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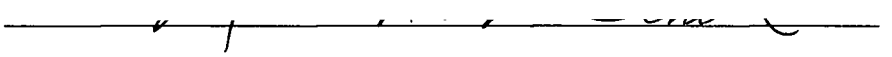

Mina R. McDaniel

Sub-threshold effects were studied in binary and tertiary mixtures comprising a panel-recognition-concentration odorant and sub-threshold odorant(s). Sub-threshold condition was maintained by controlling the sub-threshold concentration as percentages of subjects' individual detection threshold. The perceived intensities (overall intensity and several descriptors) of recognizable odorants were rated using magnitude estimation.

Sub-threshold suppression was common and concentration independent in mixtures comprising odorants with different functional groups. Suppression was observed at the lowest sub-threshold concentration tested (30% level). At sub-threshold concentrations, acetic acid suppressed the perceived intensity of

acetaldehyde and ethanol but not vice versa. Acetaldehyde and ethanol, however, suppressed each other when one was at sub-threshold concentrations in binary mixtures. Enhancement was observed in tertiary mixtures containing acetaldehyde at panel recognition concentration and was dependent on sub-threshold concentrations of acetic acid and ethanol.

In mixtures that contained aliphatic acids with different carbon chain lengths (acetic acid, propanoic acid and n-butanoic acid), sub-threshold enhancement and suppression depended on concentrations and molecular similarity of mixture components. Sub-threshold effects were not observed when the acids were two carbon-atoms different. 50% and 70% sub-threshold levels caused sub-threshold enhancement; however, higher concentrations caused decrease in intensity. Sub-threshold suppression was observed in mixtures containing n-butanoic acid as a recognizable odorants with propanoic acid at a 10% level in a binary mixture and acetic acid and propanoic acid in a 30%-30% combination in the tertiary mixture.

Sub-threshold Effects on the Perceived Intensity of Recognizable Odorants: The Roles of Functional Groups and Carbon Chain Lengths

by

Kannapon Lopetcharat

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APPROVED:

Major Professor, representing Food Science and Technology

Chair of Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kannapon Lopetcharat, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

SUB-THRESHOL EFFECTS ON THE PERCEIVED INTENSITY OF RECOGNIZABLE ODORANTS: THE ROLES OF FUNCTIONAL GROUPS AND CARBON CHAIN-LENGTH

Chapter 1

I. INTRODUCTION

Each person is different in his or her sensitivity toward odorants. The detection threshold, the minimum concentration of an odorant necessary for perceptual detection or awareness of the odorant (Lawless & Heymann, 1999), is a quantity used to measure sensitivity. Sub-threshold and suprathreshold are concentrations lower and higher than detection threshold of a given odorant. Everyday aromas emitted from many sources such as foods and flowers comprise odorants at various concentrations including sub-threshold and suprathreshold. Advances in analytical techniques such as gas chromatography, mass spectroscopy and gas-chromatography olfactometry objectively and subjectively aid scientists to identify and quantify many odorants in foods. For example, aroma active odorants are supra-threshold odorants that have an impact on aroma perception and the odorants are identified by gas chromatography olfactometric methods. However, qualitative and quantitative information on individual odorants cannot be used to predict the perception of mixtures. This unpredictable phenomenon is called

mixture interaction (Berglund et al., 1971; Berglund et al., 1973; Cometto-Muniz et al., 1997; Derby et al., 1991a, 1991b).

In supra-threshold mixtures, there are two types of mixture interactions: quality and intensity mixture interactions (Berglund et al., 1976; Derby et al., 1991a, 1991b). The former is a phenomenon that happens when the quality of mixtures changes from that of individual components. The later occurs when the intensity of mixtures or components changes, (Derby et al., 1991a). Suppression is a common phenomenon reported for intensity mixture interactions (Ache, 1989; Cain et al., 1995; Derby et al., 1985).

In addition, sub-threshold odorants are important in aroma perception but lack of research causes the limited understanding of the effects of sub-threshold odorants in mixtures. Low concentrations (often too low to be detected by instrumental measurements) and technical difficulties of sub-threshold odorants discourage researchers from studying them. Moreover, flavorists usually fail to mimic natural food aromas using only aroma active odorants (Plotto et al., 1998).

Determination of the detection thresholds of sub-threshold mixtures has resulted in indirect evidence suggesting sub-threshold effects; an additive effect was commonly reported (Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991; Patterson et al., 1993). However, in olfaction, there is no reported evidence about the effects of sub-threshold odorants on the perception of recognizable odorants. Consequently, this research was undertaken to systematically study, and to understand the effect of sub-threshold odorants on the

perception of recognizable odorants in mixtures. This research was divided into two major sections in which each section contains two parts as follows.

Section 1: Determination of the role of functional groups (carboxylic acid, aldehyde, and alcohol) in simple molecules: acetic acid, acetaldehyde and ethanol, and concentrations (percentages of individual detection thresholds) on the sub-threshold effect on the perceived intensity of recognizable odorants

- Part 1: Binary mixture study (a sub-threshold odorant and an odorant at panel-recognition concentration)
- Part 2: Tertiary mixture study (two sub-threshold odorants and an odorant at panel-recognition concentration)

Section 2: Determination of the role of carbon chain-lengths (two, three, and four carbon atoms) in simple molecules: acetic acid, propanoic acid and n-butanoic acid, and concentrations (percentages of individual detection thresholds) of sub-threshold aliphatic acids on the sub-threshold effect on the perceived intensity of recognizable aliphatic acids

- Part 1: Binary mixture study (a sub-threshold odorant and an odorant at panel-recognition concentration)
- Part 2: Tertiary mixture study (two sub-threshold odorants and an odorant at panel-recognition concentration)

Chapter 2

II. LITERATURE REVIEW

II.1 Olfaction

1. Introduction

The discriminatory power of the olfactory system is incredibly high compared to other modalities. The olfactory system is tuned to detect and identify an immense variety of odorous molecules of different shapes and sizes that may be present in an infinitesimal quantity in an environment. The present olfactory systems in vertebrates including humans have evolved unique anatomical structures in which a highly efficient information-processing system occurs. Some basic structures and processes are also shared with lower level animals such as insects and crustaceans (Ache & Restrepo, 2000; Hildebrand & Shepherd, 1997). Information-processing takes place on the olfactory epithelium in the nasal cavity where the odorous molecules (odorants) from the external environment encounter olfactory sensory neurons. Information from the odorants is transmitted to the olfactory bulb of the brain where it is relayed and transmitted further to higher-order structures of the brain.

This review discusses current knowledge concerning the olfactory system of air-breathing vertebrates, especially humans. Basic structures and anatomy of

some olfactory organs will be introduced. Furthermore, how the olfactory system transduces, encodes and processes information at various levels in the neural pathway will be discussed, followed by strategies that the olfactory system employs to handle an immense amount of information from the environment. Studying the olfactory system in invertebrates may yield further insight about the underling mechanisms of information coding in the olfactory system as genetic research approaches become more sophisticated.

2. Structure and anatomy of the olfactory system

2.1 Nasal cavity and olfactory mucosa

The olfactory system of most air-breathing vertebrates contains at least two distinct olfactory organs: olfactory mucosa and vomeronasal organ (Smith, 2000b). This section will focus exclusively on the olfactory mucosa and related topics.

The first organ is the nasal cavity (Figure II.1). There are three small passages (meatus) in the nasal cavity created by the lateral protrusion of three nasal turbinates (conchas): the superior, middle, and inferior meatus. The primary function of the nasal cavity is for the passage of air containing odorants to reach the olfactory epithelium. In conjunction with the respiratory system, the nasal cavity confines and swirls the air from the external environment within the cavity. This increases the concentration of odorous molecules and adjusts the temperature of the

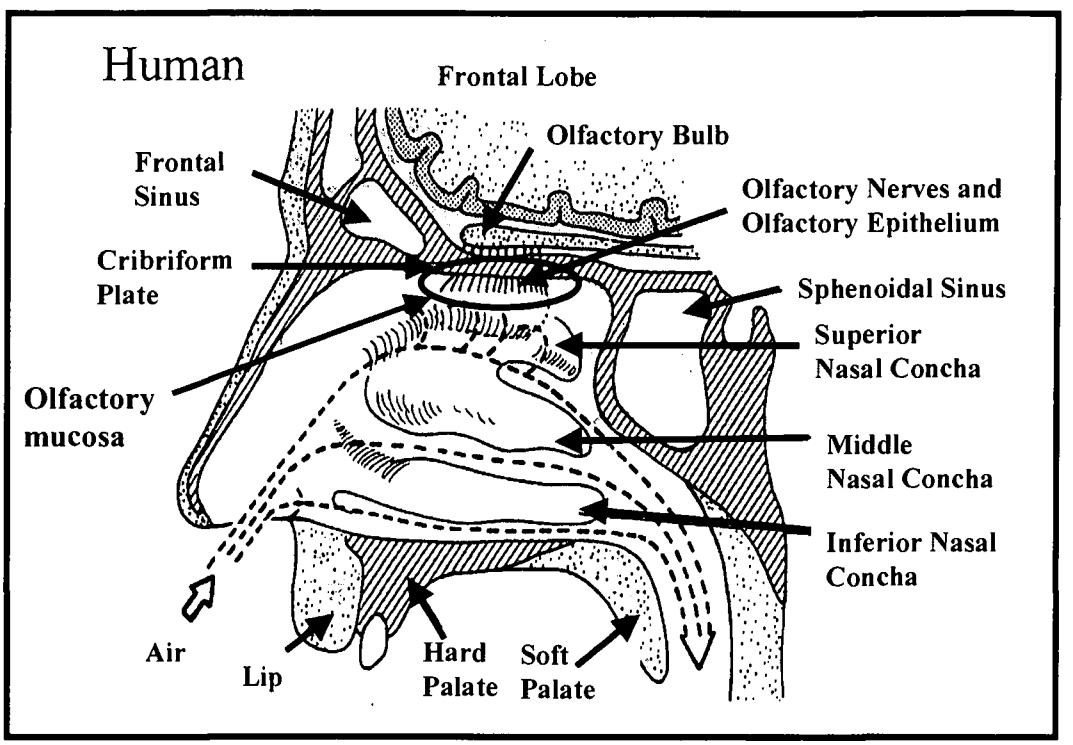

Figure II.1 Nasal cavity and olfactory mucosa

air entering the olfactory and respiratory systems to approximate body temperature (Takagi, 1989).

The olfactory mucosa consists of three layers: the olfactory epithelium, the basal lamina and the lamina propria. The olfactory mucosa covers the upper most part of the nasal cavity between the septum and the inner surface of the superior turbinate, called the olfactory cleft (Figure II.1). The olfactory mucosa in higher primates including humans (~2-6cm^2) is relatively small compared to dog (~18-21cm^2), rabbit (~9 cm^2) or other mammal olfactory epitheliums (Smith, 2000b; Takagi, 1989). The thickness of human olfactory epithelium is ~30-60 μm and the entire human olfactory mucosa is approximately 480-500 μm thick depending on the extent of the nasal venous blood flow (Takagi, 1989).

The first layer is the olfactory epithelium. There are three major types of cells in the olfactory epithelium: supporting cells, neurosensory cells (also called sensory neurons) and basal cells (Figure II.2) (Smith, 2000b; Takagi, 1989). Free nerve endings of the trigeminal system are also found in the olfactory epithelium (Takagi, 1989). The nuclei of these three cellular elements are arranged sequentially from the surface inward. The supporting cells' nuclei are found on the top layer (surface) followed by the olfactory sensory neurons' nuclei in the middle layer and the basal cells' nuclei in the bottom layer, respectively.

Supporting cells are the first type of cells in the olfactory epithelium. Supporting cells support the neurosensory cells and secrete mucus. The second cell type in the olfactory epithelium is the neurosensory cell. Neurosensory cells, also

called olfactory sensory neurons or olfactory receptor cells (ORCs), are the first component of the olfactory system that contacts the odorants. The ORCs' cell body is columnar with a round cytoplasmic body (~8-10 μm in diameter) and can be seen in the middle layer of the olfactory epithelium. Researchers have reported many types of ORCs in different species (Takagi, 1989). The ORCs are generally accepted as bipolar neurons with a single unbranched dendrite and a single axon (Buck, 1996; Smith, 2000b; Takagi, 1989). The dendrite of the ORCs squeezes between the supporting cells and forms a knob at the end of the dendrite called the olfactory knob or the olfactory vesicle (Figure II.2). The length of the ORCs' dendrites ranges from 10 to 100 μm depending on the depth of the cytoplasmic body (Takagi, 1989).

The number of cilia projected from a single olfactory knob has been reported from none to 1,000 depending on the type of ORCs and species (Smith, 2000b; Takagi, 1989). The number 10 cilia/olfactory knob in humans was reported (Kanda et al., 1973). In humans, most cilia projected from each olfactory knob lie horizontally and are embedded in the epithelial mucus forming a dense mat; however, some cilia align perpendicularly with the olfactory epithelium (Takagi, 1989). Human olfactory cilia are ~1-2 μm in length and ~0.1 μm in diameter (Takagi, 1989). Each cilium membrane contains a high density of globular particles which are believed to be the olfactory receptor sites (Smith, 2000b). Intramembrane particles (IMPs) were first discovered in bovine (Menco et al., 1976). The density of the IMPs is believed to be a differentiating index between

sensory cilia and non-sensory cilia (Takagi, 1989). Menco and others (1976) calculated the concentration of IMPs as 0.2 (in frog) to $3x10^{19}$ (in mammal) particles/liter or 3 (in frog) to 25 μM (in mammal) if each particle is assumed to be a single molecule (Takagi, 1989). The receptor sites are located in the region of the olfactory knob and proximal portion of the cilia (Getchell et al., 1980). On the contrary, the 5'-AMP-specific olfactory receptor sites were found to locate along the entire outer dendritic segment of the ORCs (Blaurstein et al., 1993).

The axons of the ORCs project directly into the olfactory bulb (OB). The ORCs are stimulated by odorants and convert the information to neuronal signals, which are later transduced at higher levels in the olfactory system and the brain, producing perceptions and responses. The number of the ORCs in many animals has been estimated with various counting methods but there is much discrepancy in the results (Takagi, 1989). Kanda and others (1973) reported 30,000 ORCs/mm^2 in human. Employing the estimated sizes of the olfactory epithelium (2.4-6.4 cm^2) and the estimated density of ORCs/mm^2, the total number of human ORCs can be extrapolated, from 7.2 to 19.2 million cells. Another report was about 6-7 x 10^6 cells in adult humans (Moran et al., 1982).

The last cell type of the olfactory epithelium is the basal cell. Basal cells function as regeneration units and constitute the base of the olfactory epithelium. Basal cells have the ability as stem cells to divide and form new functional neurons. Therefore, the degraded ORCs can be replaced with freshly generated neurons

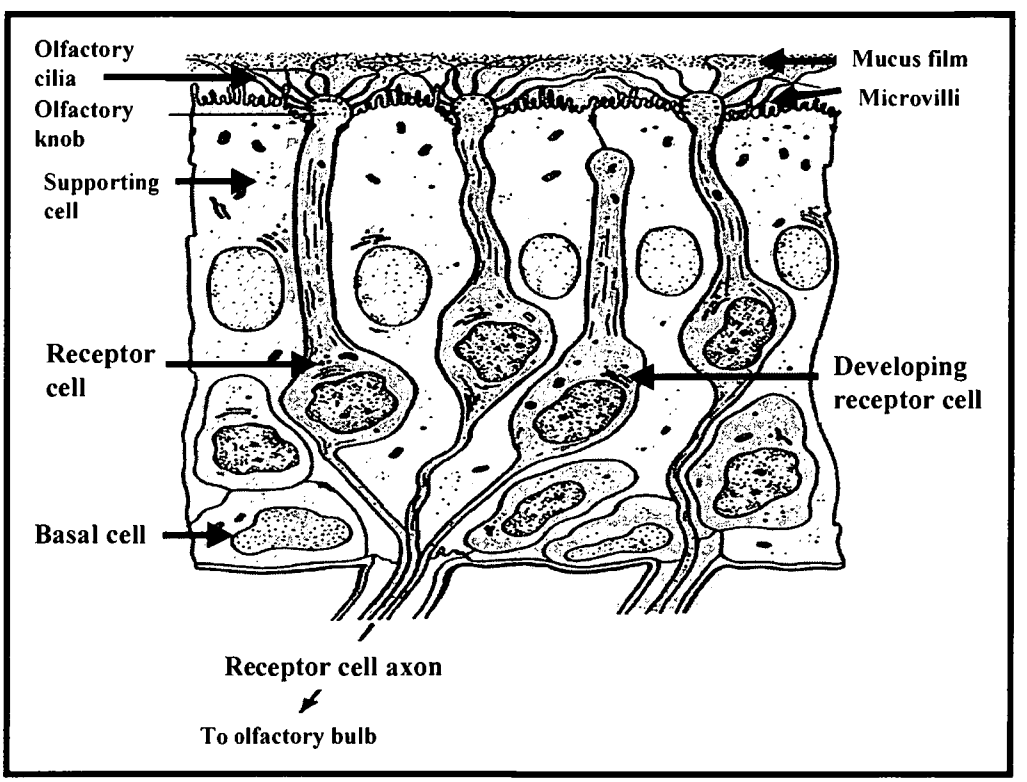

Figure II.2 Cellular organization in the olfactory epithelium

derived from the basal cells throughout life (Smith, 2000b; Takagi, 1989). Regeneration of the basal cells in pigeon and rat was reported to take 30-60 days with the regeneration cycle of the olfactory epithelium taking 30-60 days (Buck, 1996).

The second layer of the olfactory mucosa is the basal lamina layer (Figure II.2). This layer is a membrane separating the olfactory epithelium and the lamina propria. The basal cells lie on this membrane with the end of the supporting cells and the starting of the axons projected from the ORCs.

The final layer of the olfactory mucosa is the lamina propria. The lamina propria contains olfactory axon bundles, Schwann cells, blood vessels and olfactory gland or Bowman's gland. The nonmyelinated nerve fibers and the olfactory axons bundle together to form nerve bundles projecting directly to the olfactory bulb. The Schwann cells direct the olfactory nerve bundles to the designated location in the olfactory bulb. The Bowman's gland found in the lamina propria function as a mucus generator as found in supporting cell in the olfactory epithelium (Buck, 1996). However, the composition of the mucus secreted from the Bowman's gland is different from that of the olfactory supporting cells. The mucus from the Bowman's gland contains only neutral mucopolysaccharides whereas the mucus from the olfactory supporting cells contains both neutral and acidic mucopolysaccharides (Getchell et al., 1984).

The thickness of the mucus layer varies from region to region as well as from species to species. The mucus layer protects the olfactory epithelium from

dryness, extreme temperature, particles and pathogenic contamination (Takagi, 1989). In addition, the mucus layer also acts as the first odorant-screening center of the olfactory system.

The mucus is hydrophilic in nature and not homogeneous. It can be divided into several domains (Ache & Restrepo, 2000). In order to contact olfactory sensory neurons or olfactory receptor cells, all odorants (mostly hydrophobic molecules) must be able to dissolve in the olfactory mucus layer (hydrophilic layer).

The olfactory mucus also provides ions necessary for the activities of the olfactory neurons and supporting cells (Takagi, 1989). Besides protection and screening roles, the olfactory mucus also removes odorants from the olfactory epithelium by means of mucus flow, desorption of odorants into the air and uptaking odorants into the circulatory system (Getchell et al., 1984; Hornung & Mozell, 1977).

The mucus is generated from two major sources: supporting cells in the olfactory epithelium and Bowman's glands in the lamina propria. Mucus secretion in supporting cells is governed by two mechanisms: a low-level and a rapid apocrine discharge. Mucus secreted by the low-level mechanism is the protective mucus on the epithelium surface. Supporting cells secrete the protective mucus continuously to replenish the old protective mucus (Getchell et al., 1988). The rapid apocrine discharge is triggered by the stimulation from odorants. This

mechanism is believed to produce a large amount of mucus in a short time in reaction to noxious odorants (Getchell et al., 1988).

An agonist-induced mechanism is believed to govern mucus secretion in the Bowman's glands in the lamina propria layer and other parts of the body (Getchell et al., 1988). This mechanism is initiated by the stimulation of both odorant and trigeminal receptors in the olfactory epithelium, especially by noxious odorants such as volatile acids. The stimulation of odorant receptors results in transduction of the olfactory neurons. The stimulation of the trigeminal cells triggers two pathways. First, the signal from the trigeminal nerves travels through the central nervous system, signaling the Bowman's glands to secrete mucus. The other pathway is the intraepithelial release of substance-P (an immunoreactive neurotransmitter), which is secreted by "substance-P fibers" found in lamina propria layer in close association with the Bowman's glands and blood vessels. Substance-P reacts with possible receptors on the olfactory epithelium, which activate the supporting cells and the Bowman's glands resulting in secretion of mucus (Getchell et al., 1988).

In summary, there are two olfactory organs exposed to the external environment: the nasal cavity and the olfactory mucosa. The nasal cavity acts as an air passage, thermal stabilizer, and agitator. The olfactory mucosa is visually classified into three layers: the olfactory epithelium, basal lamina and lamina propria layers. There are three major olfactory cell types found in the olfactory epithelium, the supporting cells (secreting mucus), the ORCs (detecting and

sending neuronal signal) and the basal cells (cell regenerators). Bowman's glands found in lamina propria secrete mucus in conjunction with supporting cells forming a mucus layer covering the olfactory epithelium.

2.2 Olfactory bulb

The olfactory bulb is located above the olfactory epithelium (Figure II.1). The cribriform plate separates the olfactory bulb from the olfactory epithelium, however, axons from the ORCs extrude through perforations in the cribriform plate to enter the olfactory bulb.

The olfactory bulb is classified into six major anatomical layers: the olfactory nerved layer (ONL), the glomerular layer (GL), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL) and the granule cell layer (GCL) (Figure II.3).

The first layer is the olfactory nerved layer (ONL). In this layer, unmyelinated axons from the ORCs enter the olfactory bulb. The glomerular layer (GL) is the location of the complex synaptic terminals between the ORCs and mitral cells and the ORCs and tufted cells. A spherical region formed by the complex synaptic terminals is called a glomerulus with the convergence ratio approximately equal to 1,000:1 (the ORCs axons: a single mitral cell aborisation) (Figure II.3) (Smith, 2000b). The approximated number of mitral cell aborisations forming a glomerulus is 1,000-2,000. It means that there are about 25,000 axons from the ORCs that converge on a single glomerulus. There are approximately

8,000 glomeruli in each olfactory bulb in young adult human (Meisami et al., 1998).

Besides, the ORCs' axons, periglomelurus cells (PGCs) located in between glomeruli are also found in the GL layer. The PGCs' axons are short and extrude into the glomerulus layer; meanwhile, the PGCs' dendrites synapse with the neighboring glomerulus. There is good evidence to support that these PGCs are inhibitory (Smith, 2000b).

After the GL, there are three continuous layers: the external plexiform layer (EPL), the mitral cell layer (MCL) and the internal plexiform layer (IPL). In the EPL, ~500 μm thick, there are two sublayers: superficial and deep EPL. Tufted cell somata, dendrites and the mitral cell dendrites are found in the superficial EPL. In the deep EPL, secondary dendrites and cell bodies of displaced mitral cells are found (Takagi, 1989). Tufted cells are not located only in this layer but are distributed in many layers. Therefore, there are three subclasses of tufted cells based on location in the olfactory bulb: external, middle and internal (Takagi, 1989). The majority of cells in the MCL are mitral cells. The somata of the mitral cells can be found in this layer. Mitral cells are classified into two types: mitral cells and displaced mitral cells. The mitral cells are found in the mitral cell layer (MCL) and the displaced mitral cells are found in the deepest portion of the external plexiform layer (EPL) which is close to the MCL. The similarities between these two cell types are shape and their response to the lateral olfactory tract (Takagi, 1989). From this layer mitral cells project their principal dendrite to

a single glomerulus, many secondary dendrites to the EPL and an axon to the deeper layer of the olfactory bulb.

The IPL consists of axons from mitral cells and tufted cells, which are recurrent and have complex synapses between each other. The synapses between the axons of mitral cells and tufted and dendrites of granule cells start around the border of the IPL layer.

The deepest layer in the olfactory bulb is the granule cell layer (GCL). The majority of cells in this layer are granule cells (GCs) (Figure II.3). The output from this layer is specifically inhibitory for the mitral cells and tufted cells' dendrites (Smith, 2000b; Takagi, 1989).

The complicated organization of the olfactory bulb with specific cell types in each layer is a very important feature, which allows the olfactory system to manage information from the external environment.

2.3 Olfactory cortex and brain

From the olfactory bulb, the axons from the mitral and tufted cells form the olfactory tracts from which the neuronal signals are transmitted to a higher-level in the brain. The olfactory tracts terminate in many regions of the brain: orbito frontal cortex, thalamus, lateral hypothalamus, pyriform cortex, etc. In each region, the neuronal signals are processed simultaneously and the sensory signals will be perceived, transmitted and expressed as behavioral responses toward the odorants.

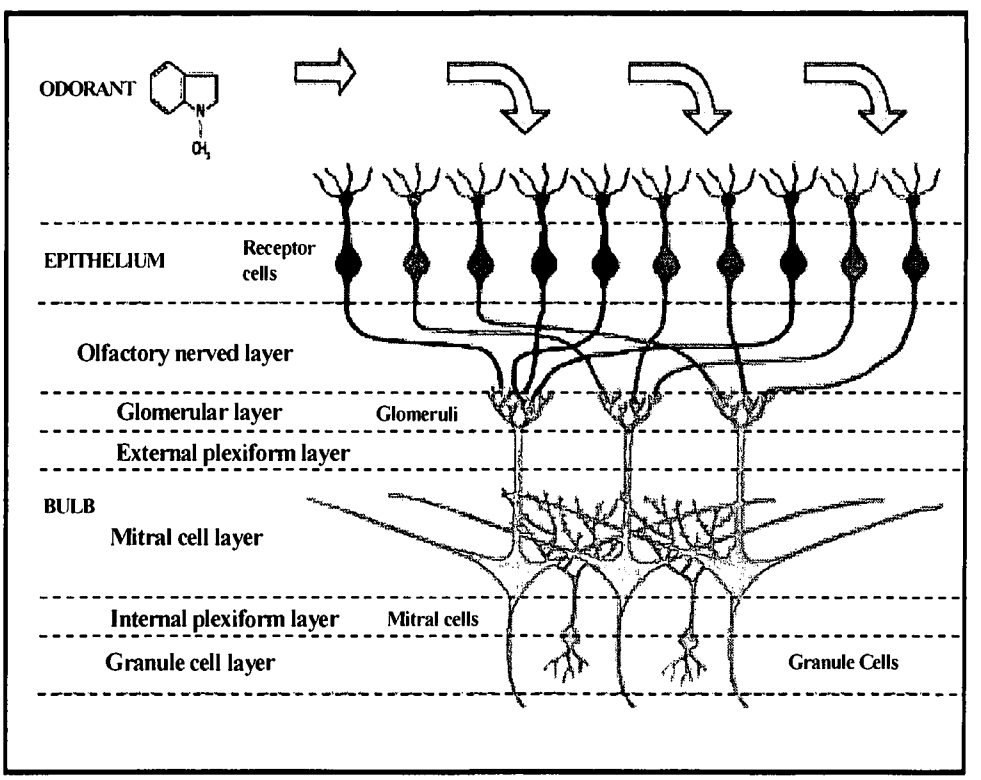

Figure II.3 Cellular organization in the olfactory bulb

The olfactory pathway within the olfactory bulb and basal forebrain was distinguished into six circuits based on multiple neuronal circuit theory (Shepherd et al., 1981). The six circuits are as follows:

(a) The pure sensory circuits: these circuits conveys pure sensory signal from the ORCs through the olfactory bulb to the olfactory cortex, the thalamus and other diencephalic areas in the brains (the hypothalamus, substantia innominata, and the septum).

(b) The circuits formed by axon collaterals of olfactory cortical neurons that terminate in various areas to the olfactory cortex and in the olfactory bulb. These circuits are believed to create the rhythmic brain waves observed in the olfactory bulb and periform cortex.

(c) The circuits formed by sensory input pathways to the limbic system. These circuits are believed to contribute to the affective quality of odor perceptions.

(d) The limbic system modulating circuits: these circuits are formed by the connection directly and indirectly of the brain regions i.e. hypothalamus, hippocampus, etc. that constitute to the limbic system to the olfactory bulb.

(e) The limbic circuits formed within the olfactory bulb and base forebrain independently of olfactory input. The reciprocal synapses between the mitral cells and the granule cells may be considered as an element in these circuits.

(f) The central olfactory-limbic-midbrain circuits: these circuits are believed to control the forebrain arousal system.

The functions and responses of these circuits are still unclear. More research is needed to solve the mysteries of the brain.

3. Information coding and signal transduction

3.1 Olfactory mucosa level

3.1.1 Perireceptor events: Olfactory mucus and odorant binding proteins

As stated in section 2.1, nasal mucus provides the ORCs with a suitable environment for changes in the ciliary membrane potential that is critical to signal generation in the ORCs (Getchell, 1974). Chloride, sodium, potassium, phosphorus, and sulfur ions are found in the olfactory mucus with different distribution within the mucus layer (Ache & Restrepo, 2000). Nasal mucus produced by the supporting cells and Bowman's glands acts as the first screening center in the olfactory system. The odorants must be able to dissolve in, partition into and traverse the nasal mucus in order to be recognized by the ORCs (Buck, 1996).

There are small protein-molecules found in the nasal mucus called the soluble odorant-binding proteins (OBPs). The OBPs belong to a large family of

ligand carrier proteins known as the lipocalin family (Buck, 1996). The OBPs are believed to shuttle hydrophobic odorants through the olfactory mucus (Sengupta & Carlson, 2000). Different OBPs are specialized to bind to different structural classes of odorants (Buck, 1996). Moreover, the OBPs might enhance the detection by presenting and/or removing the odorants from cognate receptors, which allows higher odorant concentration and rapid recovery (Breer, 1994; Pevsner & Snyder, 1990; Vogt et al., 1991). On the contrary, Lazar and others (2001) presented evidence to discredit the delivering function of the OBPs. Instead, odorant sequestering was proposed (Lazar et al., 2001).

Odorants are different in their partitioning ability because of different solubility, diffusibility and specificity to the OBPs and nasal mucus. The chromatographic-like model was proposed in order to explain different sorption behaviors of different odorants in nasal mucus (Mozell, 1964, 1970; Mozell & Jagodowicz, 1973). The chromatographic-like model could not completely explain the sorption phenomena because it failed to take into account many factors such as the role of OBPs and heterogeneity of the mucus layer. However, this model is still valid for odorants that do not depend on the OBPs and/or distribute in the homogenous zone of the mucus layer, such as some polar odorants. The exact function of the OBPs and the behavior of odorants in the mucus layer are still unknown and controversial.

3.1.2 Odorant receptors

In the early 1960s, the stereochemical theory of odor was pr0posed (Amoore et al., 1964). The stereochemical theory was derived from data gathered from specific anosmic subjects and research on stereo-isomers of many odorants. Physiological studies revealed that an olfactory neuron reacts with a variety of odorants but it may not be activated at all by certain odorants (Derby et al., 1991a; Simon & Derby, 1995). This phenomenon confirms the specificity of the olfactory receptor neuron.

Because of the specificity of the olfactory receptor neurons, the idea of ligand binding analogous to the concept of specific affinity in immunology was introduced. In this model, the olfactory receptors are defined as "those molecular structures that undertake the initial interaction with the stimulatory ligand" (Lancet, 1988). Because of the hydrophobic nature of most odorants and stereo specificity of the olfactory receptors, the olfactory receptors are presumed to be proteins, which bind the odorants, undergo changes and initiate the neuronal transduction events (Lancet, 1988).

A unique developmentally regulated protein called olfactory marker protein (OMP) was discovered (Margolis, 1972). The OMP is a product of tissue-specific gene expression for the olfaction system (Margolis, 1988). Moreover, olfactory neurons were also found to be rich in GTP-binding protein (G-protien) and adenylate cyclase (Margolis, 1988). A candidate receptor molecule was believed to be a unique transmembrane glycoprotein found in olfactory cilia (Chen et al.,

1986). The complement DNA (cDNA) of the rat olfactory receptor was first identified in 1991 by Dr. Linda Buck and Dr. Richard Axel (Buck & Axel, 1991). There are ~500-1000 olfactory receptor genes in mammals (Buck, 1996).

The olfactory receptor site is believed to be a G-protein coupled protein that belongs to the G-protein coupled receptor superfamily (Buck, 1996). The G-protein coupled receptor protein is a transmembrane protein with a unique 7-fold conformation with the N-terminus exposed to the extracellular region and the C-terminus exposed to the intracellular region. The G-protein coupled receptor protein is also called the 7-transmembrane protein (7TM protein) (Buck, 1996; McClintock, 2000). 7TM proteins are found abundantly in most chemosensory organs. Because of the unique folding conformation and genetically governed expression of amino acid sequences in the olfactory receptor transmembrane protein, selective receptor pockets can be created employing the same mechanism that governs enzyme (the receptor)-substrate (the odorant ligand) specificity (Buck & Axel, 1991). These findings suggest that specific anosmia and lacking of smell in stereo-isomer odorants are genetic problems.

