal and caffeine level on perception of green aroma and bitterness

Tables 5-6 and 5-7 provide a summary of the results obtained in experiment 2 for the attributes green flavour and bitterness respectively. Scores shown are the mean across all reps and subjects for each of the hexanal / caffeine level combinations, along with values of standard deviation in brackets.

Table 5-6 - mean green flavour scores and standard deviation for each of the samples

		Added hexanal (ppm)				
Added caffeine (g/L)		**0**	**6**	**12**	**24**	***Total***
	0	0.25 (0.72)	4.74 (3.01)	6.99 (2.90)	8.78 (1.69)	*5.19 (3.92)*
	0.25	1.23 (2.90)	5.08 (3.08)	7.01 (2.84)	8.61 (1.97)	*5.48 (3.86)*
	0.5	0.50 (1.27)	5.15 (2.78)	6.91 (2.55)	8.73 (1.95)	*5.32 (3.77)*
	1.0	0.56 (1.60)	4.50 (3.25)	6.92 (2.81)	8.76 (1.76)	*5.19 (3.92)*
	Total	0.64 (1.82)	4.87 (3.01)	6.96 (2.75)	8.72 (1.83)	

Table 5-7 - mean bitterness scores and values of standard deviation for each of the samples

		Added hexanal (ppm)				
Added caffeine (g/L)		**0**	**6**	**12**	**24**	***Total***
	0	1.67 (2.46)	1.59 (2.22)	2.12 (2.83)	2.46 (3.23)	*1.96 (2.70)*
	0.25	2.29 (2.70)	3.87 (3.21)	3.72 (3.05)	3.66 (2.72)	*3.39 (2.96)*
	0.5	6.74 (3.03)	6.03 (2.67)	6.09 (2.58)	7.19 (2.70)	*6.51 (2.76)*
	1.0	9.29 (1.41)	9.38 (1.10)	9.12 (1.68)	9.36 (1.31)	*9.29 (1.38)*
	Total	*5.00 (4.00)*	*5.22 (3.75)*	*5.26 (3.68)*	*5.67 (3.77)*	

As hexanal level increased, mean green flavour score increase (at all levels of caffeine) (across columns in table 5-6). As caffeine level increased, mean bitterness score increased (at all levels of caffeine) (down rows in table 5-7). The question in the current study was whether or not level of caffeine addition resulted in any difference in perception of green flavour intensity, or whether level of hexanal addition resulted in any significant difference in perception of bitterness intensity.

3-way ANOVA was carried out in order to answer this question, the results of which are summarised in Table 5-8.

Table 5-8 - Significance level (p) associated with hexanal level, caffeine level, subject and interactions calculated during 3-way ANOVA

	Green flavour	Bitterness
Hexanal level (A)	**<0.001 ***	0.125
Caffeine level (B)	0.669	**<0.001 ***
Subject (C)	**< 0.001 ***	**<0.001 ***
A * C interaction	**0.003 ***	0.462
B * C interaction	0.065	0.057
A * B interaction	0.904	0.135
A * B * C interaction	**0.05 ***	0.372

***** = significant effect at p=0.05

It can be seen from Table 5-8 that hexanal level had a significant effect on the green flavour score, and caffeine level had a significant effect on the bitterness score (p=<0.001 in both cases). Tukey's HSD post-hoc tests revealed that each level of hexanal and caffeine was significantly different to all others in terms of green flavour and bitterness score respectively.

Caffeine level did not have a significant effect on green flavour score (p=0.669). Likewise, hexanal level did not have a significant effect on bitterness score (p=0.125).

In the case of both attributes, a significant subject effect was present (p=<0.001), indicating differing use of the scale, and in the case of green flavour, there was a significant hexanal level–subject interaction. For neither attribute was there a hexanal level–caffeine level interaction suggesting that the effect of hexanal level did not depend upon the caffeine level and vice versa. In the case of green flavour, a three-way interaction term was significant. This is largely due to the presence of the hexanal level-subject interaction and suggests that caffeine level within this context would also result in a different ranking order of samples between subjects.

5.4.3 Experiment 3 - investigating interactions between black tea aroma and bitterness using tea consumers

For 64 subjects the minimum number of correct responses required for significance at the α=0.05 level was 41 (BSI, 2006). In cases where frequency of response for one sample exceeded this critical value, it was concluded that the subjects perceived that particular sample as being more bitter.

Table 5-9 shows the number of respondents identifying the water and volatile SAFE extract as being the most bitter sample at the four levels of caffeine addition.

Table 5-9 - Number of subjects identifying water and volatile SAFE extract as being most bitter at each of the four caffeine concentrations

	Water	**SAFE**
Caffeine level		
0 g/L	13	**51 ***
0.3 g/L	29	35
0.6 g/L	33	31
1.2 g/L	**47 ***	17

***** - value exceeding the critical number of responses (41) for α=0.05

At caffeine levels of 0.3 and 0.6 g/L the number of responses did not exceed the critical value of 41 for either of the samples (water or volatile SAFE extract). At these two caffeine levels it was concluded that subjects were unable to perceive any difference in bitterness between the two samples.

At 0 g/L caffeine, 51 subjects indicated that they found the volatile SAFE extract to be the more bitter sample. Note - in the preliminary experiment where subjects wore noseclips, neither sample (containing no caffeine) was perceived as being significantly more bitter than the other. At 1.2 g/L caffeine, 47 subjects indicated that the water was more bitter than the volatile SAFE extract.

5.5 DISCUSSION

The current study has shown only limited evidence for the presence of perceptual interactions between the volatile and non-volatile components of black tea infusions, specifically the three attributes of aroma, bitterness and astringency. The first experiment showed that the level of specific aroma notes had no effect upon the perception of bitterness or astringency intensity (i.e. no evidence of aroma-induced taste or mouthfeel enhancement or suppression). The second experiment showed that the level of green aroma note had no effect upon the perception of bitterness intensity, or the reciprocal; with level of caffeine having no effect on the perception of green aroma note intensity. The third experiment showed that at low and medium levels of bitterness, presence of black tea volatiles had no impact upon perception of bitterness intensity. These results do not agree with initial hypotheses, since some perceptual interactions had been expected.

The acquisition of taste properties by aromas has been widely reported (section 1.2.3), and it is well known that sweet smelling aromas can enhance the perceived sweetness of sucrose, as frequently shown in the case of strawberry and lemon aroma (Frank and Byram, 1988, Schifferstein and Verlegh, 1996). Prescott (1999), and Stevenson et al. (1999) observed that aromas judged to be low in smelled sweetness (e.g. peanut butter) suppressed, whereas others, perceived to be sweet smelling (e.g. raspberry) enhanced the sweetness of sucrose solutions. Stevenson et al. (1999) observed that whilst enhancing the sweetness of sucrose, caramel aroma suppressed the sourness of citric acid. An important point noted by Prescott (2004) was the fact that addition of sucrose itself to a solution of citric acid would have resulted in the same effect (i.e. a decrease in perceived sourness). This is due to the well known phenomena that sucrose suppresses the sourness of citric acid, and the reciprocal effect that citric acid suppresses the sweetness of sucrose (Schifferstein and Frijters, 1990).

Whilst contradictory results have been obtained regarding interactions of bitterness with sour and salty tastes (Kamen et al., 1961, Pangborn, 1960, Breslin and Beauchamp, 1995), it is generally accepted that an interaction exists between bitterness and sweetness, where sucrose has been shown to suppress the bitterness associated with caffeine (Kamen et al., 1961, Lawless, 1979). On this

basis, it was hypothesised that the sweet smelling fruity aroma note (described by subjects as "pear drop" flavour) would suppress the bitterness of tea infusions. Similarly, the cabbage aroma note was expected to enhance the bitterness of tea infusions, due to the bitterness frequently associated with cabbage. Evidence to support this theory came from Frank et al. (1993) who reported that both lemon and almond aroma (described as sweet-smelling) suppressed the bitterness of three concentrations of quinine hydrochloride solutions. On this basis, a combination of aroma-induced bitterness enhancement and suppression had been expected in the first experiment, depending upon the nature and level of the aroma note used.

Whilst information regarding perceptual interactions involving astringency is sparse, several workers have reported a possible interaction between astringency and sweetness, where sucrose appears to result in a reduction in the astringent sensation. In red wine, maximum intensity and total duration of astringency were shown to decrease significantly with increasing sucrose concentration (Ishikawa and Noble, 1995). Studies have also shown that sucrose resulted in a decrease in astringency elicited by alum (Breslin et al., 1993), and tannic acid (Lyman and Green, 1990). Whether or not these effects were cognitive in nature was not fully determined, the authors suggesting that the decrease in astringency was more likely caused by an increase in lubrication due to an increase in viscosity. This theory was supported by work carried out by Smith et al. (1996) where aspartame (a non-viscous sweetener) was found to have no effect on astringency perception, yet increasing viscosity using carboxymethylcellulose significantly decreased both the maximum intensity and duration. Courregelongue et al. (1999) investigated the astringency of soymilk, agreeing that sucrose addition resulted in a decrease in astringency intensity, although claimed that since aspartame behaved in the same way, that the process was more likely to be cognitive in nature. Interactions have also been observed between sourness and astringency, where increases in malic and lactic acids were shown to lead to an increase in intensity and duration of astringency (Kallithraka et al., 1997b). Again, this interaction was however not thought to be cognitive in nature, rather the result of reduction in pH meaning increase in hydrogen bonding between polyphenols and salivary proteins. Brannan et al. (2001) observed that perception of saltiness, bitterness and sourness were all reduced in the presence of astringent compounds, whereas perception of astringency was shown to decrease in the presence of each of these taste attributes. These authors however provided no indication as to whether the interactions were cognitive or physical in nature.

Whilst previous findings are inconclusive, if these observed interactions between astringency and taste qualities were cognitive in nature, it might have been expected that the sweet-smelling aroma notes (e.g. fruity) would have decreased the intensity of astringency observed in the first experiment. Similarly, the presence of cabbage aroma note may also have been expected to have resulted in a decrease in the perception of astringency intensity due to its association with bitterness.

The presence of the green aroma note was expected to enhance perceived bitterness intensity of samples in both experiments 1 and 2. This was shown to be the case in a series of experiments carried out by Caporale et al. (2004), who observed a significant enhancement in the perception of bitterness in model olive oil systems caused by the presence of a cut grass aroma. Subjects were presented with samples of model olive oil of varying bitterness intensity (4 concentrations of quinine dihydrochloride) and asked to rate the intensity of bitterness. To half of the samples, a standard amount of *Z*-3-hexen-1-ol had been added, creating a distinct green aroma. It was shown that samples were perceived as being significantly more bitter in the presence of the added aroma, irrespective of the concentration of quinine dihydrochloride.

In the third experiment of the current study, the presence of black tea volatiles in aqueous solutions of caffeine had been expected to enhance perceived bitterness intensity. This hypothesis was based on work carried out by Scharbert and Hofmann (2005), who prepared an artificial taste reconstitute of Darjeeling tea infusion in water containing 51 non-volatile compounds in their natural concentrations. Using the SAFE procedure, these workers then prepared an aqueous volatile extract of Darjeeling tea infusion, to which the same 51 compounds were added. It was observed that the presence of tea volatiles induced an increase in perception of both bitterness and astringency compared to the artificial taste reconstitute containing no volatiles. Not only did results obtained in experiment 3 not show this enhancing effect, they appeared a direct contradiction, since in solutions containing the highest level of caffeine (1.2 g/L), presence of black tea volatiles actually appeared to suppress (rather than enhance) bitterness.

The absence of any astringency-aroma interactions observed in the current study does however agree with results obtained by Sanderson et al. (1976), where the aroma was removed from black tea infusions by stripping off the volatile materials under reduced pressure. It was found, that whilst the removal of aroma reduced the

overall tea-like quality, it had virtually no effect on the level of astringency of the infusions. A similar conclusion was reached by Cayeux and Mercier (2003) who studied interactions between the sourness and astringency of four acids (lactic, citric, malic and phosphoric), and different aromas (vanilla, chocolate, lemon and apple). Whilst it was shown that sourness enhanced the perceived intensity of congruent aromas (lemon and apple), whatever the considered congruency, the presence of aroma was shown to have no effect on the perceived intensity of either sourness or astringency.

To summarise, the only evidence to suggest perceptual interactions in the current study was in the third experiment, and even here, only in the case of solutions containing the highest level of caffeine addition. This does not mean that findings of the current study, nor those reported by previous workers are "incorrect". The study of perceptual interactions is a complicated field, and there are many potential reasons for the lack of interactions observed. These will now be discussed, and although there is some overlap, can be broadly divided into two key areas; those related to the nature of the samples and subjects used, and those related to the experimental approach.

One of the most likely explanations for the results observed in the current study, (i.e. the general absence of interactions) lies in the nature of the samples used. It is widely appreciated that taste-aroma interactions are more likely to occur in the case of congruent mixtures. Schifferstein and Verlegh (1996) for example showing that strawberry and lemon, but not ham aroma enhanced the sweetness of sucrose. The aims of experiments 1 and 2 in the current study were to investigate individual notes important to the complete aroma profile of black tea. Whilst effort was made to use only volatile compounds naturally present in black tea, the aroma of infusions containing these compounds was not particularly "tea-like", especially considering that single compounds were used (methyl salicylate, hexanal, dimethyl sulfide and hexyl acetate), and at higher concentrations than those naturally present. It is therefore possible that the nature of the samples used in the current study were too far removed from genuine black tea to be considered congruent. In experiment 3, pure caffeine was added to water or volatile SAFE extract in order to produce "infusions" of varying bitterness intensity. There is no question over the volatile component of the samples resembling black tea aroma. This was shown both by GC-MS analysis (5.2.2), and by sensory evaluation. Critically however, samples did not contain any polyphenolic compounds, known to be extremely important

contributors to the taste of black tea as described in section 1.3.4. Sanderson et al. (1976) reported that tea aroma with caffeine and all other black tea solids (except the polyphenols) had a "weak, slightly bitter, greenish taste that was not recognised as black tea taste". The current study therefore contrasts with work carried out by Scharbert and Hofmann (2005) who prepared a comprehensive cocktail of 51 non-volatile compounds, assessed by a sensory panel to closely resemble the taste of black tea. The fact that all samples across all three experiments were served at room temperature in the current study may also have played a role in exaggerating their unrealistic nature, further enhancing lack of congruency.

In experiment 2 of the current study, hexanal was added to infusions in order to enhance the intensity of the green note, known to be an important contributor to the overall aroma profile of black tea. This note is not usually experienced in isolation in black tea, and was therefore unlikely to be considered a congruent combination by subjects in these samples. In contrast, a green note is frequently associated with olive oil, Monteleone et al. (1996) (referenced by Caporale et al. (2004)) reporting a significant correlation between the intensities of bitterness and the green aroma of olive oils from different cultivars and countries of origin. This may explain why green aroma was shown to enhance perceived bitterness of olive oils, yet not black tea. The fact that the subjects used by Caporale et al. (2004) were Italian, and regular consumers of olive oil is also important since they would regularly experience the two attributes (green aroma and bitterness) together, and likely have a cognitive association between the two.

This leads directly onto the subject of learned association, and cultural factors related to the subjects employed, which in turn directly impacts upon degree of congruency of certain taste-aroma interactions. In the current study, the entire trained panel and the vast majority of the 64 subjects taking part in experiment 3 were of British origin where black tea (i.e. no milk) is rarely consumed. In contrast, the 15 subjects used in the study by Scharbert and Hofmann at the University of Munster were more likely of German origin, where the consumption of black tea is widespread. It is plausible that there existed a cognitive association between Darjeeling tea aroma and bitterness and astringency in the German subjects since the stimuli are frequently associated together (i.e. are considered congruent). This is not the true of British subjects where bitterness and astringency of tea infusions are both substantially reduced by addition of milk (Sanderson et al., 1976). Since

milky tea is not usually associated with bitterness of astringency, the tea aroma and bitterness in the samples were seen as an incongruent combination.