3.1.3 Signal transduction and information coding at the receptor level

Information coding: Since the cloning of rat olfactory receptor genes by Dr. Linda Buck and Dr. Richard Axel in 1991, molecular studies in olfaction have progressed quickly. There are three important assumptions that led to the isolation of odorant receptor genes (Mombaerts, 1999):

(a) The receptors in the olfaction system are likely to be G-protein coupled receptors.

(b) Odorant receptors are likely encoded by members of a large gene family.

(c) The conservation of a certain amino acid motif within 7TM proteins expressed in the olfactory epithelium.

In humans, there are about 500-750 genes governing the expression of the olfactory receptor (OR) sites (Buck, 1996; Buck & Axel, 1991). The expression of each olfactory gene is found to be limited to a small fraction of the olfactory neurons, which leads to the assumption that each neuron directly expresses a single olfactory gene (Buck, 1996; Mombaerts, 1999). However, there is no conclusive evidence about the one gene-one neuron assumption or co-expression of multiple genes within one olfactory neuron either (Mombaerts, 1999).

Physiological studies show that many neurons in different regions of the olfactory epithelium respond to a single odorant and the number of neurons stimulated depends on the concentration of the odorants (Edwards et al., 1988).

Moreover, each olfactory neuron also reacts with many odorants (Sicard & Holley, 1984). With this evidence, the odor alphabet theory was proposed suggesting that each odor is a result of the combination of signals from many ORs (Buck & Axel, 1991).

The organization of the ORs is not random. Spatial patterns of odor receptor expression were proposed with the evidence from studies involving rats and mice (Ressler et al., 1993; Ressler et al., 1994). Ressler and others (1993) discovered four distinct spatial zones in which different sets of the OR genes were expressed. Olfactory neurons expressing the same OR genes or any OR genes that are members of the same subfamily scatter randomly within the same spatial zone and the genes presumably recognize the same odors (Buck, 1996; Ressler et al., 1993; Ressler et al., 1994). These zones patterns are bilaterally symmetrical in both nasal cavities (Buck, 1996). Therefore, odor information is encoded by the combination and the location of the ORs. The odor information is believed to be maintained when it is transmitted to the olfactory bulb (Buck, 1996).

Signal transduction: Olfactory signal transduction begins with an odor-bound receptor activating the GTP-binding protein (G-protein), which regulates the production of intracellular second messengers such as cAMP (adenosine 3'5'-cyclic monophosphate), $InsP_3$ (inositol-1,4,5-triphosphate), etc. A two-stage amplification cascade is directly or indirectly triggered by these second messengers. In the first stage, ion channels that generate the initial receptor current are activated and subsequently they trigger second ion channels that carry the

majority of the receptor current. The net receptor current depolarizes the cell generating a local receptor potential that passively spreads throughout the cell. Then it activates voltage activated "Hodgkin-Huxley" type ion channels that are capable of generating action potentials that self-propagate down the axon of the cell to the first relay center, the glomeruli in the olfactory bulb. In turn, the result of this sequence of events is the large molecular amplification of the signal at minimal expense to the signal to noise ratio of the system (Ache & Restrepo, 2000; Buck, 1996).

Signal termination: An observation supporting signal termination in the olfactory system is the fact that adaptation is an important feature of this system. Adaptation is a failure to continue smelling an odor over extended periods of exposure (Buck, 1996).

There are two key elements in the signal transduction process involving signal termination: the odorant-receptor and the cyclic nucleotide mediated ion-gates (CNGs). The odorant-receptor element involves two important components: the odorant and the receptor. Removing the odorant or stopping the receptor will terminate signal transduction. OBPs can bind to the odorants and transport the odorants from the mucus to the receptor, which in turn can transport the odorant from the receptor to the mucus (Buck, 1996). Lazar and others (2001) presented evidence to support the later function of OBPs. Changing mucus components via the secretion process will change the receptor properties and the microenvironment,

which can result in release of the odorant from the receptor (Getchell et al., 1984; Getchell et al., 1988).

Stopping the receptors is also possible. There is accumulated evidence that many types of protein kinases (enzymes involving phosphorylation of protein) engage in signal termination; for example, protein kinase A, protein kinase C or specialized G-protein receptor kinase (Buck, 1996). These molecules are believed to effect the phosphorylation of the receptors by some signal from cyclic nucleotides in the cyclic nucleotide mediation cascade (Buck, 1996).

Signal termination also can be done by inhibiting the CNGs. Evidence shows that the elevation of internal Ca^{2+} inhibits the ion-gate; however, the mechanism is still controversial (Zufall et al., 1991). Direct inhibition from Ca^{2+}-regulated protein such as calmodulin and indirect inhibition from Ca^{2+}-activated calmodulin-dependent phosphodiesterase were proposed as possible inhibition mechanisms of the CNGs (Buck, 1996).

3.2 Olfactory bulb level

3.2.1 Information coding

The anatomy of the olfactory bulb is described in section 2.2. The olfactory bulb is the first information processing site in the brain. It receives the information from thousands of ORCs' axons, which precisely project to thousands of glomeruli in the olfactory bulb (Axel, 1995; Buck, 1996; Hildebrand & Shepherd, 1997).

Glomeruli are signal-processing modules, which create a one-way excitatory synaptic connection between the dendrites from the mitral cells and tufted cells and the axons from the ORCs (Takagi, 1989).

As stated in section 3.1.3, the ORCs, which are expressed by the same gene or the same gene subfamily, are usually distributed in a close proximal area in the olfactory epithelium called a zone. Moreover, the same type of ORCs recognizes the same type of odorant ligands (Duchamp-Viret et al., 1999; Malnic et al., 1999; Sicard & Holley, 1984). Now the problem is how the information encoded in the combination of receptors is activated by the odorant ligands and conveyed to the olfactory bulb in an organized fashion. The olfactory system manages the information employing two principles of the olfactory axon projection to the olfactory bulbs: "zone-to-zone projection" and "glomerular convergence" (Mori et al., 1999).

Zone-to-zone projection: Genetically close ORCs express their receptors in the same zone (Malnic et al., 1999). The orientation of the ORCs is called zonal organization (Mori et al., 1999). The zonal organization is preserved to some extent when axons from the ORCs project to the olfactory bulb (Strotmann et al., 2001; Treloar et al., 2001). Evidence from immunohistochemical studies, in situ hybridization and anatomically tracing studies suggest that the olfactory bulb may comprise spatially segregated zones (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999; Johnson et al., 1998; Mori et al., 1999; Vassar et al., 1994). The projection of the axons is bilaterally specific

and constant across the individual in the same species. Therefore, the spatially restricted set of glomeruli are activated by an odorant and it results in a specific topographic map encoding a given odorant's quality (Vassar et al., 1994).

Glomerular convergence: Vassar and others (1994) also discovered that axons from ORCs in the same zone in the olfactory epithelium from rats converge to one or at most a few glomeruli within the olfactory bulb. More conclusive results about glomerular convergence were presented employing a gene-targeting technique (Axel, 1995; Mombaerts, 1999; Mori et al., 1999).

The zone-to-zone projection and glomerular convergence models suggest that glomeruli are specifically tuned. The results from physiological studies of single glomerulus, mitral cells and tufted cells revealed that the mitral and tufted cells are specifically tuned to detect the odorants that share characteristic structural features including the overall stereo-chemical structures of hydrocarbon chains and the types and positions of the attached function groups (Mori et al., 1999). However, the extent of differences between these structural features is still unknown. Moreover, each glomerulus is exclusively tuned to detect specific molecular features (Mori et al., 1999). Ressler and others (1994) proposed that the olfactory bulb is an epitope map for converged information from the olfactory epithelium. In 1996, Johnson and Leon mapped rat-glomeruli response patterns using 12^{d}-glucose uptake technique. The map comprised many activated clusters of glomeruli called "fields" or "modules". The distribution, shape and size of the modules were overlapping but distinct and depended on molecular length and

functional groups of odorants (Johnson & Leon, 1996, 2000b; Johnson et al., 1999; Johnson et al., 1998).

3.2.2 Information processing

Information processing in the olfactory bulb occurs as the interaction between cells in the glomeruli: mitral cell, tufted cells, periglomerulus cells and granule cells. The odorants' information received by the olfactory bulb is processed and refined prior to transmission to the olfactory cortex (Buck, 1996).

The refining process in the olfactory bulb is the result of the inhibition caused by granule cells and periglomerulus cells. Mitral cells and tufted cells stimulate granule cells and periglomerulus cells, which in turn inhibit signal transduction of mitral cells and tufted cells. This process is called dendrodendritic reciprocal synapses (Buck, 1996). The inhibition caused by granule cells via the dendrodendritic synapses is believed to mediate the synchronized oscillatory discharges of mitral and tufted cells (Mori et al., 1999) and lateral inhibition (Buck, 1996). The synchronized oscillatory discharges presumably indicate the temporal binding of signals from different odorant receptors (Mori et al., 1999). Lateral inhibition is also caused by periglomerulus cells (Buck, 1996; Getchell & Shepherd, 1975; Isaacson & Strowbridge, 1998; Mori et al., 1999). Lateral inhibition is believed to enhance the contrast between strongly and faintly activated neurons and thus it sharpens the tuning specificity of individual mitral and tufted cells.

3.3 Olfactory cortex and brain level

Knowledge about how olfactory information is relayed within the cortex is still limited. Information from the olfactory bulb is transmitted to the olfactory cortex via the axon of mitral and tufted cells traveling in the lateral olfactory tract (Buck, 1996). The olfactory cortex is divided into five areas: the anterior olfactory nucleus (AON), the periform cortex, the olfactory tubercle, the amygdala and the entorhinal area. In addition, the limbic area, which is not a part of the olfactory cortex, contains several brain circuits that connect the olfactory cortex and the limbic system. The brain circuits are explained in section 2.3.

Vassar and others (1994) discussed information processing in the olfactory cortex using the analogous model in vision. They suggested that odorant information is collected by the activation of the olfactory receptors in the olfactory epithelium. After that, the information is broken down into pieces in the olfactory bulb and then sent to the olfactory cortex to be reconstructed. Odorous stimuli are also mediated bilaterally in the brain but different stimuli were processed with different patterns (Savic & Gulyasz, 2001). There is evidence indicating the topographical projection from the olfactory bulb to many olfactory cortex areas: AON, the periform and the olfactory tubercle (Buck, 1996).

These findings suggested that information from the olfactory epithelium encoded as a spatial map in the olfactory bulb is still maintained in the cortex as several copies in different areas (Buck, 1996).

II.2 Sensation and perception

When any organ of our bodies is stimulated by an external stimulus, the energy from the external stimulus is detected and encoded into a certain process called "sensation" (Schiffman, 1996). On the other hand, "perception" is the awareness of the stimuli, whose information or energy is organized, interpreted and given meaning. In other words, perception results from psychological processes in which psychological factors such as judgment, relationship, meaning, etc. play a role (Schiffman, 1996).

The meanings of sensation and perception are distinct; however, in general, sensation and perception are inseparable or unified processes. For example, when a suprathreshold odorant contacts the olfactory epithelium, olfactory receptors can detect the existence of the odorant (sensation): nevertheless, a person also can tell what the odorant is and express feelings toward the odorant (responses caused by perception) almost instantaneously. Therefore, in a normal environment, sensation and perception are unified. In some special conditions, such as in a well-controlled laboratory, sensations and perceptions can be separated.

II.3 Sensory evaluation

Sensory evaluation is a scientific method used to evoke, quantify, analyze and interpret those responses to products as perceived through senses in order to establish lawful relationships between product characteristics and perception (Lawless & Heymann, 1999; Stone & Sidel, 1993). In order to appropriately evoke

and quantify sensation and perception through responses toward stimuli, sensory evaluation utilizes many discoveries and inventions from psychophysics.

In this section, the definition of psychophysics and its development will be introduced. Psychophysical laws, types of responses, and scales, magnitude estimation, thresholds, and threshold determinations will be described. Furthermore, mixture studies in olfaction, sub-threshold perception and sub-threshold studies in olfaction will be illustrated.

1. Psychophysics

Psychophysics is a science focusing on the quantitative relationship between physical stimuli and subjective responses or sensory experiences from subjects (Schiffman, 1996; Smith, 2000a). Psychophysics is a valuable tool with which to study sensation and perception (Schiffman, 1996).

Psychophysics is the oldest branch of experimental psychology (Lawless & Heymann, 1999). The first true psychophysical theorist was nineteenth century German physiologist, E. H. Weber, 1834, whose observations led to the formulation of Weber's law (Lawless & Heymann, 1999; Schiffman, 1996). Weber's law, also called Weber's fraction or Weber's ratio, describes a fundamental principle of relative sensitivity which can be expressed in mathematical equation as follows.

$$(\Delta I/I) = k$$

where: I is the magnitude of the physical stimulus at starting level.

ΔI is the increment of the physical stimulus intensity, when added to the stimulus intensity I, produced a just noticeable differences (JND).

k is a constant that varies with the sensory system being measured.

In 1860, Gustave Theodor Fechner published his famous book called *The Elements of Psychophysics* (Lawless & Heymann, 1999; Schiffman, 1996). Fechner proposed Fechner's law which can be expressed mathematically as follows.

$$S = k \log(I)$$

where: S is the magnitude of sensation.

I is the physical intensity of the stimulus.

k is a constant that take into account the specific Weber fraction for a given sensory dimension.

Fechner's law is based on the assumption that the sensation of the difference between two physical stimuli at the low end of the physical intensity scale that are separated by 1 JND is smaller than the difference between two physical stimuli at the high end of the physical intensity scale that are also separated by 1 JND (Schiffman, 1996).

About 100 years after Fechner's work, S. S. Stevens, an acoustical researcher at Harvard University Acoustic Research Laboratory, proposed a new psychophysical law called Steven's power law or Stevens' power function (Stevens, 1956). Fechner's law is based on the difference threshold and Weber's fraction for a given sensory dimension; however, Stevens' power law is based on a different psychophysical scale. The Stevens' power law is based on the assumption that the increase in subjective magnitudes is in proportion to the physical intensity of the stimulus raised to a power. This assumption can be mathematically expressed as follows.

$$\mathbf{S = k\, I^{b}} \qquad \textbf{or}$$

$$\mathbf{\log(S) = b[\log(I)]+[\log(k)]}$$

where: S is the magnitude of sensation.

I is the physical intensity of the stimulus.

k is a constant that takes into account the choice of units used in a given sensory dimension.

b is the "exponent" of the equation and reflects the relationship between sensory magnitude and stimulus magnitude which is specific for each sensory dimension.

The "exponent" or "b" is believed to be specific for each sensory dimension and categorized into three types, < 1, $= 1$ and > 1. If b is greater than 1, it is called expansion of the response dimension. The expansion reflects that the increase in subjective intensity is much faster than the increase in physical intensity. On the

other hand, if b is less than 1, it is called compression of the response dimension. The compression reflects that the increase in subjective intensity is much slower than the increase in physical intensity. If b is equal to 1, it reflects that the sensory magnitude grows with physical stimulus magnitude. Moskowitz (1983) reported the exponents of many sensory dimensions (Moskowitz, 1983). The exponents of odorants usually range from 0.1 to 0.7 and it is very rare to find the exponent of odorants to exceed 1.

2. Sensory responses and sensory response measuring techniques

2.1. Types of responses and how to measure them

Sensory response measurements are methods of applications of numbers to quantify sensory experience (Lawless & Heymann, 1999; Meilgaard et al., 1991). Numbers collected from any measurements are called responses. There are four general types of responses based on the character of the basic empirical operations performed (Stevens, 1946). The results of the operation performed are reflected in the information contained in the four responses: nominal, ordinal, interval and ratio responses (Meilgaard et al., 1991).

2.1.1. Nominal response

The basic empirical operation of nominal responses is determination of equality (Stevens, 1946). Therefore, the nominal response contains the least

amount of information among the four response types. The only information contained in the nominal response is pure qualitative information such as name, gender, etc. The qualitative information gained from the nominal responses does not associate with any degree of differences between groups or numbers assigned to any group. For example, if gender is assigned as 0 = male and 1 = female, it does not mean that male and female are numerically different and the difference is 1. If the assigned number changes to 0 = male and 500 = female, the meanings are always the same as 0 and 1, which are male and female. There is no association between numbers and their numerical values in nominal responses.

The sensory measurement given to the nominal response is *classification* (Meilgaard et al., 1991). The task is asking subjects to classify objects or stimuli into groups or classes. For example, the experimenter gives a subject a stimulus and asks the subject to tell that "the subject can detect any existence of any substances in the stimulus or not". The answer will be "yes" or "no". Asking the subject to expresses that the subject can detect or cannot detect the stimuli is equivalent to asking the subject to classify the stimulus into two categories: yes, something exists or no, there is nothing. The classification is very useful in sensory evaluation, especially in discrimination tasks such as duo-trio test, triangle test, threshold determination, etc.

2.1.2. Ordinal response

Ordinal responses contain a little bit more information than the nominal responses. The basic empirical operation of the ordinal response is determination

of greater or less (Stevens, 1946). Therefore, information contained in the ordinal response is the order of intensities or levels of stimuli; however, it does not contain any numerical magnitudes of differences between stimuli. For example, subjects are asked to order the length of a 2"-cigarette, 7"-pen and 12"-ruler in the order of the shortest (1) to the longest (3). The order will be the cigarette=1 (shortest), the pen=2 (middle) and the ruler=3 (longest); however, magnitude of differences between 1, 2 and 3 do not reflect to the physical differences in the length of those 3 objects. Therefore, the only information gained is the order but not the numerical magnitude of real differences between objects.

The sensory measurements producing ordinal responses are ordering, ranking and category scaling without knowing the magnitude of differences between categories. Ordering and ranking are tasks that subjects perform ordering or ranking stimuli based on magnitude of a certain quality or attribute of interest. However, in the category scaling without knowing the magnitude of differences, subjects will be asked to place stimuli in an assigned category that is pre-ranked and labeled based on magnitude of a certain quality, liking or descriptor of interest. The unknown magnitude of differences between any categories is the most important characteristic of ordinal responses that differentiate the ordinal responses from interval responses (Lawless, 1999).

Ordering and ranking are commonly used in preference work (Lawless, 1999). However, ordering and ranking are very time consuming and tedious for subjects. Problems such as fatigue and changing discrimination criteria occur,

especially with strong stimuli. Nonetheless, the uneven-interval category scaling is popular in marketing and more common for strong stimuli because it is a less tedious task.

2.1.3. Interval response

Interval responses contain more information than nominal and ordinal responses. Stevens (1946) stated that the interval response is basically employed to determine the equality of intervals or differences. Therefore, the numerical magnitude of differences is contained in the interval responses along with other information gained from the nominal and ordinal responses. Good examples for this type of response are temperature units. In temperature measurement, both °C and °F can be measured; however, both units do not have a real zero point. Both °C and °F have their own arbitrary-zero points. However, these two units are inter-convertible as the following equation: (°C/5) = ((°F-32)/9).

The sensory measuring technique for this type of responses is category scaling with known magnitude of differences between categories. However, in order to achieve the true interval responses, the differences between categories employed in the category scaling must be known. So far, only the 9-point hedonic scale (Peryam & Girardot, 1952) is acceptable to produce the interval hedonic response (Lawless, 1999). Lawless and Heymann (1999) provide more information about the 9-point hedonic scale and how it was developed. However, other types of

scales are assumed to give interval responses such as the 16-point intensity scale used by the Spectrum® method and categorized line scale (Meilgaard et al., 1991).

2.1.4. Ratio response

Similar to the interval response, the ratio response is used to determine the equality of ratios. The ratio response contains the most information about stimuli: equality or identity, rank-order, equality of intervals and equality of ratios. The ratio response has a true-zero value and linearly increase in intensity. The most common examples are physical units such as length, mass and temperature in Kelvin unit. These physical values have their true-zero points and the relationship between units in the same continuum is linear. For example, the ratio of differences between 2.54 cm and 5.08 cm is 2. This relationship is still true in inch units: 2.54 cm = 1 inch and 5.08 cm = 2 inch. The relationship between centimeter and inch is 2.54 cm = 1 inch.

A sensory response measurement that is believed to yield the ratio response is magnitude estimation (Stevens, 1956). The linear property of the ratio responses gathered from magnitude estimation is still controversial because of contextual effects and a biased number assignment process (Lawless & Heymann, 1999).

In summary, there are four types of responses: nominal, ordinal, interval and ratio. These responses are different from each other because of their empirical operations, which result in different information contained in each response. The

nominal, ordinal, interval and ratio contain information from the least to the most, respectively.

2.2. Magnitude estimation and the Power function

Magnitude estimation was invented by S. S. Stevens and his colleagues in early 1950s (Moskowitz, 1983). In general, magnitude estimation is the unrestricted application of numbers to represent sensation ratios (ASTM, 1999c; Lawless, 1999; Meilgaard et al., 1991; Moskowitz, 1983; Moskowitz & Jacobs, 1988). There are two primary variations of magnitude estimation: assigned modulus and modulus-free magnitude estimation (ASTM, 1999c; Lawless, 1999; Moskowitz, 1983).

Assigned modulus magnitude estimation involves the use of an external reference-sample with an arbitrarily pre-assigned numerical value (also called modulus) for the comparison task (ASTM, 1999c; Moskowitz, 1983). Because of employing a modulus, the instruction is as follow.

"In front of you is a series of ...solutions. Your task is to tell how strong they smell. The intensity of the reference stimulus is 10 (the number, 10, can be changed to any number the experimenter wishes for the modulus, and the instructions need to be changed accordingly). Please smell the stimuli from left to right. If the first stimulus smells 2 times as strong as the reference, assign it the number 2 times as large. That number equals to 10 x 2 = 20. If it smells one-tenth as strong as the reference, then assign it a number 1/10 as large. That number

equals to 10 x (1/10) = 1.0. You can use any numbers, fractions, and decimals. Make each assignment proportional to the intensity of the aroma, as you smell it. Remember, 10 equals you reference's aroma intensity."

The other type is modulus-free magnitude estimation (Moskowitz, 1983) or internal modulus magnitude estimation (ASTM, 1999c). The modulus-free magnitude estimation uses the first sample as a reference. Therefore, if the presentation orders of all stimuli are randomized for each subject, the reference stimulus for each subject will be different. The instruction for modulus-free magnitude estimation is as follows.

"You will be presented with a series of stimuli in random order. Your task is to tell how intense they seem by assigning numbers to them. Call the first stimulus any number that seems appropriate to you. Then assign successive numbers in such a way that they reflect your subjective impression. There is no limit to the range of numbers that you may use. You may use whole numbers, decimals, or fraction. Try to make each number match the intensity, as you perceive it. (Stevens, 1975)"

The intensity of modulus used in the assigned modulus magnitude estimation has some effect on ratio responses obtained. If the modulus is at the top of the stimulus range, it tends to narrow the range of the numbers that subjects will assign. In addition, if the modulus is at the bottom of the stimulus range, the slope of power function tends to increase because of increasing in ranges of numbers used by subjects. Furthermore, if the modulus is in the middle range of the

stimulus, the distribution of the data tends to be distorted around the region of the modulus (Moskowitz, 1983). Using no modulus as in modulus-free magnitude estimation with appropriate presentation order should produce the best data but analysis of the data will be much more tedious than the assigned modulus method (Moskowitz, 1983).

Because of variation in scaling between subjects, it becomes necessary to rescale each subject's data to bring the data into the same scale before further analysis. The rescaling process is called normalization although it has nothing to do with the normal distribution or Z-scores. A common normalization method is geometric mean normalization or modulus equalization. The process is as follow. First, calculate the geometric mean, the n^{th} root of the product of n numbers, of each subject's ratings across his or her entire data of descriptor of interest. Second, calculate the grand geometric mean of the entire data set. Third, calculate the ratio of individual geometric mean and grand geometric mean called the normalization factor, which is unique for each subject. Forth, multiply all the data for each subject with their individual normalization factor in order to obtain the normalized data (Lawless, 1999; Moskowitz, 1983). This results in a new geometric mean for each individual which is equal to zero.

The data obtained from magnitude estimation are usually submitted to log-transformation after normalization because the data tend to be log-normally distributed or at least positively skewed (Lawless, 1999).

However, if the data contain zero, any transformation or geometric mean normalization will not be possible because log of zero is undefined. ASTM (1997) suggested replacing the zeros for each subject with small positive value such as 1/10 of the smallest number given by each subject; however, replacing the zeros will influence the data set. Alternatives to replacing zeros are employing the arithmetic mean or median in normalization (Lawless, 1999).

In conjunction with an appropriate experimental design, after normalization, the data from magnitude estimation are usually used to construct the power function of the stimuli. According to Stevens' law, the relationship between subjective responses (S) and physical intensity (I) in the Power function, $\mathbf{S = k\, I^b}$, is linear after log-transformation of both sides of the Power function. The results of log-transformation is **log (S) = b [log (I)]+[log (k)]**. If log(s), y-axis, is plotted against log (I), x-axis, it yields a linear line with slope = "b" and y-intercept = "log (k)". The meanings of both "b" and "log (k)" are explained in the Psychophysics section above.

Besides exploring the exponent, b, of any sensory continuum, the exponent can also be used as a subject's scaling performance index in sensory evaluation. The exponent for each subject reflects the range of numbers assigned to test stimuli by each subject. If the exponent for subject A is bigger than that of subject B, it means that subject A used a bigger range of numbers than subject B. Therefore, the exponent can be used as a scaling index for each subject and helps the panel leader to determine the amount of training necessary for panel in order to achieve the

purpose of training; calibrating all subjects to be accurate sensory measurement machines. Besides the exponent, ordering ability is also observed from the power function and can be used to determine ordering ability of subjects.

Usually magnitude estimation is used for only one descriptor such as overall intensity (Lawless, 1999). Magnitude estimation also can be applied in descriptive analysis for a profiling task (Lawless, 1999). If both exponent and ordering ability between subjects turn out to be different, there is a high probability of misunderstanding the definition of the descriptor of interest. Therefore, more discussion about attribute definitions is required before any scaling task can proceed.

In summary, magnitude estimation is believed to yield a ratio response and follow Stevens' law or the Power law. There are two primary types of magnitude estimation: assigned modulus and modulus free magnitude estimation. The intensity level of the modulus method influences the results but is a less tedious task than the modulus free method. The relationship between log (S) and log (I) is linear with slope or exponent (b) that is believed to be unique for each sensory modality and y-intercept (log (k)). The power function is also a useful tool for the sensory panel leader to evaluate subjects' scaling performance.

3. Thresholds and threshold determination

Literally, the word "threshold" or "limen" means "the point at which a physiological or psychological effect begins to be produced" (Anonymous, 1994).

Threshold determination has been a center of Fechner's psychophysics for a long time (Lawless & Heymann, 1999). So far, there are four types of thresholds: detection threshold, recognition threshold, difference threshold and terminal threshold (Brown et al., 1978).

3.1. Detection threshold

Hypothetically, the detection or absolute threshold is the minimum physical energy level of a stimulus necessary for perceptual detection or awareness (ASTM, 1999a; Brown et al., 1978; Lawless & Heymann, 1999; Schiffman, 1996). The hypothetical definition of detection threshold implies that there is a level of stimulus energy below which detection never occurs and above which the detection always occurs. However, with the smooth nature of psychometric functions, the plot of positive-response frequency as a function of physical stimulus concentration, such a level cannot be found. Therefore, many empirical definitions for detection threshold are established, such as the stimulus that has a probability of 0.5 of being detected under the condition of the test (ASTM, 1999b; Lawless & Heymann, 1999). Consequently, the empirical definitions of detection thresholds are dependent on methods used (Lawless & Heymann, 1999).

The incongruity between hypothetical and empirical definitions is still controversial. Many methods have been developed to determine the detection threshold of any stimulus based on which definition a researcher adopts. Besides

the detection threshold, other thresholds such as recognition, difference and terminal threshold also depend on the definition adopted.

One of the simplest methods to determine the detection threshold is "method of limits", which has been used since Fechner's time (Fechner, 1864). This method requires subjects to test a series of stimuli in ascending or descending order and report at which level the subjects detect the stimulus. Many problems are encountered employing this method even though it seems straightforward. Fatigue, sensory adaptation, expectation and habituation to method are examples of such problems. In order to minimize these effects, the staircase method was introduced in 1960s (Schiffman, 1996). With the staircase method, the experimenter uses the same presentation scheme as in the method of limits; however, when a subject gives a correct response the experimenter reverses the presentation direction for the next stimulus. The experimenter will use this reverse-when-correct scheme until the experimenter obtains a constant up-down pattern response (Schiffman, 1996).

Moreover, because of the many replications required for each subject, the discrimination criteria adopted by subjects to distinguish between detectable and non-detectable stimulus level is changed over time. Therefore, the data obtained result from a mixing of subject sensitivity with response bias. In order to get around this problem, a forced-choice element is used at each level or concentration step in conjunction with the classical method of limits. The most common example for this type of method is the standard method developed by ASTM called Forced-choice ascending concentration series method of limits (ASTM, 1999b).

In order to determine the threshold level from the test, the researcher needs to decide which definition, hypothetical or empirical, of the detection threshold the researcher will use. If the hypothetical definition is adopted, the individual detection thresholds can be defined in many ways such as: 1) the geometric mean of the ending level and the next (higher or lower) level step that would have been given had the series been extended, 2) the lowest correct stimulus level or the geometric mean of the last incorrect concentration step and 3) the first correct concentration step (ASTM, 1999b; Lawless & Heymann, 1999). After obtaining individual thresholds, the group detection threshold is defined as the geometric mean of the individual threshold (ASTM, 1999b).

If the empirical definition is adopted, ASTM (1997) suggests using both graphical and logistic modeling methods obtained from a relationship between the % correct above chance and log of stimulus level which can be found in ASTM procedure E 1432-91 (ASTM, 1999a). The individual detection threshold is the level that the probability of correct responses is 0.5. The group threshold for the empirical case is also the geometric mean of the individual thresholds or calculated from the rank/probability method (ASTM, 1999a).

Beside presentation schemes such as: ascending, descending or staircase, and forced-choice presentation, 2-AFC, 3-AFC, 2 out of 5, etc, another concern is the number of stimulus levels presented to the subjects. Lawless and Heymann (1999) discussed this problem statistically and pointed out the importance of the number of the samples within the series, which they called the "stopping rule".

The number of the samples within the series depends on what forced-choice presentation method is selected, the number of replications, and the confidence level the researcher wants to keep (Lawless, 1999).

There are several other techniques for detection threshold determination. The method of constant stimuli (Schiffman, 1996), semi-ascending pair-difference method (Lundahl et al., 1986), estimation from dose-response curves (Marin et al., 1991) and 2-AFC with five replications (Stevens & O'connel, 1991) are some of the others. For a more detailed list of methods Brown and others (1978) and Lawless and Heymann (1999) are recommended.

3.2. Recognition threshold

Conceptually, recognition threshold is the minimum level of physical stimuli at which a stimulus is *correctly identified*. In addition, as in the detection threshold, the empirical definition is also defined as the level of physical stimuli at which the probability of *correct identification* is 0.5. Usually the recognition threshold is a little higher than the detection threshold; however, the difference depends on stimulus used (Lawless & Heymann, 1999).

Recognition threshold determination methods are the same as detection threshold determination methods, the difference being in questions asked to subjects (ASTM, 1999b). In detection threshold determination, subjects will be asked to detect differences; however, in the recognition threshold determination, subjects will be asked to *detect* and *identify* test stimuli. Therefore, the difference

between the recognition threshold and the detection threshold is the *identification task.* The most commonly used recognition threshold determination method is the ASTM procedure E 679-91 (1999) called 3-AFC ascending concentration series method of limits.

3.3. Difference threshold and JND

As in the detection threshold, conceptually, difference threshold or difference limen is the amount of change in physical stimulus necessary to produce a just noticeable difference (JND) in sensation (Schiffman, 1996). However, the empirical definition is also applied as in the detection threshold. The JND, empirically, is the amount of change in physical stimuli that subjects can detect 50% of the time (Stone, 1963, 1964; Stone et al., 1962; Stone & Sbosley, 1965). Both Weber's fraction and Fechner's law are involved in the difference threshold.

The Weber's fraction fundamental principle is that an increase in the difference threshold is required when the initial stimulus involved in the difference threshold increases. Based on Weber's concept, Fechner discovered that a greater change in physical stimulus intensity is required at high intensity level, than at a low intensity level, in order to produce one JND.

There are many methods to determine the difference threshold such as the method of limits, method of constant stimuli and method of adjustment or method of average error (Schiffman, 1996). The presentation schemes of these methods are the same as in detection threshold determinations with some modification in the

forced-choice element and task performed by subjects. In the difference threshold determination, the forced-choice element is always employed because of the nature of the question asked to subjects. The subjects are asked to *compare* and to *find* the *difference* between a given stimulus and a *reference* stimulus.

If the reference stimulus is at 0-level or blank, the increase in stimulus level responsible for perceptual changes compared to the reference stimulus is the detection threshold. Therefore, the detection threshold is a special case of the difference thresholds when the reference stimulus is at 0-level or at undetectable level (Lawless & Heymann, 1999). Because of the requirement of the undetectable level of reference stimulus for the detection threshold, in a background media situation, i.e. beer and wine, the difference threshold and detection threshold are the same entity (Brown et al., 1978; Lundahl et al., 1986).

3.4. Terminal threshold

The terminal threshold is the minimum level of physical stimuli at which no changes are perceived (Lawless & Heymann, 1999). In practice, this level is rarely approached; however, in some products such as hot sauce, perfumery compounds, very sour or very sweet candy, it would be an exception. There are many problems when the stimulus level approaches the terminal threshold especially when many other sensations are evoked automatically. For example, irritation and pain are usually perceived along with concentrated odorants and results in mucus production. In turn, mucus obscures perception of descriptors of interest in

conjunction with distraction from irritation and pain (Cain & Murphy, 1980; Getchell et al., 1988). Moreover, changes in perceived quality at different concentrations are a common phenomenon in olfaction and this makes studies along the odor perception continuum more difficult (Pause et al., 1997).

4. Mixture suppression studies in olfaction

Attempts to understand how the olfactory system works have been conducted for decades by many branches of science. Psychophysicists are attempting to understand and formulate a rule to predict the olfactory perceptions and responses. Meanwhile, physiologists are trying to understand the underlying physiology of the olfaction system in order to explain the behaviors observed by psychophysicists and behavioralists.

Because of legal and moral limitations, studying human olfaction is limited to the psychophysical level or at most the clinical level. Therefore, most of physiological understandings of olfaction were from investigation of other animal models such as fruit fly, lobster, frog, mice and monkey (Ache, 1989; Derby et al., 1985; Laska & Freyer, 1997; Laska & Galizia, 2001). Human subjects have also been employed in some recent studies (Hari et al., 2001).

When several odorants are mixed together, there are two possible changes: changing in intensity and changing in quality (Berglund et al., 1971). Changing in intensity is usually called intensity mixture interaction (Derby et al., 1991a) and changing in quality is called quality mixture interaction (Wise & Cain, 2000) or

pattern mixture interaction (Derby et al., 1991b). Components in mixtures determine which interaction will occur in final mixtures (Berglund et al., 1971). In this thesis, intensity mixture interaction is a major topic.

In general, intensity mixture interaction is a phenomenon that deviates from perfect additivity of individual component in the mixtures (Bartoshuk, 1975; Hyman & Frank, 1980). Most studies indicated suppression in mixtures (Derby et al., 1985), however, enhancement was also found in some situations (Rifkin & Bartoshuk, 1980). Mixture hyper addition, enhancement or synergistic interaction is a phenomenon that the intensity of mixtures is greater than the sum of the intensity of each component in the mixtures. On the other hand, mixture suppression can be classified into three classes: subtraction, strongest-suppression and hypo-additivity (Berglund et al., 1976; Laffort, 1989). Subtraction is a phenomenon where the overall perceived intensity is smaller than the perceived intensity of one of the two components in the mixture, in a binary system case (Berglund et al., 1976). The strongest-suppression is a case where the perceived intensity of mixtures is the perceived intensity of the strongest component in the mixture (Laffort, 1989). The third type, hypo-additivity, is a condition where the intensity of mixtures is less than simple additivity of responses of the components in the mixture but the intensity of the mixture is higher than individual component's intensity (Derby et al., 1985; Laffort, 1989). The hypo-additivity is the most common case of suppression. A situation has not been found where the intensity of

mixtures is less than intensities of all components in the mixture, however, this case could happen.

This section will describe several important findings about olfactory sensation, perception and responses, especially in mixture interactions. The section is classified into two sub-sections: physiological studies and psychophysical studies of mixture suppression level.

4.1 Physiological studies of mixture suppression

Because animals in different phyla share the basic function and organization of chemical senses (Hildebrand & Shepherd, 1997; Sengupta & Carlson, 2000), many researchers use insects, crustaceans, and vertebrates such as salamander, catfish, and rat in their studies about the physiological level of olfaction.

There are three broad mechanisms involved in mixture interaction: peri-receptor, receptor-level and central mechanism. Peri-receptor and receptor-level mechanism are collectively called the peripheral mechanism. The peripheral mechanism comprises receptors, stimuli and other factors that interact directly and indirectly with the receptors and the stimuli. However, the peripheral mechanism does not directly interact with high-level components in signal transduction pathways such as interneurons, glomeruli and the brain. Alternatively, the central mechanism involves any physiological components in signal transduction at a higher level than receptors beginning at the olfactory bulb.

In olfaction, suppression in mixtures is a common phenomenon. Using a lobster model, (Carr et al., 1989) has shown evidence of peri-receptor suppression, caused by antagonistic reactions, a type of non-competitive peripheral suppression from neighboring receptors. The neighboring receptors produced inhibitory substances for other receptors such as enzyme-catalyzed dephosphorylation (Carr et al., 1989; Miller, 1971). Aside from the antagonistic reactions, (Getchell et al., 1984) there have been some suggestions of mucus preventing odorants from reaching the receptors. The mucus is produced by indirect activation of Bowman's gland and direct activation supporting cells or odorant binding proteins (Buck, 1996). According to the chromatographic-like model in the olfactory mucosa, in a mixture system, temporal pattern differences between components cause peri-receptor suppression (Atema et al., 1989; Hornung & Mozell, 1977; Hornung et al., 1980; Mozell, 1964, 1970, 1971; Mozell & Jagodowicz, 1973). An odorant that reaches receptors first can act as an inhibitor for other receptors in nearby proximity by indirect inhibition. In addition to receptor-related peri-receptor suppression, direct chemical interaction and alteration of mucus environment such as ionic strength and pH were also proposed (Derby et al., 1985; Schafer et al., 1984).

In addition to the peri-receptor mechanisms, the receptor-level mechanism is also possible in mixture suppression. Bell and others (1987) suggested that mixture suppression between polar and non-polar components in a binary mixture is primarily a peripheral event. Ache (1989) suggested a competition between

suppressants and stimulants for common receptor sites as demonstrated in a lobster model system. Moreover, Ache (1989) also reported the ability of overcoming suppression by increasing the concentration of a target stimulant. Other competitive suppressions, such as competing for common receptor sites between two stimulants and receptor-binding ability differences between two odorants were also possible (Ache, 1989; Derby et al., 1985). Failure to overcome suppression when concentration of stimulants increased reveals the differences in binding-ability of suppressants and stimulants (Ache, 1989). According to the possible mechanisms for competitive peripheral suppression, it is quite safe to note that competitive suppression is concentration dependent regardless of the degree of dependency on the concentration of the suppression effect.

In addition to competitive suppression at the receptor level, non-competitive receptor-level suppression was also proposed (Derby et al., 1985). Dependency on concentration differentiates non-competitive mechanisms from competitive mechanisms (Daniel & Derby, 1991b; Derby et al., 1991a). However, binding ability on receptor sites of odorants is non-competitive but depends on concentration (Olson & Derby, 1995). Non-competitive suppression can be explained as two or several receptor sites on one receptor cell being stimulated by many odorants (Daniel et al., 1994; Kashiwayanagi et al., 1996). In this case, suppression can occur when one odorant contacts the same receptor cell as other odorants but at a different receptor site causing suppression from within the receptor cell. There are several proposed mechanisms of the within receptor cell

suppression of mixtures such as activation of opposing ionic conductances, changing conformation of other receptor sites to become unsuitable for other odorants and activate inhibition pathways (Daniel et al., 1994; Doolin et al., 2001; Michael, 1995; Olson & Derby, 1995). A direct effect on ion channels or on second messenger metabolism of a suppressant was also proposed for the within receptor cell suppression (Kurahashi et al., 1994). Kurahashi and others (1994) also opposed the alteration of receptor site condition idea and defined the suppression caused by alteration of ion channels or second messenger metabolism as true suppression. Inhibition caused by "cross-talk" between two excitatory transduction pathways activated by different components within cell was also proposed (Anholt & Rivers, 1990; Simon & Derby, 1995). Another possible mechanism is direct peripheral inhibition synapses between receptors; however, no evidence of this type of suppression is available (Derby & Ache, 1984).

Using lobster as an animal model, when a non-competitive predictive model was used, signal intensity of mixtures was better predicted than the prediction from competitive predictive models (Daniel et al., 1996). Many peripheral components (excitatory and inhibitory transduction corrections, the number of receptor sites/transduction processes per cell, the specificity of receptor cell and the contribution of the magnitude of response of each receptor cell to the overall response magnitude of the population of all receptor cells, and binding interactions between receptors) were also incorporated into the non-competitive model to improve the prediction accuracy (Daniel & Derby, 1991a; Gentilcore & Derby,

1998). From these models, the evidence supports non-competitive suppression rather than competitive suppression.

Central suppression or synaptic inhibition is an intensity mixture interaction that happens in signal transduction at higher-level rather than at receptor-level and results in the reduction of sensory signals (Cain, 1975; Derby et al., 1985). Derby and Ache (1984) reported interneuron-level suppression in lobsters that response toward crab extracts by electrophysiological studies. Activation of separate filament from the olfactory organ of lobster provided evidence of central suppression in lobsters (Derby et al., 1985). According to physiological organization of the olfactory bulb (Section 2.2 & 3.2 in Physiological section), lateral inhibition is a common phenomenon in glomeruli of vertebrates' olfactory bulb (Buck, 1996; Getchell & Shepherd, 1975; Isaacson & Strowbridge, 1998; Mori et al., 1999). Gamma-aminobutyric acid (GABA) and histamine-mediated inhibition was reported at the glomerular output neurons of the spiny lobster olfactory lobe (Wachowiak & Ache, 1998). Specific excitatory responses and non-specific inhibitory responses in the olfactory bulb were reported (Duchamp & Sicard, 1984). The differences in specificity of both response types were caused by synaptic organization of the olfactory bulb (Duchamp & Sicard, 1984). Because of tremendous convergence of the ORCs to a glomerulus, and differences in sensitivity toward odorants of receptors and glomeruli, it is more likely that the central suppression is concentration independent.

In summary, deviation from perfect additivity is mixture interaction. In olfaction, suppression is common. Peripheral (peri-receptor and receptor-level) and central mechanisms play important roles in suppression. Peripheral mechanisms are both concentration dependent (competitive mechanisms) and concentration independent (non-competitive mechanisms); however, the central mechanism depends less or does not depend on concentration. The relative contributions of peripheral and central mechanisms in intensity mixture interaction are still unknown. The ratios of contribution of both mechanisms seem to be specific for any specific mixture systems.

4.2. Psychophysical studies of mixture suppression

There are two foci in psychophysical studies of mixtures. One is focusing on changes in the "quality of the mixture" and the other one is focusing on the "intensity of the mixture". Wise and others (2000) gave an excellent review of the methodologies of quantification of quality of odorants. Threshold determination or force-choice procedures are examples for the quantification of quality of odorants (Wise & Cain, 2000). Threshold determination has been employed by many researchers in sub-threshold effect investigations.

Descriptive analysis or scaling the intensities of descriptors profiled by subjects is usually employed in quantification of the intensity of mixtures. Even though descriptive analysis provides a lot of information, there are some cautions regarding the application of descriptive analysis for complex odors, such as

correlation between attributes and assuming an independent intensity scale (Lawless, 1999). A realization of the limitations of intensity-based descriptive approaches and the correlation between responses obtained from subjects is very important for interpretation of results (Lawless, 1999). This section will summarize many findings in psychophysical studies of the intensity of mixtures.

Studies of mixture interactions in olfaction have been carried out by many researchers around the world for more than 100 years (Laffort, 1989). Before the development of magnitude estimation in the 1960s, psychophysical studies about olfaction involved areas of threshold determination, however, threshold data did not predict supra-threshold intensity (Engen, 1965; Engen & McBurney, 1964).

Since then, many models have been developed in order to predict the perceived intensity of binary mixtures. Early models developed in 1970s involve solely the perceived intensities of components in mixtures (Berglund et al., 1971, 1976; Berglund et al., 1973; Cain & Drexler, 1974; Koster, 1969). Berglund and others (1973) defined the qualitative nature of odorant mixtures as homogeneous percept and heterogeneous percept. The former is described as a new odor quality being formed or synthesized when the individual components are not perceivable in the mixture, for example wine aromas or food aromas. Occasionally this phenomenon is called homogeneous percept of synthesis phenomenon (Berglund et al., 1973). The later is a phenomenon where individual component quality is still perceived in mixtures. Berglund and others (1973) also suggested using careful instruction when investigating the perceived intensity of mixtures for both

homogeneous and heterogeneous mixtures because of different continua of the intensities.

Many models developed in the 1980s incorporated both perceived intensities and exponents of all components in mixtures (Laffort, 1989; Laffort et al., 1989). However, applications of these models for more complicated mixtures are still underdeveloped. Rabin and Cain (1989) addressed the decrease in an intensity compared to the summation of individual components' intensities and the lack of some quality in a binary mixture. Actually, the human can identify a maximum of three to four components in mixtures because of new qualities synthesized in a more complex mixture (Laing & Livermore, 1992) and the identification ability depends on the experience of subjects (Laska & Hudson, 1992). However, discrimination ability does not increase with training (Laing & Livermore, 1996). In addition, Wise and Cain (2000) reported that the temporal component is involved in odor discrimination in mixtures, which depends on available working memory (Laing et al., 2001).

Laing (1989) pointed out another way to look at suppression instead of the intensity-difference aspect. Suppression was classified based on the effect of each component to the other in a binary mixture (Laing, 1989). Reciprocal suppression is the condition where both components have an effect on each other and the intensity of each component is important. Non-reciprocal suppression is the condition where only one component has an effect on the other, and both intensities and qualities of components play an important role on the mixture intensity. Laing

(1989) also reported that lower intensity odorants tend to suppress higher intensity odorants in binary mixtures.

The reciprocal and non-reciprocal suppression was partially explained by the chromatographic-like model proposed by Mozell and Jagodowicz (1973). According to the model, odorants are selectively adsorbed by the olfactory epithelium (Mozell & Jagodowicz, 1973). Polar odorants such as acids, alcohols and ketones are adsorbed at anterior area of the olfactory epithelium. Non-polar odorants such as hydrocarbons are adsorbed at both the anterior and posterior area (Mozell, 1964, 1970, 1971; Mozell & Jagodowicz, 1973). Laing (1988 & 1989) suggested that suppression takes place at the epithelium and glomeruli level. The mixtures of odorants that are absorbed in the anterior or posterior area tend to have reciprocal suppression; however, mixtures of anterior absorbed and anterior-posterior absorbed odorants tend to have non-reciprocal suppression.

The absorbed location and polarity are not good predictors for the intensity of mixtures (Bell et al., 1987; Laing, 1988). Edward and Jurs (1989) reported correlation of odor intensities with structural properties of odorants. This work was only carried out in single component systems (Edwards & Jurs, 1989). More recently the zonal organization of the olfactory epithelium was discovered (Ressler et al., 1993; Vassar et al., 1993). Specificity of the olfactory receptors toward odorants has been confirmed by many studies (Axel, 1995; Buck & Axel, 1991; Duchamp-Viret et al., 1999; Duchamp-Viret et al., 2001; Enomoto & Shoji, 1992; Kashiwayanagi & Nagasawa, 1995; Laska et al., 2000; Sullivan et al., 1996; Turin,

1996; Vassar et al., 1993; Wang et al., 1998) and molecular receptive range of an aldehyde-activated olfactory receptor was reported (Firestein et al., 2001). Firestein and others (2001) concluded that the receptive range is highly discriminating for specific ligand features in some odorant molecules and very tolerant to others.

Because chemical features of odorant molecules reflect the chemical and physical properties of odorants, the chemical features are believed to influence suppression. Therefore, the relationships between odorant chemical structures and odor perception have been investigated intensively. Dr. Amoore and Dr. Beets recognized the importance of the chemical structures of odorants and proposed the Sterochemical Theory of Olfaction (Amoore, 1964; Amoore et al., 1964; Johnston, 1965). Studies about the relationship between odorant structures and odor perception in conjunction with molecular physiological evidence led to molecular bases of odor discrimination theories (Edwards & Jurs, 1989; Malnic et al., 2001). For example, floral and fruity aromas were reported to be dependent on hydrocarbon chain-length instead of functional group through alteration of functional group in floral and fruity odorants (Anselmi et al., 2001).

In addition to chemical structures and polarities of odorants, temporal implication in odor perception for both single component and mixture systems is believed to occur (Laing et al., 1994). Laing and others (1994) and Pause and others (1997) suggested that fast odorants (the odorants that reach the receptor fist) tend to suppress slow odorant (the odorants that reach the receptor later). The

processing time can be changed by altering the concentration of components in mixtures causing the fast component to be suppressed less than the slow component in binary mixtures. Moreover, Laing and others (1994) also suggested the central mechanism involvement in suppression. These findings are also supported by the differential absorption findings, which are closely related with polarity and the chemical features of odorants.

The evidence from psychological studies point out that suppression of intensity and changes in quality of mixtures are common and are most likely caused by both peripheral and central mechanisms. Peripheral mechanisms involve differential absorption, closely related to chemical features of odorants and specificity of receptor sites. Central mechanisms are involved in temporal information of mixtures. However, how close chemical features of odorants are to be "close enough" is still unknown, and controversial because of very high discriminative power and sensitivity of the olfactory system.

5. Sub-threshold perception

The existence of sub-threshold or subliminal perception has been a subject of controversy for several decades. The sub-threshold effect has been studied extensively in the visual and auditory systems (Cheesman & Merikle, 1984; Dixon, 1971; Dixon, 1981; Duncan, 1985; Erdelyi, 1974; Fowler et al., 1981; Harris et al., 1996; Toth et al., 1996). According to Harris's critique (1996) of Merikle's study (1988), in certain conditions, the sub-threshold effect is not found in the auditory

system (Harris et al., 1996). In this section, evidence about the sub-threshold effect in visual and auditory systems will be given along with some considerations regarding the sub-threshold effect.

Starting with the visual system which has similar physiological organization and signal transduction mechanisms to the olfaction system (Buck, 1996), most of the sub-threshold effect research has been done at a cognitive level. For example, the masking effect on the meaning perception by another word that is below theirs detection thresholds was reported (Fowler et al., 1981). In addition, a study provided evidence of the ability of individuals to use parafoveal visual information which was considered as a sub-threshold stimulus in the study (Balota & Rayner, 1983). Additive effect and an inhibition were reported. They provided the evidence that sub-threshold information processing starts at the peripheral level (location of retina stimulated). In 1996, Toth and others reported sub-threshold facilitation and suppression in the primary visual cortex. Sub-threshold facilitation was defined as the excitatory and inhibitory effect of sub-threshold visual stimuli on the perception of suprathreshold visual stimuli (Toth et al., 1996). This study suggested that the influence of sub-threshold stimuli happened at high- or central-level information processing in the visual systems such as the visual cortex region in brain.

In the auditory system, sub-threshold messages were actually a big concern in the music industry (Vokey & Read, 1985). Merikle's study in 1988 (Reingold & Merikle, 1988) is often quoted regarding the sub-threshold effect in the auditory

system (Harris et al., 1996). Merikle's study (1988) reported a sub-threshold effect in commercially processed audiocassettes. However, several researchers disagreed with the sub-threshold effect in audiocassettes. Vokey and Read (1985) studied "backward message in rock music" and found no effect of the message on the rock music listeners. The backward message was treated as a sub-recognition threshold message in this particular case (Vokey & Read, 1985).

In 1996, Harris and others replicated Merikle's earlier study with some modifications to avoid some previous pitfalls (no threshold measurement, identification task instead of detection task, etc.) found in Merikle's study. No sub-threshold effect was reported (Harris et al., 1996). Harris and others (1996) stated that "it is too early to draw a conclusion regarding the effectiveness of commercially produced sub-threshold audiocassettes".

Some concerns regarding the methodologies employed in these studies were mentioned (Duncan, 1985; Miller, 1991, 2000). Duncan (1985) recommended avoiding forced-choice identification, which is a technique that forces subjects to report the detection of stimuli. The force-choice technique in studies about sub-threshold effects in word awareness was not recommended because of an unknown chance level of detection of randomly chosen stimuli (Duncan, 1985). In addition to the unknown chance level involved in the force-choice technique, other concerns were variations of threshold values among subjects, and within subjects over time and the measurement error estimation from experiments based on signal detection theory (Miller, 1991, 2000).

In summary, the sub-threshold effect in both the visual and auditory system is still controversial. However, in the visual system, which shares some common physiological organization and signal transduction mechanisms with the olfactory system, the sub-threshold effect is more likely to be real and happen at both the peripheral and the central level of the visual system (Balota & Rayner, 1983; Toth et al., 1996). However, methodologies used and variations of threshold values have to be carefully taken into consideration.

6. Sub-threshold research in olfaction

Even though the sub-threshold effect in the visual and auditory system has been investigated quite extensively, there are far fewer studies about the sub-threshold effect in olfaction. Studies about the interaction between sub-threshold and peri-threshold components in mixtures are limited and there are even fewer studies about the interaction between sub-threshold or peri-threshold components and the perception of suprathreshold components. In this section, several studies involving sub-threshold odorants and some examples of the sub-threshold effect in flavor and taste will be discussed in chronological order.

Studies about interaction among sub-threshold components started in the early 1970s. The additive effect on flavor thresholds of many sub-threshold and peri-threshold saturated and unsaturated monocarbonyl compounds in autoxidized milk fat samples was proposed (Lillard & Day, 1961). Simple-correlation coefficients between the reciprocals of flavor thresholds and individual carbonyl

compound contents were calculated and compared with a multiple-correlation coefficient calculated from a multiple carbonyl compound equation. The multiple-correlation coefficient was higher than all simple-correlation coefficients calculated from individual carbonyl compound equation and the additive effect of sub-threshold components was suggested (Lillard & Day, 1961).

Even though the results from Lillard and Day (1961) were indirect, they were reasonable. In 1962, Rosen and others reported supporting evidence from their studies of threshold determination in pure solutions and mixtures. Rosen and others (1962) determined odor thresholds of phenol, o-cresol, m-cresol, p-cresol, o-xylene, m-xylene, p-xylene and 1-butanol and the mixtures of 1-butanol, p-cresol and pyridine. At each solution threshold, each individual constituent in the solution was determined. The concentrations of individual constituents in the mixtures at the mixture threshold were lower than each individual concentration in pure solutions at individual thresholds (Rosen et al., 1962). The additive effect and synergistic effect were concluded depending on the mixtures (Rosen et al., 1962). The threshold determination of mixtures has since been employed by many researchers.

Nawar and Fagerson (1962) also concluded a synergistic effect of sub-threshold constituents in a methyl ketone solution employing a triangle test. The mixture of butanone, pentanone, hexanone, heptanone and octanone, all at sub-threshold, was detected by a panel of six subjects when it was presented against a

water blank. Therefore, the synergistic effect of a mixture consisted of several methyl ketones at sub-threshold was proposed (Nawar & Fagerson, 1962).

In 1963, Day and others presented evidence to support the additive effect of sub-threshold carbonyl compounds on the flavor threshold of milk lipids as was proposed in 1961. The experiment planned to prove the additive effect by mixing equal weight compounds in solution; however, the additive effect was not common across the mixtures prepared in the study. Only the mixtures of n-nonanal/n-decanal and n-octanal/n-decanal showed an additive effect but the mixtures of n-hept-2,4-dienal/n-non-2-enal, n-nonanal/n-non-2-enal, n-heptanal/propanal, n-hex-2-enal/n-non-2-enal and n-hexanal/n-hex-2-enal were not significantly different from the individual constituents (Day et al., 1963). In addition to the additive effect, this study also pointed out many interesting findings. One such finding was changes in flavor detection thresholds as a function of carbon chain-length (Day et al., 1963).

Also in 1963, Guadagni and others presented evidence to support the additive effect between sub-threshold odorants. Employing the threshold determination of sub-threshold mixtures, which consisted of many linear and branched aldehydes and dimethyl sulphide, butyric acid and butylamine in many ratios, the actual odor detection thresholds was compared to theoretical odor detection thresholds based on the additive effect assumption. The actual detection threshold and the theoretical threshold of a mixture prepared were equal or close to each other, therefore, the additive effect was concluded (Guadagni et al., 1963b).

Langler and Day (1964) proposed a synergistic effect of sub-threshold methyl ketones in mixtures of homogenized milk, employing the detection threshold determination for flavor. The synergistic conclusion was made because of the much lower concentrations of individual ketones in the mixture detection threshold than the concentrations of individual ketones at their threshold in a single component system (Langler & Day, 1964).

Keith and Powers (1968) reported the effect of sub-threshold compounds on the detection of the other compounds at threshold level; however, this effect was not common. Only the mixture of isovaleric aldehyde (at sub-threshold), benzaldehye (at threshold) and 2-methyl butyric acid (at threshold) exhibited the sub-threshold effect, but the other twenty-three mixtures showed no sub-threshold effect (Keith & Powers, 1968). The additive effect of sub-threshold mixtures was also confirmed to be uncommon and depended upon the ratios and classes of individual components. This phenomenon was also reported by Day and others (1963) in the mixture of sub-threshold compounds with different functional groups. The additive effect was confirmed when the experiment was conducted as in Guadagni and others (1963) at 80% of threshold level of each compound. Keith and Powers (1968) also commented about the variability between subjects which made it very difficult to prove the sub-threshold additive effect.

Besides flavor and aroma, the additive effect of sub-threshold compounds was also reported in taste. The additive effect was reported in perception of bitterness of limonin and naringin mixtures (Guadagni et al., 1974b). In addition to

the interaction between sub-threshold taste stimuli, Guadagni and others (1974) also investigated the effect of two different taste stimuli, naringin (bitter) and sweeteners, both at sub-threshold level, on the bitterness perception of limonin, at supra-threshold level. No sub-threshold effect was found in that particular experiment (Guadagni et al., 1974b).

Studies of stimulation of unitary responses from the frog olfaction system provided supporting evidence about differential threshold influence caused by peri-threshold odorants on odor discrimination (Van Drongelen, 1978). Van Drongelen (1978) introduced the idea of summated activity of frog receptor neurons stimulated with sub-threshold isoamyl acetate. The sub-threshold and peri-threshold odorant was important in odor detection and most likely happened in the olfactory bulb (Van Drongelen, 1978).

Laska and Hudson (1991) compared the detection thresholds of odor mixtures and their components. They concluded a hyper-additivity (synergistic) effect of sub-threshold mixtures; however, they also point out that this phenomenon was true for only 53% of the subjects involved in the study. They also reported that the hyper-additive effect was not easily perceived at peri-threshold (Laska & Hudson, 1991). More stable thresholds of the mixtures than thresholds of individual components were also reported (Laska & Hudson, 1991).

Complete additivity effect was confirmed again in the mixtures of 1-butanol, 2-pentanone and butyl acetate employing the detection threshold

determination (Patterson et al., 1993). In this case, there was a three-fold gain in sensitivity of the mixture compared to individual component sensitivity.

Cometto-Muniz and others (1997) reported the additive effect, also called agonistic effect in the study, of sub-threshold odorants in sub-threshold mixtures employing threshold determinations. The additive effect reported was calculated after taking into account individual subject's detection thresholds for each constituent in the sub-threshold mixtures (Cometto-Muniz et al., 1997).

Studies of the sub-threshold effect in sub-threshold mixtures are few. Interestingly, studies about the effect of sub-threshold stimuli on suprathreshold perception are even fewer, especially in olfaction. So far, there is only one study regarding this matter published. Bult and others (2001) reported the concept-influenced detection probability of detecting sub-threshold and peri-threshold components in suprathreshold and water background of apple aroma. The sub-threshold and peri-threshold components were not detected better in the suprathreshold background compared to in the water background (Bult et al., 2001). These results suggest a suppression mechanism in olfaction. However, when subjects were given a well-defined concept of the sub-threshold and peri-threshold stimuli, the probability to detect the sub-threshold or peri-threshold stimuli was better in the suprathreshold background than in the water background (Bult et al., 2001). This result suggested cognitive-level influenced odor detection especially semantic information as reported in the visual system (Bult et al., 2001).

Moreover, lateralization improvement in odor detection thresholds of humans, which happened unconsciously, was reported (Radil & Wysocki, 2001).

From those studies, the additive effect of sub-threshold odorants in sub-threshold mixtures is foremost supported by much evidence. However, it is important to note that the evidence was found mostly in the mixtures of odorants that are in the same functional groups or close in shape and size. The effect is uncommon in the mixtures of odorants with different functional groups, shapes and sizes (Guadagni et al., 1963b; Keith & Powers, 1968). The effect of sub-threshold odorants on perception of suprathreshold odorant exists (Bult et al., 2001) but much more evidence (both psychophysical and physiological) is needed to understand this phenomenon.

7. Stimulus-presentation procedures

Besides physiological, psychological and chemical aspects of the olfaction system, stimulus-presentation procedure is another important aspect in olfactory studies. Stimuli should be presented in a consistent manner with reliable equipment (Doty & Kobal, 1995). Therefore, stimulus-presentation procedure includes how and with what equipment researchers present a stimulus to subjects.

Stimulus-presentation procedures are classified into two categories: static and dynamic stimulus presentations (Berglund et al., 1986). In a static presentation-procedure, odorants are in closed containers of various types and/or only the movement caused by inhalation and diffusion occurs. On the other hand, a

dynamic presentation-procedure requires a continuous flow of odorants; researchers are able to control the concentrations of odorants by adjusting the flow of the odorants (Berglund et al., 1986). This section includes the descriptions of some static and dynamic stimulus-presentation procedures used by researchers in olfactory studies.

7.1. Static stimulus-presentation

Sniffing from a container or dosage system is the most common procedure used in olfactory studies (Berglund et al., 1986). Glass-sniff-container or open-and-sniff method is the most popular container used the sniffing procedure (Acree et al., 1985; Cain, 1977; Engen, 1960; Hudry et al., 2002; Hulshoff Pol et al., 1998; Lawless et al., 1995; Lehrner et al., 1999; Martin et al., 2000; Moncrieff, 1956; Olsson & Friden, 2001). The open-and-sniff method is used in a standard clinical test to study the pleasantness rating of amyl acetate (Doty et al., 1995).

In addition to glass containers, some mediums can carry odorants from closed container to the nose of subjects such as a cotton pad (Davidson & Murphy, 1997), a cotton ball on a stick (Engen & McBurney, 1964; Henion, 1971), a strip of blotter paper (Theimer & McDaniel, 1971) and a small hollow-tube (Hummel et al., 1997). The cotton pad was used in the Alcohol Sniff Test for rapid administration in clinical testing. T&T olfactometer test, a standardized olfactory test widely used in Japan, uses a strip of blotter paper to present odorants to subjects (Takagi, 1989);

Sniffin' Sticks test, a North American standardized olfactory test, uses the hollow-tube method (Hummel et al., 1997).

A plastic squeeze bottle is another odorant dispenser widely used in olfactory studies (Algom & Cain, 1991; Ayabe-Kanamura et al., 1998; Cain et al., 1988; Cometto-Muniz & Cain, 1995; Cometto-Muniz et al., 2000; Distel & Hudson, 2001; Guadagni et al., 1963a; Guadagni et al., 1963b; Laska et al., 1997; Pierce et al., 1995; Stevens & Cain, 1985; Takeoka et al., 1996). In 1983, a squeeze bottle with a cap with a pop-up spout was used in clinical tests for the first time (Amoore & Ollman, 1983). Two standard clinical tests, the Connecticut Chemosensory Clinical Research Centre (CCCRC) olfactory test (Cain & Rabin, 1989) and Smell Threshold Test™ (Doty, 2001), are performed by means of the squeeze bottle (Dawes, 1998; Doty & Kobal, 1995; Hummel et al., 1997).

Another presentation procedure in the static category is a "scratch and sniff" technique. This technique uses paper that is adhered with microencapsulated odorants and the odorants are released by scratching the surface of the paper (Hummel et al., 1997; Wysocki & Gilbert, 1989). Using microencapsulated odorant minimizes odor preparation time and increases stability of odorants over time. The University of Pennsylvania Smell Identification Test(UPSIT) that uses encapsulated odorants as an administering procedure is the most distributed standardized olfactory test in North America ((Dawes, 1998; Doty, 1984, 1997, 2001; Doty & Kobal, 1995; Doty et al., 1995).

Besides small containers, researchers use a scented room as an odorant container and subjects walk into the room to sniff the odorant (Degel et al., 2001; Knasko, 1995). A scented room provides the most realistic situation; however, it is inconvenient and expensive to prepare, clean, control and maintain the condition in the room. A smaller version such as a hood that fits a subject's head has been developed to minimize cost and gain more control in testing (Berglund et al., 1986).

Disadvantages of the static stimulus-presentation are the slow control of odorant concentrations and environmental interferences such as temperature and humidity. The environmental interference led to the development of the squeeze bottle method to replace the open-and-sniff method. The squeeze bottle method was reported to give a more reproducible threshold than the open-and-sniff method (Guadagni et al., 1963a). However, the open-and-sniff method is still widely used because it is much cheaper and provides a more realistic situation than the squeeze bottle method. Moreover, with care and advancement in environmental control technology, the environmental interference problems can be minimized regardless of method used. In addition, Doty and others (1995) reported higher test-retest reliability of an open-and-sniff method than a squeeze bottle method.