Another possible reason for the lack of interactions observed in the current study lies in the experimental approach employed. It is well known that an association between taste and aroma can be made during repeated tasting, resulting in the aroma developing taste qualities, and developing the ability to enhance specific taste characteristics when in solution. This has been clearly demonstrated by Prescott (1999) who observed that aromas that initially had no impact on sucrose sweetness (water chestnut) or suppressed sucrose sweetness (peanut butter), actually enhanced sweetness of sucrose in solution after a period of 12 repeated exposures. It is also known that association between particular tastes and aroma can be made over a shorter period of time, Prescott et al. (2004) showing that as little as one co-exposure can be sufficient to form an association, subsequently creating an enhancement of sweetness. On this basis it initially appears odd that no such association appears to have been made between the taste and aroma components of samples in the current study, specifically in the first two experiments. Training was carried out over a period of four months, during which time subjects were frequently exposed to the specific aromas in combination with bitter and astringent tea infusions. On the basis of learned association, it would be expected that by the time of the actual experiment, subjects would have associated particular aroma notes with bitterness and astringency, leading to an enhancement effect. Prescott (1999) does however report that the effect of repeated exposure on taste enhancement appeared to be odourant dependent, and whilst peanut butter and water chestnut aroma both enhanced sweetness of sucrose solutions following co-exposure (perceived as low and moderately sweet aromas in a pre-test respectively), there appeared to be no learned association in the case of Oolong tea aroma. It is possible that the specific aromas used in the current study were in this second category, where repeated exposure did not increase degree of congruency.

Another explanation for the absence of any apparent taste-aroma interactions lies with the high degree of training received by subjects. The cognitive strategy employed by subjects is thought to be largely dependent upon training, and it is generally assumed that whilst naïve subjects operate a synthetic approach (taking flavour as a whole), trained subjects adopt a more analytical approach (taking individual attributes separately) (Prescott, 2004). Data showing the effect of training (and so cognitive strategy employed) on presence of taste-aroma

interactions is however contradictory. Some studies e.g. Bingham et al. (1990) have shown training to result in a decrease in the ability of subjects to perceive taste enhancement (as would be expected with an analytical approach). Whilst untrained subjects perceived sucrose-maltol mixtures to be sweeter than sucrose alone, a trained descriptive analysis panel found no such enhancement. In contrast, Hort and Hollowood (2004) found that degree of training had relatively little impact on taste-aroma interactions, and with a few exceptions, experienced and naïve subjects scored similarly during a time-intensity procedure. Training was also shown to have relatively little effect on taste enhancement in work carried out by Stevenson (2001), and Stevenson and Case (2003), where subjects were specifically trained to differentiate between the sensations of taste and aroma in the mouth. One explanation proposed by the authors was that the period of training used was too short for effects to become apparent, (training was limited to two sessions over a two week period). This compares to a period of four months used in the current study, and it is possible that use of a highly trained panel led to the results obtained in experiments 1 and 2. In studies on Darjeeling tea (Scharbert and Hofmann, 2005), and olive oil (Caporale et al., 2004) where taste-aroma interactions were observed, the extent of panel training was considerably lower (limited to "familiarisation sessions") and may have been insufficient for subjects to have automatically adopted an analytical approach.

Whilst Stevenson (2001) found training *per se* to have little effect on taste enhancement, when the trained subjects were later specifically instructed to use their newly developed skills to split the mixture into its component parts and concentrate only on sweetness, subjects reported significantly less enhancement. This suggests that even with appropriate knowledge and ability to use an analytical approach (i.e. training), subjects still by default tend to adopt a synthetic approach (unless specifically instructed otherwise). This could account for the result observed by Hort and Hollowood (2004), where highly experienced subjects were shown to score similarly to naïve subjects, both appearing to adopt synthetic strategies.

Whilst the naïve (untrained) tea consumers used in the third experiment would normally have been expected to adopt a synthetic approach, the instructions given immediately prior to the test may well have led to them adopting an analytical approach. Subjects were told to indicate which sample they found the most bitter of the two "focussing only on the bitterness, ignoring anything else which may differ between the samples". If this is indeed the case, it supports the findings of

Stevenson (2001) who suggested that specific instructions given to subjects may be more important than training itself.

As well the effects of training, Stevenson (2001), and Stevenson and Boakes (2003) also investigated the effect of pre-exposure of individual stimuli (i.e. taste and aroma in isolation) on the extent of taste enhancement. Stevenson (2001) observed that pre-exposure of stimuli led to a significant decrease in enhancement when using trained subjects. Stevenson and Case (2003) also reported that pre-exposure had a marked impact upon the acquisition of taste properties by aromas showing to lead to no increase in sweetness or sourness of aromas paired with sucrose or citric acid respectively. It was thought that pre-exposure of stimuli in isolation helped subjects to appreciate the separate nature of the taste and aroma attributes, aiding their ability to rate them independently in subsequent experiments. This finding is of relevance to the current study since during the training stage of experiments 1 and 2, a significant proportion of time was devoted to enabling subjects to effectively rate the attributes of interest. Samples of aqueous caffeine and alum were used in order to familiarise subjects with bitterness and astringency respectively, and subjects were also presented with aqueous samples of the four aroma notes in isolation. During the testing stage, subjects were presented with reference samples of low and high bitterness (and astringency) to ensure that the scales were being used appropriately, and in experiment 2 subjects were also presented with an aqueous solution of hexanal. Use of these reference samples effectively equated to pre-exposure of the individual stimuli, and certainly based on the findings of Stevenson (2001), and Stevenson and Case (2003) may well have had a significant effect upon the taste-aroma interactions observed.

Training of subjects for experiments 1 and 2 in the current study is therefore likely to have resulted in several counteracting effects. On the one hand training is likely to have resulted in a degree of learned association between the taste and aroma attributes, making the presence of perceptual interactions more likely. On the other hand, the period of training itself, specifically the pre-exposure of taste and aroma stimuli in isolation is likely to have resulted in subjects adopting a more analytical approach, thus making the presence of perceptual interactions less likely.

On the basis of the above discussion, it might be hypothesised that had untrained continental European subjects been used in the three experiments of the current study, and had samples been more tea-like, perceptual interactions such as those

seen by Scharbert and Hofmann (2005) would have been observed. It is however essential at this stage of the discussion to mention several weaknesses of the approach used by these workers, which may well account for the interactions observed.

The first important point to make is that subjects rated attribute intensity on scales of 0 to 3, a very small range, not normally considered appropriate for sensory rating procedures. This compares to the use of unstructured line scales used in the first two experiments of the current study where subjects were free to rate attributes at any point (within the limit set by the reference samples). It is also worth noting that whilst 15 subjects were used in the study by Scharbert and Hofmann (2005), no mention of any replication is made. Whilst the presence of tea volatiles was shown to lead to an increase in the mean bitterness and astringency scores of +0.2 and +0.1 respectively, this small difference in score could simply have been caused by a single subject giving a different score to one of the samples. The authors have made no mention of statistical analyses, and without the raw data it is impossible to confirm whether these differences are statistically significant.

On the subject of scales, another possibility to consider is the phenomenon of halo-dumping. This is where inadequate provision of appropriate attribute scales leads subjects to dump sensations they experience into alternative categories. Subjects were asked to rate five attributes (astringent, bitter, sour, sweet, umami and salty) of the artificial aqueous taste reconstitute both with and without the presence of tea volatiles. Crucially, no provision was made for subjects to express the additional sensation of the tea aroma when present. Subjects may simply have dumped the aroma attribute in alternative categories (i.e. sweet, bitter and astringent in this case). A similar effect was observed by Frank et al. (1993) who showed that almond aroma enhanced the bitterness of bitter quinine hydrochloride solutions when bitterness was the only attribute rated yet actually suppressed bitterness when an additional seven attribute ratings, including “almondness” were included. Similar observations have been observed by many workers investigating taste-aroma interactions and are reviewed in section 1.2.3. In the current study, both taste and aroma attributes were rated in experiments 1 and 2, minimising the potential for halo-dumping to occur. Had subjects rated only bitterness and astringency, it is quite possible that the increasing level of aroma notes would have led to an increase in the scores of these two attributes.

Further evidence to suggest the occurrence of halo-dumping in work by Scharbert and Hofmann (2005) comes from the fact that subjects rated the aqueous volatile SAFE extract (minus non-volatile compounds) as being tasteless when evaluated using noseclips, yet as being slightly bitter in their absence. This suggests that the aroma attribute had either been dumped, or confused with bitterness. A similar explanation may well apply to the result observed in experiment 3 of the current study where no significant difference in bitterness was found between distilled water and volatile SAFE extract when assessed by subjects wearing noseclips, yet in their absence, significantly more subjects scored the volatile SAFE extract as being the more bitter sample of the two. Whilst not necessary being "dumped" (as a paired comparison, rather than rating procedure was used), it makes sense that given the choice of a completely tasteless solution (distilled water), and one with a strong tea-like aroma, subjects would be more inclined to pick the latter as being the most bitter. Although probably not confusing aroma with bitterness, subjects were simply opting for the sample with at least "something" present.

An alternative explanation is related to the theory of learned association as discussed above. It is possible that the tea aroma of the volatile SAFE extract evoked memories associated with consumption of (bitter) black tea, and as such subjects did indeed perceive a certain degree of bitterness in the sample. The fact that there was no significant difference in the perceived bitterness of the two samples at both low and medium levels of caffeine addition however appears to contradict this theory. Had subjects genuinely associated the aroma of the volatile SAFE extract with the bitterness of black tea, it would be expected that significantly more subjects would also have also chosen the volatile SAFE extract as being the more bitter in samples containing added caffeine.

Whilst the current study showed very little evidence for any form of perceptual interactions between the volatile and non-volatile components of black tea infusions, the notable exception was shown in experiment 3 where there was an apparent suppression of bitterness caused by tea volatiles in solutions containing high (1.2 g/L) levels of caffeine. As previously mentioned, this result directly contrasts to results of a similar study carried out by Scharbert and Hofmann (2005) where tea volatiles were found to enhance bitterness. Results of the current study do however appear to agree with findings of Frank et al. (1993) who reported that both lemon and almond aroma suppressed the bitterness of quinine hydrochloride solutions. Unlike Scharbert and Hofmann, these workers controlled the halo-dumping effect by

asking subjects to rate all relevant attributes. These findings can potentially be explained by the "sweetness" associated with lemon and almond aroma, due to their frequent co-occurrence with sucrose in foods. Since sucrose is known to suppress bitterness (Lawless, 1979, Kamen et al., 1961), it is possible that these "sweet" aromas caused a suppression of bitterness, similar to the observation that sweet smelling aromas can suppress perceived sourness of citric acid solutions (Stevenson et al., 1999). This theory is supported by a high proportion of subjects who described the volatile SAFE extract as having a sweet smelling aroma.

A key problem with this theory however is the fact that it was only observed in the sample containing the highest level of caffeine addition, Frank et al. (1993) reporting the suppression effect to occur irrespective of actual bitterness level, where three different concentrations of quinine hydrochloride were used. Schifferstein and Verlegh (1996), whilst looking at the sweetness of sucrose solutions did however observe that aroma induced sweetness enhancement decreased with increasing sweetness level and it is possible that the same is true for bitterness, resulting in suppression only appearing at the highest bitterness level.

An alternative hypothesis to explain the apparent suppression effect occurring at the highest caffeine addition level is related to the unpleasant nature of the samples. Whilst the low and medium bitterness levels were generally quite acceptable, the high level was chosen to resemble a very bitter tea infusion which many subjects, not used to such high intensity of bitterness may have found objectionable. Whilst both samples contained equal concentrations of caffeine, (and in theory exhibited the same degree of bitterness), the volatile SAFE extract (with its pleasant aroma) was considered slightly less objectionable to many subjects (confirmed in informal trials). Subjects assigning the more unpleasant sample as the most bitter sample is an example of a "horn" effect (Clark and Lawless, 1994). This is where subjects tend to associate certain sensations (bitter in this case) with unpleasantness, and so score accordingly. (The reverse effect is known as the "halo" effect, where for example subjects have been shown to rate sweeter samples as being more pleasant).

There now follow several other important discussion areas relevant to the current study. The first of which is performance of the trained panel used in experiments 1 and 2.

Since taste and smell are commonly confused, and retronasal aromas and tastes are often perceived as being located in the same place at the same time (Rozin, 1982), it seems logical that confusion between the two is most likely to occur where the taste and aroma are considered to be similar. In work by Frank et al. (1991), subjects rated the perceived similarity of paired mixtures involving four tastants and six odourants. It was observed that that perceived similarity of the stimulus pairs was a good predictor of aroma induced taste enhancement.

In experiment 2 of the current study, two subjects (Gill and Dawn) appeared to show an element of confusion between bitterness and green aroma. Both subjects gave high scores for green flavour in two of the three reps (as well as in the practice session) for the sample containing no hexanal, yet a low (0.25 g/L) level of caffeine. In rep 1 for example, Dawn and Gill gave green flavour scores of 9.4 and 9.9 (out of 10) respectively (scores between 0 and 0.2 were given by the remaining 9 subjects). It is interesting that Dawn and Gill appeared only to show confusion with this single sample (i.e. this hexanal / caffeine level combination). It is suggested that the very subtle level of bitterness exhibited by 0.25 g/L caffeine was mistaken for a very high level of hexanal. At the higher levels of caffeine addition (0.5 and 1.0 g/L), the bitterness of the sample was very distinct, and given the training undertaken, it was obvious to the subjects that the sensation was that of bitterness as opposed to the green flavour. The reciprocal of this confusion also occurred, where in rep 1 the same two subjects gave the highest bitter scores of the whole panel for the sample containing no caffeine, but a medium (12 ppm) level of hexanal. In rep 2, Gill gave a bitterness score of 9.9 for this sample, even though there was in fact no caffeine present. Whilst it is not appropriate to remove these two subjects from the data analysis, it is interesting to note that had they been removed, overall results of the ANOVA would have remained unchanged.

The fact that only two subjects appeared to confuse attributes, only for two samples, and even here only occasionally, suggests that the training sessions effectively enabled subjects to clearly discriminate between the green aroma note and bitterness. The slight confusion that was present however does clearly indicate the potential for confusion between these two attributes, and had subjects received less training may have been more widespread. In such case, taste-aroma interactions may well have been seen, particularly where levels of bitterness of aroma note were subtle enough to be mistaken. Whilst there are many other reasons for the taste enhancing effect of green aroma observed by Caporale et al. (2004) (e.g. olive oil as

opposed to tea, more congruent samples, cultural effects, level of training), it is at least possible that confusion between attributes played a part, especially given the lack of training subjects received.

Another key point worth mentioning on the subject of panel performance is the widely different portion of scale utilised by subjects in both experiments 1 and 2 when rating the bitterness and astringency attributes. An example of this is shown below in figure 5-4 which shows the astringent scores given by all ten subjects for samples containing four different levels of hexanal addition (green aroma note).