7.2. Dynamic stimulus-presentation

The continuous flow of odorants distinguishes dynamic stimulus-presentation procedures from the static methods (Berglund et al., 1986); however, in practice,

the ability to change odorant concentration quickly without changing odorant container is a major criteria to distinguish them. Equipments that researchers have been using to facilitate the changes of odorants are called olfactometers. By this definition, an olfactometer can be used in both static and dynamic situations. However, in this thesis, the definition of an olfactometer will be limited to equipment that can facilitate rapid changes in the concentration of odorants in a dynamic stimulus-presentation.

The first recognized dynamic procedure is the draw-tube olfactometer of Hendrick Zwaardemaker that was developed in the late 19th century (Berglund et al., 1986; Dawes, 1998; Doty & Kobal, 1995). The concentration of odorants administered by the Zwaardemaker olfactomer has been controlled manually by subjects; however, olfactometers used today are computerized and controlled by researchers (Berglund et al., 1971; Cheesman, 1964; Dravnieks et al., 1981; Engen, 1965; Gregson et al., 1969; Jinks & Liang, 2001; Laing, 1994; Laing, 1988; Murphy et al., 1994; Stone, 1963).

Even though sophisticated olfactometers allow researchers full control of odorant concentration, researchers still have the same problems with sniffing as in the static procedures. In order to minimize those problems, special sniffing masks or hoods were developed (Berglund et al., 1971). The hood represents a more realistic situation than the sniffing masks but it added more expense to the already expensive systems. Another disadvantage compared to the static procedures is

subject's odor perception may be affected by the movements of the odorants (Berglund et al., 1971).

II.4 The odorants

Aroma is a result of sensations stimulated by odorants followed by perceptions and then responses from the brain. Structural features of odorants are responsible for the perception of the aromas. However, there is no physical and chemical measurement that can accurately predict aroma quality and intensity of odorants.

Acetic acid, acetaldehyde, butanoic acid, ethanol and propanoic acid were selected in the current studies because of their close molecular features. Acetic acid, acetaldehyde and ethanol contain two carbon atoms connected with each other by a single covalent bond. The major differences between acetic acid, acetaldehyde and ethanol are functional groups: carboxylic, carbonyl and hydroxyl groups, respectively.

On the other hand, acetic acid, propanoic acid and n-butanoic acid have the same functional group, a carboxylic group, but with different numbers of carbon atoms: two, three and four, respectively. Some physical and chemical properties of the selected acids are reported in Table 1.

However, in real live situations, food aromas are complicated perceptions as results from tremendous combinations between odorants in both suprathreshold and sub-threshold levels. Acetic acid, acetaldehyde, butanoic acid, ethanol and

propanoic acid are not the exception. These odorants are usually found in food aromas as dominant or minor odorants. The examples of food products containing these odorants are shown in Table 2.

1. Acetic acid

Glacial acetic acid or pure acetic acid is rarely used as a flavoring (Reineccius, 1981); however, vinegar is commonly used. There are six types of vinegars, cider, wine, malt, sugar, glucose and spirit vinegar, which are recognized by the United States Food and Drug Administration (Furia, 1972). Vinegar must contain at least 4 grams of acetic acid per 100 ml (Furia, 1972). Vinegar is considered differently in different regions around the world (Reineccius, 1981). For example, vinegar generally means cider vinegar in the United States but it

Table II.1 Physical and chemical properties of selected odorants

	Acetic acid	Acetaldehyde	Butanoic acid	Ethanol	Propanoic acid
Structural formula	CH_3COOH	CH_3COH	$CH_3(CH_2)_2COOH$	CH_3CH_2OH	CH_3CH_2COOH
Molecular weight	60	44	88	46	74
Functional group	Carboxyl	Carbonyl	Carboxyl	Hydroxyl	Carboxyl
Number of Carbon atoms	2	2	4	2	3
Boiling point (°C)	118	21	163	78	141
Density (g/cm^3)	1.05	0.78	0.96	0.79	1.41
Solubility (in water, %)	100	100	100	100	100
Oxidative power	High	Medium	High	Low	High
Reductive power	Low	Medium	Low	High	Low
Polarity[a]	1^{st}	5^{th}	3^{rd}	4^{th}	2^{nd}
Dielectric constant (ε)[b]	6.15 @20°C	2.1 @10°C	2.97 @20°C	24.3 @25°C	3.30 @10°C

"a" = 1^{st} is being the highest and 5^{th} is being the lowest

"b" = from (Weast, 1975)

Table II.2 Examples of foods containing acetic acid, acetaldehyde, butanoic acid, ethanol and propanoic acid

Product	AA[a]	ACT[b]	BA[c]	EtOH[d]	PA[e]	Reference
Beverage						
Green Tea			X (buttery, rancid[f])			(Guth & Grosch, 1993)
Black Tea			X (buttery, rancid)			(Guth & Grosch, 1993)
Strawberry	X (sour)		X (sweaty, rancid)			(Schieberle & Hofmann, 1997)
Grape juice			X			(Sefton et al., 1993)
Vegetable (Fermented)						
Kimchi	X (vinegar)		X	X		(Cha et al., 1998)
Fish						
Emerald Shiners (fresh)	X			X		(Josephson et al., 1984)
Salmon (canned)	X	X				(Girard & Nakai, 1991)
Cod (boiled)		X (sweet)				(Milo & Grosch, 1995)
Trout (boiled)		X (sweet)				(Milo & Grosch, 1995)
Fish sauce	X	X	X (cheesy)		X	(McIver et al., 1982; Peralta et al., 1996; Sanceda et al., 1992; Shimoda et al., 1996)

"a" = Acetic acid
"b" = Acetaldehyde
"c" = Butanoic acid
"d" = Ethanol
"e" = Propanoic acid
"f" = Descriptor(s) used in the reference(s)

Table II.2 (continued) Examples of foods containing acetic acid, acetaldehyde, butanoic acid, ethanol and propanoic acid

Product	AA[a]	ACT[b]	BA[c]	EtOH[d]	PA[e]	Reference
Meat						
Dry-cured Ham	X (vinegar)			X		(Flores et al., 1997)
Dairy						
Skim milk	X		X	X	X	(Shiratsuchi et al., 1995)
Milk (off-flavors)	X		X			(Marsili, 1999)
Dommiati Cheese		X		X		(Collin et al., 1993)
Cheddar Cheese	X (sour[f])	X (sweet, pungent)	X (sweet, sweaty)		X	(Dacremont & Vickers, 1994; Jack et al., 1993; Milo & Reineccius, 1997)
Rice						
Cooked Rice		X		X		(Buttery et al., 1988; Maga, 1984)
Scent Rice		X		X		(Buttery et al., 1988; Maga, 1984)
Raw rice		X		X		(Buttery et al., 1988; Maga, 1984)
Fruit						
Caja	X		X			(Allegrone & Barbeni, 1992)
Raspberry	X		X			(Pabst et al., 1991)

"a" = Acetic acid
"b" = Acetaldehyde
"c" = Butanoic acid
"d" = Ethanol
"e" = Propanoic acid
"f" = Descriptor(s) used in the reference(s)

Table II.2 (continued) Examples of foods containing acetic acid, acetaldehyde, butanoic acid, ethanol and propanoic acid

Product	AA[a]	ACT[b]	BA[c]	EtOH[d]	PA[e]	Reference
Vinegar						
Wine Vinegar	X	X	X	X	X	(Blanch et al., 1992)
Sherry Vinegar	X	X	X	X	X	(Blanch et al., 1992)
Alcoholic beverage						
Wine	X		X		X	(Ferreira et al., 1993; Villen et al., 1995)
Tequila	X	X		X		(Benn & Peppard, 1996)
Beer	X (acetic[f])	X (green leaves, fruity)	X (butanoic)	X (alcohol)	X (acetic, milky)	(Meilgaard, 1982; Meilgaard, 1975; Siebert, 1999)

"a" = Acetic acid
"b" = Acetaldehyde
"c" = Butanoic acid
"d" = Ethanol
"e" = Propanoic acid
"f" = Descriptor(s) used in the reference(s)

means malt vinegar in the United Kingdom (Reineccius, 1981). Vinegar is used in many products such as pickles, sauces and relishes.

Vinegar can be produced from carbohydrate fermentation by acetobacter bacteria. Acetobacter fermentation converts ethyl alcohol produced from yeast fermentation to acetic acid. Acetic acid content in vinegar is expressed as grain: 60 grain vinegar being 6 percent acetic acid.

In addition to vinegar, acetic acid is also found in many foods as a minor constituent such as fruit flavor (Reineccius, 1981), fish aromal (Josephson et al., 1984), fermented fish (McIver et al., 1982; Peralta et al., 1996; Sanceda et al., 1992; Shimoda et al., 1996) and dairy products (Collin et al., 1993; Dacremont & Vickers, 1994; Jack et al., 1993; Milo & Reineccius, 1997; Shiratsuchi et al., 1995). For example, in dairy products, acetic acid also is produced by lactic acid bacteria such as *Leuconostoc sp.* through heterofermentation, which produces lactic acid, acetic acid, ethanol, carbon dioxide and other metabolites (Reineccius, 1981).

Acetic acid is a predominant odorant in vinegar or aerobically fermented products produced from carbohydrate sources. In several foods, acetic acid exists as a minor odorant but still contributes to overall perception of the food. It provides pungent, sour, and vinegar aroma, astringency and sour taste to foods and related products (Civille & Lyon, 1996). The olfactory threshold of acetic acid in water is reported in Table 3.

Table II.3 Olfactory thresholds of selected odorants[a]

Odorant	Threshold (ppm)[b]
Acetic acid	100
Acetaldehyde	0.0007-0.21
Butanoic acid	7.0
Ethanol	0.25-9.23
Propanoic acid	20

"a" = from (ASTM, 1978)
"b" = in water

2. Acetaldehyde

Acetaldehyde is an important compound, widely found in fruits such as orange or persimmon (Bruemmer, 1986; Lindsay, 1996) and cultured dairy products especially yogurt.

It is commercially produced as a final product of ethanol or glucose by the yeast *Candida utilis*. Acetaldehyde also can be produced from fermentation of alcohol by *Penicillium pastoris* (Reineccius, 1981). *Leuconostoc citrovorum* produces acetaldehyde along with other metabolites by using citric acid as raw material (Lindsay, 1996; Walsh & Cogan, 1973). Moreover, homoferementation from *Lactobacillus lactis* and *Streptococcus thermophilus* yields acetaldehyde, which is a character-impact compound found in yogurt (Gonzalez et al., 1994; Lindsay, 1996). In addition, acetaldehyde is also produced along with ethanol in wheat dough leavening. Oxidation of alcohol is catalyzed by alcohol dehydrogenase in yeast fermentation to produce acetaldehyde (Lortie et al., 1992).

Acetaldehyde was reported to cause astringency in persimmons (Pesis et al., 1988). In citrus fruit, acetaldehyde is produced through the decarboxylation of pyruvate catalyzed by pyruvate decarboxylase during maturation of fruits (Bruemmer, 1986).

Besides astringency, acetaldehyde provides sharp, penetrating, choking fruity aroma, leafy green taste in fruits especially orange, apple, and butter (Civille & Lyon, 1996; Furia, 1980). Moreover, acetaldehyde helps to create naturalness, fruitiness and juiciness in flavoring agents (Furia, 1980). The olfactory threshold of acetaldehyde in water is reported in Table 3.

3. Butanoic acid

Butanoic acid is commonly found in dairy products such as cheese and butter as a result from lypolysis of fats or bacterial metabolism of carbohydrates and amino acids (Reineccius, 1981; Sinki & Schlegel, 1990). Hydrolysis of milk fats under acidic condition results in butanoic acid, which gives a cheesy, milky and buttery flavor (Lindsay, 1996). Butanoic acid gives characteristic flavor to Romano and Provolone cheese (Gatfield, 1986). In Cheddar cheese, n-butanoic acid is one of major volatile fatty acids produced by the starting culture of *Lactobacilli* or *Pediococci* (Dacremont & Vickers, 1994; Jack et al., 1993; Milo & Reineccius, 1997; Reineccius, 1981; Scharpf et al., 1986). Butanoic acid is produced in butter by *Clostridium butyricum* using dextrose as a substrate (Sinki & Schlegel, 1990).

Butanoic acid is desirable in dairy products but not in fermented products such as fish sauce and soy sauce (Lopetcharat, 1999). A cheesy note is an indicator of low quality fish sauce, which is believed to be produced by excessive oxidation of fatty acids by non-enzymatic reactions and/or microbial reactions, especially from *Bacillus*, during fermentation (McIver et al., 1982; Peralta et al., 1996; Sanceda et al., 1992; Shimoda et al., 1996). In addition, butanoic acid is also reported in beer (Meilgaard, 1975), vinegar (Blanch et al., 1992), tea (Guth & Grosch, 1993), strawberry juice (Schieberle & Hofmann, 1997), grape juice (Sefton et al., 1993), and more.

Butanoic acid contributes to cheesy and buttery notes in cultured dairy products; however, excessive amount of butanoic acid will give sour and rancid notes. Moreover, trigeminal sensations such as a sharp and pungent smell are perceived at high levels of butanoic acid (Furia, 1980; Sinki & Schlegel, 1990). Lypolysis of lipids or fermentation of carbohydrates and proteins produces butanoic acid. Because of its low olfactory threshold and cheese-like aroma, in some products such as tea or fish sauce, it is undesirable even though it is present in a small amount. The olfactory threshold of butanoic acid in water is reported in Table 3.

4. Ethanol

Ethanol is one of the final products from fermentation of the yeast *Saccharomyces cerevisiae*, using starches or sugars as raw materials under

anaerobic or oxygen-limited conditions. Generally, ethanol is consumed in the form of alcoholic beverages such as beer and wine. Food grade ethanol is commercially produced by fermentation of starches and sugars from many different sources such as corn, wheat, rye and barley by microorganisms such as *Saccharomyce cerevisiae* and *Zymomonas mobilis* or enzymatic reaction of invertase (Kirk & Doelle, 1993; Wade, 1991). Therefore, ethanol is sometimes called "grain alcohol".

Besides its use as a main ingredient in alcoholic beverages, ethanol is also widely used as an extraction solvent, preservative and flavor carrier (Baranowski, 1990; Furia, 1980). It is also produced as a final product of fermentation and reduction of carbohydrates and acids such as lactic acid and acetic acid. For example, cheese and yogurt contain low levels of ethanol (Dacremont & Vickers, 1994; Marsili, 1999; Shiratsuchi et al., 1995) (Jack et al., 1993; Milo & Reineccius, 1997). Fermentation of lactic acid bacteria such as *Leuconostoc citrovorum* yields low levels of ethanol (in part per million) (Lindsay, 1996). Fermentation or thermal treatment of vegetables, grains, and fruits under limited-oxygen condition also yield ethanol in final products such as kimchi (Cha et al., 1998) and cooked rice (Buttery et al., 1988; Maga, 1984). Ethanol is naturally generated during maturation of citrus fruits by metabolism of malate, pyruvate and acetaldehyde under anaerobic conditions (Bruemmer, 1986). In addition to natural foods, ethanol is used in candy manufacture as a carrier of candy glaze material. Ethanol is later evaporated leaving a coating of the glaze on the candy (Baranowski, 1990).

Ethanol provides an alcoholic, pungent, and solvent note in alcoholic beverages (Civille & Lyon, 1996; Meilgaard, 1982; Meilgaard, 1975). At low concentrations it provides sweet notes. Ethanol is also found at very low or non-detectable levels in final products when it is used as a flavor carrier for flavoring agents. The olfactory threshold of ethanol in water is reported in Table 3.

5. Propanoic acid

Propanoic acid is used as a preservative because of its fungistatic properties (Furia, 1972). It is found in many foods such as baked goods, cheese, confections and frosting, gelatins, candy, non-alcoholic beverage, etc. *Bacillus mesentericus* causing ropiness in baked good is prevented by adding propionate salts in baked products (Jay, 1992; Reineccius, 1981). In dairy products, propanoic acid is used as a dipping agent for solid cheeses such as cheddar cheese to prevent mold growth. Propanoic acid is also naturally found in Swiss cheese in which it is produced by *Propionivacteriuj shermanii* (Furia, 1972; Lindsay, 1996). It is also naturally generated in the rumen of ruminant animals such as the cow (Furia, 1972).

Besides its direct use in food, propanoic acid is also widely used on packaging materials such as lids, bags, containers, caps and wrappers as antimycotics (Furia, 1972). Propanoic acid derivatives are widely used in food packaging in polymeric coatings and paper and paper board in contact with dry food (Furia, 1980).

Propanoic acid has a strong odor somewhat reminiscent of acetic acid and butanoic acid. Its aroma tends to resemble acetic acid, sour, pungent and vinegar when in concentrated and butanoic acid, buttery and cheesy, when in diluted forms (Furia, 1980; Reineccius, 1981). There is no legal limit of using propanoic acid; however, its use is limited because of its undesirable sensory properties (Doores, 1990). The olfactory threshold of propanoic acid in water is shown in Table 3.

Chapter 3

III. FUNCTIONAL-GROUP DEPENDENT SUB-THRESHOLD SUPPRESSION OF THE PERCEIVED INTENSITY OF RECOGNIZABLE ODORANTS IN BINARY MIXTURES

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III.1 Abstract

The perceived intensities, overall intensity and two or three additional descriptors, of a recognizable odorant in the presence of a sub-threshold or a peri-threshold odorant was studied using magnitude estimation. All possible binary combinations of acetic acid, acetaldehyde and ethanol were prepared to address the effect of sub-threshold and peri-threshold odorants with different functional groups. Sub-threshold and peri-threshold concentration was maintained and its effect was tested by expressing the sub-threshold and peri-threshold odorants as percentages of individual detection thresholds (30%, 50%, 70%, 90%, 100%, 110%, and 120%). Concentration independent sub-threshold suppression was initiated at 30% of individual detection thresholds, which suggests a non-competitive mechanism. The reciprocal sub-threshold suppression was found in ethanol/acetaldehyde mixtures and non-reciprocal sub-threshold suppression was found in mixtures containing acetic acid at panel-recognition or sub-threshold concentrations. Acetic acid at sub-threshold level suppressed the intensities of the descriptors of ethanol and acetaldehyde. Ethanol at sub-threshold levels suppressed only overall intensity of acetaldehyde at group recognition concentration, but acetaldehyde at sub-threshold levels suppressed all intensities of the descriptors of ethanol.

III.2 Introduction

Aromas are complex mixtures of numerous odorants at different concentrations. The ability of the olfactory system to recognize and discriminate between enormous numbers of odorants is remarkable. Suppression is one of the mechanisms that the brain uses to manage this vast amount of information. The intensity of suprathreshold odor mixtures has been studied with respect to both physiological and psychological aspects (Ache, 1989; Cain et al., 1995; Derby et al., 1985). A chromatographic-like process governing the distribution of odorants across the olfactory epithelium in frog and the spatial and temporal processing of odor information was proposed (Mozell, 1964, 1970, 1971; Mozell & Jagodowicz, 1973). Peripheral and central neural components governing suprathreshold mixture suppression were discovered using a lobster model (Derby et al., 1985). Evidence suggested that suppression in suprathreshold odor mixtures is primarily a peripheral event and depends on the polarity of the component in the mixtures (Ache et al., 1987; Bell et al., 1987). Laing (1988) concluded that the interaction between odorants in suprathreshold mixtures could be partially predicted by the polarity of each component in the mixtures. Non-reciprocal and incomplete reciprocal suppression among polar and non-polar odorants were discovered (Laing, 1988).

Few studies of the perception of sub-threshold mixtures have been reported. The most common outcome reported is an additive or agonistic effect (Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991; Patterson et al., 1993). In some cases, a synergistic effect was reported (Laska & Hudson, 1991;

Nawar & Fagerson, 1962). These studies employed detection threshold determination of odor mixtures and discovered that, at the mixture's detection threshold, the individual component concentration in the mixture is lower than the individual component concentration at its individual detection threshold. Therefore, researchers concluded the effect of a sub-threshold mixture to be additive or synergistic. However, some studies indicated that additive and synergistic effects are not common phenomena (Guadagni et al., 1974b; Keith & Powers, 1968).

Laska and Hudson (1991) reported both additive and synergistic effects in odor mixtures, but they also pointed out that there were only 53% of subjects who demonstrated increased sensitivity toward the mixtures. The disagreement among subjects' detection thresholds of odor mixtures should be caused by differences in sensitivity between subjects. Commetto-Muniz and Cain (1997) took into account each subject's detection threshold for each component in the mixtures during the analysis step and concluded that the effect was agonistic (additive).

Most odor-mixture research focused on the perception of either suprathreshold or sub-threshold mixtures (Berglund et al., 1971, 1976; Cain et al., 1995; Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991). However, aromas encountered everyday are comprised of both sub-threshold and supra-threshold odorants. Research determining the effect of sub-threshold odorants on supra-threshold odorants or vice versa is very limited. In taste, Guadagni and others (1973) reported an enhancing effect of naringin

dihydrochalcone at detection threshold on bitterness of a dilute limonin solution. However, neohesperidin dihydrochalcone at detection threshold did not increase the bitterness of the dilute limonin solutions (Guadagni et al., 1974a). In vision, sub-threshold stimuli were reported to contribute to observed intrinsic signal activity in a cat's visual cortex and it was believed to result from metabolic activity in dendrites or at synapses (Toth et al., 1996).

In olfaction, there is only one published study aimed at the investigation of the perception of the mixtures of sub-threshold and supra-threshold odorants (Bult et al., 2001). Bult and others (2001) employed a duo-trio method to test discrimination ability of sub-threshold mixtures of ethanol, 1-butanol, and 1-hexanol in a water and supra-threshold apple mixture background. Discrimination ability in the water background condition was higher than in the supra-threshold apple mixture background condition (Bult et al., 2001). However, the discrimination ability of the sub-threshold mixtures in the supra-threshold apple background was increased when a refined concept of an apple aroma was introduced to the subjects. The sub-threshold components were not detected better in the apple-like mixture background than the water background. From these results, Bult and others (2001) concluded that sub-threshold discrimination started from cognitive processing.

This study was designed to investigate the effect of sub-threshold and peri-threshold odorants on the intensities of recognizable odorants (acetic acid, acetaldehyde and ethanol) in binary mixtures. In addition to overall intensity, the

effect of sub-threshold odorants on the intensities of specific descriptors of recognizable odorants was investigated. In addition, this study was performed to determine whether the extent of the effect depends on the functional groups and the concentrations of the odorants tested.

III.3 Materials and methods

1. Odorants and odorant presentation

Food grade ethanol, acetic acid and acetaldehyde (99% purity), purchased from Aldrich (Milwaukee, WI) were used as odorants in this study (Table III.1). Double-distilled deionized water served as the solvent for all odorants. Odorants were presented in 125-ml wide-mouth column clear glass bottle (inside diameter 4.8 cm) with screw cap containing five 4-ml vials. The bottle caps were lined with a Teflon® liner. Five vials were needed in order to fill up the space in the bottle but the number of vials used depended on the experiments. The headspace ranged from approximately 108 ml to 112 ml depending on the experiments.

In threshold determination and panel-recognition concentration determination experiments, one vial contained 4-ml of an odorant solution or 4-ml of deionized water (blind control and the other four vials were empty. In the binary mixture study, two vials contained odorants and three vials were empty. One of the two vials contained 4-ml of a recognition-concentration odorant solution, the other vial contained 4-ml of a sub-threshold odorant solution or 4-ml of deionized water

(blind control). All bottles and vials were washed with distilled water and baked at 130°C for at least five hours before use. The plastic caps and Teflon® liners were boiled for 10 minutes in distilled water and air-dried. All bottles were labeled with 3-digit random codes.

In all experiments, the bottles containing the solutions were placed in an odor free room at ambient temperature (~25°C-27°C) for at least 2 hours prior to the experiment to allow for the saturation of the odorants in the bottles' headspace. All subjects were required to wear white cotton gloves that had been washed with only hot water and tumble dried before contacting any bottles.

2. Screening test and subjects

Twenty volunteers were screened based on their ability to detect and recognize ethanol, acetic acid and acetaldehyde using 3-AFC ascending concentration series method of limits (ASTM, 1999). Subjects whose detection and recognition thresholds were within two SDs from the geometric mean of the group threshold were selected.

Fourteen female and three male subjects (mean age = 27, SD = 4, range 22 to 35) participated in the study after informed consent. The subjects were paid for their participation. Subjects were not under medication and were non-smokers. They did not suffer from any acute or chronic illness of the respiratory system (self-reported). Subjects were instructed not to use odorous personal products on the day of the experiment.

3. Subject training

3.1 Odorants' descriptor definition

Standard ethanol, acetic acid and acetaldehyde solutions at different concentrations were introduced to subjects. Subjects were asked to describe all the solutions and consensus descriptors were selected via discussion (Table III.1).

3.2 Intensity rating training

Magnitude estimation (ME) was employed to rate the intensity of the odorants. First, subjects were familiarized with ME using acetaldehyde as a sample odorant and n-butanol as a modulus. Then an acetaldehyde standard was introduced as the modulus instead of n-butanol in order to evaluate all descriptors (overall aroma intensity, sweetness, sourness and green).

Table III.1 Odorants and descriptors accepted by subjects

Compounds	MW	Purity	Descriptors	Concentrations*
Ethanol	60	99.9%	Sweetness Alcohol	438.6 and 877.2 mM
Acetic acid	44	99.9%	Sweetness Sourness Vinegar	8.9 and 17.9 mM
Acetaldehyde	46	99.9%	Sweetness Sourness Green	546.3 and 1092.7 nM

* in double distilled deionized water

Individual overall-intensity power functions of acetaldehyde were constructed using five concentration levels. The concentration levels decreased 2-fold for each dilution step. Individual subject's performance was evaluated using their acetaldehyde power function. Subjects were ready for testing when they could correctly rate and order the intensity of four out of five concentrations and using performed magnitude estimation appropriately. All tests were done in triplicate.

3.3 Individual detection threshold and recognition threshold determination

Each individual has a different detection and recognition threshold for any odorant. The main objective of this study was to investigate the effect of sub-threshold and peri-threshold concentrations of an odorant on the intensity of the recognition level of another odorant. In order to ensure that the sub-threshold, peri-threshold and recognition concentration condition were maintained, the detection threshold and recognition threshold of each subject had to be measured.

Detection and recognition threshold determinations were conducted after the training sessions because the sensitivity of subjects tends to improve after training (Engen, 1960; Laska & Hudson, 1991; Lawless et al., 1995). Determination of both detection and recognition thresholds was performed for all odorants: acetic acid, ethanol and acetaldehyde in three separate sessions. Seventeen doubling concentration steps of each odorant were prepared. One vial

containing 4 ml of an odorant was placed in 125-ml bottle with four empty vials (headspace ~ 112 ml). Bottles were closed and left at 25°C-27°C for at least 2 hours before testing.

Individual detection thresholds were determined using the 3-AFC ascending concentration series method of limits (ASTM, 1999). Subjects were introduced to the reference odorants before the test. An individual's detection threshold was defined as the geometric mean of the geometric means from four replications. The replication geometric means of detection threshold were calculated from the highest incorrect concentration and the lowest correct concentration followed by three consecutive correct concentrations. An individual's recognition threshold was defined as in the same way as the individual detection threshold, but the replication geometric means of recognition threshold were calculated from the highest *incorrectly identified* concentration and the lowest *correctly identified* concentration followed by three correctly identified consecutive concentrations. The individual's detection threshold and recognition thresholds are in Tables III.2 and III.4.

Subjects were grouped by their individual detection thresholds because of the close proximity of individual detection thresholds between subjects. Because the data tend to have log-normal distributions, (ASTM, 1999) the grouping was performed on log10 of the individual thresholds employing ANOVA followed by Duncan's multiple range test. The geometric mean of the individual's thresholds of subjects from the same group was calculated and assigned as a group detection

threshold. The group detection thresholds were also assigned as the individual detection thresholds for subjects in the same group. The group detection thresholds for all odorants are shown in Table III.3.

3.4 Determination of panel-recognition concentration of all odorants

The panel-recognition concentration of an odorant is defined as the concentration at which all subjects can 100% correctly identify the odorant. Highest individual recognition thresholds for all odorants among all subjects were used as starting concentrations. For example, from Table III.4, the highest individual recognition threshold for acetic acid was 6.33 mM; this concentration was used as the starting concentration to determine panel-recognition concentration of acetic acid. The 3-AFC technique was employed to confirm the concentrations.

Table III.2 Individuals' detection thresholds for ethanol, acetic acid and acetaldehyde*

	Ethanol (mM)		Acetic acid (mM)		Acetaldehyde (nM)	
Subject	**Median**	**SD**	**Median**	**SD**	**Median**	**SD**
1	3.04	0.03	1.26	0.03	3.80	0.03
2	1.21	0.06	0.20	0.03	2.40	0.03
3	12.21	0.07	1.26	0.03	9.58	0.03
4	38.76	0.14	0.16	0.04	30.42	0.03
5	1.53	0.07	0.63	0.07	0.50	0.03
6	0.96	0.04	0.47	0.08	3.02	0.02
7	1.44	0.03	5.02	003	1.79	0.04
8	2.42	0.07	0.03	0.04	1.27	0.07
9	0.86	0.04	0.63	0.04	12.07	0.05
10	0.38	0.03	0.79	0.04	0.90	0.05
11	6.11	0.03	0.14	0.04	1.07	0.06
12	8.15	0.17	0.40	0.04	9.58	0.03
13	0.43	0.03	3.16	0.04	2.54	0.07
14	1.53	0.05	1.99	0.03	1.20	0.05
15	0.86	0.06	0.02	0.03	0.60	0.03
16	0.86	0.04	0.47	0.04	0.95	0.03
17	1.44	0.04	1.00	0.04	1.51	0.06

* in double distilled deionized water

Table III.3 Group detection thresholds for ethanol, acetic acid and acetaldehyde*

Ethanol detection threshold (mM)			
Group	**Median**	**SD**	**Subjects**
1	0.40	0.03	10,13
2	1.15	0.30	9,15,16,6,2,7,17,5,14
3	4.38	2.68	8,1,11,12
4	12.21	0.07	3
5	38.76	0.14	4

Acetic acid detection threshold (mM)			
Group	**Median**	**SD**	**Subjects**
1	0.016	0.026	15
2	0.025	0.035	8
3	0.163	0.030	11,4,2
4	0.704	0.332	12,6,16,5,9, 10,17,1,3
5	1.992	0.026	14
6	3.162	0.035	13
7	5.020	0.260	7

Acetaldehyde detection threshold (mM)			
Group	**Median**	**SD**	**Subjects**
1	0.53	0.09	5,15
2	0.90	0.05	10
3	1.27	0.31	16,11,14,8, 17,7
4	2.90	0.63	2,13,6,1
5	9.58	0.00	3,12
6	12.07	0.05	9
7	30.42	0.03	4

* in double distilled deionized water

Table III.4 Individual recognition thresholds and panel-recognition concentrations for ethanol, acetic acid and acetaldehyde*

	Recognition threshold		
Subject	**Ethanol (mM)**	**Acetic acid (mM)**	**Acetaldehyde (nM)**
1	12.21	1.58	9.58
2	48.84	0.40	12.07
3	155.07	1.26	76.66
4	195.37	0.31	76.66
5	12.21	2.51	6.04
6	2.42	1.88	3.02
7	155.07	5.02	136.58
8	620.27	6.33	24.14
9	109.65	1.26	24.14
10	7.69	1.58	60.84
11	155.07	1.33	3.02
12	11.53	0.40	9.58
13	97.69	3.16	17.07
14	155.07	1.99	2.40
15	13.71	1.00	193.16
16	155.07	1.12	17.07
17	9.69	1.58	8.54
Panel reg. conc.	**620.27**	**6.33**	**193.16**

* in double distilled deionized water

One bottle containing an odorant solution and two bottles containing deionized water (a set) were introduced to subjects. Subjects were asked to select the odd bottle and identify the solution. Three sessions of five sets were presented with 30-second set-interval and 10-minute session-interval. All odorants were randomly presented within session. Upon completion of three sessions, each odorant was presented to each subject five times. Overall, each odorant was presented at each presentation order three times across all subjects and sessions. If any subject failed to identify any odorant 100% correctly, the next higher doubling concentration step was introduced. Panel recognition concentration for each odorant was the concentration that all subjects 100% correctly identify the odorant used which was the highest individual recognition threshold for this panel (Table III.4).

3.5 The effect of sub-threshold and peri-threshold on perceived intensity of panel-recognition concentration in binary mixtures (binary mixture study).

A binary system was obtained by placing a 4-ml vial containing a sub-threshold or peri-threshold solution of one odorant with another 4-ml vial containing another odorant at its panel-recognition concentration in a 125-ml bottle with 3 empty 4-ml vials. The headspace was ~ 108 ml. All possible binary combinations of three odorants (ethanol, acetic acid and acetaldehyde) were used as sub-threshold and peri-threshold level solutions. Eight concentrations of individual detection thresholds (0 (blind control), 30, 50, 70, 90, 100, 110, 120% of individual detection thresholds) were used as the sub-threshold level and peri-threshold level

concentrations in this study. The concentrations at and near threshold level were prepared in order to cover the variation in threshold level of individuals (Lawless et al., 1995). All six possible treatment combinations were tested as shown in Table III.5.