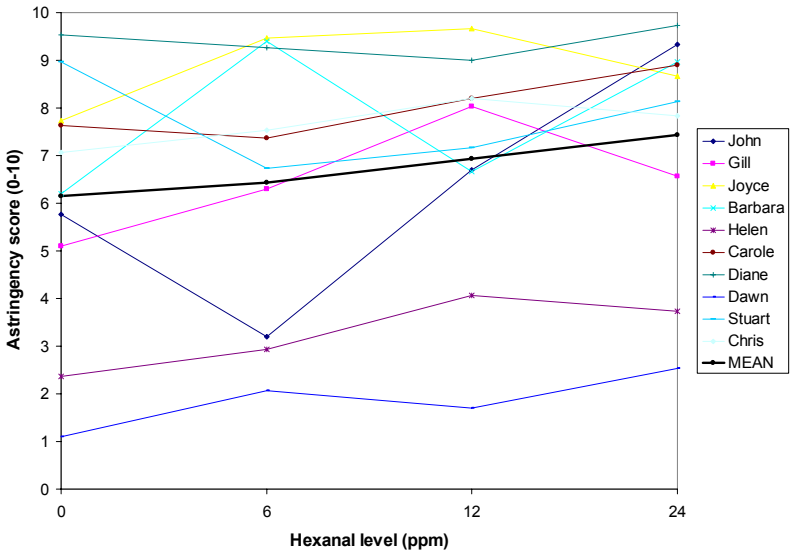


Figure 5-4 - Astringency scores given by subjects in experiment 1 for four levels of hexanal addition illustrating widely different scale usage

It is clear from figure 5-4, that some subjects (e.g. Dawn) used a much lower portion of the scale than others (e.g. Diane). All subjects were presented with the same reference samples (alum and caffeine solutions) to anchor the scale, and subsequently received the same experimental samples. It had also previously been proven that subjects were able to successfully rate the bitterness and astringency of pure solutions of caffeine and alum between the two reference samples (low and high) provided. This is shown in figure 5-5 which shows the mean scores for bitterness (a) and astringency (b) (3 reps) given by each of the subjects for five concentrations between the two reference samples.

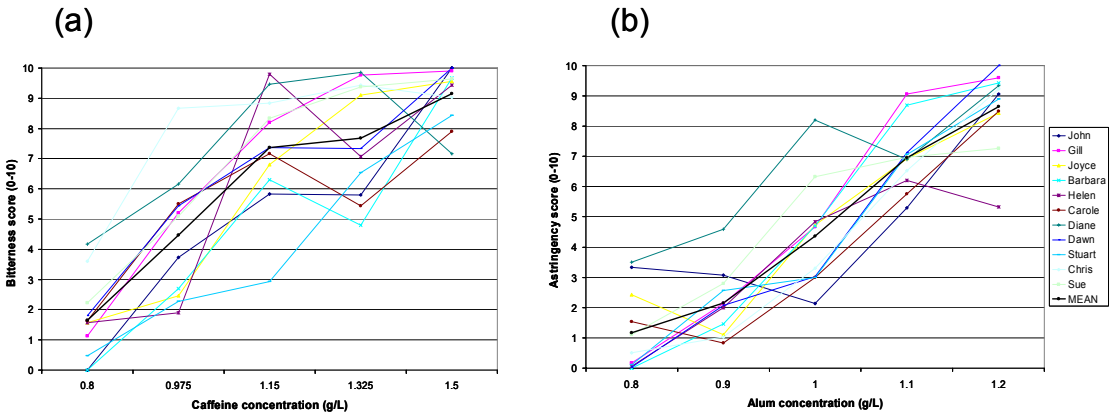


Figure 5-5 - Bitterness (a) and astringency (b) scores (average of 3 reps) given by subjects rating the intensity of these attributes in solutions of caffeine and alum

This data confirms that subjects were able to discriminate between levels of bitterness and astringency provided by the very tight reference samples, and that different scale usage seen for the experimental samples (e.g. figure 5-4) was not simply due to inability to rate these two attributes.

The most likely explanation for the widely different scale usage lies in the choice of compounds used as reference samples. The bitterness and astringency associated with tea infusions, whilst still subject to considerable debate is thought to be composed of a combination of many different compounds, including catechins, theaflavins, thearubigins, amino acids and caffeine (section 1.3.4). Since the vast majority of these compounds exhibit both bitterness and astringency, they were not appropriate for attribute references which needed to display pure bitter or astringent sensations. It was also found that many of these compounds (especially the catechins) had a strong associated aroma, which would have proved confusing for subjects.

Alum (a multivalent cation salt) has been described as the most suitable reference sample for use in sensory evaluation of black tea infusions (Drobna et al., 2004), and was chosen for this study. Peleg et al. (1998) have however shown alum to behave differently to the catechins, suggesting that it cannot be used interchangeably with phenolic astringents in psychophysical studies. Alum inevitably produces the astringent sensation according to a different mechanism than that of the polyphenols naturally present in tea (i.e. absence of protein-tannin

interactions). Coupled with the effect that salivary composition and flow rate has on astringency perception (i.e. low saliva flow rate subjects tend to rate astringency higher and for a longer duration (Ishikawa and Noble, 1995)), and the fact that these physiological aspects may affect different compounds in different ways, it is possible that different subjects experienced the astringency of the tea samples differently to the alum reference samples. For some subjects (e.g. Dawn), the alum reference samples may have appeared very astringent relative to the compounds naturally present in the tea samples and so accordingly these subjects rated the tea samples using only the lower end of the scale. For other subjects (e.g. Diane) the opposite may have been true, these being more sensitive to polyphenol-induced astringency relative to that of alum. These subjects therefore rated all of the tea samples using only the upper end of the scale.

The same theory can also be applied to bitterness, and whilst caffeine does contribute to the bitterness of tea infusions, there are many other compounds also known to play a part, particularly the catechins. A subject quite insensitive to the bitterness of caffeine may have found the bitterness associated with the tea samples to be quite high (given the additional contribution of other bitter compounds to which they may be much more sensitive to).

Another key feature worth discussing is the fact that the sample containing no green aroma note was scored significantly higher in terms of its green flavour than the corresponding attributes associated with the other three aroma notes (experiment 1) even though all samples were identical. There are several plausible explanations for this, one being that the sample (whilst containing no added hexanal) did still contain an element of green flavour. Although the reconstituted non-volatile SAFE infusion contained significantly less aroma than a complete infusion, a small amount of residual aroma did remain (which could possibly be described as green). Although the terms “none” and “high” were used as word anchors on the scale, these words were chosen based upon panel discussion as is standard practice in the QDA approach. Even if only one subject perceives none of a specific attribute, it is necessary to use the word “none” as an anchor. An alternative explanation could lie in the lingering effect of hexanal (relative to the other aroma notes), where despite the use of palate cleansers, some flavour remained and was transferred to subsequent samples.

Whilst this study has shown that neither bitterness or astringency were affected by level of aroma compound, it is well known that bitterness and astringency are two very commonly confused attributes, and getting subjects to reliably distinguish between the two (especially in complex mixtures) is extremely difficult. This has also been observed by Fischer and Noble (1994) with regards to the bitterness and astringency associated with wine, and Lea and Arnold (1978), looking at bitterness and astringency of cider go so far as describing the two as "twin sensations". Even with several years training, independently rating bitterness and astringency has still been shown to be problematic (Unilever, personal communication). Given this difficulty, it was useful to gain an idea as to whether subjects were indeed rating these two attributes independently in experiment 1. Figure 5-6 is a scatter plot showing scores of bitterness and astringency for each of the samples across the whole experiment.

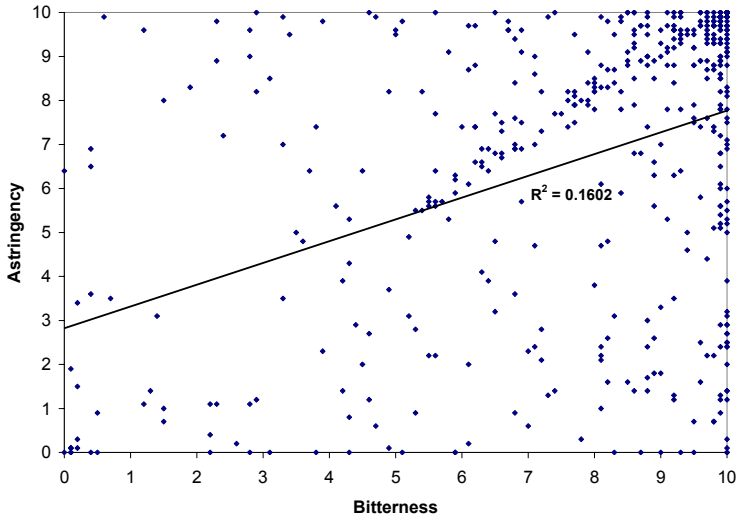


Figure 5-6 - Evidence to support the independent rating of bitterness and astringency by subjects in experiment 1

Whilst it cannot be conclusively proven that all panellists were correctly differentiating bitterness and astringency in all samples, it is clear from figure 5-6 that subjects were not merely giving bitterness and astringency identical scores, clearly highlighting the effectiveness of the training procedure used.

5.6 FUTURE WORK

This chapter has provided a valuable insight into some of the potential perceptual interactions occurring as a result of the delicate balance of volatile and non-volatile compounds in black tea infusions, and whilst the presence of perceptual interactions may not have been observed in this study, the results are still important, highlighting important aspects of methodology.

A considerable amount of future work could be carried out to further investigate the results of this study. Most valuable would be to repeat experiments 1 and 2 using naïve subjects. Whilst probably not solely responsible, the high level of training is thought to have contributed to the absence of observed interactions. On this basis, the use of naïve subjects may yield a different result, with a greater chance of seeing interactions. Naïve subjects are more likely to adopt a synthetic approach, and also more likely to become confused between the taste and aroma attributes (as appeared to occur to a minor extent in the case of two subjects in the current study). The results of a study using naïve subjects would be useful as it would provide information representing the perception of tea consumers as opposed to specially trained subjects.

Other work could be carried out to make the tea samples used more realistic, so increasing the degree of congruency. Rather than using single volatile compounds, more complex notes could be developed by combining those compounds naturally found in tea. By enhancing the realism of the samples, it is hypothesised that perceptual interactions are more likely to be seen.

Cultural effects, and their effect on congruency should also be further investigated by repeating the experiments using subjects more used to black tea consumption (i.e. without the addition of milk). It would be interesting to see whether repeating experiment 3 using European subjects would lead to a similar result to that observed by Scharbert and Hofmann (2005), with an enhancement of bitterness caused by the presence of black tea volatiles.

Considerable evidence exists that taste-aroma interactions not only affect maximum intensity of attributes, but also their persistence. It was observed by Cliff and Noble

(1990) that whilst increasing the sweetness of glucose solutions flavoured with peach essence failed to increase the intensity of fruitiness, it did increase the persistence of this attribute. Similar findings have been reported for bitterness, and in the work on olive oil carried out by Caporale et al. (2004) it was observed that as well as maximum bitterness intensity, values of T_{half} (time to reach half maximum intensity), and T_{end} (time to disappear completely) for bitterness were greater in samples containing added green aroma. In addition, whilst not confirmed to be cognitive in nature, Ishikawa and Noble (1995) showed that increasing the sucrose concentration in red wine increased the duration of the astringent sensation. In experiment 3 of the current study, for two of the three caffeine levels subjects did not indicate either the SAFE extract or distilled water sample as being the most bitter. Several subjects however later commented that whilst they had been unable to detect any difference in the actual intensity of bitterness, they had found the bitterness more prolonged in the sample containing the tea aroma. This should be explored further by adopting a time-intensity approach. Utilising a similar approach and repeating experiments 1 and 2 would also be interesting. It may be that although the presence of aroma notes had no impact upon perception of bitterness or astringency intensity (and the presence of caffeine, no effect upon green flavour intensity), the duration of these sensations was affected.

It is also important to emphasise that both bitterness and astringency build up over time, and have prolonged after tastes. Although precautions were taken to minimise these effects as far as possible (forced breaks, balanced design, palate cleansing), it is nevertheless possible that a build-up of one or both of these attributes caused problems for subjects rating them. Specifically investigating the impact of this build-up would however provide valuable information representing the real-life consumption black tea infusions, where build-up of these sensations does occur due to repeated sips over the time course of consumption.

Whilst no interactions related to astringency were observed in the current study, it is important to appreciate the complexity of this mouthfeel characteristic. Sanderson et al. (1976) has described the astringency of tea as consisting of two components; namely tangy and non-tangy, these differing in the type of sensations and after effects. Lee and Lawless (1991) observed that the four tactile sensations of drying, puckery feeling, roughing, and overall astringency may well not be interchangeable, and Gawel et al. (2001), whilst attempting to characterise the astringency of red wine came up with a total of 24 different astringency attributes. It is therefore clear

that the overall astringency rated in the current study was very broad in terms of definition, perhaps too much so, resulting in changes in some astringent sub-qualities being missed. This is an area of future work, although considerable more training would however be required in order for subjects to reliably sub-divide the overall astringent sensation.

6 OVERALL CONCLUSIONS

The topics covered in this thesis have been broad in nature, covering analytical techniques, psychophysics (signal detection theory), and perceptual interactions. This final section aims to bring these findings together, providing an overall conclusion of the work carried out.

Chapter 2 described the GC-EI/APCI-MS technique, successfully applied to freshly prepared black tea infusion headspace in order to assign 15 ions present on APCI-MS spectra to volatile compounds released. Six compounds were unequivocally assigned to single ion masses enabling them to be monitored and quantified with certainty. In other cases, it was only possible to assign ions to groups of compounds, as was the case for isobaric compounds such as heptanal and heptanone (*m/z* 115), and stereoisomers such as *E*-2-heptenal and *Z*-4-heptenal (*m/z* 113). In some cases, although ions were assigned to compounds known to be important contributors to the overall aroma of black tea infusions (e.g. β-damascenone and β-ionone), some unknown compounds also contributed. The compounds represented by the 15 ions covered a range of physicochemical properties, formation mechanisms, and aroma descriptors. Crucially, many of the monitored compounds were known to be important contributors to the aroma of black tea infusions.

A novel system was then developed, enabling the volatile compounds represented by the 15 ions to be monitored from above mugs of freshly prepared, hot, black tea infusions using APCI-MS. The system was a compromise between one representing the real-life system, and one attempting to maintain a reasonable level of control. Data obtained from this analytical system were found to be reproducible, with confidence variation values for the mean cumulative ion count generally below 5 % for the ions (over 25 replicate analyses of tea headspace).

A typical application of this novel approach was described in chapter 3, namely the effect preparation method had on volatile release from freshly prepared black tea infusions. Both infusion water temperature and concentration (ratio of leaf to water) were shown to significantly affect the amount of volatiles released into the headspace; the higher the infusion water temperature and concentration, the greater

the level of release. Infusion duration appeared to play a less significant role, although this was suggested to be a result of the experimental protocol, specifically the large range of infusion durations used.

In terms of infusion concentration, there appeared to be a relatively straight-forward explanation for the increase in release, where irrespective of the physicochemical properties of the compound, the higher the infusion concentration, the greater the extraction of volatile compounds from the leaves into the aqueous phase. Partitioning of volatiles between the aqueous and gas phase was then mainly a function of the air-water partition coefficient, which was largely unaffected by differences in the concentration of the volatile compounds in the aqueous phase. This explains why infusion concentration affected all monitored compounds in a similar way.

In contrast, the effect of infusion water temperature was shown to be considerably more complex, with differences in behaviour of the different compounds evident. Overall volatile release from tea infusions was due to the combined effect of temperature on several different mechanisms, including extraction from the leaf matrix into the aqueous phase, and partitioning between the aqueous and gas phases. By carrying out a series of experiments it was possible to start to decouple the effects of these two main mechanisms, providing some possible explanations for differences in behaviour of the different compounds.

GC-MS analysis confirmed that concentration of selected volatile compounds in the aqueous phase was directly correlated to level of release into the headspace, proving that increases in release caused by increases in infusion water temperature were not merely the result of differences in phase partitioning of the volatiles caused by differences in the non-volatile composition of infusions. Furthermore, on the basis that all compounds appeared to be affected to a similar extent as a function of infusion concentration, it was suggested that differences in behaviour of the compounds as a function of infusion water temperature were most likely due to differences in extraction efficiency of the volatiles out of the leaf matrix into the aqueous infusion.

Differences in extraction efficiency of the compounds was suggested to be due to differences in their physicochemical properties, with very efficient extraction of some compounds such as the more polar, water soluble Strecker aldehydes

(2-methyl propanal, 2- and 3-methyl butanal), and less efficient of the more hydrophobic compounds such as β-damascenone and β-ionone. It was suggested that infusion water temperature increase had the lowest impact on release of the efficiently extracted volatiles, since even with relatively cool water (i.e. 40 °C), a large proportion of the available pool was extracted, increasing only slightly further with increasing temperature. In contrast, increase in release of the inefficiently extracted compounds as a function of increasing infusion water temperature was much greater, it taking considerably higher temperatures to extract these into the aqueous phase.

Differences in hydrophobicity and water solubility alone did not appear to be able to fully explain differences in behaviour of the different compounds, and whilst the location of volatile compounds within the leaf matrix has not yet been elucidated, it was thought that this may have also played a key role in extraction efficiency (as has been previously shown to be the case for the non-volatiles). It is possible that some volatile compounds were more accessible to the infusion water, and so extracted more efficiently than others deep within the leaf matrix. Other factors are also likely to play a role in extraction efficiency, such as association with leaf constituents which may hinder the diffusion process. In the case of several compounds (dimethyl sulfide, 2-methyl propanal and the methyl butanals), additional generation was shown to occur during the time-course of the experiment. The marked increase in release of dimethyl sulfide following infusion with 100 °C for example was most likely due to generation of this compound during the infusion process.

Extraction efficiency of volatile compounds from the leaf matrix, and any additional generation determined the concentration in the aqueous phase, and so the amount available to partition between it and the gas phase. Temperature was also shown to have a significant effect on this phase partitioning, with clear differences in the temperature dependence between the different volatile compounds. For some, such as the methyl butanals, vapour pressure was shown to increase fairly linearly with increasing temperature, whereas for others such as β-damascenone and β-ionone, vapour pressure increased more exponentially.

The overall release of volatile compounds from infusions was therefore concluded to be a combination of all of these effects, each contributing to greater or lesser extent depending upon the particular compound. Providing absolute explanations for

differences in behaviour based on APCI-MS data alone was impossible, especially since in some cases several compounds contributed to particular ion masses. Nevertheless, this work has clearly illustrated the importance infusion preparation method has on the volatile release from black tea infusions.

Whilst APCI-MS data was able to provide clear evidence that infusion preparation method played a key role in determining the volatile release (and so aroma) from black tea infusion, it provided no indication of the relevance of the differences observed in terms of the real-life situation. The experiments described in chapter 4 were designed to address this area, exploring the relevance of the APCI-MS data in terms of the orthonasal aroma perceived by tea consumers. The orthonasal discriminability of a selection of infusions prepared according to different preparation methods (water temperature and concentration) was determined. A signal detection approach was utilised, negating the effect of response bias usually associated with the same-different discrimination test, and providing a measure of the discriminability of samples (d' and P(A)).

It was shown that the differences in volatile release as a function of tea infusion preparation method could to a large extent be detected by consumers. In many cases, even very small differences in infusion water temperature (e.g. 90 vs. 100 °C), and infusion concentration (e.g. 0.25 vs. 0.5 %w/v) resulted in perceivable differences in the orthonasal aroma of infusions. The claim made by previous workers that a value of d' of 1.0 has to be exceeded in order for differences to be detected (O'Mahony and Rousseau, 2002) applied fairly well to the data since of the four pairs where it was shown that subjects were unable to discriminate between the two samples, values of d' were less than 1.0 in three cases. It is clear that infusion preparation method not only plays a key role in the release of volatile compounds from tea infusions, but also leads to differences that can be detected by consumers via the orthonasal route.

In terms of infusion concentration, discriminability could be largely explained by relative differences in concentration of the infusions. Given the similar effect infusion concentration had on volatile release (chapter 3), level of discriminability could be related to differences in volatile release from the infusions. Values of d' for the pairs (0.25 vs. 0.5 %w/v and 0.5 vs. 1.0 %w/v) were identical, supporting the theory of Weber's law, and indicating the samples within the two pairs were equally discriminable.

Some exceptions were present, such as the higher value of d' obtained for the pair of samples 1.0 vs. 2.0 %w/v (d' 1.81), which according to Weber ought to have yielded similar values of the 0.25 vs. 0.5 %w/v and 0.5 vs. 1.0 %w/v. A similar anomaly was seen where 1.0 vs. 1.5 % infusions appeared to be more discriminable than 0.5 vs. 1.0 %w/v infusion (d' 1.50 and 1.34 respectively), even though relative differences in infusion concentration and volatile release were greater in the case of the latter. One suggestion is that these apparent contradictions are related to the experimental variation surrounding the values of d', which although different may not be statistically significant and should be explored further.

In terms of infusion water temperature it was shown that samples at the higher temperature range (90 vs. 100 °C; d' 1.58) were more discriminable than samples at the lower temperature range (40 vs. 50 °C and 40 vs. 60 °C; d' 0.95 and 0.99 respectively), despite the absolute and relative difference in infusion concentration (total solids) being greater in these latter two pairs. It was hypothesised that these differences in discriminability were brought about by larger differences in the release of key volatile compounds at higher temperatures. Unlike infusion concentration, due to the wide variation of behaviour of different compounds as a function of infusion water temperature (chapter 3) it was not appropriate to average APCI-MS volatile release data.

It was hypothesised that differences in the behaviour of volatile compounds would lead to differences in the aroma profile of infusions experienced by consumers prepared according to different preparation methods. Depending upon the specific preparation method employed, infusions were likely to contain relatively higher or lower intensities of specific aroma notes. The implications of these differences in aroma profile in terms of perceptual interactions between the volatile and non-volatile components of black tea infusions were investigated in chapter 5. Specific attention was paid to interactions involving bitter taste and the tactile sensation of astringency.

The SAFE procedure was used to split complete black tea infusions into volatile and non-volatile fractions. The concentration of key aroma notes (green, cabbage, medicinal and fruity) in the non-volatile infusions was shown to have no effect on perceived intensity of bitterness or astringency. It was also shown that concentration of green note had no effect on bitterness perception, and that

concentration of caffeine had no effect on the perception of green note in this non-volatile infusion.

Disagreeing with initial expectations, these results provided no evidence for the presence of perceptual interactions between the volatile and non-volatile components studied. Whilst several different explanations were provided, it is likely that two key factors played the biggest part in this result; the nature of the samples used, and the nature of the experimental protocol. The samples used, whilst intended to represent tea infusions containing elevated levels of specific notes were not particularly tea-like, partly due to the nature and concentration of the compounds added. This is likely to have had a direct impact upon the perceived congruency of the taste and aroma components, exemplified by cultural aspects of the (British) subjects, not used to bitter or astringent tea infusions, so lacking the cognitive association between these and tea aroma. The degree of training provided was also suggested to have played a part in the results, where it was possible that the extended training led to subjects adopting an analytical approach, as was pre-exposure of the individual attributes during training.

An additional experiment was carried out using naïve tea consumers in order to determine whether the presence of complete tea aroma caused any difference in perceived intensity of aqueous caffeine solutions. Tea aroma had no effect on perceived bitterness of solutions containing low and medium concentrations of caffeine, so supporting the findings of the study using trained panellists. However, at high concentrations of caffeine, the presence of tea aroma appeared to suppress the perceived intensity of bitterness. These results contrasted with the findings of other workers (Scharbert and Hofmann, 2005) where it had been observed that tea aroma enhanced bitterness of solutions of tea non-volatiles. This may have been partly due to differences in the nature of the samples used in the two studies, or by differences in the experimental protocols. The suppression of bitterness by tea volatiles in the current study was also suggested to be possibly due to a “horn” effect due to the unpleasant nature of the samples.

Overall, this research has provided a valuable insight into the aroma of black tea infusions, its release as a function of infusion preparation method, and the interactions which may or may not occur between the volatile and non-volatile components of infusions.

6.1 FUTURE WORK

Areas of potential future work have been described and discussed in detail in the relevant preceding chapters. However, to bring everything together, a brief summary of the key areas of future work, along with some not previously mentioned is provided below.

6.1.1 Analytical work

From an analytical point of view, a key area of future work would involve further improvement to the assignment of compounds to ions on APCI-MS spectra. Although this could be carried out in one of several ways, the two most worth exploring are use of alternative transfer re-agents and ion-trap mass spectroscopy. Enhancing the reality of the headspace sampling system, allowing the temporal profile of volatile compounds released from tea infusions to be followed is the logical next step.

The APCI-MS approach described in the current study has provided a rapid means of investigating the effect preparation method had on release of selected volatile compounds from hot, fresh, black tea infusions. A more in depth study utilising a GC-MS approach should be carried out in future, enabling the rate of extraction of volatile compounds out of the leaf matrix into the aqueous infusion to be determined. This would add valuable information to that already obtained, helping to explain potential reasons for differences in behaviour of the compounds.

In terms of the effect of preparation method, this study has focussed exclusively on the orthonasal aspect of release (i.e. release into the headspace of infusions). A complementary study should build upon these results, investigating the effect preparation method has on the volatiles perceived retronasally. Although the APCI-MS used in the current study lacked the sensitivity to monitor retronasal release, this should be investigated further, possibly making use of the recently purchased time-of-flight PTR-MS.

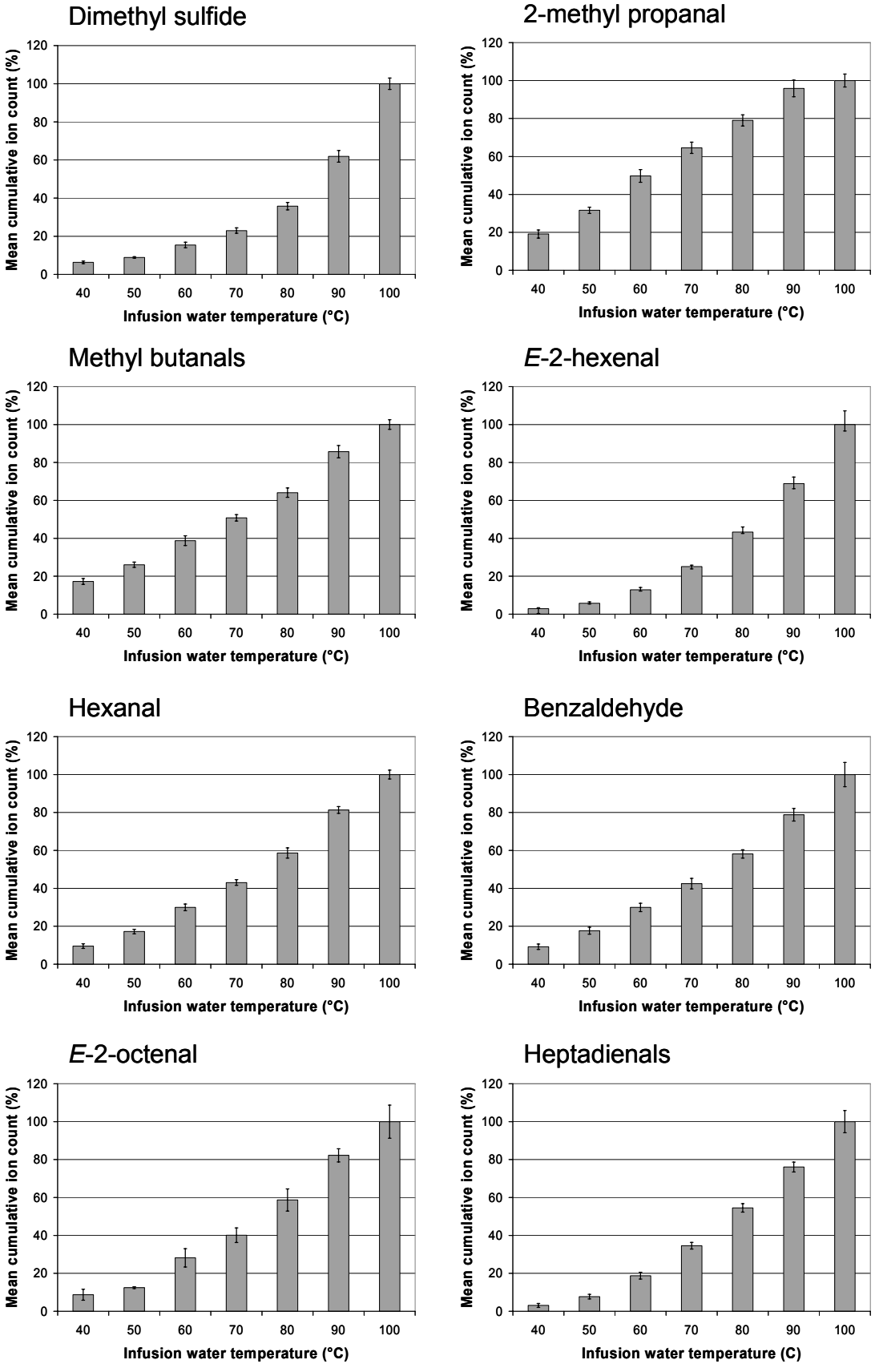
6.1.2 Sensory work

The orthonasal disriminability of infusions prepared according to different preparation methods has been successfully determined in the current study. These results should now be built upon, utilising descriptive analysis approaches to provide reasons for the discriminability of samples. Carrying out consumer preference testing on the various infusions is also an essential area of future work, particularly if the results of this study are to be applied to the commercial sector. As well as aroma, it is well known that many other factors are affected by infusion preparation method. Investigating the discriminability of infusions based upon other attributes such as taste and mouthfeel would yield valuable further information. Investigating the retronasal discriminability of infusions should also be explored, especially if retronasal release were studied using direct mass spectrometric techniques as described above.

In terms of perceptual interactions between key volatile and non-volatile constituents of tea infusions, the largest area of future work should be to explore potential reasons for the results obtained. Particular attention should be applied to enhancing the realism of samples, investigating the effect of subject training, and exploring cultural effects on congruency. Future work should also be carried out to further explore the effects of perceptual interactions in black tea infusions. This should involve investigating time-intensity procedures to look at maximum intensity and persistence of various attributes. It would also be appropriate to further explore the sensation of astringency, looking at the different sub-qualities, rather than the sensation as a whole.