A completely randomized block design with factorial structures within blocks was employed. Subjects were treated as blocking factors. Each treatment combination was tested in a separate session. Each session had eight sample bottles (seven concentrations and one blind control), which were randomly presented to subjects. Overall, each of eight sample bottles was presented in each testing position between sixteen to seventeen times across all subjects. The treatment combinations were also randomly assigned to subjects in a balanced fashion. The testing order for each treatment combination was also balanced across all subjects. The control bottle contained double distilled deionized water instead of a sub-threshold solution and was used as a blind control. Thus, it had only a panel-recognition-concentration odorant vial and a water vial. Subjects were instructed to smell standard odorants (moduli) with a designated intensity of 10 (modulus bottles contained one vial with 4-ml of an odorant solution at panel-recognition concentration along with four empty vials) before testing. Subjects sniffed a sample bottle and rated the overall intensity and descriptors' intensity using ME. The matching modulus was provided to subjects at all times. For example, if the recognition concentration tested was acetic acid, an acetic acid

Table III.5 Six possible treatment combinations in the binary mixture study

Recognition level odorant	**Sub-threshold level odorant**
Ethanol	Acetic acid
Ethanol	Acetaldehyde
Acetic acid	Ethanol
Acetic acid	Acetaldehyde
Acetaldehyde	Ethanol
Acetaldehyde	Acetic acid

modulus was provided to subjects for that test. Subjects were informed what recognition concentration was tested in order to increase subjects' sensitivity toward each odorant (Bult et al., 2001). Subjects continued testing with a 30-second sample-interval and a 5-minute session-interval. Subjects were allowed six testing sessions a day. Four replications were accomplished within four days.

4. Data analysis

Since the subjects used different scales and the data tend to follow a log-normal distribution, results from the binary mixture study were normalized within each subject employing a geometric mean normalization (Moskowitz & Jacobs, 1988; Stevens, 1956). Three-way univariate analysis of variance (ANOVA) for concentrations, subjects, replications, all 2-way interaction and a 3-way interaction (concentrations x subjects x replications) were conducted for each individual descriptors within each experiment separately. Subjects and replications were treated as a random effect (Lundahl et al., 1986) and concentrations as a fixed effect. Backward elimination was used to select a final most parsimonious model, which were used to analyze all descriptors within each experiment. The final model consisted of main effects (subjects and concentrations) and two 2-way interactions (subjects x concentration, and subjects x replications). The effect of concentrations was tested against the effect of a 2-way interaction (subjects x concentrations). Dunnett's and Tukey-Kramer multiple comparison procedures were employed to detect the difference from all comparisons against a blind control

(0%) and general pair-wise differences between treatments, respectively. All analysis was performed on the log10 of the normalized data at α=0.05, when otherwise, it will be stated. The six treatment combinations were analyzed separately. Analyses were conducted using General Linear Model in SPSS® V. 10.0 (Chicago, IL).

III.4 Results

1. Odorants and their descriptors

Even though the odorants used in this study were pure compounds, subjects still reported different descriptors for the same odorant due to different experiences with the odorants (Ayabe-Kanamura et al., 1998). Moreover, differences in individual detection and recognition thresholds also cause different perception between individuals. For example, one subject described acetaldehyde as sweet, sour and green apple but others described it as sweet, sour and yogurt. Through discussion sessions, the consensus descriptors except for overall intensity were agreed upon and are shown in Table III.1.

2. Detection thresholds and panel-recognition concentrations

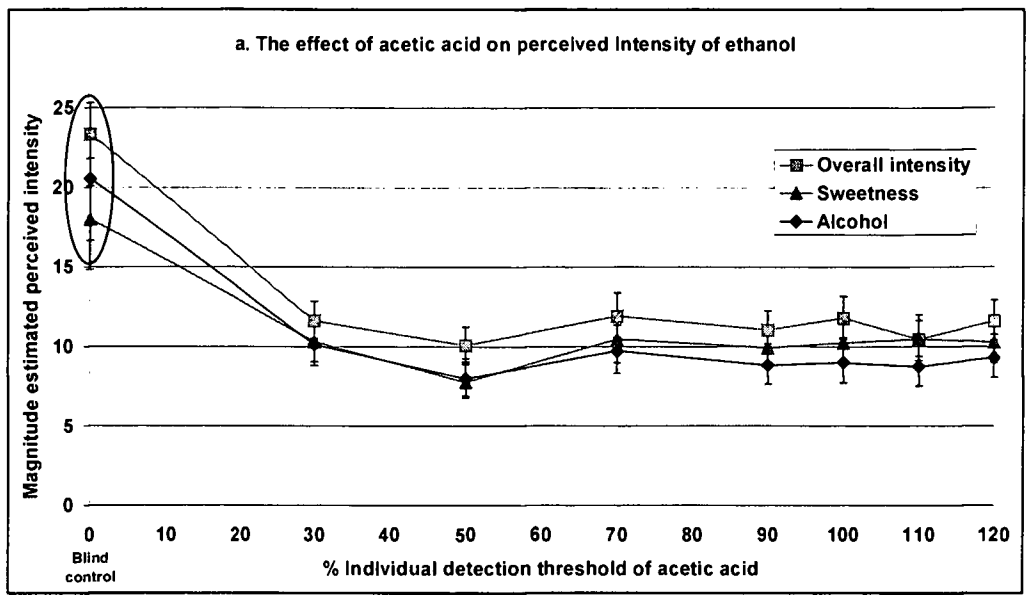
Wide ranges of individual sensitivities to ethanol, acetic acid and acetaldehyde were found among the screened subjects (Table III.2). Detection thresholds of ethanol, acetic acid and acetaldehyde spanned a factor of 1×10^2, 3.14×10^2 and 6.4×10^1 with medians 1.44 mM, 0.63 mM and 1.80 nM, respectively. Group detection thresholds for all odorants are listed in Table III.3. Panel recognition concentrations were 620.27 mM, 362.04 mM and 193.16 nM for ethanol, acetic acid and acetaldehyde, respectively (Table III.4).

3. Binary mixture study

3.1 The effect of acetic acid and acetaldehyde on ethanol perceived intensity

Acetic acid and acetaldehyde at sub-threshold and peri-threshold suppressed the intensities of ethanol (Figure III.1). The intensities of all ethanol descriptors were significantly lower than the blind control in the presence of acetic acid at sub-threshold and peri-threshold levels: [$F(7, 109) = 12.56$, $p < 0.001$ for overall intensity], [$F(7, 105) = 8.88$, $p < 0.001$ for sweetness] and [$F(7, 100) = 9.16$, $p < 0.001$ for alcohol intensity]. The same phenomena were observed in the presence of acetaldehyde at sub-threshold and peri-threshold levels: overall intensity [$F(7, 107) = 8.27$, $p < 0.001$], sweetness [$F(7, 109) = 4.23$, $p = 0.001$] and alcohol intensity [$F(7, 96) = 6.75$, $p < 0.001$]. The suppression did not depend on the concentrations of acetic acid and acetaldehyde after taking into account subjects' individual detection thresholds (Tukey's HSD, $p > 0.05$), but the suppression began at the lowest concentration tested, 30% of subjects' individual detection threshold level.

Because of the non-significant difference between sub-threshold and peri-threshold concentrations on the intensity of all descriptors of ethanol, it is reasonable to average the degree of reduction across all concentration ranges. Approximately, 50% reduction (from magnitude estimate of 24 to 12) of the magnitudes of all descriptors was observed in the presence of sub-threshold and



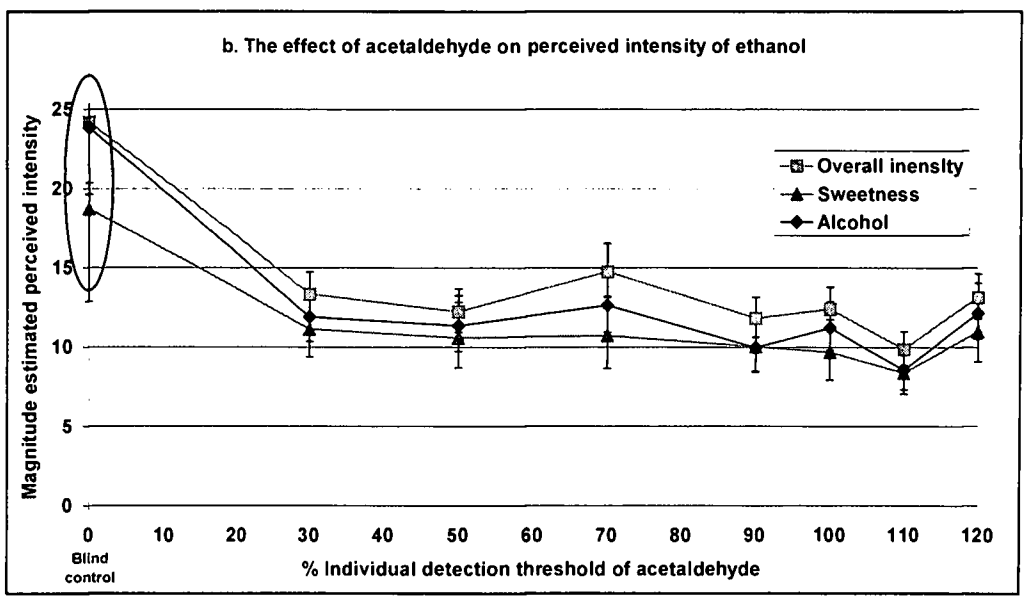


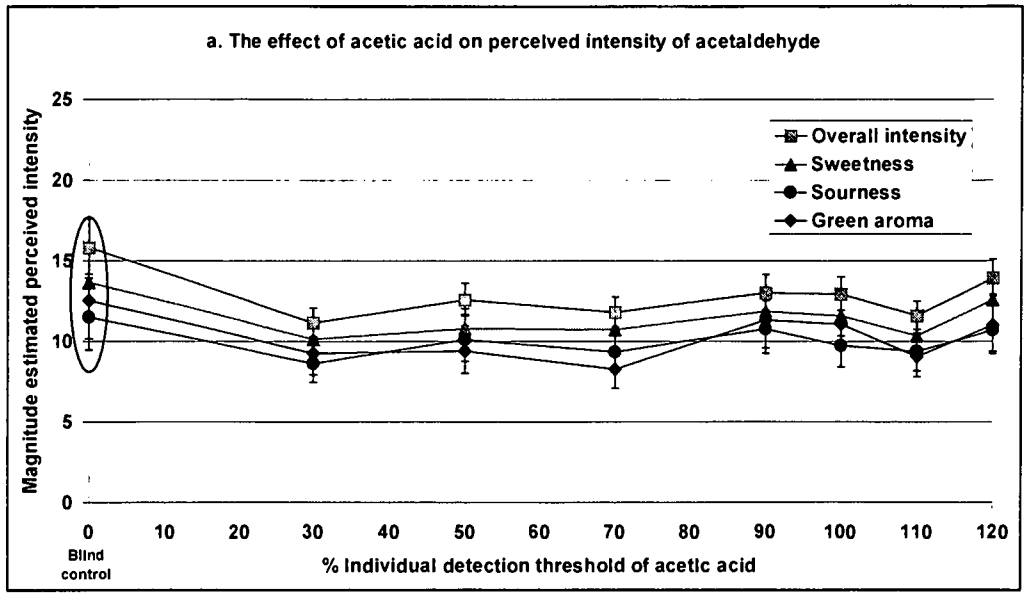
Figure III.1 The effects of (a) acetic acid and (b) acetaldehyde at sub- and peri-threshold concentrations (% individual detection threshold) on the perceived intensity of ethanol (at panel-recognition concentration) in binary mixtures. Values are medians and bars indicate ± 95% confidence interval. The oval indicates significant difference between the blind control and the other samples within descriptor at α=0.05. Seventeen subjects with four replications provided the data for the median estimation.

peri-threshold concentrations of acetic acid (Figure III.1a). The highest reduction happened with alcohol intensity (55%); meanwhile, overall intensity and sweetness were reduced by 52% and 45% respectively. With acetaldehyde (Figure III.1b), the highest reduction observed was for overall intensity (48%). Sweetness and alcohol intensity were reduced by 45% and 44% in the presence of acetaldehyde, respectively.

Because the studies of the effect of acetic acid and acetaldehyde were performed separately, statistical comparison between these two studies was not appropriate. However, by observation, the intensity orders of the descriptors were different between the acetic acid study and the acetaldehyde study. In the blind control, alcohol intensity was higher than sweetness intensity and it maintained the order when acetaldehyde at sub-threshold levels was added (Figure III.1b). On the contrary, when acetic acid was added at 70% or more of subjects' individual detection threshold, sweetness intensity of ethanol was higher than alcohol intensity, which was suppressed the most (~55%) by acetic acid (Figure III.1a).

3.2 The effect of acetic acid and ethanol on acetaldehyde perceived intensity

The effects of acetic acid and ethanol on acetaldehyde were different (Figure III.2). The significant suppression of overall intensity (~21% reduction) of acetaldehyde was caused by the presence of acetic acid regardless of concentration, [$F(7,102) = 2.26$, $p = 0.04$] (Figure III.2a). Suppression began at the



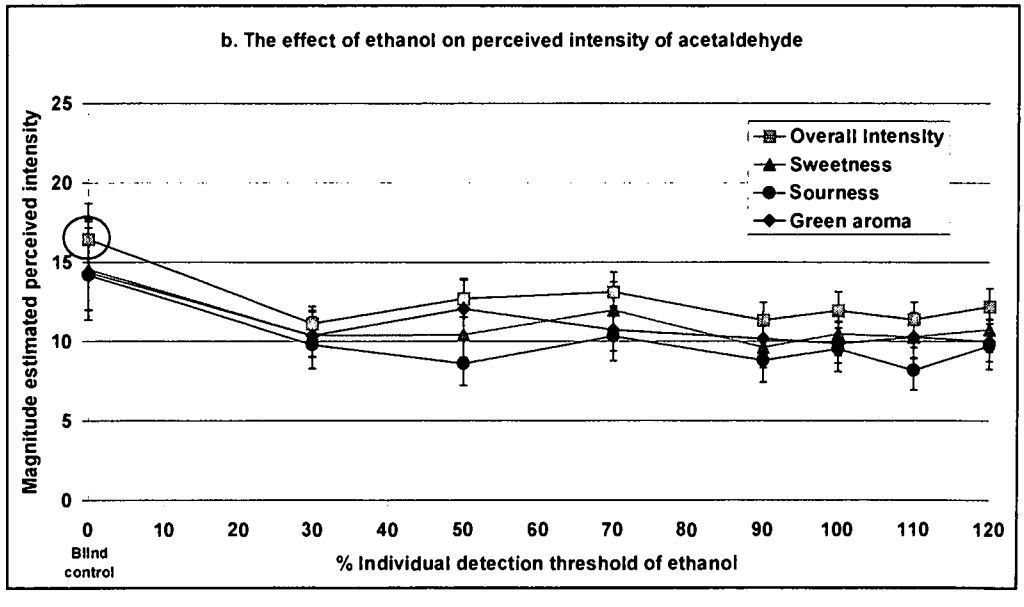


Figure III.2 The effects of (a) acetic acid and (b) ethanol at sub- and peri-threshold concentrations (% individual detection threshold) on the perceived intensity of acetaldehyde (at recognition concentration) in binary mixtures.
Values are medians and bars indicate ± 95% confidence interval. The oval indicates significant difference between the blind control and the other samples. Seventeen subjects with four replications provided the data for the median estimation.

lowest concentration studied, 30% of the individual detection threshold. Higher concentrations of acetic acid did not alter the magnitude of suppression significantly (Tukey's HSD, $p > 0.05$). Sourness, sweetness and green aroma were not significantly affected by acetic acid at all concentrations, [$F(7, 104) = 0.89$, $p = 0.52$], [$F(7, 104) = 1.82$, $p = 0.09$] and [$F(7, 104) = 2.06$, $p = 0.06$], respectively (Figure III.2a).

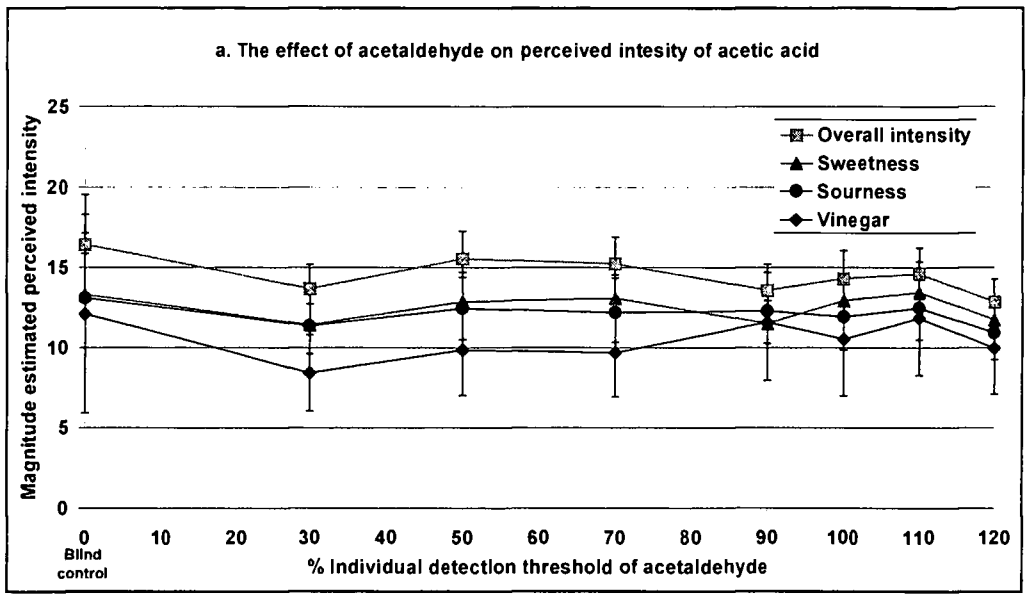
In the presence of ethanol, only sourness intensity was significantly decreased (~27% reduction) [$F(7, 94) = 2.18$, $p = 0.04$] (Figure III.2b). Overall intensity, sweetness and green aroma descriptor intensities were not significantly decreased, [$F(7, 99) = 2.06$, $p = 0.05$], [$F(7, 94) = 1.52$, $p = 0.17$] and [$F(7, 87) = 1.23$, $p = 0.30$] even though a reduction trend was observed (Figure III.2b). The suppression of sourness intensity began at 30% of the individual detection threshold. Increasing ethanol concentration did not alter the suppression significantly as the intensities of all descriptors were not significantly different when the concentration was increased (Tukey's HSD, $p > 0.05$).

By observation, the order of the intensities of the acetaldehyde descriptors in the blind control was overall intensity > sweetness ≥ green ≥ sourness (Figure III.2a and III.2b). Increasing acetic acid concentration changed the order of sourness and green intensities while sweetness maintained its position. In the presence of ethanol, sourness was still the lowest in intensity but sweetness and green intensities were interchanged.

3. 3 The effect of acetaldehyde and ethanol on acetic acid perceived intensity

Overall, both acetaldehyde and ethanol at sub-threshold and peri-threshold level did not have a significant effect on the intensities of acetic acid descriptors (Figure III.3). Acetaldehyde did not significantly change the intensities of any descriptors of acetic acid: [$F(7, 99) = 0.53$, $p = 0.81$ for overall intensity], $F(7, 92) = 0.29$, $p = 0.96$ for sweetness], $F(7, 108) = 0.12$, $p = 0.99$ for sourness] and [$F(7, 100) = 0.41$, $p = 0.89$ for vinegar] (Figure III.3a). Even though acetaldehyde concentrations were increased to 120% of threshold level, intensity at that condition did not significantly decrease from the blind control as in the previous conditions (Tukey's HSD, $p > 0.05$).

Introducing ethanol at sub-threshold and peri-threshold levels into acetic acid at recognition concentration did not suppress the intensity of any of the descriptors of acetic acid: [$F(7, 96) = 1.10$, $p = 0.37$ for overall intensity], [$F(7, 96) = 1.15$, $p = 0.34$ for sweetness], [$F(7, 96) = 0.80$, $p = 0.59$ for sourness] and [$F(7, 90) = 0.68$, $p = 0.69$ for vinegar] (Figure III.3b). Increasing concentration of ethanol did not affect intensity of acetic acid at recognition level (Tukey's HSD, $p > 0.05$). By observation, the order of the descriptors was maintained in both cases as sweetness $\geq$ sourness $>$ vinegar.



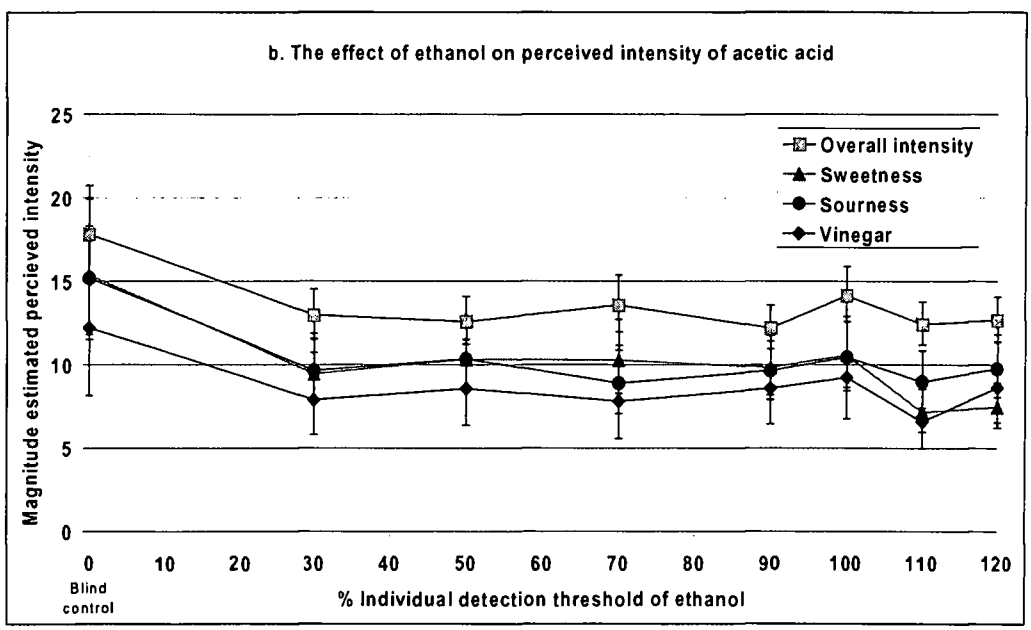


Figure III.3 The effects of (a) acetaldehyde and (b) ethanol at sub- and peri-threshold concentrations (% individual detection threshold) on the perceived intensity of acetic acid (at recognition concentration) in binary mixtures. Values are medians and bars indicate ± 95% confidence interval. Seventeen subjects with four replications provided the data for the median estimation.

III.5 Discussion

1. Methodologies in sub-threshold effect research

The sub-threshold effect in mixtures was generally studied by determination of group detection thresholds of sub-threshold mixtures (Guadagni et al., 1963b; Laing et al., 1994; Nawar & Fagerson, 1962; Patterson et al., 1993; Rosen et al., 1962). These researches investigated the interaction between sub-threshold odorants and usually reported the group detection thresholds of sub-threshold mixtures to be lower than the group detection thresholds of individual odorants. The decrease in the group threshold led to the conclusion of an additive effect (or synergistic effect) of odorants at sub-threshold level. Laska and Hudson (1991) reported some additive effect in mixture systems; however, they also observed only 53% of the panel reporting this effect. Employing group detection thresholds causes aggregation of the data and neglects differences in individual detection thresholds. The individual differences possibly caused the non-consensus results in Laska and Hudson (1991). Cometto-Muniz and others (1997) incorporated individual detection thresholds in the analysis step and concluded a simple additive effect on the group detection threshold of sub-threshold mixtures.

This study investigated the effect of sub-threshold and peri-threshold odorants on the intensities of the descriptors of recognizable odorants. This type of study has not been investigated in olfaction. Employing magnitude estimation in conjunction with tailored treatments for each individual based on their own

detection thresholds revealed the suppression of acetic acid and acetaldehyde on ethanol intensity, and acetic acid and ethanol on acetaldehyde intensity in binary mixtures. In this study, the concentrations employed as treatments were not absolute but were individually relative in terms of percentages of individual detection thresholds. For example, a 50% concentration is equivalent to 250 mM for subject A, whose detection threshold is 500 mM, but is a 30% concentration for subject B, whose detection threshold is 833.33 mM. Employing this technique allows the researchers to minimize a major confounding factor, which is difference in individual detection thresholds (Stevens et al., 1988) and ensure sub-threshold condition for all subjects.

2. Concentration independent sub-threshold and peri-threshold suppression of the perceived intensities of recognizable odorants in binary mixtures

The effect of sub-threshold compounds in odor, taste and flavor perception has been investigated but the exact effect is still controversial. Many researchers proposed an additive effect (Bennett et al., 1965; Day et al., 1963; Guadagni et al., 1963b; Guadagni et al., 1974a; Keith & Powers, 1968; Langler & Day, 1964; Lillard & Day, 1961; Patterson et al., 1993). In addition, Nawar and Fagerson (1962) and Rosen et al. (1962) concluded a synergistic effect. However, a synergistic effect of sub-threshold compounds was not common (Guadagni et al., 1974a; Keith & Powers, 1968; Nawar & Fagerson, 1962; Rosen et al., 1962).

The effect of sub-threshold components on the perception of components at recognition concentration has never been reported in olfaction. The only relevant study published is the study conducted by Bult and others (2001), which was to investigate the effect of suprathreshold component on the detection of sub-threshold components in an apple-aroma mixture. Bult and others (2001) reported that detection probability of sub-threshold concentration odorants with an apple aroma background was less than the detection probability with a water background. However, the detection probability with an apple aroma background increased when an apple concept was introduced to subjects (Bult et al., 2001). Besides olfaction, the effect of sub-threshold stimuli on supra-threshold response was also reported in the visual system, which shares common processes with olfaction (Toth et al., 1996). Toth and others (1996) proposed facilitation of a sub-threshold effect in vision. This facilitation was a phenomenon where sub-threshold visual inputs caused an excitatory or inhibitory signal for suprathreshold visual inputs in the cat visual cortex (Toth et al., 1996).

In this study, odorants at sub-threshold or peri-threshold generally suppressed the intensity of recognizable odorants (four out of six cases studied) in binary mixtures of a sub-threshold odorant and a recognizable odorant. Intensity suppression was found to be a common phenomenon in suprathreshold mixtures (Derby et al., 1991a; Laing, 1989) and it seems to apply to sub-threshold and peri-threshold suppression. The sub-threshold and peri-threshold suppression, however, was functional group specific, and did not increase in magnitude when the

concentration of sub-threshold components increased from 30% to 120% of individual detection thresholds. The suppression magnitude remained constant across the concentrations tested (Figure III.1 and III.2). The concentration independent suppression revealed by this study suggests the non-competitive nature of sub-threshold and peri-threshold suppression. Many studies reported this phenomenon in binary mixtures of supra-threshold odorants (Daniel & Derby, 1991a; Steullet & Derby, 1997). Sub-threshold level inhibition, which occurs naturally, was suggested to influence mixture suppression (Ache, 1989). The sub-threshold suppression in sub-threshold/supra-threshold mixtures was proposed to be a result of co-activated pathway inhibition caused by sub-threshold activated suppressant cells (Ache, 1989).

Mixture suppression has been suggested to be caused by central mechanisms or central suppression (Ache, 1989; Derby et al., 1985; Kurahashi et al., 1994) and peripheral mechanisms or peripheral suppression (Ache, 1989; Ache et al., 1987; Bell et al., 1987; Derby et al., 1985; Duchamp-Viret et al., 1999; Duchamp-Viret et al., 2001; Gentilcore & Derby, 1998; Miller, 1971). From this study, the concentration-independent suppression suggested non-competitive suppression mechanisms. The non-competitive suppression can be explained by both central and peripheral suppression.

Evidence of central suppression was reported in spiny lobster by introducing stimulant and suppressant to separate receptor cells in different antennae of lobsters (Derby et al., 1985). The central suppression mechanism was

suggested to operate at the glomerulus level in rats for mixture suppression (Bell et al., 1987). The organization of olfactory receptor axons to a glomerulus is necessary for central suppression to be possible. The discovery of zonal organization of olfactory neurons in olfactory epithelium, zone-to-zone projection of the olfactory receptor axons into glomeruli and genetically governed gene expression of the olfactory receptor neurons support central suppression (Ressler et al., 1993; Ressler et al., 1994; Wang et al., 1998).

Besides central suppression, peripheral suppression has been explored more extensively (Ache, 1989; Ache et al., 1987; Bell et al., 1987; Daniel et al., 1996; Daniel & Derby, 1991b; Daniel et al., 1994; Derby et al., 1985; Derby et al., 1991a, 1991b; Duchamp-Viret et al., 1999; Duchamp-Viret et al., 2001; Gentilcore & Derby, 1998; Miller, 1971; Moulton & Beidler, 1967; Olson & Derby, 1995; Simon & Derby, 1995). Peripheral suppression involves both intercellular and intracellular mechanisms (Simon & Derby, 1995). Simon and others (1995) excluded intercellular mechanisms as candidate mechanisms in mixture suppression because of unsatisfactory evidence. Simon and others (1995) suggested many possible intracellular mechanisms involved in peripheral suppression in mixtures such as inhibitory signal of odorants toward others from different receptor sites within the same receptor neuron (non-competitive) (Ache, 1994; Daniel et al., 1996; Gentilcore & Derby, 1998; Olson & Derby, 1995), inhibition of binding of one odorant on its receptor site by other odorants (competitive) (Ache et al., 1987; Laing & Livermore, 1992; Simon & Derby,

1995), direct suppressive effect on ion channels or second messenger metabolism (non-competitive) (Kurahashi et al., 1994; Miller, 1971), and "cross-talk" between two excitatory transduction pathways (non-competitive) (Anholt & Rivers, 1990). However, the contribution of peripheral and central mechanisms in intensity suppression is still unknown.

3. The role of functional groups on sub-threshold and peri-threshold suppression

In addition to the concentration independent aspect, the results also reveal interesting suppression directions between functional groups. Acetic acid at sub-threshold and peri-threshold levels suppressed both acetaldehyde and ethanol at recognition level (Figure III.1a and III.2a); however, acetaldehyde and ethanol at sub-threshold and peri-threshold did not suppress acetic acid (Figure III.3a and III.3b). The one-way suppression was named non-reciprocal suppression (Laing, 1988). By contrast, reciprocal suppression, a mixture interaction that each component suppresses each other (Laing, 1988), was found between acetaldehyde and ethanol (Figure III.1b and III.2b). The reciprocal and non-reciprocal suppression found in different binary mixtures suggest the role of functional groups in sub-threshold and peri-threshold suppression.

All odorants in this study, acetic acid, ethanol and acetaldehyde, are polar with two single-bonded carbon atoms. The only difference between these odorants is their functional groups. Therefore, it is reasonable to conclude that the direction of sub-threshold and peri-threshold suppression depends on functional groups of

components in binary mixtures of a sub-threshold or peri-threshold and a recognizable odorant. There are many studies to support the importance of functional groups in olfaction (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999; Johnson et al., 1998; Laing, 1988; Laing et al., 1994; Laska et al., 2000; Laska & Teubner, 1998, 1999; Laska et al., 1999; Rossiter, 1996).

Many studies reported suppression that was partially influence by polarities of odorants in suprathreshold binary mixtures (Cometto-Muniz et al., 1997; Laing, 1988, 1989). The polarities of the odorants used in this study in aqueous environment order as follows: acetic acid > ethanol>acetaldehyde (Gritter et al., 1985; Heftmann, 1975). Laing (1988) studied the suppression effects in suprathreshold mixtures of limonene (non-polar), octane (non-polar), carvone (polar), butanol (polar) and propanoic acid (polar) on each other. Reciprocal suppression occurred in propanoic acid/n-butanol system but not in propanoic acid/carvone and n-butanol/carvone system (Laing, 1988). Laing (1988) results supported reciprocal sub-threshold and peri-threshold suppression between ethanol and acetaldehyde that both are polar odorants and very close in chemical structures. The results from this study also suggest that reciprocal suppression could occur when the odorants are not very different in polarity such as ethanol and acetaldehyde.

Moreover, non-reciprocal suppression was found in some suprathreshold mixtures of odorants with different functional groups (Bell et al., 1987; Laing,

1988). Bell and others (1987) reported non-reciprocal suppression of (+)-limonene (non-polar) and α-pinene (non-polar) on propanoic acid (polar). This study provides the evidence that changing functional groups from a hydroxyl group (ethanol) to a carboxylic group (acetic acid) was enough change to cause non-reciprocal suppression in the acetaldehyde intensity (Figure III.1b, III.2a, III.2b, and III.3a).

In addition to suppression direction, the results reveal descriptor specific suppression, which is dependent on functional groups of components in the mixtures studied. In the non-reciprocal suppression case, acetic acid at sub-threshold and peri-threshold suppressed the intensities of all descriptors of ethanol and acetaldehyde at panel-recognition concentration (Figure III.1a and III.2a). In the reciprocal suppression case, acetaldehyde at sub-threshold and peri-threshold suppressed the intensities of all descriptors of ethanol at panel-recognition concentration, but ethanol at sub-threshold and peri-threshold significantly suppressed only sourness intensity of acetaldehyde at panel-recognition concentration. These results suggest that sub-threshold and peri-threshold odorants influence the intensity of specific quality in binary mixtures; however, the significance of this effect on mixture quality is still unknown.