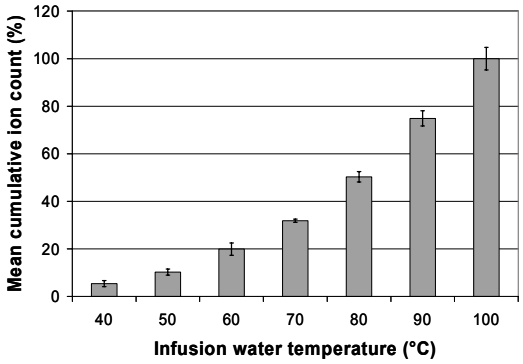
APPENDICES

Appendix 1 – Overall effect of infusion water temperature on release of selected volatile compounds from tea headspace

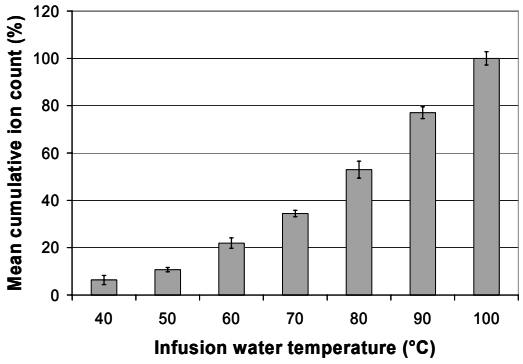


Appendix 1 (continued)

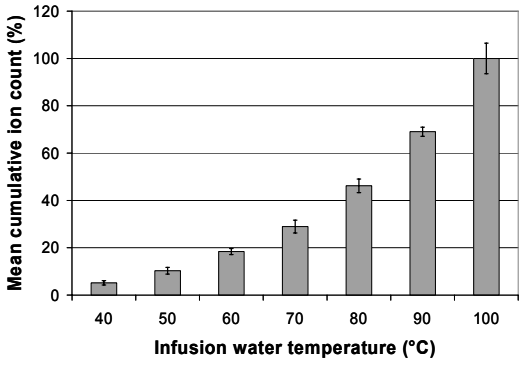
Heptenals



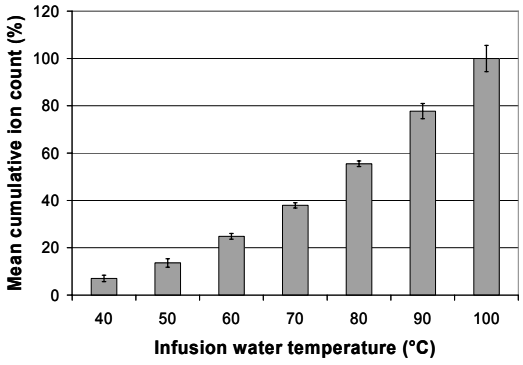
Heptanal



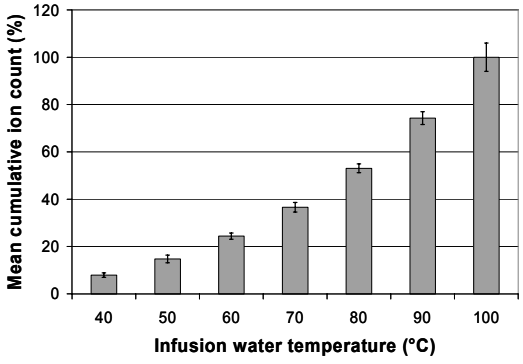
Phenylacetaldehyde



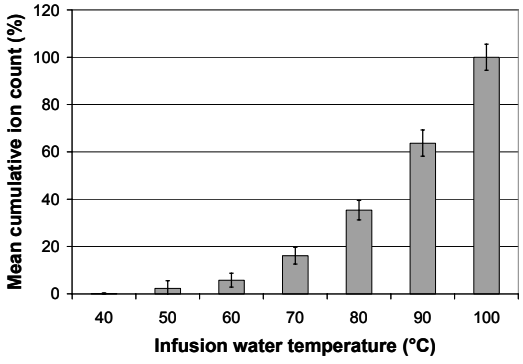
Linalool



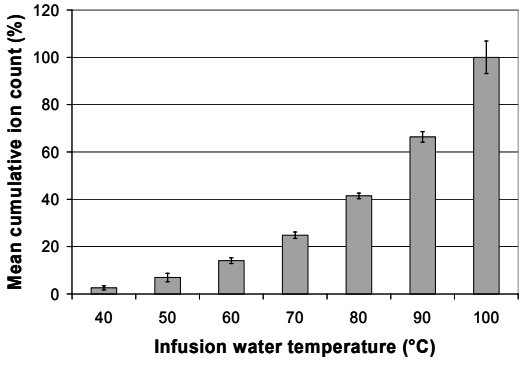
Methyl salicylate



β-damascenone

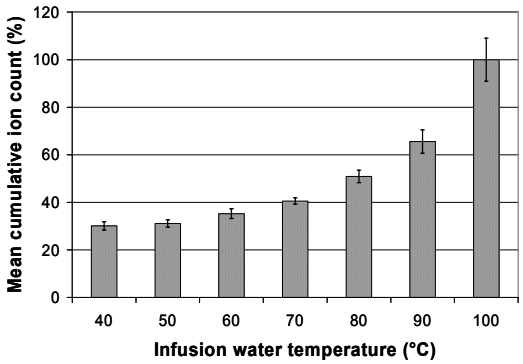


β-ionone

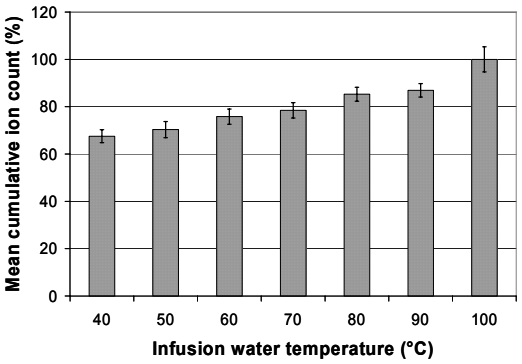


Appendix 2 – Release of volatile compounds from tea infusion headspace following 30 min incubation at 60 °C

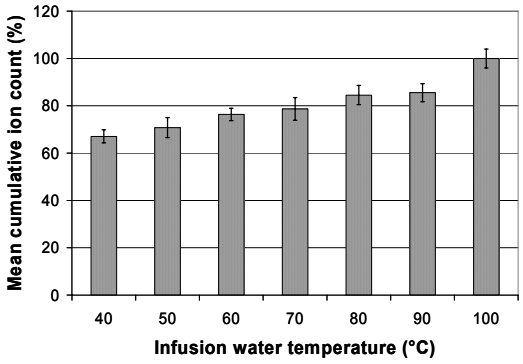
Dimethyl sulfide



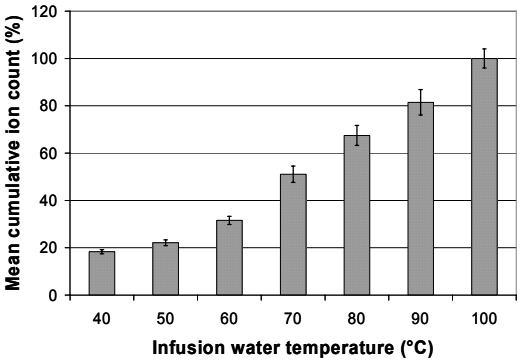
2-methyl propanal



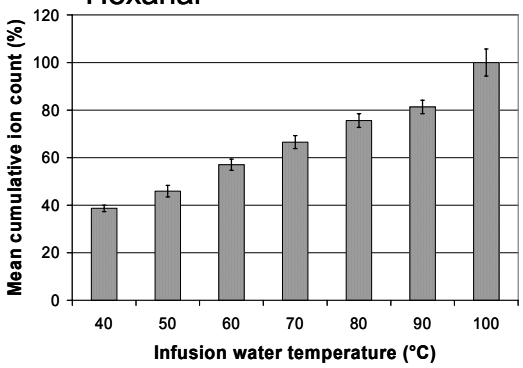
Methyl butanals



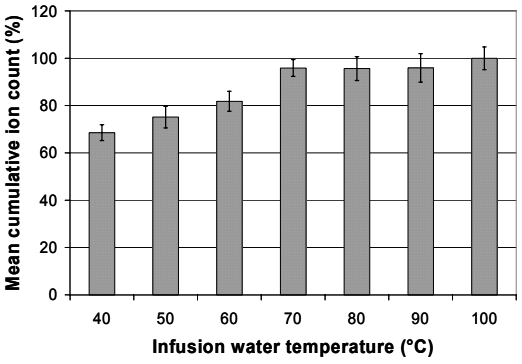
E-2-hexenal



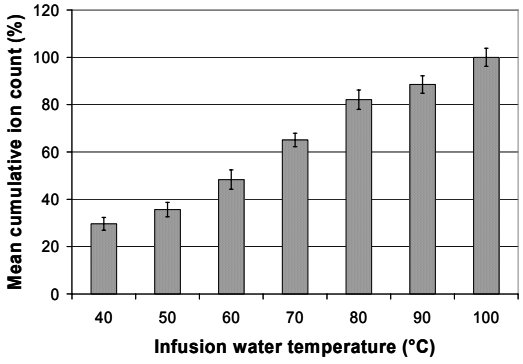
Hexanal



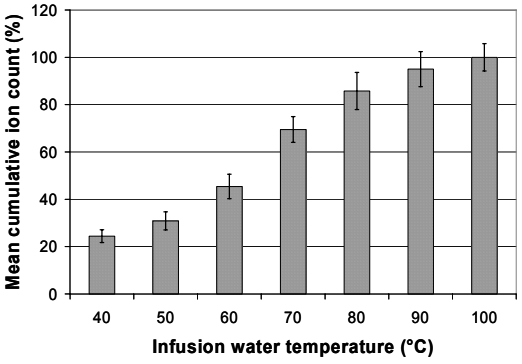
Benzaldehyde



E-2-octenal

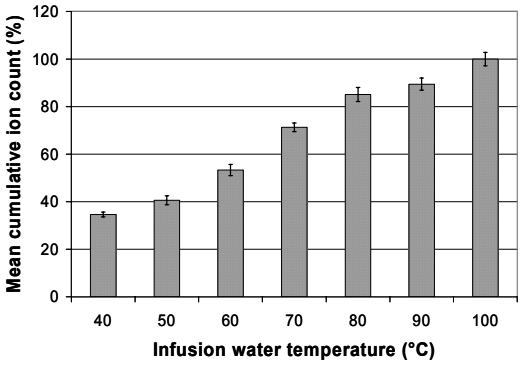


Heptadienals

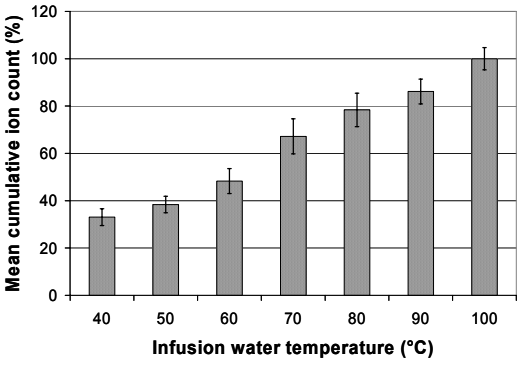


Appendix 2 – continued

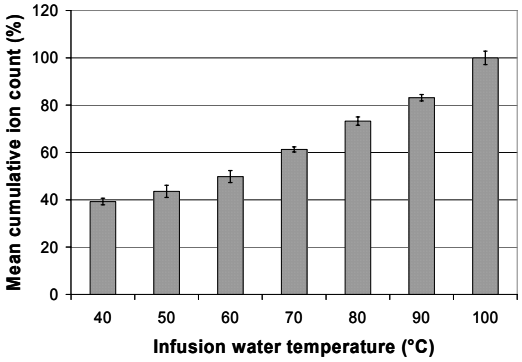
Heptenals



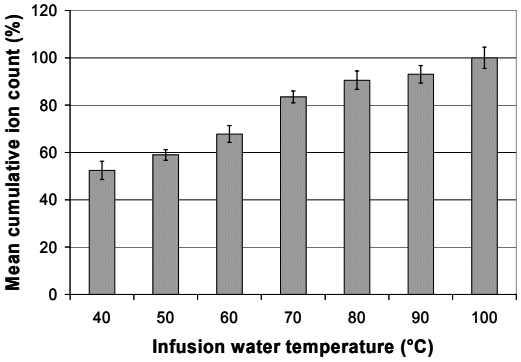
Heptanal



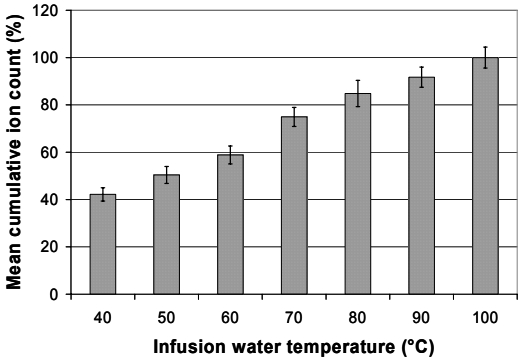
Phenylacetaldehyde



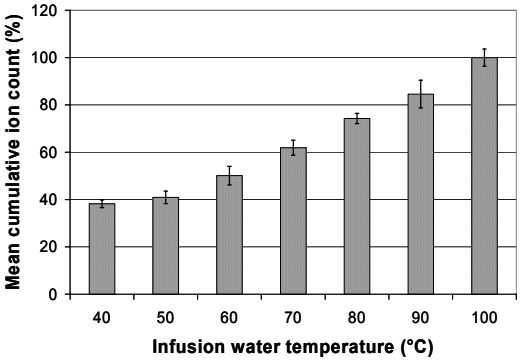
Linalool



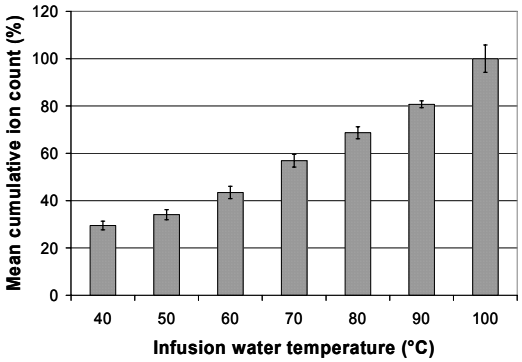
Methyl salicylate



β-damascenone

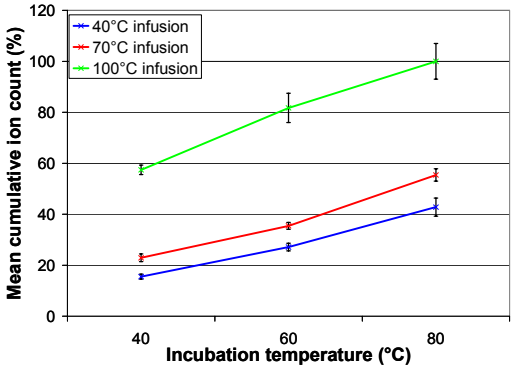


β-ionone

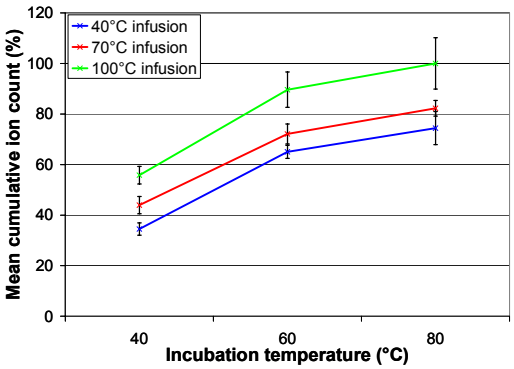


Appendix 3 - Relative effects of infusion and incubation temperature on release of volatile compounds from tea infusion headspace