Besides character-specific intensity suppression, changes in descriptors' intensity patterns of the odorants at recognition level were also observed. The changes in descriptors' intensity patterns were interesting, even though, the intensities of all descriptors along the concentration gradient from 30% to 120% of

individual detection threshold were not significantly different from each other and the study was not designed for this purpose. All descriptors' intensities decreased when a sub-threshold and peri-threshold level odorant was introduced (except in acetaldehyde at sub-threshold and acetic acid at recognition level). Then the intensities of recognizable odorants tended to increase when the concentration of sub-threshold odorants reached 120% of individual detection threshold and different functional groups affected the patterns differently in some cases.

For example, alcohol intensity was less than sweetness intensity when acetic acid at sub-threshold level was introduced to the ethanol system, but was higher than sweetness intensity when acetaldehyde at sub-threshold level was introduced instead of acetic acid (Figure III.1a and III.1b). The sweet descriptor was common between acetic acid, acetaldehyde and ethanol, but this descriptor was not altered much by adding another odorant into the system, except in the case of acetic acid at recognition concentration with ethanol at 110% and 120% level (Figure III.3b). Therefore, adding an odorant with different functional groups into mixtures may alter mixture quality, but the quality alteration may depend on concentration, and specific molecular features of components in the mixtures. The importance of functional groups in the discrimination ability of supra-threshold odors was reported (Laska et al., 2000). Changes in across-neuron patterns created by combined patterns of signals discharged by the olfactory receptor neurons, which were activated by mixtures (pattern mixture interaction) was proposed to contribute to quality discrimination of odorants in lobsters (Derby et al., 1991b).

Quality discrimination was believed to be presented in a form of spatial maps, results from activated across-neuron patterns, registered in glomerulus layer in olfactory bulb (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999; Johnson et al., 1998; Ressler et al., 1993; Ressler et al., 1994; Wang et al., 1998). Johnson and others (2000) reported that rat glomerulus-layer activity patterns differ systematically when odorants with different functional groups were presented to the rat. The effect of sub-threshold components on intensity and quality of recognizable odorants, however, needs more research to understand the effect.

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III.7 References

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Chapter 4

IV. FUNCTIONAL-GROUP DEPENDENT SUB-THRESHOLD EFFECTS ON THE PERCEIVED INTENSITY OF RECOGNIZABLE ODORANTS IN TERTIARY MIXTURES

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IV.1 Abstract

Tertiary mixtures comprising acetic acid, acetaldehyde, and ethanol were studied. In the mixtures, an odorant was at a panel-recognition concentration and the other two odorants were at sub-threshold concentrations. Controlling concentrations of the odorants as percentages of subjects' individual detection thresholds allowed the researchers to investigate the effect of the sub-threshold odorants. The sub-threshold mixtures of acetic acid and acetaldehyde suppressed the overall intensity and alcohol intensity of ethanol; however, the sub-threshold mixtures of ethanol and acetic acid enhanced the overall intensity, sourness, and sweetness of acetaldehyde. Acetaldehyde and ethanol, both at sub-threshold levels, did not have any effect on the perception of acetic acid at panel-recognition concentration. Failure to affect some descriptors by sub-threshold mixtures suggested the influence of central mechanisms on sub-threshold effects on the perception of a recognizable odorants; however, concentration-dependent suppression and enhancement suggested the contribution of peripheral mechanisms. The sub-threshold effects observed may be due to a non-competitive suppression caused by the stimulation of multiple receptor sites within a receptor neuron.

IV.2 Introduction

Everyday aromas are complex mixtures comprising odorants that are different in types (functional groups, chemical structures, etc.) and concentrations (sub-threshold, suprathreshold, etc). Odorants, concentration ratios or both qualitatively and quantitatively affect aromas of mixtures; however, the outcomes (aroma quality and intensity) are difficult to predict by individual component information. Many researchers named this unpredictable phenomenon "mixture interaction" (Berglund et al., 1971; Berglund et al., 1973; Cometto-Muniz et al., 1997; Derby et al., 1991a, 1991b).

Mixture interaction causes changes in aroma quality, intensity or both. Quality changes were defined as pattern mixture interactions (Derby et al., 1991b). If a new odor quality is created, it is called a homogenous odor, but if the odor quality of each component does not change, it is called a heterogeneous odor (Berglund et al., 1976). Intensity changes were defined as intensity mixture interactions (Derby et al., 1991a), regardless of odor quality. For binary mixtures, intensity mixture interactions of homogenous odors were classified into six categories: complete addition, hypo-addition, partial addition, compromise, compensation, and hyper-addition (Berglund et al., 1976; Cain & Drexler, 1974); and the interactions of heterogeneous odors were classified into three categories: synergism, independence, and antagonism (Berglund et al., 1976). Antagonism or suppression is a common phenomenon in mixtures; nevertheless, synergism and

independence are also reported (Berglund et al., 1971; Borroni et al., 1986; Cain et al., 1995; Carr, 1978; Shelton & Mackie, 1971).

Peripheral and central neural mechanisms are believed to govern mixture intensity interactions. Evidence, however, supported the peripheral mechanisms (Ache, 1989; Bell et al., 1987; Derby et al., 1985). Based on a chromatographic-like model (Mozell, 1964, 1970, 1971; Mozell & Jagodowicz, 1973), the polarities of odorants in mixtures partially influence how odorants are adsorbed on the olfactory epithelium. Differences in the adsorption behavior of odorants cause the spatial and the temporal processing of odor information. Therefore, the polarities of odorants partially govern how odorants suppress each other in mixtures: reciprocal or non-reciprocal suppressions(Ache et al., 1987; Bell et al., 1987; Laing, 1988).

Few studies of the perception of sub-threshold mixtures have been reported. The most common result reported is an additive or agonistic effect (Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991; Patterson et al., 1993); however, in some cases, a synergistic effect was reported (Laska & Hudson, 1991; Nawar & Fagerson, 1962). These studies used detection threshold determination of mixtures and discovered that at a mixture's detection threshold, the concentrations of individual odorants in the mixtures were lower than that of the individual component at their individual detection thresholds. Therefore, researchers concluded the effect of sub-threshold mixture to be additive or synergistic. Some

studies, however, indicated that additive and synergistic effects are not common phenomena (Guadagni et al., 1974b; Keith & Powers, 1968).

Laska and Hudson (1991) reported both additive and synergistic effects in suprathreshold mixtures, but only 53% of subjects demonstrated higher sensitivity toward the mixtures. Differences in sensitivity between subjects should cause the disagreement among subjects' mixture detection thresholds. Commetto-Muniz and Cain (1997) took into account subject's detection threshold for each component in the mixtures during data analysis and concluded that the effect was additive.

The perception of either suprathreshold mixtures or sub-threshold mixtures was a focus of many researchers (Berglund et al., 1971, 1976; Cain et al., 1995; Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991), but aromas encountered everyday comprise odorants at various concentrations including sub-threshold and supra-threshold. The perception of sub-threshold stimuli in supra-threshold background has been known as a subliminal effect. In auditory and visual systems, the subliminal effect has been studied extensively at the cognitive level (Cheesman & Merikle, 1984; Dixon, 1971; Dixon, 1981; Duncan, 1985; Erdelyi, 1974; Fowler et al., 1981; Greenwald & Daraine, 1997; Harris et al., 1996; Merikle, 1988; Vokey & Read, 1985), but in olfaction, the studies of the effect of sub-threshold odorants on the perception of supra-threshold odorants are very limited.

Lopetcharat and McDaniel (2002) reported suppression of intensity of a supra-threshold odorant by a sub-threshold odorant in sub-threshold/suprathreshold

binary mixtures of acetic acid, acetaldehyde, and ethanol. The sub-threshold suppression was concentration independent and reciprocal sub-threshold suppression depended on functional groups of the odorants in the mixtures (Lopetcharat & McDaniel, 2002). Bult and others (2001) reported an increase in detection probability of sub-threshold odorants in apple-aroma mixtures when a defined concept was introduced to subjects. However, the apple-aroma background did not increased the detection probability of the sub-threshold odorants in the mixtures compared to water background (Bult et al., 2001).

Primarily, this study was to investigate the effects of two sub-threshold odorants on the intensities of another odorant at panel-recognition concentration in tertiary mixtures. The odorants were different only in functional groups. Therefore and secondly, the study was to investigate the influence of functional groups on sub-threshold effects. Finally, overall intensity and two or three additional descriptors were rated in order to investigate the effect of sub-threshold odorants and their interaction on the specific qualities of recognizable odorants.

IV.3 Materials and methods

1. Odorants and odorant presentation

Food grade ethanol, acetic acid and acetaldehyde (99% purity), purchased from Aldrich (Milwaukee, WI) were used as odorants in this study. Double distilled deionized water served as the solvent for all odorants. Presentation

scheme and test conditions for all experiments were explained in a previous study (Lopetcharat & McDaniel, 2002).

2. Screening test and subjects

Subjects who participated in a similar previous study (Lopetcharat & McDaniel, 2002) were used. Screening methodology, criteria, and subject information were described in Lopetcharat and McDaniel (2002).

3. Subject training

3.1 Odorants' descriptor definition

Subjects smelled standard ethanol, acetic acid, and acetaldehyde solutions at different concentrations and described the solutions (Table IV.1). The subjects discussed the descriptors and only descriptors whose definitions were agreed upon were used in this study (Table IV.1).

Table IV.1 Odorants and descriptors accepted by subjects

Compounds	Descriptors	Concentration*
Ethanol	Sweetness Alcohol	438.6 and 877.2 mM
Acetic acid	Sweetness Sourness Vinegar	8.9 and 17.9 mM
Acetaldehyde	Sweetness Sourness Green	546.3 and 1092.7 nM

* in double distilled deionized water

3.2 Intensity rating training

Magnitude estimation (ME) with a modulus was used to rate the intensities of the descriptors of the odorants. Subjects were trained to use ME with a modulus as described in Lopetcharat and McDaniel (2002).

3.3 Individual detection threshold and recognition threshold determination

The detection and the recognition thresholds of each subject were measured in order to ensure sub-threshold and panel-recognition condition. The determination of detection and recognition thresholds was performed using the 3-AFC ascending concentration series method of limits (ASTM, 1999). The procedures, definitions of individual's detection and recognition thresholds, individual detection thresholds and the grouping of subjects using the individual detection thresholds were reported in Lopetcharat and McDaniel (2002).

3.4 Determination of panel-recognition concentration of all odorants

Panel recognition concentration of an odorant is defined as the concentration at which all subjects can 100% correctly identify the odorant. The highest individual recognition thresholds for all odorants among all subjects were used as starting concentrations. The 3-AFC technique was used to confirm the concentrations. The group recognition concentrations for all odorants were reported in Lopetcharat and McDaniel (2002).

3.5 The effects of sub-threshold odorants on the perceived intensity of an odorant at panel-recognition concentration in tertiary odor mixtures (tertiary mixture study).

A tertiary system was obtained by placing two 4-ml vials in a 125-ml bottle with each vial containing 4 ml of different odorants at sub-threshold concentrations. Another vial contained 4 ml of an odorant, which was different from the other two odorants, at panel-recognition concentration. Two empty 4-ml vials were placed in the bottle to fill up the space. The headspace was ~ 104 ml.

One odorant was selected as a recognition concentration odorant and the other two were selected as sub-threshold odorants. Therefore, three experiments were independently conducted because of differences in the recognizable odorants. Within each experiment, three concentrations of sub-threshold odorants (30, 50, and 70% of individual detection thresholds) were used. Nine treatment combinations were created from three sub-threshold concentrations of two odorants (Table IV.2). A factorial structure within a completely randomized block design was used. Each experiment was tested in a separate session. Each session had ten sample bottles (nine treatments and a blind control), which were randomly presented to subjects. The blind control contained double-distilled deionized water instead of sub-threshold odorants. Overall, each of ten sample bottles was presented 16 to 17 times across all subjects in each testing position. The treatment combinations were also randomly assigned to subjects in a balanced fashion within each session. The testing order for each experiment was also balanced across all subjects.

Table IV.2 Three possible treatment combinations in the tertiary mixture study

Experiment	Recognition level odorant	Sub-threshold odorants
1	Ethanol	Acetic acid*
		Acetaldehyde*
2	Acetic acid	Ethanol*
		Acetaldehyde*
3	Acetaldehyde	Ethanol*
		Acetic acid*

*9 combinations created from the factorial structure of 3 sub- threshold concentrations (30%, 50%, and 70% of each odorant's individual detection threshold)were used in order to study the interaction effect between sub-threshold odorants in each experiment.

The testing protocol was described in a previous study (Lopetcharat & McDaniel, 2002). At least a 30-second sample-interval and a minimum 5-minute session-interval were mandatory. Subjects were allowed three testing sessions a day. Four replications were accomplished within four days.

4. Data analysis

Since the subjects used different scales and the data tend to follow a log-normal distribution, results from this study were normalized within each subject employing geometric mean normalization (Moskowitz & Jacobs, 1988; Stevens, 1956). Univariate analysis of variance (ANOVA) with a mixed model was employed for each descriptor response within each experiment separately. A 3^2-factorial structure within a completely randomized block design was implemented. The original general linear model of the mixed model comprised main factors: subjects (blocking factor treated as random effect) (Lundahl & McDaniel, 1988), first sub-threshold odorant (fixed effect), second sub-threshold odorant (fixed effected), replications (random effect), all 2-way interaction factors, and a 3-way interaction factor (subjects x first sub-threshold odorant x second sub-threshold odorant). Backward elimination was used to select the most parsimonious model used in this analysis. The final model comprised subjects, replications, first sub-threshold odorant, second sub-threshold odorant, 2-way interaction factors [(first sub-threshold odorant x second sub-threshold odorant) and (subjects x replications)], and a 3-way interaction (subjects x first sub-threshold odorant x

second sub-threshold odorant). Dunnett's and Bonferroni multiple comparison procedures were used to detect the differences from all comparisons against the blind control (0%) and general pair-wise differences between treatments, respectively. All analyses were performed on the log10 of the normalized data at α=0.05, when otherwise it will be stated. Analyses were conducted using General Linear Model in SPSS® V. 10.0 (Chicago, IL).

IV.4 Results

1. Tertiary mixture study

1.1 The effects of acetic acid (AA) and acetaldehyde (AC) at sub-threshold concentrations on the perceived intensity of ethanol

The sub-threshold mixtures of acetic acid and acetaldehyde generally suppressed the overall intensity of ethanol with the mixtures compared to the blind control (without sub-threshold mixture) (Figure IV.1). Suppression was dependent on concentration (the percentages of individual detection thresholds) of both acetic acid and acetaldehyde as determined by significant interaction term in ANOVA [$F(4, 144) = 2.10$, $p = 0.08$]. The overall intensity of the blind control (15.3) was significantly higher than that of the 30%AC/50%AA mixture (12.1), the

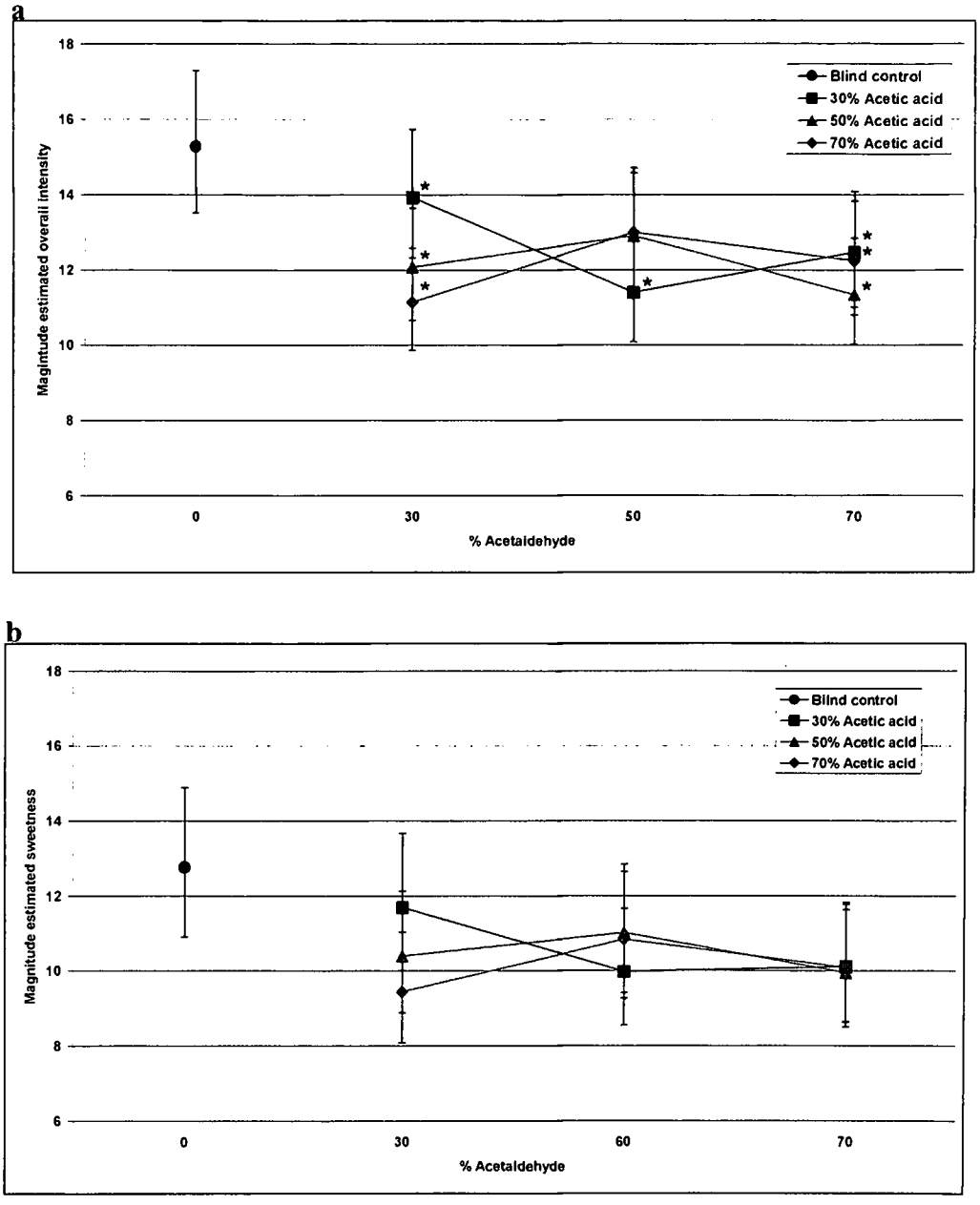

Figure IV.1 Estimated medians of the perceived overall intensity (a), sweetness (b), and alcohol (c) of ethanol at panel recognition concentration in the presence of sub-threshold mixtures of acetic acid and acetaldehyde. Acetic acid and acetaldehyde were at 30%, 50%, and 70% of subjects' individual detection thresholds. The asterisks indicate significant suppression of the sub-threshold mixtures compared to the blind control at α=0.05. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Seventeen subjects with four replications provided the data for the median estimation

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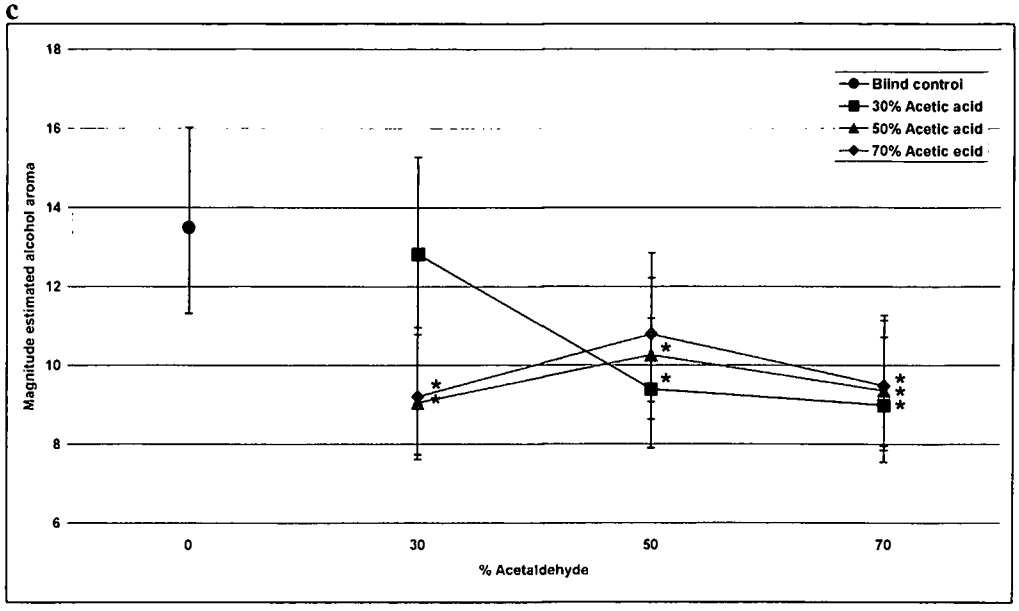


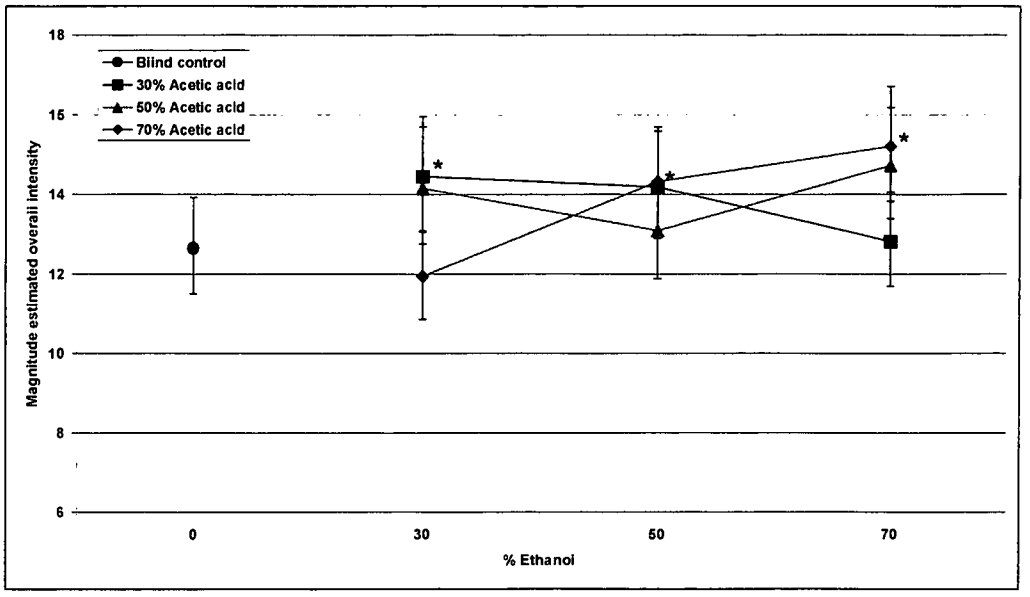
Figure IV.1 (continued) Estimated medians of the perceived overall intensity (a), sweetness intensity (b), and alcohol intensity (c) of ethanol at panel recognition concentration in the presence of sub-threshold mixtures of acetic acid and acetaldehyde. Acetic acid and acetaldehyde were at 30%, 50%, and 70% of subjects' individual detection thresholds. The asterisks indicate significant suppression of the sub-threshold mixtures compared to the blind control at α=0.05. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Seventeen subjects with four replications provided the data for the median estimation

30%AC/70%AA mixture (11.1), the 50%AC/30%AA mixture (11.4), the 70%AC/30%AA mixture (12.5), the 70%AC/50%AA mixture (11.4) and the 70%AC/70%AA mixture (12.2) (Figure IV.1a). Although, the six mixtures were not significantly different as indicated by Bonferroni test. Unlike overall intensity, sweetness intensity was not affected by either acetic acid [$F(2, 144)=0.17$, $p=0.8$], acetaldehyde [$F(2, 144)=0.5$, $p=0.6$] or both [$F(4,144)=0.89$, $p=0.5$] (Figure IV.1b). The alcohol aroma of some mixtures was significantly lower than that of the blind control (13.5) and suppression depended on both acetic acid and acetaldehyde concentration [$F(4, 135) = 2.54$, $p < 0.04$]. The mixtures that were less intense than the blind control were the 30%AC/50%AA, the 30%AC/70%AA, the 50%AC/30%AA, the 50%AC/50%AA, the 70%AC/30%AA, the 70%AC/50%AA and the 70%AC/70%AA mixtures with 9.1, 9.2, 9.4, 10.3, 9.0, 9.4, 9.5 scores, respectively (Figure IV.1c). Bonferroni test did not reveal significant differences among the seven mixtures.

1.2 Effect of acetic acid (AA) and ethanol (ET) at sub-threshold concentration of the perceived intensity of acetaldehyde

Both acetic acid and ethanol at sub-threshold concentrations significantly affected the overall intensity of acetaldehyde at the group recognition concentration [$F(4, 142)=3.0$, $p=0.02$] (Figure IV.2a). The overall intensity of the 30%AA/30%ET mixture (14.4) was significantly higher than that of the blind

a



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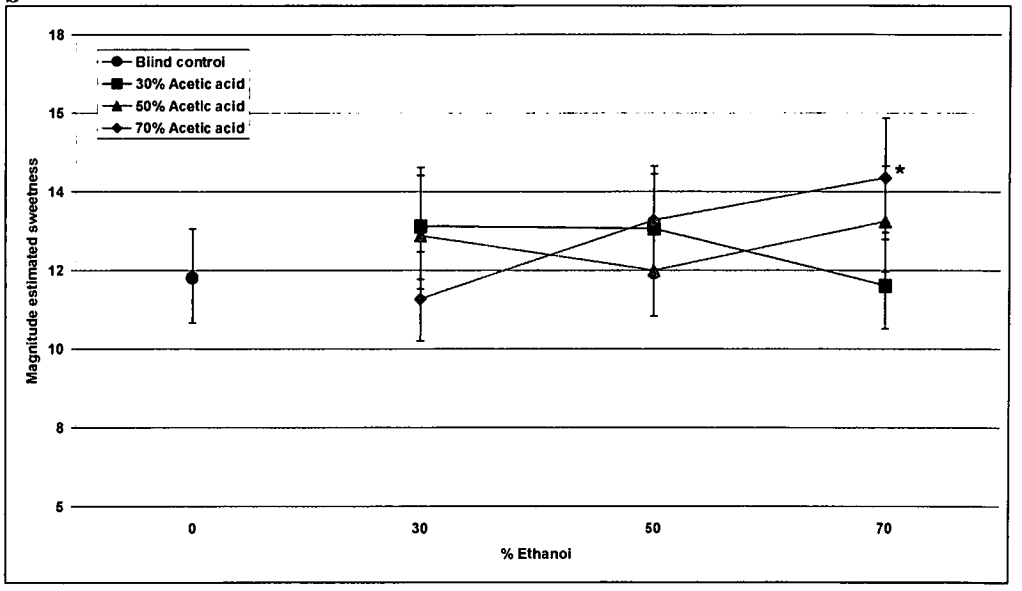

Figure IV.2 Estimated medians of the perceived overall intensity (a), sweetness (b), sourness (c), and green aroma (d) of acetaldehyde at panel-recognition concentration in the presence of sub-threshold mixtures of acetic acid and ethanol. Acetic acid and ethanol were at 30%, 50%, and 70% of subjects' individual detection thresholds. The asterisks indicate significant enhancement of the sub-threshold mixtures compared to the blind control at α=0.05. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Seventeen subjects with four replications provided the data for the median estimation

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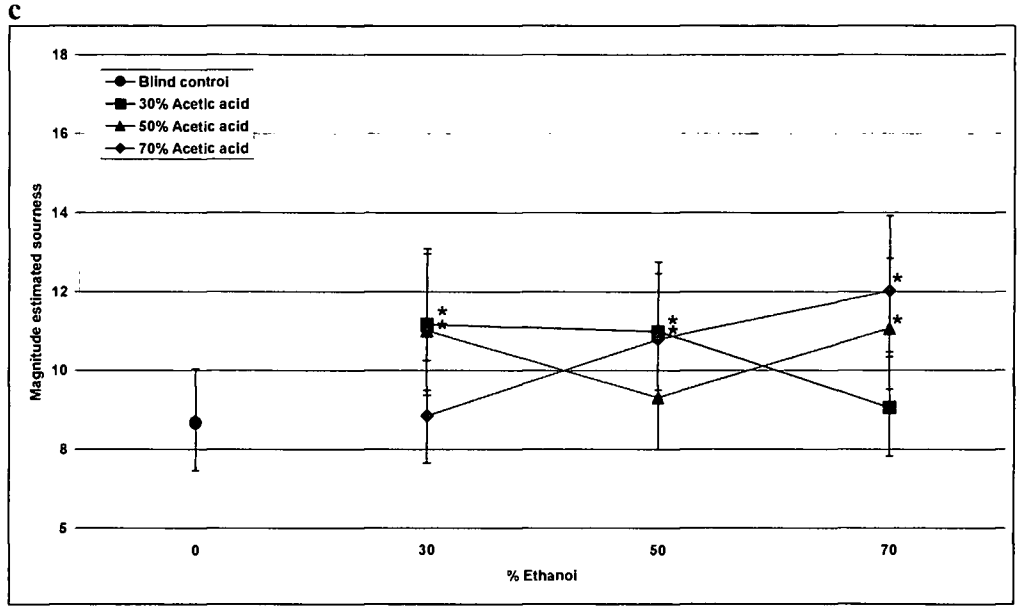

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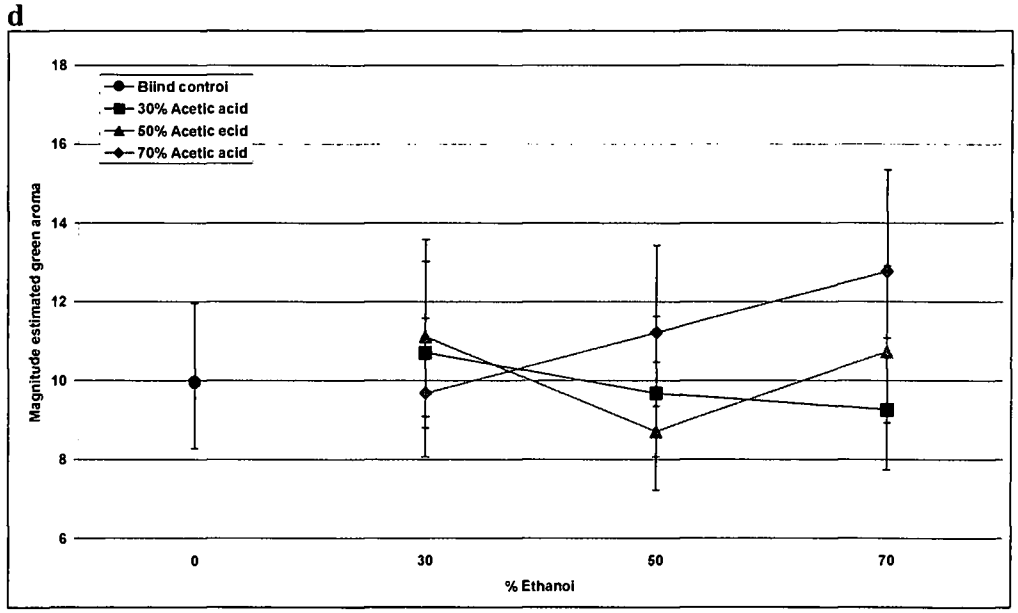

Figure IV.2 (continued) Estimated medians of the perceived overall intensity (a), sweetness (b), sourness (c), and green aroma (d) of acetaldehyde at panel recognition concentration in the presence of sub-threshold mixtures of acetic acid and ethanol. Acetic acid and ethanol were at 30%, 50%, and 70% of subjects' individual detection thresholds. The asterisks indicate significant enhancement of the sub-threshold mixtures compared to the blind control at α=0.05. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Seventeen subjects with four replications provided the data for the median estimation

control (12.6), and the increase was 13% (Figure lV.2a). Similarly, the 70%AA/50%ET mixture (14.3) and the 70%AA/70%ET mixture (15.2) were significantly rated higher than the blind control (12.6) (Figure IV.2a). The other six mixtures were not significantly different in overall intensity compared to the blind control. The overall intensity of the 30%AA/70%ET mixture (12.8) was significantly lower than that of the 30%AA/30%ET (14.4), the 70%AA/50%ET mixture (14.3), and the 70%AA/70%ET (15.2); otherwise the overall intensity of mixtures was not significantly different.

The blind control was significantly less sour than six out of nine mixtures (30%AA/30%ET, 30%AA/50%ET, 50%AA/30%ET, 50%AA/70%ET, 70%AA/50%ET and 70%AA/70%ET) [F(4,135)=2.93, p=0.02]. The mixtures were rated 11.2, 11.0, 11.0, 11.1, 10,8 and 12.0, respectively; meanwhile, the blind control was rated 8.7 in sourness intensity (Figure IV.2b). Average increase in intensity of the mixtures were 28.9% and the significant increases ranged from 24.4% for 70%AA/50%ET mixture to 38.7% for 70%AA/70%ET mixture. The mixtures that were rated significantly higher in sourness intensity than the blind control were not significantly different as indicated by Bonferroni test.

Sweetness intensity was significantly different between the blind control and nine mixtures [F(4, 143)=2.79, p=0.03] (Figure IV.2c). 70%AA/70%ET mixture (14.4) was significantly sweeter than the blind control (11.8), but the other eight mixtures were not significantly different from the blind control (Figure IV.2c). Most of the mixtures were not significantly different in sweetness intensity

except 30%AA/70%ET-70%AA/70%ET pair. Bonferroni test determined that 30%AA/70%ET mixture (11.6) was significantly less sweet than 70%AA/70%ET mixture (14.3). Green-aroma intensity was not affected by either acetic acid [$F(2, 144)=2.1$, $p=0.12$], ethanol [$F(2, 144)=0.9$, $p=0.42$] or both [$F(4,144)=2.2$, $p=0.1$] (Figure IV.2d).

1.3 Effect of acetaldehyde (AC) and ethanol (ET) at sub-threshold concentrations on the intensity of acetic acid

Acetaldehyde or ethanol alone at sub-threshold concentrations did not have significant effect on the intensity of most descriptors of acetic acid except the intensity of sweetness (Figure IV.3). Acetaldehyde at sub-threshold concentration significantly altered the acetic acid sweetness intensity [$F(2,135)=4.03$, $p=0.02$]. The significant difference was between 30% level (11.5) and 50% level (9.6) in the presence of ethanol at sub-threshold concentrations and the difference was 16%. The sub-threshold mixtures of acetaldehyde and ethanol did not significantly effect overall intensity [$F(4,135)=1.53$, $p=0.20$], sourness [$F(4,135)=1.01$, $p=0.41$], sweetness [$F(4,135)=1.53$, $p=0.20$] and vinegar [$F(4,144)=0.38$, $p=0.82$].

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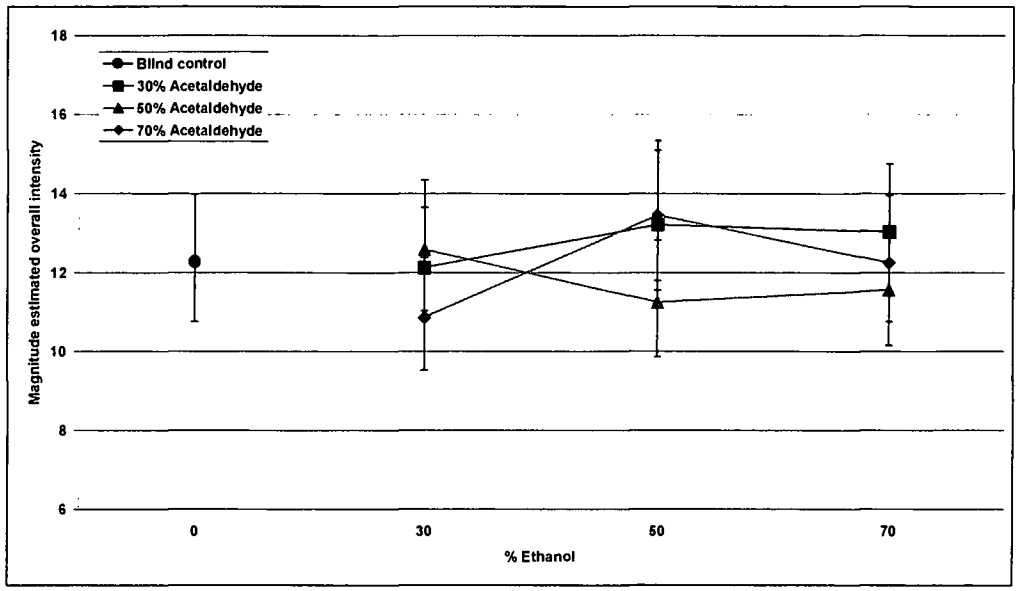

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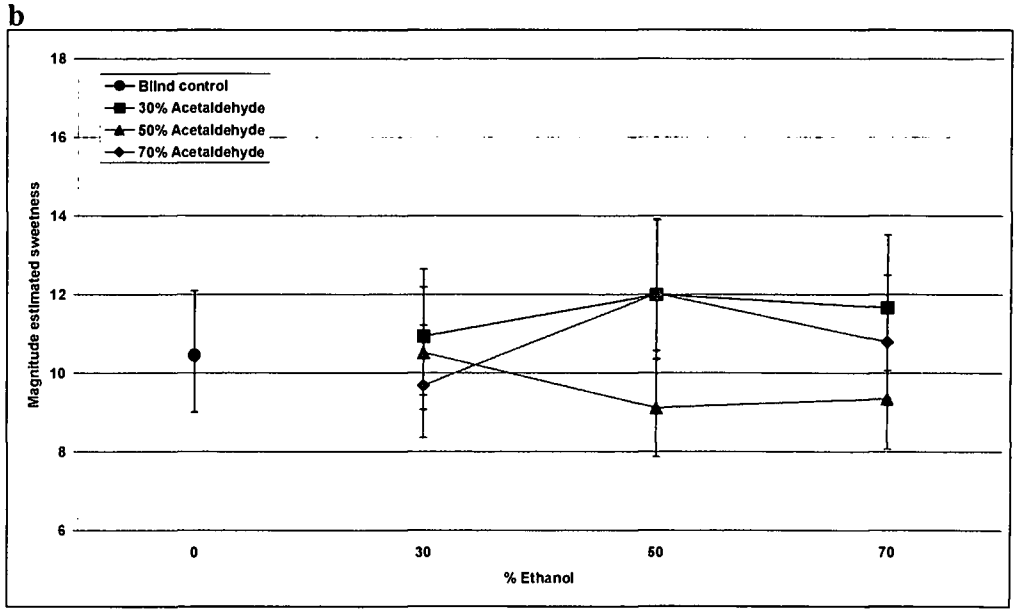

Figure IV.3 Estimated medians of the perceived overall intensity (a), sweetness (b) sourness (c) and vinegar (d) of acetic acid at panel recognition concentration in the presence of sub-threshold mixtures of acetaldehyde and ethanol. Acetaldehyde and ethanol were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

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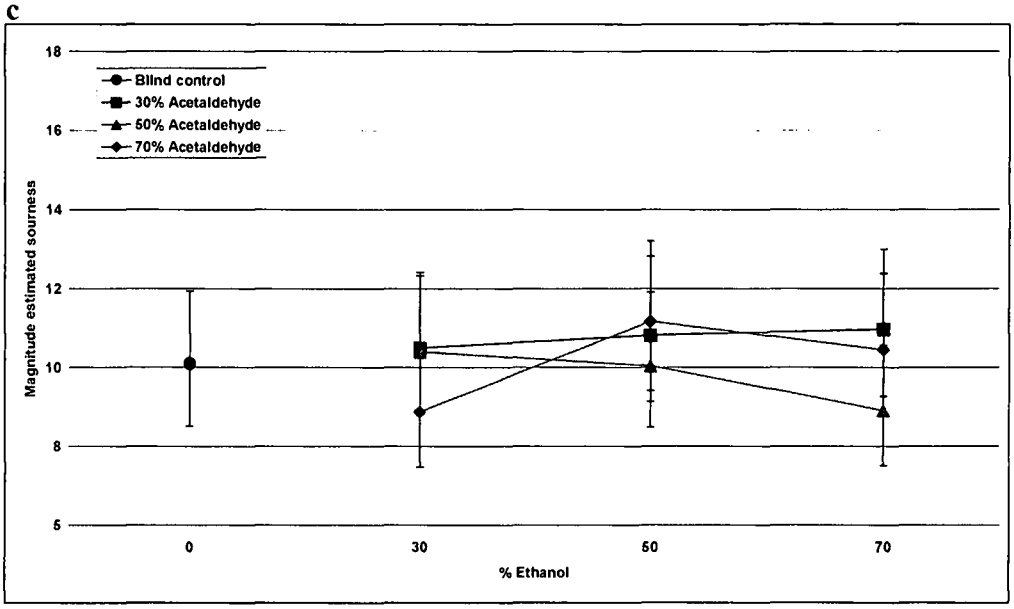

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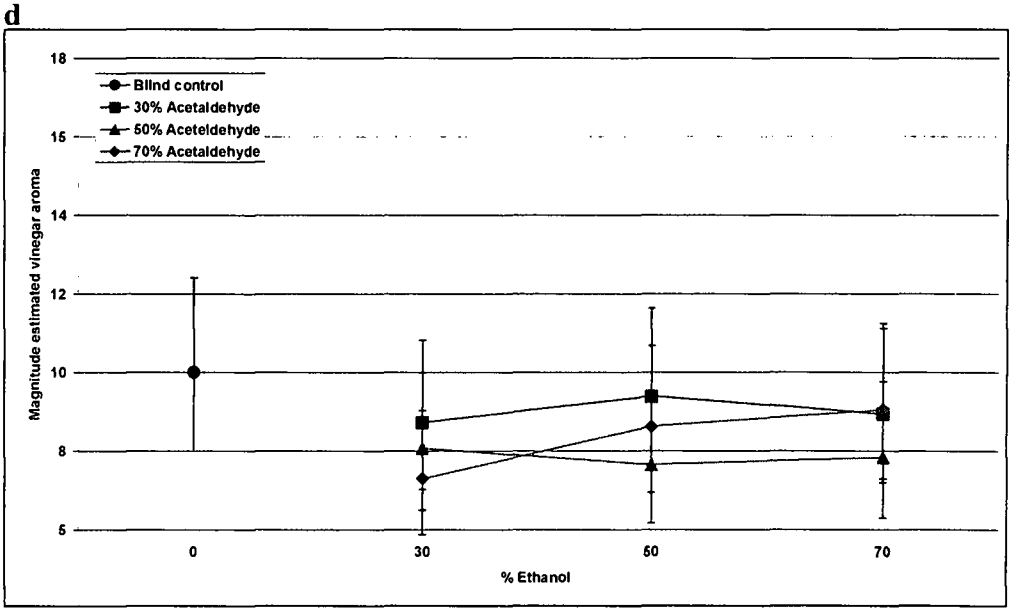

Figure IV.3 (continued) Estimated medians of the perceived overall intensity (a), sweetness (b) sourness (c) and vinegar (d) of acetic acid at panel recognition concentration in the presence of sub-threshold mixtures of acetaldehyde and ethanol. Acetaldehyde and ethanol were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

IV.5 Discussion

1. The effects of the sub-threshold mixtures of acetic acid and acetaldehyde on the perceived intensity of ethanol at panel-recognition concentration

In binary mixtures comprising an odorant at the panel-recognition concentration and an odorant at a sub-threshold concentration, acetic acid and acetaldehyde at sub-threshold concentrations suppressed the intensities of ethanol; although, ethanol at sub-threshold concentrations suppressed only the overall intensity of acetaldehyde but not acetic acid (Lopetcharat & McDaniel, 2002). These results suggested the influence of functional groups (perhaps relative polarity) between odorants in the mixtures (Cometto-Muniz et al., 1997; Laing, 1988, 1989).

In this study, the same design was used to investigate the effects of functional groups by controlling other chemical features. All odorants used in this study have two adjacent carbon atoms bonded by a single-covalent bond. In tertiary mixtures comprising two sub-threshold odorants and a recognizable odorant, the sub-threshold mixtures of acetic acid and acetaldehyde suppressed the overall intensity and alcohol intensity of ethanol at recognition concentration as expected; but the mixtures did not significantly suppress sweetness.

The sub-threshold suppression was functional group and concentration dependent. For example, at 30% acetic acid, increasing acetaldehyde concentration to 50% and 70% induced more suppression than 30% acetaldehyde, but both the

50% and 70% levels were not significantly different in suppression magnitude. When the concentration of acetic acid increased to 50% and 70% of the individual detection threshold, the suppression varied and depended on acetaldehyde concentration (Figure IV.1a and IV.1c). These results suggested that functional groups influenced the concentration dependency of the observed sub-threshold suppression. Moreover, the concentration dependency diminished when either acetaldehyde or acetic acid was higher than 30% of individual detection threshold. Therefore, at least 50% of individual detection threshold of either acetic acid or acetaldehyde was needed in order to effectively suppress the overall intensity and the alcohol aroma of ethanol.

The least suppression occurred in the 30%AA/30%AC mixture which suggested an antagonistic effect between acetaldehyde and acetic acid. Lopetcharat and McDaniel (2002) reported sub-threshold suppression of acetic acid on acetaldehyde but acetaldehyde had no effect on acetic acid perception. Moreover, in binary mixtures, sub-threshold suppression was concentration independent (Lopetcharat & McDaniel, 2002). However, the antagonistic effect of acetaldehyde on acetic acid diminished when acetic acid concentrations increased.

From the antagonistic phenomenon that was limited-concentration dependent and from functional group dependent suppression, results suggest that sub-threshold suppression caused by sub-threshold mixtures of acetic acid and acetaldehyde on ethanol perception involves a non-competitive peripheral mechanisms. Non-competitive mechanisms was favored because, physically,

acetaldehyde molecules were ~1000 fold less than acetic acid molecules as reported in Lopetcharat and McDaniel (2002). There are many possible non-competitive mechanisms proposed, for example, an intracellular suppression caused by multiple receptor sites (Ache, 1994; Daniel et al., 1996; Gentilcore & Derby, 1998; Lopetcharat & McDaniel, 2002; Olson & Derby, 1995) and an intracellular inhibitory signal generated from suppressing receptor within the same receptor neuron (Ache, 1994; Daniel et al., 1996; Gentilcore & Derby, 1998; Olson & Derby, 1995). The existence of multiple receptor sites within an olfactory neuron was confirmed (Cromarty & Derby, 1997; Kashiwayanagi et al., 1996). In addition, a direct suppressive effect on ion channels or second messenger metabolism (non-competitive) (Kurahashi et al., 1994; Miller, 1971), and "cross-talk" between multiple excitatory transduction pathways (non-competitive) (Anholt & Rivers, 1990) were also proposed.

Another interesting result was a failure to suppress the sweetness intensity in the sub-threshold mixtures; however, the trend was decreasing as other descriptors. In binary mixtures of acetaldehyde or acetic acid at sub-threshold significantly suppressed the sweetness of ethanol at recognition concentration (Lopetcharat & McDaniel, 2002). The discrepancy between sweetness perception, and alcohol perception indicated that the sub-threshold mixtures of acetic acid and acetaldehyde possibly affected both intensity and quality of ethanol at panel-recognition concentration.

The effect of sub-threshold mixtures on specific quality of odorants has never been reported. Quality perception is believed to result from both peripheral mechanisms: pattern mixture interactions (Derby et al., 1991b) and central mechanisms: spatial map of glomeruli and brain (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999; Johnson et al., 1998; Ressler et al., 1994). Bult and others (2001) reported a cognitive effect on sub-threshold detection ability. Therefore, sub-threshold mixtures possibly have an effect in both peripheral and central levels.

2. The effects of the sub-threshold mixtures of acetic acid and ethanol on the perceived intensities of acetaldehyde at recognition concentration

Acetic acid and ethanol at sub-threshold concentration suppressed the intensities of acetaldehyde at recognition concentration in binary mixtures of a sub-threshold and a recognizable odorant, but the suppressions were different in both magnitude and what quality they suppressed (Lopetcharat & McDaniel, 2002). Differences in relative polarity between acetaldehyde and its suppressors were believed to partially influence the suppression (Cometto-Muniz et al., 1997; Laing, 1988, 1989).

Unlike in binary mixtures, the sub-threshold mixtures of acetic acid and ethanol at some ratios significantly enhanced the intensities of acetaldehyde at recognition concentration (Figure IV.2a, IV.2b and IV.2c); however, this enhancement was not common for all descriptors. The sub-threshold mixtures did

not significantly alter green aroma intensity, and only at one level, 70% acetic acid with 70% ethanol, enhanced sweetness intensity. The sub-threshold mixtures enhanced overall intensity and sourness intensity when acetic acid and ethanol were at 30% of individual detection thresholds, and when ethanol and acetic acid were at least 50% of individual detection thresholds; otherwise, the overall intensity and the sourness intensity were not significantly changed.

From these results, acetic acid and ethanol at sub-threshold concentration interacted and resulted in enhancement at specific ratios instead of the suppression observed in binary mixtures (Lopetcharat & McDaniel, 2002). From the observation of the data, the ratios of ethanol to acetic acid that caused the enhancement ranged from 0.7 to 1.4; otherwise, no significant change detected.

Many researchers reported enhancement or synergism as an uncommon phenomenon in supra-threshold mixtures using animal and human models (Atema et al., 1989; Borroni et al., 1986; Carr et al., 1984; Cometto-Muniz et al., 1989; Derby et al., 1991a; Guadagni et al., 1974a; Koster, 1969; Laffort et al., 1989; Shelton & Mackie, 1971; Zimmer-Faust et al., 1984). Zimmer-Faust and others (1984) and Borroni and others (1986) reported higher responses from complex mixtures than the response of less complicated mixtures in lobster models, and concluded that suppressants caused suppression in binary mixtures and non-complex mixtures, but special mixture combinations can surpass the suppression. Zimmer-Faust and others (1984) suggested that different chemoreceptor sites simultaneously activated caused enhancement in chemoreception, and the

enhancement happened by means of central mechanisms such as in the central nervous system. Toth and others (1996) reported supporting evidence of sub-threshold facilitation including synergism in cat visual cortex.

The enhancement in intensity did not occur for all descriptors, and the result suggested the roles of central mechanisms. Further investigations are needed to test this hypothesis. This study supported only the intensities of specific descriptors, but not overall perception of odorants as an entity.

3. The effect of the sub-threshold mixtures of acetaldehyde and ethanol on the perceived intensities of acetic acid at recognition concentration

The sub-threshold mixtures of acetaldehyde and ethanol did not significantly alter the intensity acetic acid descriptors at recognition concentration in tertiary mixtures as expected (Figure IV.3). Even though, vinegar intensity was reduced but the suppression was not significant (Figure IV.3d). In binary mixtures, Lopetcharat and McDaniel (2002) reported the same phenomenon. Results suggest the influence of polarity on the interaction among odorants (Cometto-Muniz & Cain, 1990; Laing, 1988, 1989).

The non-reciprocal effect that acetic acid had on acetaldehyde and ethanol confirmed that functional groups or certain properties related to functional groups such as relative polarity influenced the directions of the interactions among these odorants (Bell et al., 1987; Hornung & Mozell, 1977; Hornung et al., 1980; Mozell, 1964, 1970; Mozell & Jagodowicz, 1973). However, still questionable is that what

mechanisms govern this phenomenon, peripheral mechanisms, central mechanisms or both.

Johnson and Leon (2000) presented the relationship between peripheral components (chemical structures) and central component (overlapping glomerulus-activity maps) in olfactory information processing. They published overlapping activity maps of rat glomerulus layer stimulated by pentanoic acid, 1-pentanol and pentanal. The maps also revealed that odorants that share some chemical features activate the same receptor types that stimulate the same region in glomeruli. However, the degree of overlapping depended on concentration of odorants (Johnson & Leon, 2000a). Therefore, it seems that ethanol and acetaldehyde were not concentrated enough to stimulate any receptors that the odorants share with acetic acid in order to have a significant effect on acetic acid. Moreover, if the peripheral-governed suppressions, which were supported by many studies (Ache et al., 1987; Bell et al., 1987; Laing, 1988), do exist for acetic acid stimulated receptors, it seems that those receptors are not stimulated by acetaldehyde and ethanol. Another possibility is that receptor sites that suppress acetic acid signal transduction are not stimulated by ethanol and acetaldehyde at sub-threshold levels studied.

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Chapter 5

V. SUB-THRESHOLD EFFECTS ON THE PERCEIVED INTENSITY OF RECOGNIZABLE ODORANTS IN BINARY MIXTURES: THE ROLE OF CARBON CHAIN-LENGTH AND CONCENTRATION

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V.1 Abstract

The effects of sub-threshold aliphatic acids (S) on the perceived intensities, overall intensity and two or three additional descriptors, of recognizable aliphatic acids (R) in binary mixtures of an S and an R with different carbon chain-lengths were studied using magnitude estimation. All possible binary combinations of acetic acid, propanoic acid, and n-butanoic acid were studied. Sub-threshold and peri-threshold concentration were maintained and their effects were tested by expressing the sub-threshold concentrations as percentages of individual detection thresholds (10%, 20%, 30%, 50%, 70%, 90%, and 100%). The sub-threshold effects depended on differences in carbon chain-lengths and concentrations. However, the effects were not observed when the difference was two carbon atoms. With one carbon atom difference, both suppression and enhancement were observed, but increasing sub-threshold concentration tended to enhance the intensities of recognizable odorants. The results suggest that both suppression and enhancement occurred simultaneously but at sub-threshold concentrations, the activity gained by enhancement overcame suppression and resulted in increased intensities.

V.2 Introduction

Aromas encountered everyday comprised of odorants at sub-threshold and supra-threshold concentrations. Most odor-mixture research has focused on the perception of either suprathreshold mixtures or the detection of sub-threshold mixtures (Berglund et al., 1971, 1976; Cain et al., 1995; Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991). In supra-threshold mixture studies, mixture interactions were defined as changes in quality and/or intensity of suprathreshold mixtures (Berglund et al., 1971; Derby et al., 1991a; Wise et al., 2000). Suppression is commonly observed in both physiological and psychological studies of changes in intensity of suprathreshold mixtures; those changes are called intensity mixture interactions (Ache, 1989; Cain et al., 1995; Derby et al., 1985). The contribution of peripheral and central mechanisms underlining intensity mixture interactions is still poorly understood.

Peripheral and central neural mechanisms governing suprathreshold mixture suppression were discovered using a lobster model (Derby et al., 1985). Evidence suggested that suppression in suprathreshold odor mixtures is a result of peripheral events (Ache et al., 1987; Bell et al., 1987) and depends on the polarity of the component in the mixtures (Bell et al., 1987; Laing, 1988) based on a chromatographic-like process (Mozell, 1964, 1970; Mozell & Jagodowicz, 1973). However, many studies also reported evidence of the contribution of central mechanisms (Cain, 1975; Derby et al., 1985).

In sub-threshold mixture studies, changes in the detection threshold of sub-threshold mixtures compared to the detection threshold of pure odorants were used to conclude the effects of sub-threshold concentrations in odor, taste and flavor; however, the results are not consistant. The most common outcome reported is an additive effect of sub-threshold mixtures (Bennett et al., 1965; Day et al., 1963; Guadagni et al., 1963b; Guadagni et al., 1974a; Keith & Powers, 1968; Langler & Day, 1964; Lillard & Day, 1961; Patterson et al., 1993). In addition, Nawar and Fagerson (1962), Laska and Hudson (1991), and Rosen et al. (1962) reported a synergistic effect. However, a synergistic effect of sub-threshold compounds was not common.

Laska and Hudson (1991) reported both additive and synergistic effects in odor mixtures, but they also pointed out that only 53% of the subjects demonstrated increased sensitivity toward the mixtures. The disagreement among subjects' detection thresholds of odor mixtures could be caused by differences in sensitivity between subjects. Commetto-Muniz and Cain (1997) took into account each subject's detection threshold for each component in the mixtures during the analysis step and concluded that the effect was agonistic (additive).

Research determining the effect of sub-threshold odorants on supra-threshold odorants or *vice versa* is very limited. In taste, Guadagni and others (1973) reported enhancing effect of naringin dihydrochalcone at detection threshold on bitterness of a dilute limonin solution (Guadagni et al., 1974a). In vision, sub-threshold stimuli were reported to contribute to observed intrinsic signal activity in

a cat's visual cortex and it was believed to result from metabolic activity in dendrites or at synapses (Toth et al., 1996).

In olfaction, Bult and others (2001) employed a duo-trio method to test discrimination ability of subjects on sub-threshold mixtures of ethanol, 1-butanol, and 1-hexanol in a water and supra-threshold apple mixture background. Subjects could discriminate sub-threshold mixtures better when a refined concept was introduced to them, and the involvement of cognitive processing in sub-threshold detection was suggested (Bult et al., 2001).

In 2002, Lopetcharat and McDaniel reported sub-threshold effects on the intensities of descriptors of recognizable odorants in binary and tertiary mixtures comprised of sub-threshold and panel-recognition concentration odorants. Lopetcharat and McDaniel maintained sub-threshold conditions in their studies by expressing sub-threshold concentrations as percentages of subjects' individual detection thresholds, and the intensities of the descriptors of recognizable odorants were rated using magnitude estimation. The studies reported that suppression was prominent in both binary and tertiary mixtures of acetic acid, acetaldehyde and ethanol; however, enhancement was observed in some tertiary mixtures. Suppression and enhancement observed in the mixtures affected the intensities of different descriptors of recognizable odorants depending on functional groups of the mixtures' components (Lopetcharat & McDaniel, 2002a, 2002b).

This study employed the same technique successfully used in the previous studies to investigate the effects of sub-threshold aliphatic acids on the intensities

of recognizable aliphatic acids in binary mixtures. The mixtures comprised two aliphatic acids, one at sub-threshold and the other at panel-recognition concentration, that were different in carbon chain-lengths (acetic acid, propanoic acid and n-butanoic acid). In addition to overall intensity, the effect of sub-threshold acids on the intensities of specific descriptors of recognizable acids was investigated. Moreover, this study was performed to determine whether the extent of the effect depends on the carbon chain-lengths and the concentrations of the acids tested.

V.3 Materials and methods

1. Odorants and odorant presentation

Food grade acetic acid, propanoic acid and n-butanoic acid (99% purity), purchased from Aldrich (Milwaukee, WI) were used as odorants in this study (Table V.1). Double-distilled deionized water served as the solvent for all odorants. Odorants were presented in a 125-ml wide-mouth column clear glass bottle (inside diameter 4.8 cm) with screw cap containing five 4-ml vials. The bottle caps were lined with a Teflon® liner. Five vials were needed in order to fill the space in the bottle but the number of vials used depended on the experiments. The headspace ranged from approximately 108 ml to 112 ml depending on the experiments. All methodologies and testing condition were described in details in previous study (Lopetcharat & McDaniel, 2002b)

2. Screening test and subjects

From twenty volunteers screened, seventeen subjects were selected based on their ability to detect and recognize acetic acid, propanoic acid and n-butanoic acid using 3-AFC ascending concentration series method of limits (ASTM, 1999). Subjects whose detection and recognition thresholds were within two SDs from the geometric mean of the group threshold were selected.

Fourteen female and three male subjects (mean age = 27, range 22 to 35) participated in the study after informed consent. Sixteen subjects had participated in previous studies (Lopetcharat & McDaniel, 2002a, 2002b). The subjects were paid for their participation. Subjects were not under medication and were non-smokers. They did not suffer from any acute or chronic illness of the respiratory system (self-reported). Subjects were instructed not to use odorous personal products on the day of the experiment.

3. Subject training

3.1 Odorants' descriptor definition

Standard acetic acid, propanoic acid and n-butanoic acid solutions at different concentrations were introduced to subjects. Subjects described all the solutions and, through discussion, consensus descriptors were selected (Table V.1).

Table V.1 Odorant and descriptors accepted by subjects

Compounds	Purity	MW	Descriptors	Concentrations
Acetic acid	99%	60	Sweetness Sourness Vinegar	8.9 and 17.9 mM
Propanoic acid	99%	74	Sweetness Sourness Saltiness	273.7 and 547.4 mM
n-Butanoic acid	99%	88	Sweetness Sourness Spoiled dairy note	21.4and 85.7 mM

3.2 Intensity rating training

Magnitude estimation (ME) was employed to rate the intensity of the odorants. The same training procedure was used as previous studies (Lopetcharat & McDaniel, 2002b); however, an acetic acid standard was used in training instead of acetaldehyde.

Subject performance was evaluated through power functions of the overall intensity of acetic acid based on their scaling and ordering abilities. Subjects were ready for testing when they could correctly rate and order the intensity of four out of five concentrations.

3.3 Individual detection threshold and recognition threshold determination

Individual detection and recognition thresholds were determined in order to ensure sub-threshold and recognition concentrations. Determination of detection and recognition thresholds for all odorants, acetic acid, propanoic acid and n-butanoic acid, were conducted after the training sessions because the sensitivity of subjects tends to improve after training (Engen, 1960; Laska & Hudson, 1991; Lawless et al., 1995). Threshold determinations were conducted in three replications.

The 3-AFC ascending concentration series method of limits (ASTM, 1999) was used and the details of actual procedure was described elsewhere (Lopetcharat & McDaniel, 2002b). Subjects were introduced to the reference odorants before the test. An individual's detection threshold was defined as the geometric mean of

the geometric means from four replications. The replication geometric means of detection thresholds were calculated from the highest incorrect concentration and the lowest correct concentration followed by three consecutive correct concentrations. An individual's recognition threshold was defined as in the same way as the individual detection threshold, but the replication geometric means of recognition thresholds were calculated from the highest *incorrectly identified* concentration and the lowest *correctly identified* concentration followed by three correctly identified consecutive concentrations. The individual's detection and recognition thresholds are in Table V.2. Subjects were grouped by their individual detection thresholds as described in previous study (Lopetcharat & McDaniel, 2002b) (Table V.3) and the individual's recognition thresholds are in Table V.4.

3.4 Determination of group recognition concentration of all odorants

The panel-recognition concentration of an odorant is defined as the concentration at which all subjects can 100% correctly identify the odorant. Highest individual recognition thresholds for all odorants among all subjects were used as starting concentrations. Panel recognition determination was described in

Table V.2 The medians of individual detection thresholds for acetic acid, propanoic acid and n-butanoic acid*

	Detection threshold (μM)					
	Acetic acid		Propanoic acid		n-Butanoic acid	
Subject	Median	SD	Median	SD	Median	SD
1	139.88	0.11	534.54	0.08	7.40	0.03
2	665.42	0.06	899.02	0.05	35.18	0.07
3	665.42	0.03	899.02	0.03	118.33	0.03
4	117.63	0.14	112.38	0.03	5.23	0.15
5	1882.04	0.03	2542.76	0.02	7.40	0.10
6	470.51	0.07	899.02	0.04	35.18	0.03
7	139.88	0.09	2138.17	0.02	59.17	0.06
8	279.75	0.06	534.54	0.05	29.59	0.04
9	941.07	0.02	1797.95	0.04	140.72	0.02
10	559.54	0.07	94.50	0.10	140.72	0.04
11	665.42	0.05	1512.02	0.12	49.75	0.08
12	69.94	0.04	317.86	0.03	59.17	0.02
13	470.51	0.06	635.69	0.06	4.40	0.26
14	117.63	0.03	449.49	0.06	99.51	0.03
15	470.51	0.03	449.49	0.19	14.79	0.04
16	559.54	0.06	534.54	0.03	70.36	0.02
17	166.35	0.05	534.54	0.02	49.75	0.03

* in double distilled deionized water

Table V.3 The medians of grouped detection thresholds for acetic acid, propanoic acid and n-butanoic acid*

Acetic acid detection threshold (μM)			
Group	**Median**	**SD**	**Subjects**
1	69.94	0.04	12
2	344.43	0.04	1,2,3,4,6,7,8,9,10,11,13,14,15,16,17
3	1882.04	0.03	5

Propanoic acid detection threshold (μM)			
Group	**Median**	**SD**	**Subjects**
1	620.87	0.03	All

n-Butanoic acid detection threshold (μM)			
Group	**Median**	**SD**	**Subjects**
1	40.88	0.04	All

* in double distilled deionized water

Table V.4 The individual recognition thresholds and highest group recognition threshold for acetic acid, propanoic acid and n-butanoic acid*

	Recognition threshold (μM)					
	Acetic acid		Propanoic acid		n-Butanoic acid	
Subject	**Median**	**SD**	**Median**	**SD**	**Median**	**SD**
1	719.25	0.13	1663.56	0.04	17.59	0.02
2	1438.43	0.04	2797.59	0.04	70.36	0.03
3	855.26	0.03	3326.95	0.03	118.33	0.03
4	604.80	0.05	349.71	0.03	35.18	0.02
5	2876.71	0.05	5595.47	0.06	49.75	0.07
6	359.60	0.06	1398.86	0.06	35.18	0.03
7	719.25	0.09	6654.26	0.03	167.35	0.02
8	2876.71	0.02	9409.85	0.03	167.35	0.02
9	1209.55	0.02	4705.15	0.02	140.72	0.02
10	1017.09	0.03	5595.47	0.03	167.35	0.03
11	1438.43	0.06	6654.26	0.08	70.36	0.05
12	508.57	0.03	3326.95	0.02	167.35	0.02
13	846.58	0.03	2352.45	0.10	140.72	0.04
14	1710.61	0.06	6654.26	0.06	99.51	0.03
15	359.60	0.03	2797.59	0.10	14.79	0.04
16	1710.61	0.07	3956.49	0.13	70.36	0.02
17	508.57	0.03	6654.26	0.08	70.36	0.03
Highest conc.	**2876.7**		**9409.8**		**167.4**	

* in double distilled deionized water

details in previous study (Lopetcharat & McDaniel, 2002b). Panel-recognition concentrations were the individual recognition thresholds and they are in Table V.4.