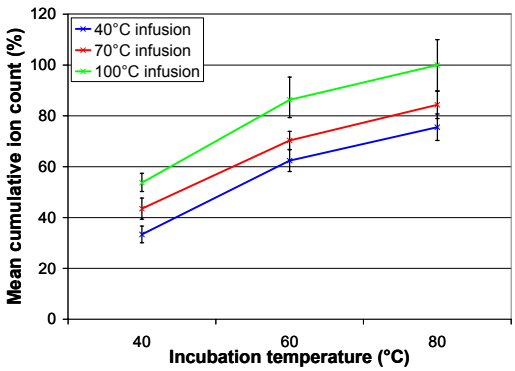
Dimethyl sulfide



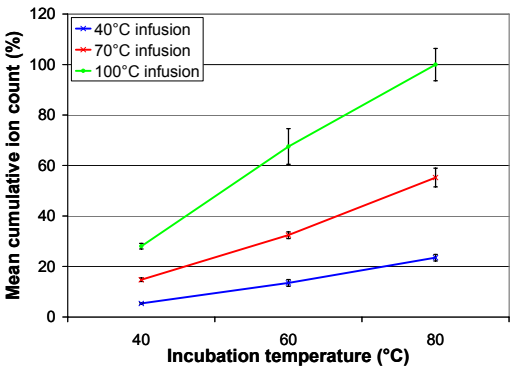
2-methyl propanal



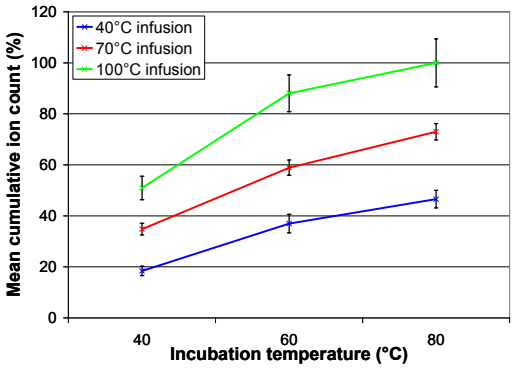
Methyl butanals



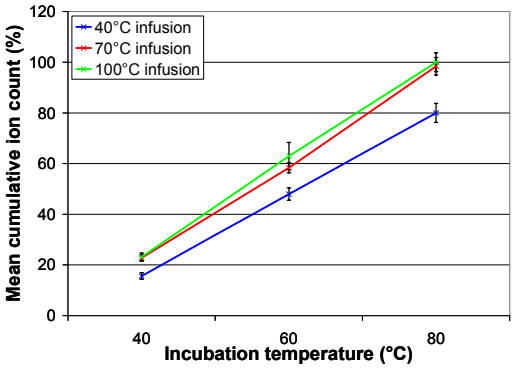
E-2-hexenal



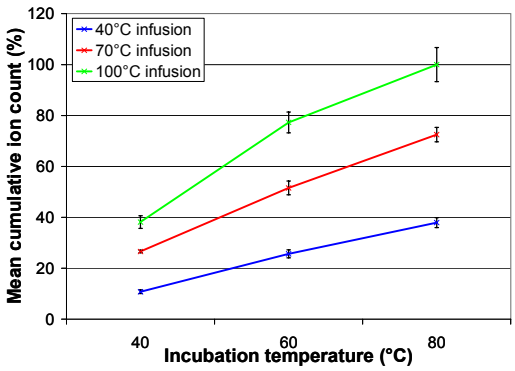
Hexanal



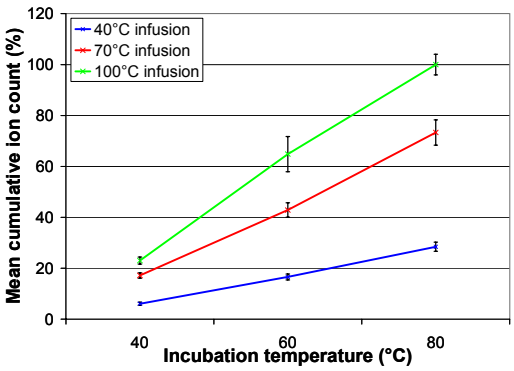
Benzaldehyde



E-2-octenal

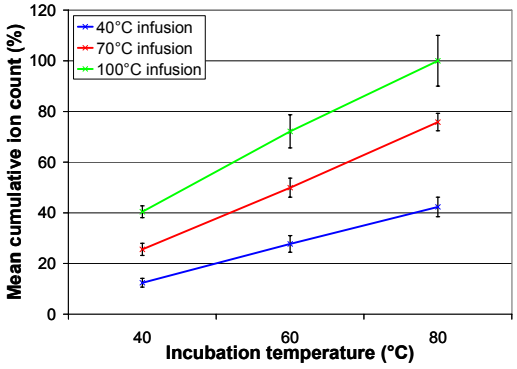


Heptadienals

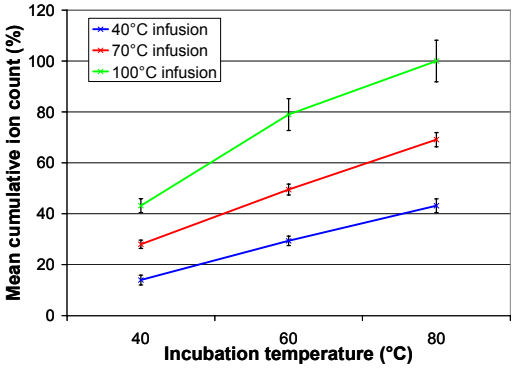


Appendix 3 – continued

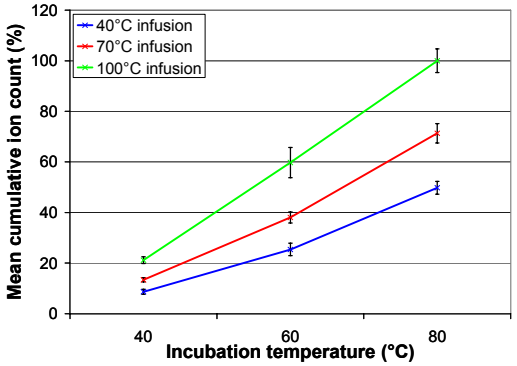
Heptenals



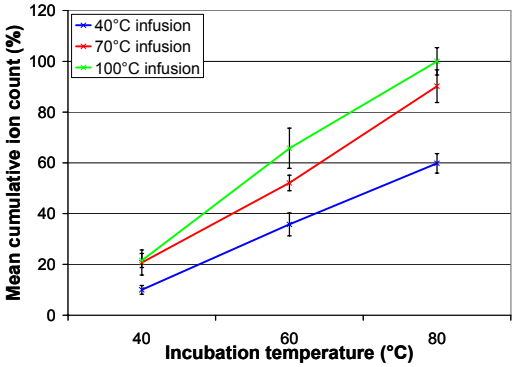
Heptanal



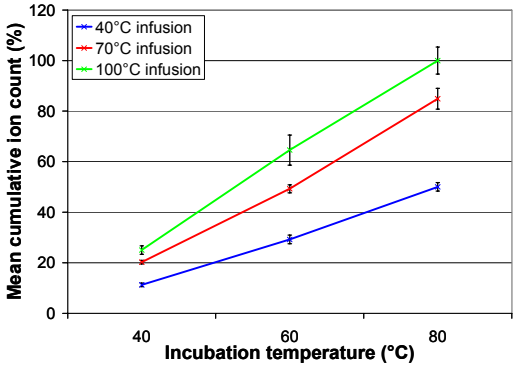
Phenylacetaldehyde



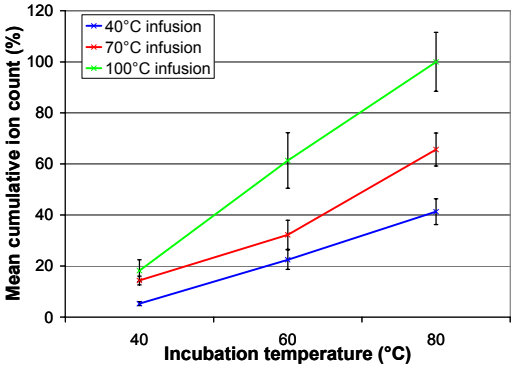
Linalool



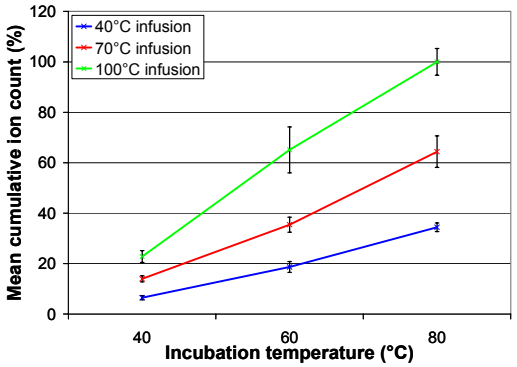
Methyl salicylate



β-damascenone

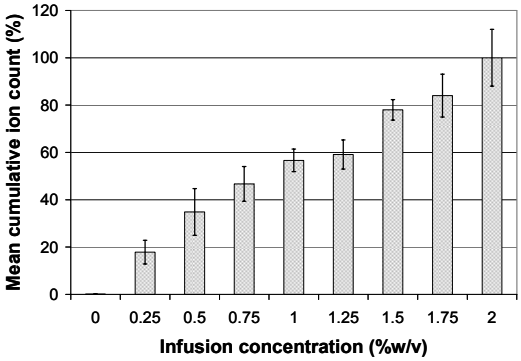


β-ionone

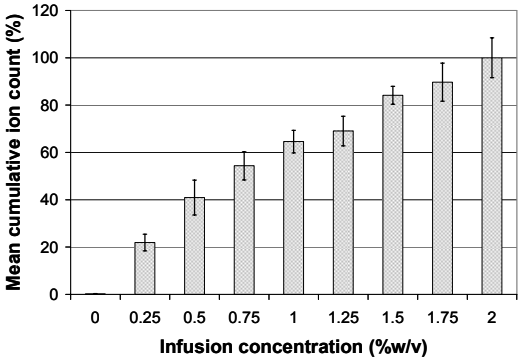


Appendix 4 – Effect of infusion concentration on release of volatile compounds from tea infusion headspace

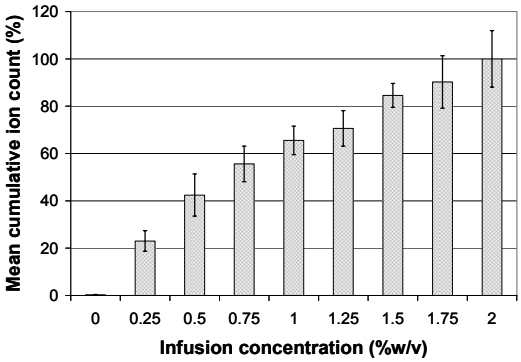
Dimethyl sulfide



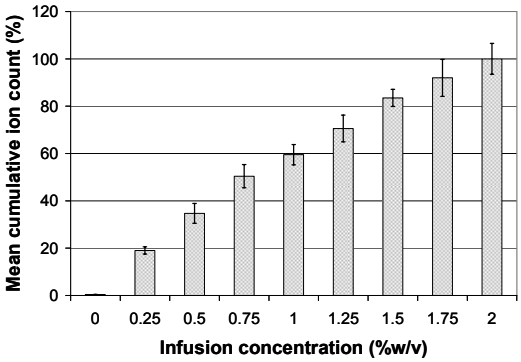
2-methyl propanal



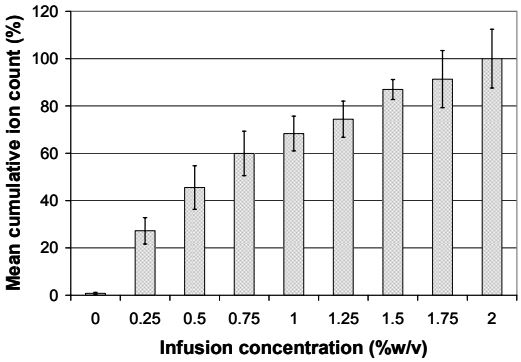
Methyl butanals



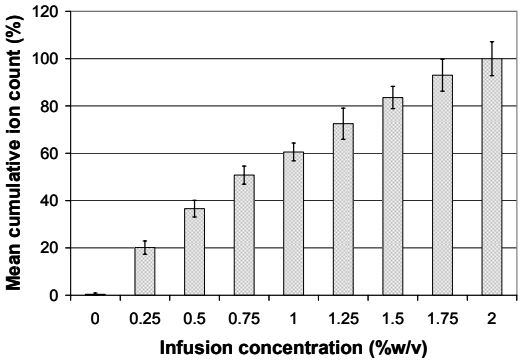
E-2-hexenal



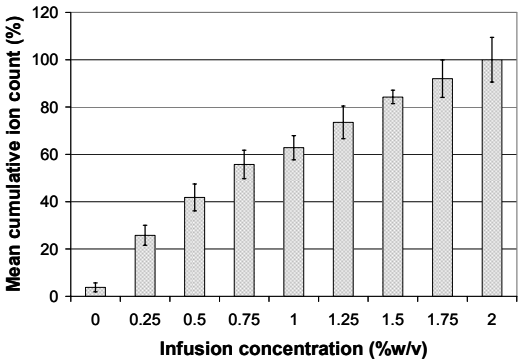
Hexanal



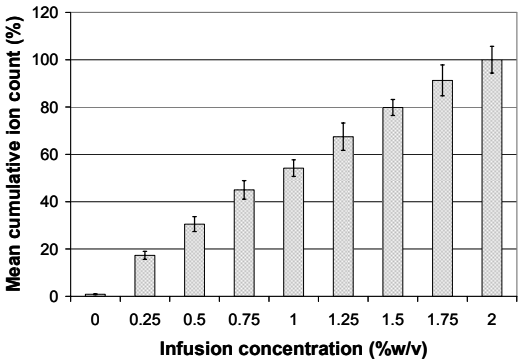
Benzaldehyde



E-2-octenal

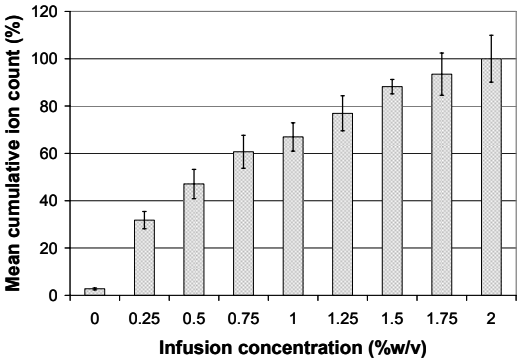


Heptadienals

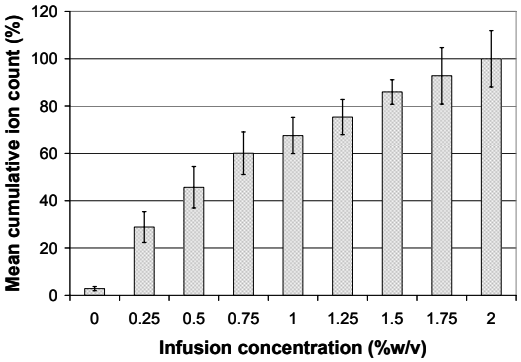


Appendix 4 - continued

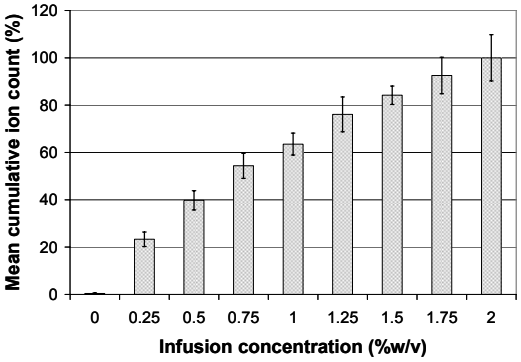
Heptenals



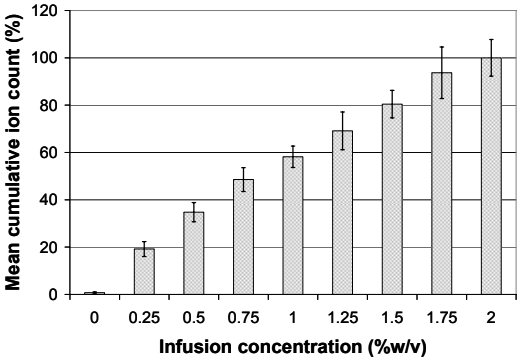
Heptanal



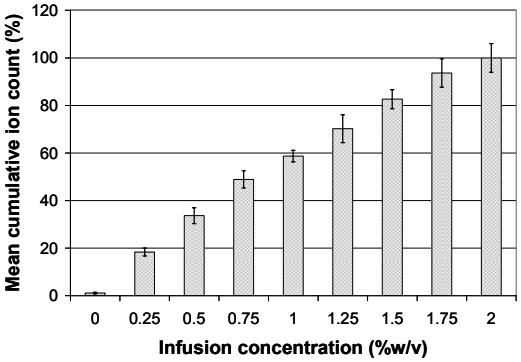
Phenylacetaldehyde



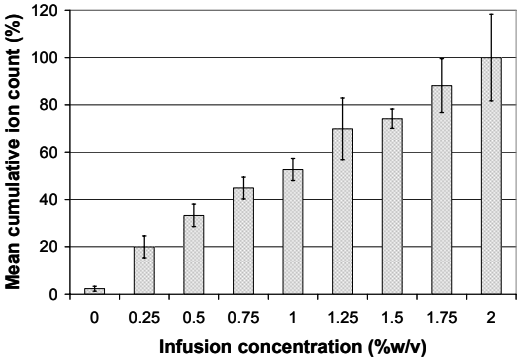
Linalool



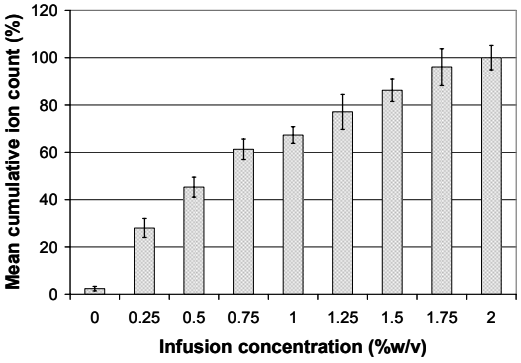
Methyl salicylate



β-damascenone

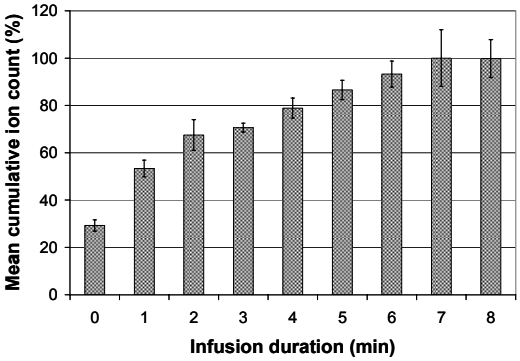


β-ionone

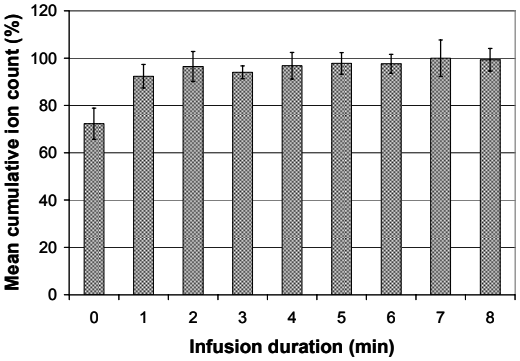


Appendix 5 – Effect of infusion duration on release of volatile compounds from tea infusion headspace

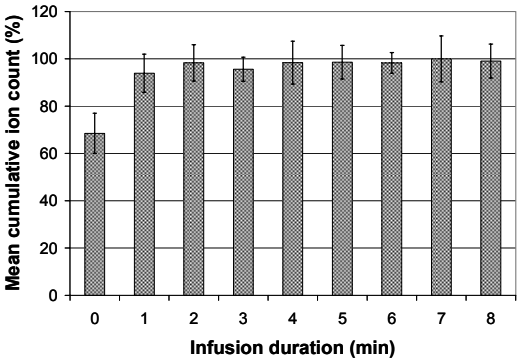
Dimethyl sulfide



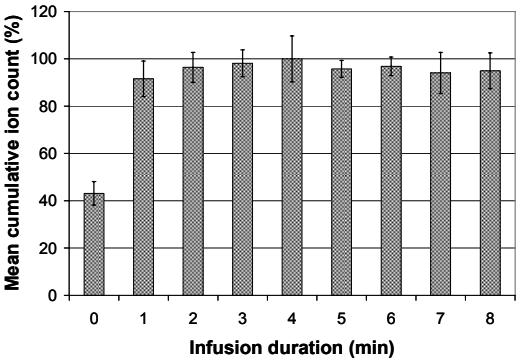
2-methyl propanal



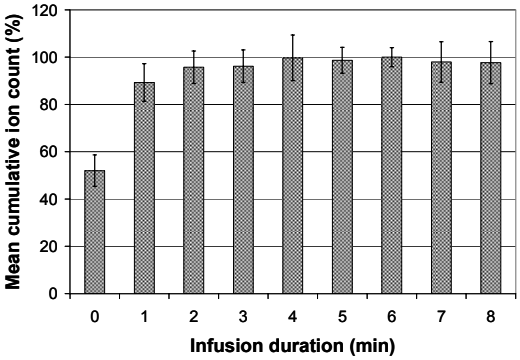
Methyl butanals



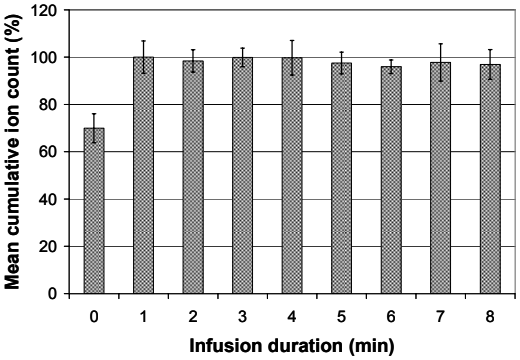
E-2-hexenal



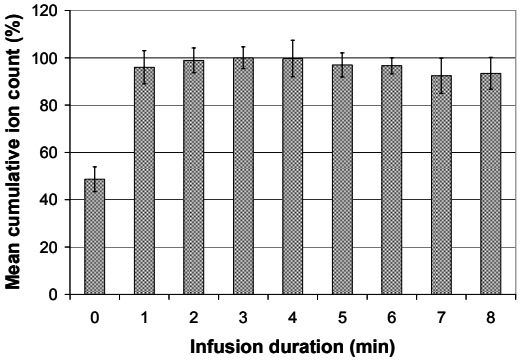
Hexanal



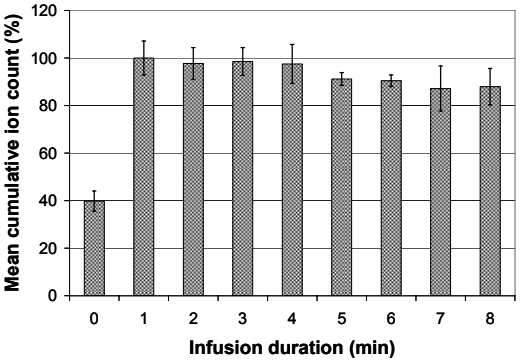
Benzaldehyde



E-2-octenal

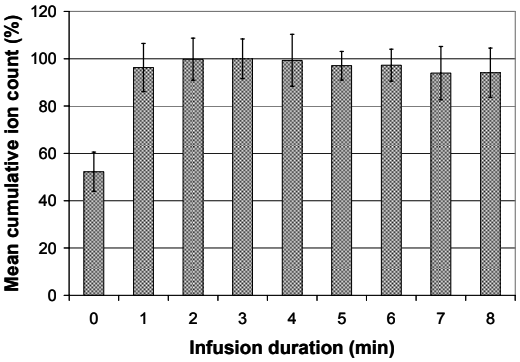


Heptadienals

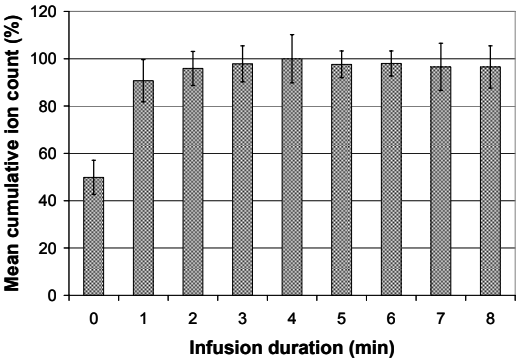


Appendix 5 – continued

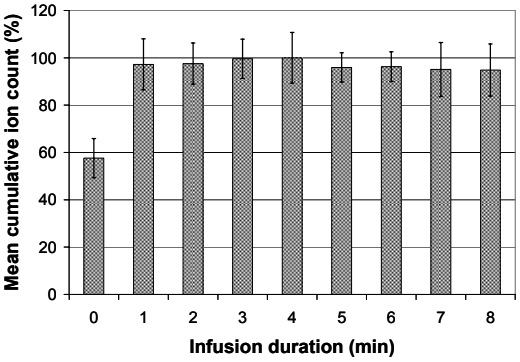
Heptenals



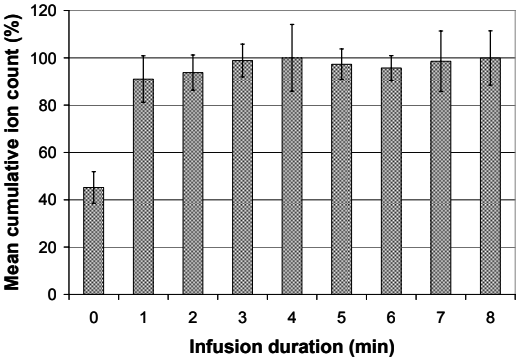
Heptanal



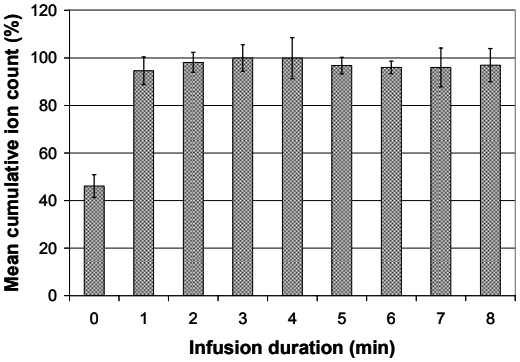
Phenylacetaldehyde



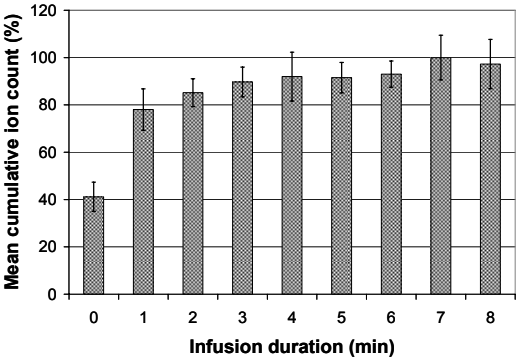
Linalool



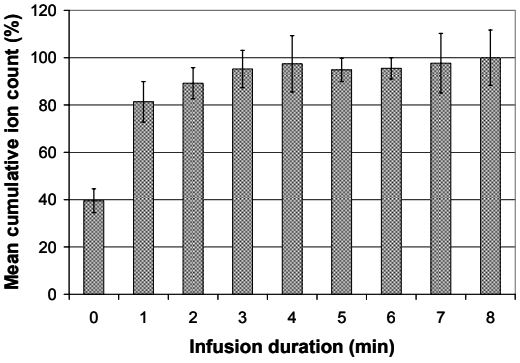
Methyl salicylate



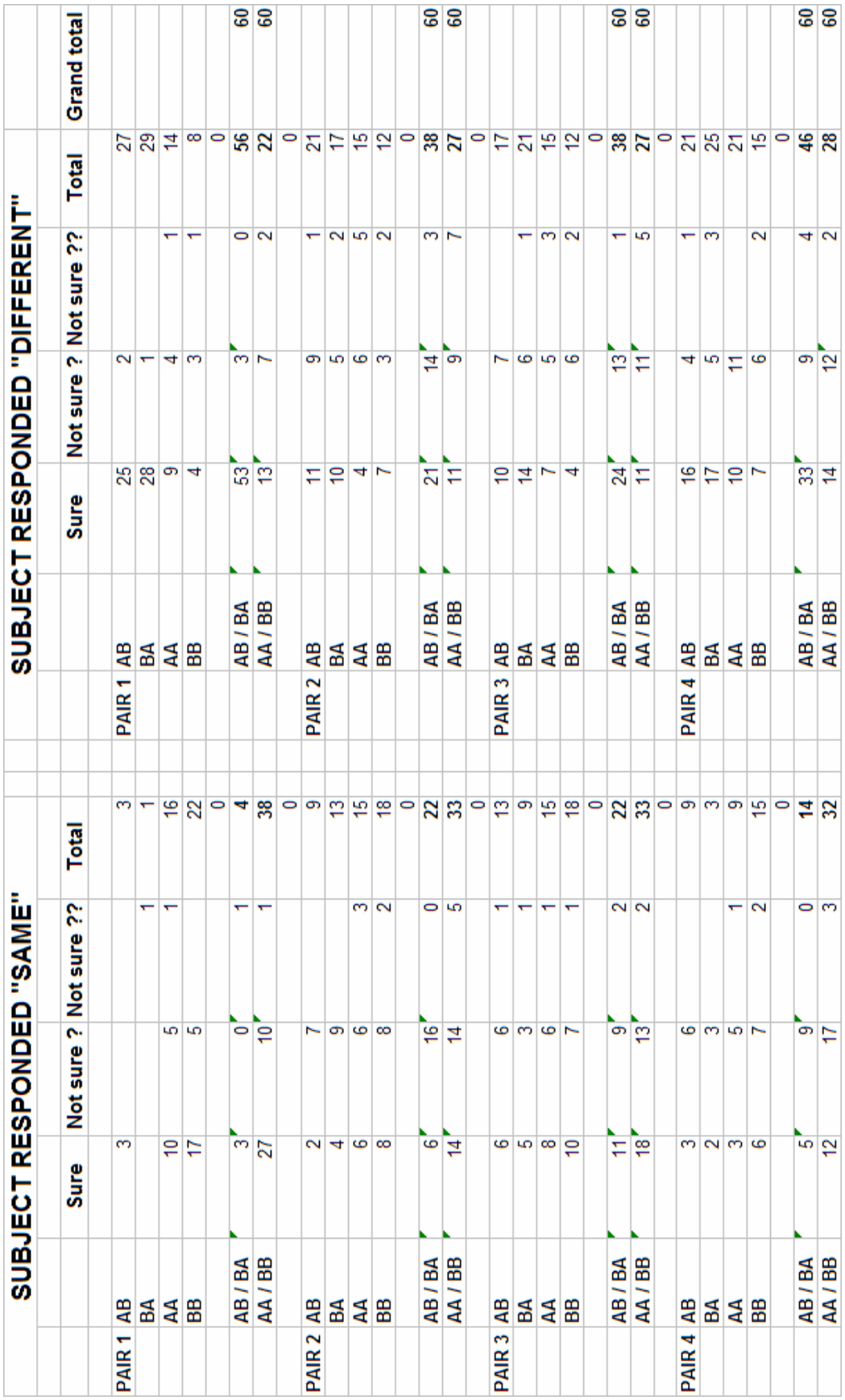
β-damascenone



β-ionone



Appendix 6 – Full breakdown of concentration discrimination test results

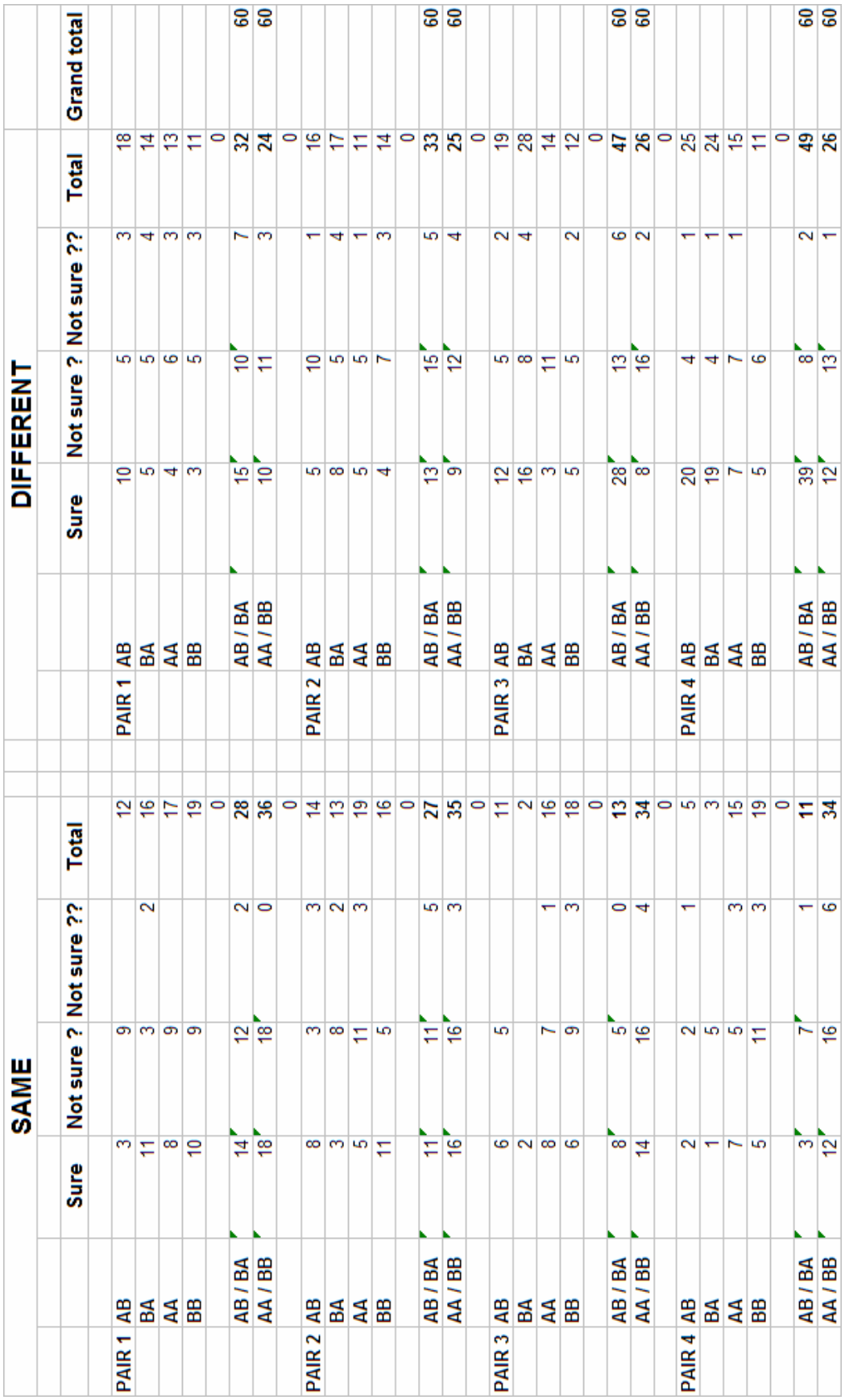


		SUBJECT RESPONDED "SAME"					SUBJECT RESPONDED "DIFFERENT"					
		Sure	Not sure ?	Not sure ??	Total		Sure	Not sure ?	Not sure ??	Total	Grand total
PAIR 1	AB	3			3	PAIR 1 AB	25	2		27	
	BA			1	1	BA	28	1		29	
	AA	10	5	1	16	AA	9	4	1	14	
	BB	17	5		22	BB	4	3	1	8	
					0					0	
	AB / BA	3	0	1	4	AB / BA	53	3	0	56	60
	AA / BB	27	10	1	38	AA / BB	13	7	2	22	60
					0					0	
PAIR 2	AB	2	7		9	PAIR 2 AB	11	9	1	21	
	BA	4	9		13	BA	10	5	2	17	
	AA	6	6	3	15	AA	4	6	5	15	
	BB	8	8	2	18	BB	7	3	2	12	
					0					0	
	AB / BA	6	16	0	22	AB / BA	21	14	3	38	60
	AA / BB	14	14	5	33	AA / BB	11	9	7	27	60
					0					0	
PAIR 3	AB	6	6	1	13	PAIR 3 AB	10	7		17	
	BA	5	3	1	9	BA	14	6	1	21	
	AA	8	6	1	15	AA	7	5	3	15	
	BB	10	7	1	18	BB	4	6	2	12	
					0					0	
	AB / BA	11	9	2	22	AB / BA	24	13	1	38	60
	AA / BB	18	13	2	33	AA / BB	11	11	5	27	60
					0					0	
PAIR 4	AB	3	6		9	PAIR 4 AB	16	4	1	21	
	BA	2	3		3	BA	17	5	3	25	
	AA	3	5	1	9	AA	10	11		21	
	BB	6	7	2	15	BB	7	6	2	15	
					0					0	
	AB / BA	5	9	0	14	AB / BA	33	9	4	46	60
	AA / BB	12	17	3	32	AA / BB	14	12	2	28	60

Appendix 6 – continued

SUBJECT RESPONDED "SAME"								SUBJECT RESPONDED "DIFFERENT"							
		Sure	Not sure ?	Not sure ??	Total				Sure	Not sure ?	Not sure ??	Total	Grand total
PAIR 5	AB	3	1		4	PAIR 5	AB		18	8		26	
	BA		1		1		BA		28	1		29	
	AA	6	7	2	15		AA		8	6	1	15	
	BB	8	6		14		BB		8	8		16	
					0							0	
	AB / BA	3	2	0	5		AB / BA		46	9	0	55	60
	AA / BB	14	13	2	29		AA / BB		16	14	1	31	60
					0							0	
PAIR 6	AB	7	6	1	14	PAIR 6	AB		7	8	1	16	
	BA	2	3		5		BA		16	7	2	25	
	AA	5	10	2	17		AA		7	5	1	13	
	BB	6	10	2	18		BB		7	4	1	12	
					0							0	
	AB / BA	9	9	1	19		AB / BA		23	15	3	41	60
	AA / BB	11	20	4	35		AA / BB		14	9	2	25	60
					0							0	
PAIR 7	AB	7	6	1	14	PAIR 7	AB		9	5	2	16	
	BA	8	5	1	14		BA		7	6	3	16	
	AA	8	5		13		AA		10	6	1	17	
	BB	7	10		17		BB		6	7		13	
					0							0	
	AB / BA	15	11	2	28		AB / BA		16	11	5	32	60
	AA / BB	15	15	0	30		AA / BB		16	13	1	30	60

Appendix 7 – Full breakdown of water temperature discrimination test results



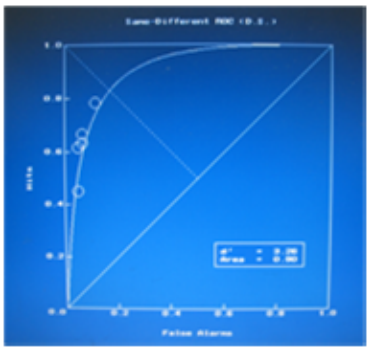
		SAME						DIFFERENT						
		Sure	Not sure ?	Not sure ??	Total			Sure	Not sure ?	Not sure ??	Total	Grand total
PAIR 1	AB	3	9		12	PAIR 1	AB	10	5	3	18	
	BA	11	3	2	16		BA	5	5	4	14	
	AA	8	9		17		AA	4	6	3	13	
	BB	10	9		19		BB	3	5	3	11	
					0						0	
	AB / BA	14	12	2	28		AB / BA	15	10	7	32	60
	AA / BB	18	18	0	36		AA / BB	10	11	3	24	60
					0						0	
PAIR 2	AB	8	3	3	14	PAIR 2	AB	5	10	1	16	
	BA	3	8	2	13		BA	8	5	4	17	
	AA	5	11	3	19		AA	5	5	1	11	
	BB	11	5		16		BB	4	7	3	14	
					0						0	
	AB / BA	11	11	5	27		AB / BA	13	15	5	33	60
	AA / BB	16	16	3	35		AA / BB	9	12	4	25	60
					0						0	
PAIR 3	AB	6	5		11	PAIR 3	AB	12	5	2	19	
	BA	2			2		BA	16	8	4	28	
	AA	8	7	1	16		AA	3	11		14	
	BB	6	9	3	18		BB	5	5	2	12	
					0						0	
	AB / BA	8	5	0	13		AB / BA	28	13	6	47	60
	AA / BB	14	16	4	34		AA / BB	8	16	2	26	60
					0						0	
PAIR 4	AB	2	2	1	5	PAIR 4	AB	20	4	1	25	
	BA	1	5		3		BA	19	4	1	24	
	AA	7	5	3	15		AA	7	7	1	15	
	BB	5	11	3	19		BB	5	6		11	
					0						0	
	AB / BA	3	7	1	11		AB / BA	39	8	2	49	60
	AA / BB	12	16	6	34		AA / BB	12	13	1	26	60

Appendix 7 – continued

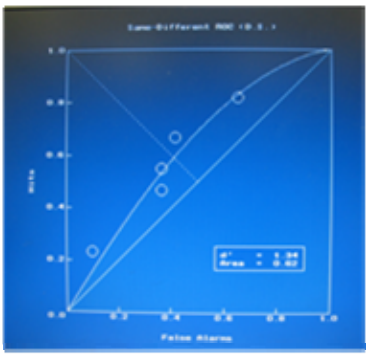
		SAME						DIFFERENT						
		Sure	Not sure ?	Not sure ??	Total			Sure	Not sure ?	Not sure ??	Total	Grand total
PAIR 5	AB	3	3		6	PAIR 5	AB	16	8		24	
	BA	4	3		7		BA	17	4	2	23	
	AA	10	4	3	17		AA	10	2	1	13	
	BB	12	5	1	18		BB	5	5	2	12	
					0						0	
	AB / BA	7	6	0	13		AB / BA	33	12	2	47	60
	AA / BB	22	9	4	35		AA / BB	15	7	3	25	60
					0						0	
PAIR 6	AB	4	4		8	PAIR 6	AB	18	2	2	22	
	BA	7	7	1	15		BA	8	6	1	15	
	AA	8	9	1	18		AA	5	4	3	12	
	BB	7	12		19		BB	5	5	1	11	
					0						0	
	AB / BA	11	11	1	23		AB / BA	26	8	3	37	60
	AA / BB	15	21	1	37		AA / BB	10	9	4	23	60
					0						0	
PAIR 7	AB	4	7	2	13	PAIR 7	AB	9	5	3	17	
	BA	8	6	1	15		BA	8	5	2	15	
	AA	11	9		20		AA	2	3	5	10	
	BB	8	8	1	17		BB	6	4	3	13	
					0						0	
	AB / BA	12	13	3	28		AB / BA	17	10	5	32	60
	AA / BB	19	17	1	37		AA / BB	9	13	1	23	60

Appendix 8 – ROC curves for the seven infusion concentration pairs

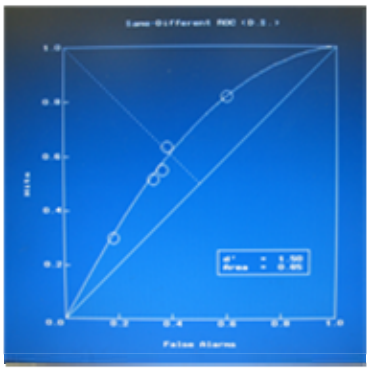
Pair 1



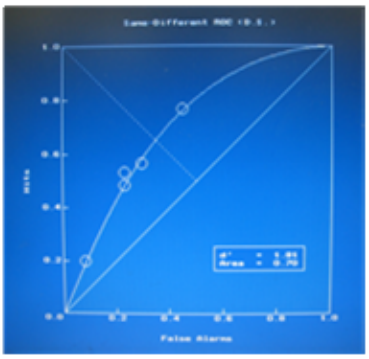
Pair 2



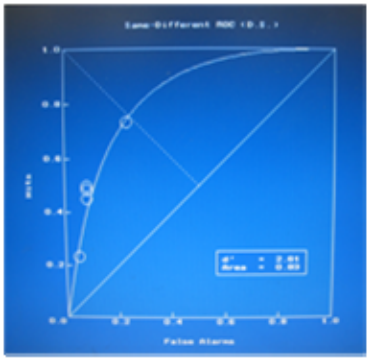
Pair 3



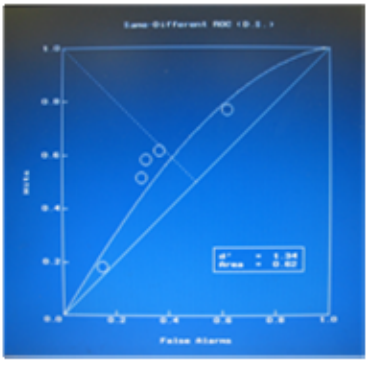
Pair 4



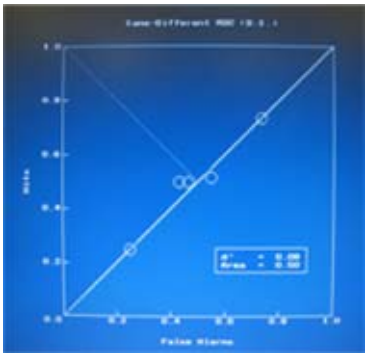
Pair 5



Pair 6

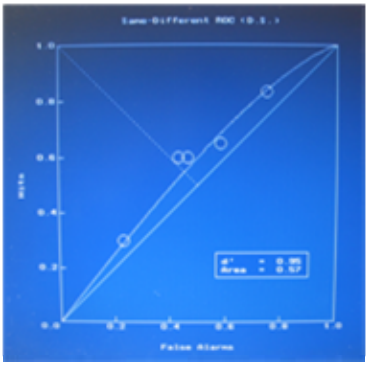


Pair 7

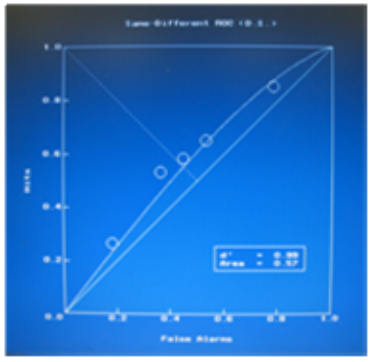


Appendix 9 – ROC curves for the seven infusion water temperature pairs

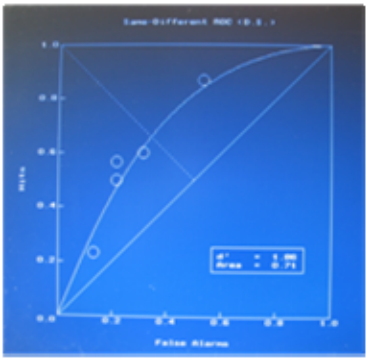
Pair 1



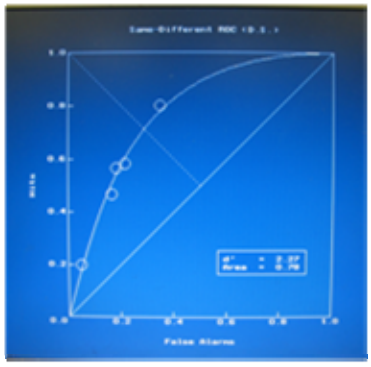
Pair 2



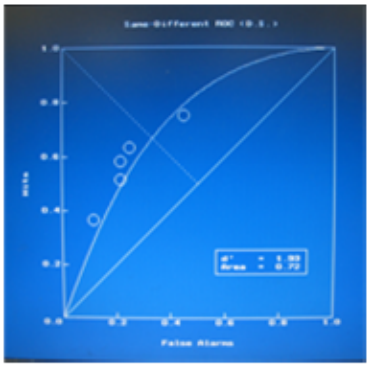
Pair 3



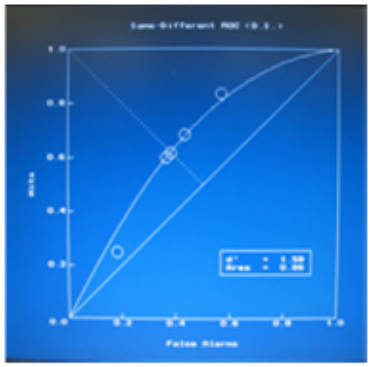
Pair 4



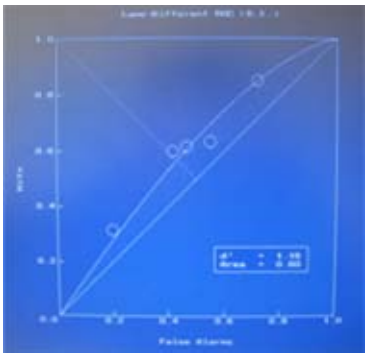
Pair 5



Pair 6



Pair 7



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