3.5 The effect of sub-threshold on perceived intensity of panel-recognition concentration in binary mixtures (binary mixture study)

A binary system was obtained as described in the previous study (Lopetcharat & McDaniel, 2002b). All possible binary combinations of three odorants (acetic acid, propanoic acid and acetaldehyde) were used as sub-threshold solutions. Eight concentrations of individual detection thresholds (0 or blind control, 10, 20, 30, 50, 70, 90, 100% of individual detection thresholds) were used. Sub-threshold suppression was reported at 30% of individual detection thresholds in binary mixtures of odorants with different functional groups (Lopetcharat & McDaniel, 2002b). Therefore, 10% and 20% concentrations were selected in order to investigate sub-threshold effects more thoroughly. All six possible treatment combinations were tested as shown in Table V.5.

A completely randomized block design was employed. Subjects were treated as blocking factors. The experiments were design to be balanced across all subjects (Lopetcharat & McDaniel, 2002b). The treatment combinations were also randomly assigned to subjects in a balanced fashion. The control bottle contained water instead of a sub-threshold solution and was used as blind control. Thus, it had only a panel-recognition-concentration odorant vial and a water vial. Subjects

Table V.5 Six possible treatment combinations in the binary mixture study

Recognition level odorant	**Sub-threshold level odorant**
Acetic acid	Propanoic acid
Acetic acid	n-Butanoic acid
Propanoic acid	Acetic acid
Propanoic acid	n-Butanoic acid
n-Butanoic acid	Acetic acid
n-Butanoic acid	Propanoic acid

were instructed to smell standard odorants (moduli) with a designated intensity of 100 (modulus bottles contained one vial with 4-ml of an odorant solution at panel-recognition concentration along with four empty vials) before testing. Subjects sniffed a sample bottle and rated the overall intensity and descriptors' intensity using ME. The matching modulus was provided to subjects at all times. The details of testing procedure were described in a previous study (Lopetcharat & McDaniel, 2002b). The testings were done in four replications.

4. Data analysis

Since the subjects used different scales and the data tend to follow a log-normal distribution, results from the binary system study were normalized within each subject employing a geometric mean normalization (Moskowitz & Jacobs, 1988; Stevens, 1956). Three-way univariate analysis of variance (ANOVA) for concentrations, subjects, replications, all 2-way interaction and a 3-way interaction (concentrations x subjects x replications) were conducted for each individual descriptors within each experiment separately. Subjects and replications were treated as a random effect (Lundahl et al., 1986) and concentrations as a fixed effect. Backward elimination was used to select a final most parsimonious model, which were used to analyze all descriptors within each experiment. The final model consisted of main effects (subjects and concentrations) and a two 2-way interactions (subjects x concentration). The effect of concentrations was tested against the effect of the 2-way interaction. Dunnett's and Tukey-Kramer multiple

comparison procedures were employed to detect the difference from all comparisons against a blind control (0%) and general pair-wise differences between treatments, respectively. All analysis was performed on the log10 of the normalized data at α=0.05, when otherwise, it will be stated. The six treatment combinations were analyzed separately. Analyses were conducted using General Linear Model in SPSS® V. 10.0 (Chicago, IL).

V.4 Results

1. Odorants and their descriptors

Subjects reported sweetness and sourness aroma for all odorants; however, each odorant possessed some unique quality (Table V.1). In addition to sour and sweet notes, the subjects described acetic acid as vinegar, propanoic acid as salty and n-butanoic acid as spoiled-dairy. The subjects agreed upon the descriptors of acetic acid and propanoic acid, but not on the spoiled-dairy note of n-butanoic acid. Even though the subjects described n-butanoic acid differently, the descriptors used involved bad dairy smell such as rotten yogurt and baby vomit. Therefore, through discussion, they agreed to use the spoiled-dairy note as a collective descriptor. Differences in experience and sensitivity were believed to cause heterogeneous perception (Ayabe-Kanamura et al., 1998; Lopetcharat & McDaniel, 2002b).

2. Detection thresholds and panel-recognition concentrations

Detection thresholds of acetic acid, propanoic acid, and n-butanoic acid spanned a factor of 2.7×10^1, 2.7×10^1, and 3.2×10^1 with medians 356.6 μM, 629.2 μM and 33.8 μM, respectively (Table 2). Panel detection thresholds for all odorants are listed in Table V.3.

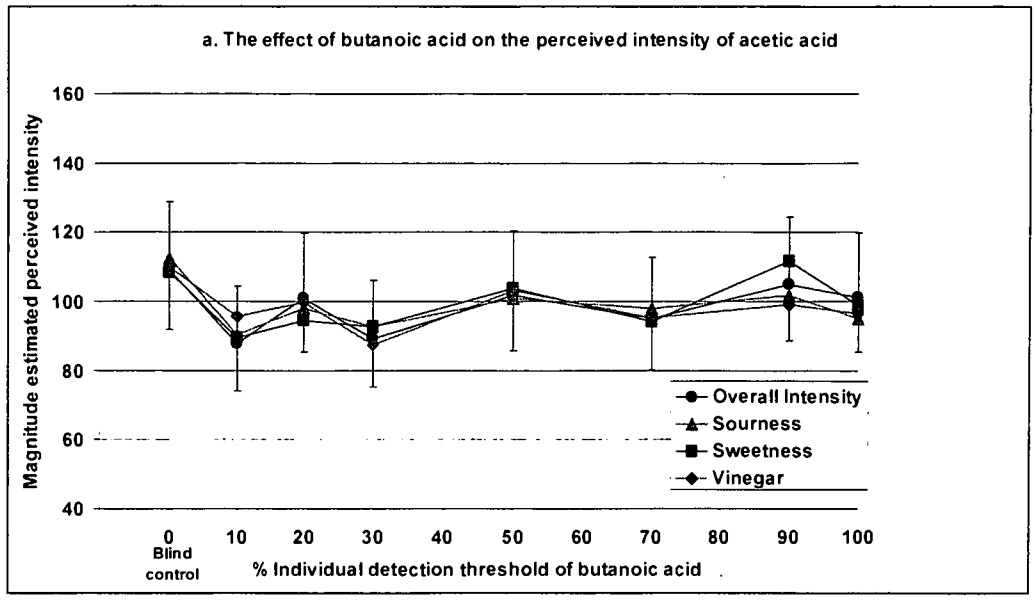
Panel recognition concentrations were the highest individual recognition threshold, which were 2876.7 μM, 6654.3 μM and 167.4 μM for acetic acid, propanoic acid and n-butanoic acid, respectively (Table V.4). The panel-recognition concentration of acetic acid also reduced from 6,330 μM (Lopetcharat & McDaniel, 2002b) to 2,880 μM with decreased variation.

3. Binary mixture study

3.1 The effects of n-butanoic acid and propanoic acid on acetic acid perceived intensity

N-butanoic acid and propanoic acid at sub-threshold did not have significant effects on the intensities of acetic acid in binary mixtures; however, there were different trends observed in both conditions (Figure V.1). The intensities of all descriptors of acetic acid at panel-recognition concentration were not significantly different from the acetic acid blind control when n-butanoic acid at all sub-threshold concentrations (% of individual detection threshold) was introduced: [$F(7, 114) = 1.18$, $p = 0.32$ for overall intensity], [$F(7, 114) = 0.92$, $p = 0.49$ for sourness], [$F(7,114)=1.26$, $p = 0.28$ for sweetness] and [$F(7, 114) = 0.70$, p

= 0.67 for vinegar] (Figure V.1a). By observation, the intensities of the descriptors decreased in the presence of n-butanoic acid at sub-threshold and the reduction began at the lowest concentration tested, 10% of subjects' individual detection threshold level (Figure V.1a).



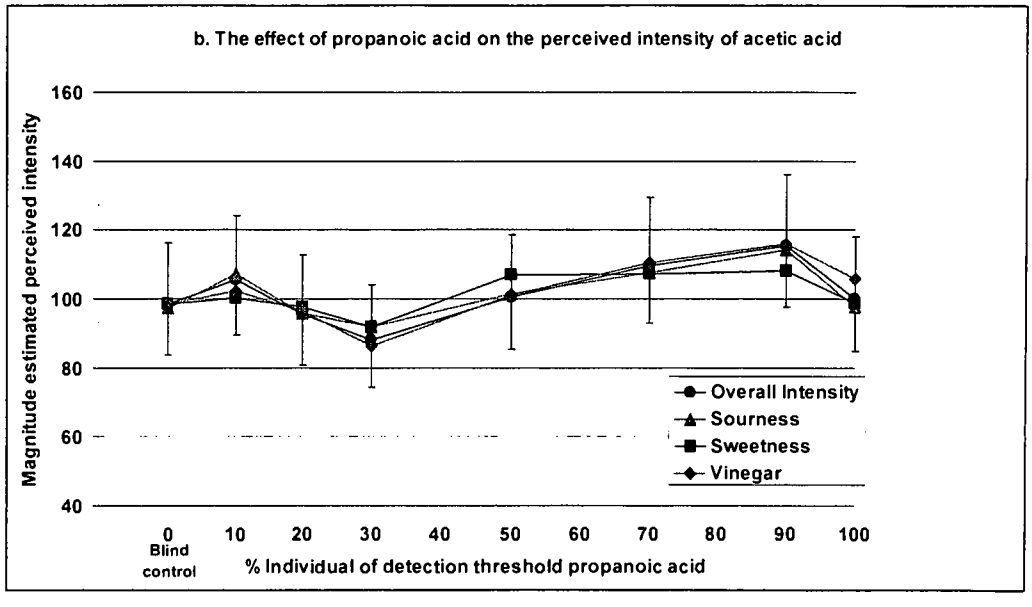
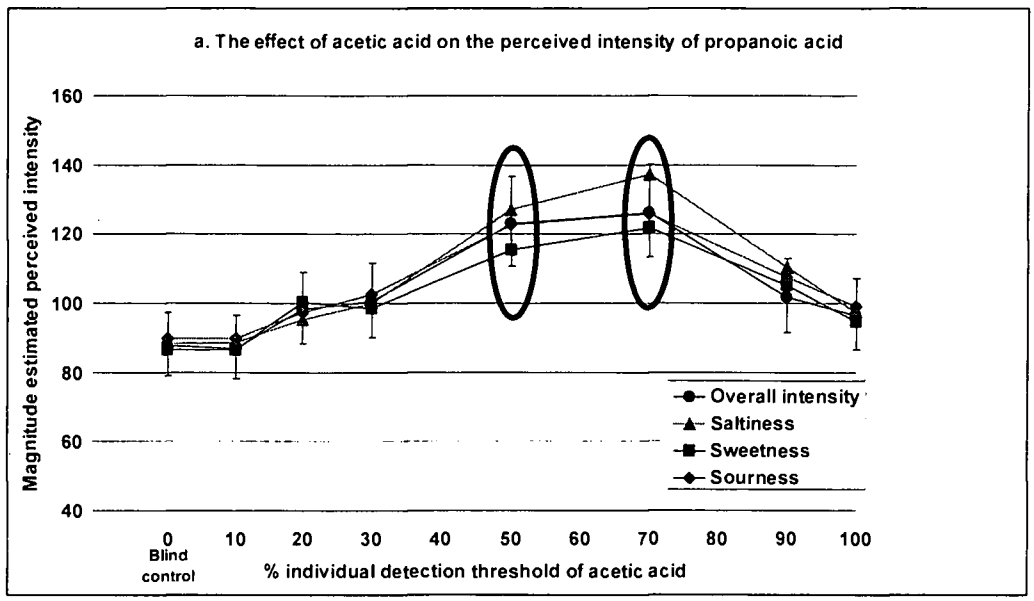


Figure V.1 The magnitude estimated perceived intensity of acetic acid descriptors at panel-recognition concentration in the presence of n-butanoic acid (a) and propanoic acid (b) at sub-threshold concentration (% of individual detection thresholds) in binary mixtures. The bars indicate 95% confidence interval. Seventeen subjects with four replications provided the data for the median estimation.

Propanoic acid at all sub-threshold concentrations did not significantly affect the intensities of acetic acid: [$F(7, 114) = 1.28$, $p = 0.27$ for overall intensity], [$F(7, 113) = 0.82$, $p = 0.58$ for sourness], [$F(7,113)=0.56$, $p = 0.79$ for sweetness] and [$F(7, 114) = 1.20$, $p = 0.31$ for vinegar] (Figure V1b). However, the trend observed differed from that of n-butanoic acid. The intensities of all descriptors increased in the presence of propanoic acid at 10% of subjects' individual detection threshold, but decreased at 20% and 30%. The intensities of all acetic acid descriptors increased with increasing sub-threshold concentrations of propanoic acid from 50% to 90%. However, at 100% threshold, the increasing magnitude reduced.

3.2 The effects of acetic acid and n-butanoic acid on propanoic acid perceived intensity

The effects of acetic acid and n-butanoic acid at sub-threshold concentrations on the intensities of propanoic acid descriptors were different (Figure V.2). Increasing acetic acid sub-threshold concentrations caused enhancement of the intensities of propanoic acid descriptors. Acetic acid at 50% and 70% of individual detection thresholds significantly enhanced the intensities of propanoic acid descriptors: [$F(7,86)=6.38$, $p = 0.00$ for overall intensity], [$F(7,86)=5.81$, $p = 0.00$ for sourness], [$F(7,86)=4.61$, $p = 0.00$ for sweetness] and [$F(7,86)=7.10$, $p = 0.00$ for saltiness]. The overall intensity increased from 88 of the propanoic acid blind control to 123 and 126 of the 50% and 70% levels, and the increases were 40% and 43%,respectively (Figure V.2a). At 90% and 100% the



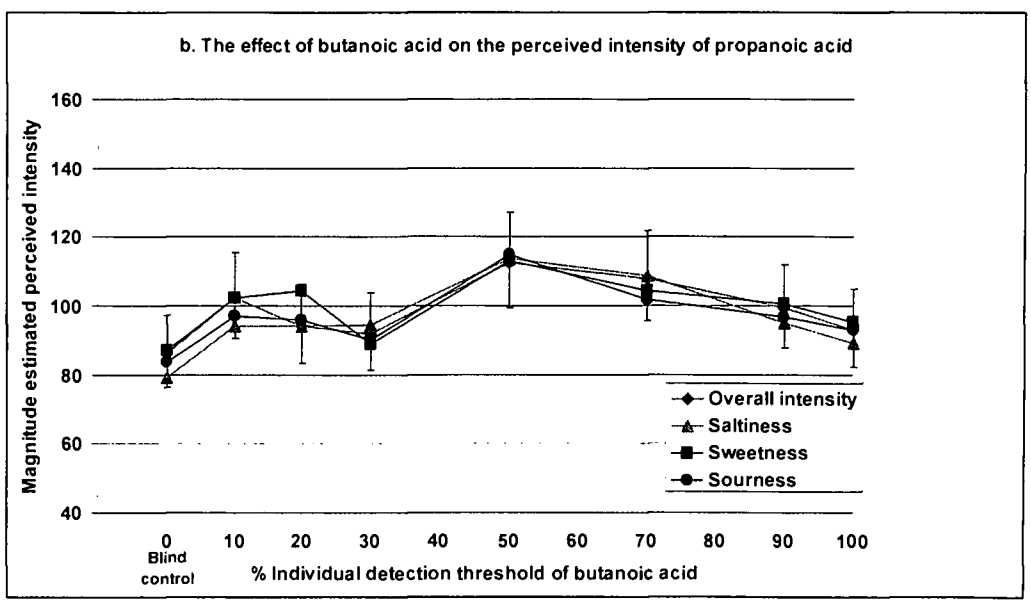


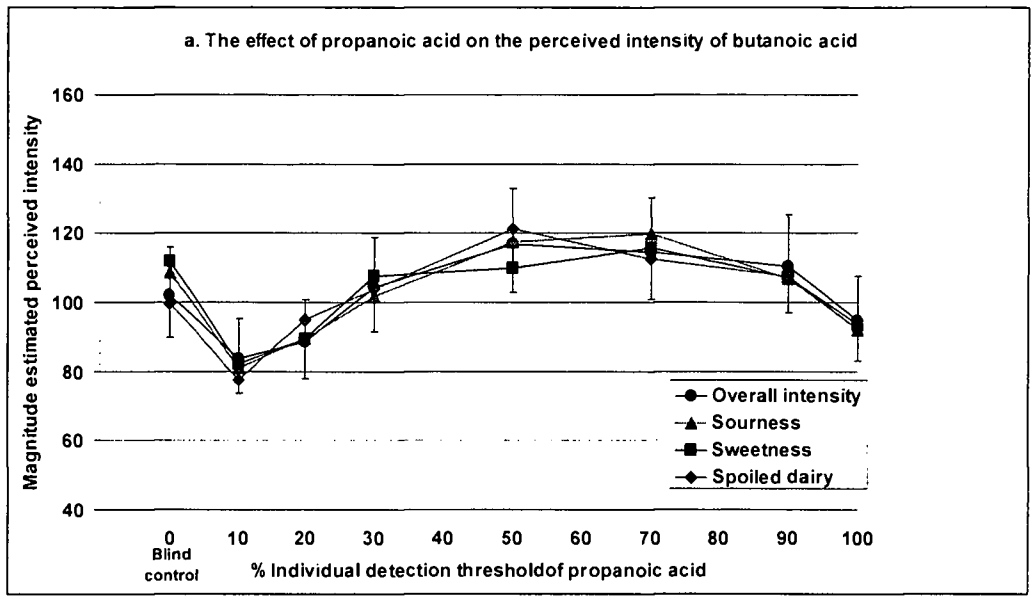
Figure V.2 The magnitude estimated perceived intensity of propanoic acid descriptors at panel-recognition concentration in the presence of acetic acid (a) and n-butanoic acid (b) at sub-threshold concentrations (% of individual detection thresholds) in binary mixtures. The bars indicate 95% confidence interval. The ovals indicate significantly different from the blind control at α=0.05 from Dunnett test. Seventeen subjects with four replications provided the data for the median estimation.

intensities of propanoic acid descriptors decreased from the peak at 70% to non-significantly different from the blind control (Dunnett $p > 0.05$).

In the presence of n-butanoic acid, at 50% and 70% levels, only saltiness intensity was significantly different from the propanoic blind control [$F(7, 86) = 2.32$, $p = 0.03$] (Figure V.2b). Overall intensity, sourness, and sweetness intensities were suggestively affected at significant level of 0.05: [$F(7, 86) = 1.80$, $p = 0.10$ for overall intensity], [$F(7, 86) = 1.72$, $p = 0.11$ for sourness] and [$F(7, 86) = 1.73$, $p = 0.11$ for sweetness] and an increasing trend was observed (Figure V.2b). The increase in intensities of the propanoic descriptors began at the lowest concentration tested (10% of individual detection threshold) and peaked at the 50% level. Increasing n-butanoic acid concentration to 70%, 90% and 100% caused the reduction in intensities of all descriptors compared to those of 50% level; however, the intensities were higher than, but not significantly different from those of the blind control (Figure V.2b).

3.3 The effects of acetic acid and propanoic acid on n-butanoic acid perceived intensity

Propanoic acid had a significant effect on the intensities of n-butanoic acid descriptors, but acetic acid did not (Figure V.3). ANOVA indicated significant differences in the intensities of n-butanoic acid descriptors caused by different



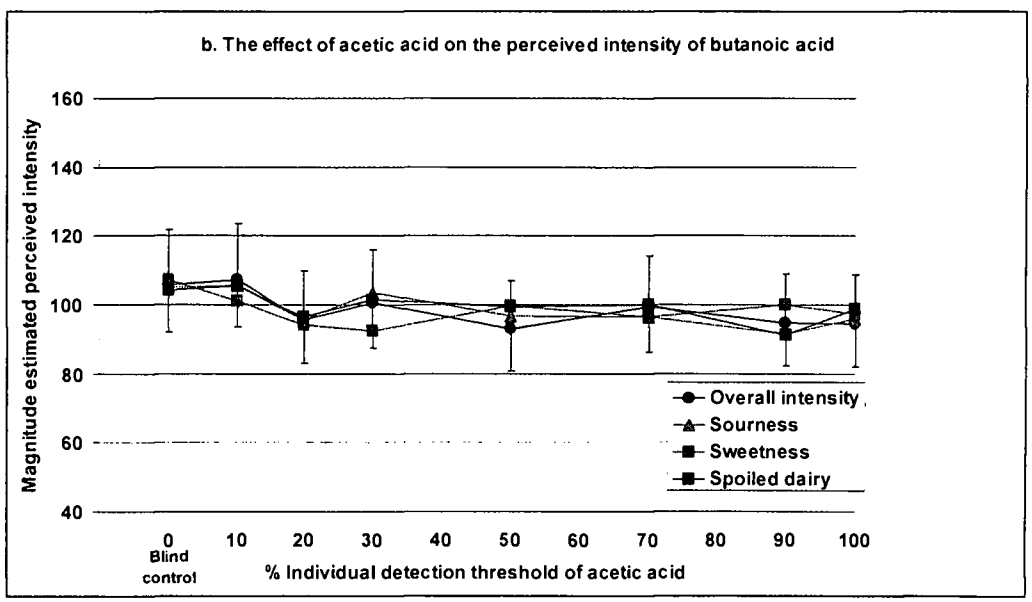


Figure V.3 The magnitude estimated perceived intensity of n-butanoic acid descriptors at panel-recognition concentration in the presence of propanoic acid (a) and acetic acid (b) at sub-threshold concentrations (% of individual detection thresholds) in binary mixtures. The bars indicate 95% confidence interval. Seventeen subjects with four replications provided the data for the median estimation.

sub-threshold concentrations of propanoic acid: [$F(7, 98)=4.44$, $p \leq 0.001$ for overall intensity], [$F(7,98)=3.15$, $p = 0.005$ for sourness], [$F(7, 98)=3.15$, $p = 0.005$ for sweetness], and [$F(7,98)=2.74$, $p = 0.01$ for spoiled-dairy aroma]. In general, propanoic acid at 10% of subjects' individual detection thresholds significantly suppressed the intensities of n-butanoic acid; however, the intensities increased when the sub-threshold concentrations of propanoic acid increased (Figure V.3a). The significant differences detected by ANOVA were the differences between the 10% and 50% levels, and between the 10% and 70% levels depending on descriptors. Overall intensity, sourness and spoiled-dairy aroma of the 10% level were significantly less intense that those of the 50% level with 28% ($p = 0.01$), 30% ($p = 0.03$) and 36% ($p = 0.01$) differences, respectively. In the same way, the intensities of overall intensity, sourness and sweetness of the 10% level were significantly less intense than those of 70% level with 27% ($p = 0.03$), 32% ($p = 0.01$) and 28% ($p = 0.01$) difference, respectively. Only the sweetness of the 10% level was significantly less than that of the n-butanoic blind control (0% level) with a 26% reduction ($p = 0.04$); otherwise, there was no significant difference between the blind control and other levels.

Acetic acid at sub-threshold concentrations did not significantly alter the intensities of n-butanoic acid descriptors, even though there was a subtle reduction trend observed: [$F(7,98)=0.85$, $p = 0.55$ for overall intensity], [$F(7,98)=0.66$, $p = 0.70$ for sourness], [$F(7,98)=0.59$, $p = 0.77$ for sweetness] and [$F(7,98)=0.34$, $p = 0.92$ for spoiled-dairy aroma] (Figure V.3b). Overall intensity reduced from 106

for the n-butanoic acid blind control to approximately 98 (the median estimated intensity of all sub-threshold levels). In the same way, the intensity of sourness, sweetness and spoiled-dairy aroma reduced from 105 to 98, 107 to 97, and 104 to 99, respectively; however, these changes were not significantly different (Figure V.3b).

V.5 Discussion

1. Sub-threshold effects on the intensities of recognizable odorants in binary mixtures: roles of carbon chain-length

Previous studies (Lopetcharat & McDaniel, 2002a, 2002b) reported sub-threshold effects on the intensity of recognizable odorants in binary and tertiary mixtures comprised of odorants with different functional groups and concentrations. The direction and magnitude of the effect depended on the functional groups of the odorants in the mixtures (Lopetcharat & McDaniel, 2002a, 2002b) and the polarity of odorants was believed to partially influence the effect, which was supported by other suprathreshold mixtures studies (Bell et al., 1987; Laing, 1988). The results of this study demonstrate another chemical aspect, carbon chain-length, governing the sub-threshold effect on the intensity of recognizable odorants in binary mixtures (aliphatic carboxylic acids with different number of carbon atoms and concentrations).

In this study, sub-threshold effects depended on the carbon chain-length (number of carbon atoms) of the aliphatic carboxylic acids. When the acids were

different by one carbon atom, an enhancement trend was observed. However, only acetic acid at sub-threshold significantly enhanced the intensities of propanoic acid descriptors (Figure V.2a); otherwise, the enhancements were very subtle and not significant (Figure V.1b & V.3a). Interestingly, in acetic acid/n-butanoic acid mixtures, sub-threshold odorants in the mixtures did not have an effect on the intensities of recognizable odorants (Figure V.1a & V.3b).

The enhancement and the elimination of the sub-threshold effect (Figure V.1a and V.3b) can be partially explained by the studies of the chemotropic map in the glomerular layer (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999; Johnson et al., 1998). Johnson and Leon (2000) reported molecular length of carboxylic acid as an important aspect of the olfactory code studied by [^{14}C]2-deoxyglucose (2DG)-uptake in the glomerular layer of rat olfactory bulbs. Small differences in carbon number produced drastic differences in 2DG-uptake patterns (Johnson et al., 1999). Acetic acid, propanoic acid and n-butanoic acid produced overlapping but distinct 2DG-uptake pattern in the rat glomerular layer (Johnson et al., 1999). The distinction of degree of stimulation (measured by 2DG-uptake) within each field stimulated by acetic acid and n-butanoic acid was more pronounced than the distinctions between acetic acid and propanoic acid, and propanoic acid and n-butanoic acid (Johnson et al., 1999). These distinctions possibly caused the directional sub-threshold effect observed in this study when the difference in carbon number was two.

In addition to the distinctions between glomerular fields stimulated by the acids, the directional sub-threshold effect related to differences in polarities that inversely related to number of carbon atoms. The order of the polarities of the odorants in an aqueous environment is acetic acid > propanoic acid > n-butanoic acid. A difference of more than one carbon atom could possibly cause enough difference to eliminate the sub-threshold effect (Figure V.1a and V.3b). The influence of the polarity of odorants on the direction and the magnitude of mixture interaction was reported in sub-threshold/suprathreshold mixtures (Lopetcharat & McDaniel, 2002a, 2002b) and supra-threshold mixtures (Bell et al., 1987; Laing, 1988). Many studies provided evidence to relate polarity and stimulated field in the glomerular layer (Bell et al., 1987; Bozza & Kauer, 1998; Laing, 1988; Mozell, 1964, 1970; Mozell & Jagodowicz, 1973; Ressler et al., 1993; Ressler et al., 1994; Sato et al., 1994; Vassar et al., 1994; Vassar et al., 1993; Wang et al., 1998). In turn, both assumptions, differences in polarities and distinctions between stimulated glomerular field, can explain sub-threshold effect in concert.

In addition to the intensity aspect, we designed this study to observe changes in qualities of recognizable odorants by asking subjects to rate two to three additional descriptors along with overall intensity. Most responses were correlated , however, different descriptors were affected differently by sub-threshold level aliphatic acids (Figure V.1 and V.3). For example, in the case of propanoic acid as a recognizable odorant, acetic acid and n-butanoic acid at sub-threshold levels affected the intensities of propanoic acid descriptors differently (Figure V.2).

Acetic acid enhanced the intensity of saltiness of propanoic acid the most; meanwhile, n-butanoic acid tended to enhance the intensity of sweetness of propanoic acid. This phenomenon was supported by overlapping but distinct stimulated glomerular fields and recruiting of olfactory neurons (Johnson et al., 1999; Malnic et al., 1999). Acetic acid and n-butanoic acid possibly stimulate different-but-close classes of neurons that stimulate glomeruli at different locations which results in the effect on different qualities of propanoic acid.

2. The roles of concentration on sub-threshold effects

In addition to the number of carbon atoms, the sub-threshold effect depended on the concentrations of sub-threshold odorants that were percentages of subjects' individual detection threshold. However, the sub-threshold effect was restricted to levels that the difference in number of carbon atoms was only one. In general, the intensities of recognizable odorants increased when the concentrations of sub-threshold odorants increased; however, the trends were different in different binary mixtures (Figure V.1b, V.2 & V.3a). When the concentrations of sub-threshold odorants approached 100% (detection threshold) the intensities of recognizable odorants decreased but the intensities were higher than the intensities of the blind controls (no sub-threshold odorant). Enhancement caused by detection threshold-level concentration stimuli was reported in taste. Naringin dihydrochalcone, having a bitter taste at low concentration, at detection threshold

enhanced bitterness of limonin and naringin (Guadagni et al., 1963b; Guadagni et al., 1973).

The concentration-dependent sub-threshold enhancement of the intensities of recognizable odorants was possibly the result of recruiting olfactory neurons and overlapping stimulated glomerular fields. Even though olfactory neurons are specifically tuned for certain molecular features (Sato et al., 1994), the neurons that respond to structurally similar odorants locate near each other in olfactory epithelium (Ressler et al., 1993). Increasing odorant concentrations increase the recruitment of lower affinity olfactory neurons for the odorants (Malnic et al., 1999). In turn, recruiting of olfactory neurons causes an increase in glomerular activity and/or alteration of stimulated glomerular fields (Johnson & Leon, 2000a). Glomeruli that respond maximally to an aliphatic acid are located near glomeruli that respond maximally to acids with slightly different carbon chain-lengths (Johnson et al., 1999).

Therefore, increasing concentrations of sub-threshold aliphatic acids, which were only one carbon atom different from recognizable aliphatic acids, caused recruiting of nearby olfactory neurons that activated the same glomerular fields as the recognizable acids. From the results, suppression occurred in all cases; however, it was not statistically significant in only one case (propanoic acid at the 10% level and n-butanoic acid at panel-recognition concentration) (Figure V.3a). Moreover, the intensities of recognizable odorants decreased when sub-threshold concentrations approached 100%. These results suggest that suppression and

enhancement occurred simultaneously, but at sub-threshold level, neuronal activity gained was higher than suppression signals and resulted in the trends observed.

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Chapter 6

VI. SUB-THRESHOLD EFFECTS ON THE PERCEIVED INTENSITY OF RECOGNIZABLE ODORANTS IN TERTIARY MIXTURES: ROLE OF CARBON CHAIN-LENGTH AND CONCENTRATION

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VI.1 Abstract

All possible combinations of three aliphatic acids (acetic acid, propanoic acid and n-butanoic acid) were studied in tertiary mixtures. The tertiary mixtures comprised two aliphatic acids at sub-threshold concentrations, and an aliphatic acid at panel-recognition concentration. By varying concentrations of the sub-threshold components in the mixtures as the percentages of individual detection thresholds (30%, 50% and 70%), the sub-threshold level was maintained. This study demonstrates that similarity in carbon chain-lengths between sub-threshold and recognizable acids influenced sub-threshold effect in tertiary mixtures. The sub-threshold effect observed only in mixtures comprising n-butanoic acid (at panel-recognition concentration) and acetic acid and propanoic acid (both at sub-threshold concentrations) non-linearly depended on the concentrations of the sub-threshold acids. The contribution of propanoic acid concentrations to the sub-threshold effect was more than that of acetic acid concentrations. Acetic acid, however, at sub-threshold level was necessary for propanoic sub-threshold effect on overall intensity of n-butanoic acid. In the n-butanoic acid system, sub-threshold suppression and enhancement occurred simultaneously, and the observed outcome was dependent on the ratio between the degree of suppression and enhancement.

VI.2 Introduction

Mixture interaction is a phenomenon where aroma quality and intensity of mixtures cannot be predicted by the information obtained from individual components in the mixtures (Berglund et al., 1971; Berglund et al., 1973; Cometto-Muniz et al., 1997; Derby et al., 1991a, 1991b). Changes in odor quality were defined as pattern mixture interactions (Derby et al., 1991b), which were classified into two categories, homogenous and heterogeneous odors. Homogenous odor is a phenomenon when mixtures have a new aroma, different from individual component's aroma; while heterogeneous odor is described when aromas of mixture components are still perceivable (Berglund et al., 1976)

Intensity mixture interactions are phenomena that occur when the intensity of mixtures change regardless of odor quality (Derby et al., 1991a). In binary mixtures, six categories were defined for intensity mixture interactions of homogeneous odors: complete addition, hypo-addition, partial addition, compromise, compensation, and hyper-addition (Berglund et al., 1976; Cain & Drexler, 1974), and three categories for that of heterogeneous odors: synergism, independence, and antagonism (Berglund et al., 1976). Antagonism or suppression is a common phenomenon in mixtures; nevertheless, synergism and independence are also reported (Berglund et al., 1971; Borroni et al., 1986; Cain et al., 1995; Carr, 1978; Shelton & Mackie, 1971).

Peripheral and central neural mechanisms contribute to mixture intensity interactions. However, most evidence has supported the peripheral mechanisms

(Ache, 1989; Bell et al., 1987; Derby et al., 1985). According to the chromatographic-like model (Mozell, 1964, 1970, 1971; Mozell & Jagodowicz, 1973), odorants are adsorbed differently on the olfactory epithelium and the different adsorption behaviors cause spatial and temporal processing of odor information. Polarity of odorants partially governs how odorants are adsorbed and, in turn, it governs intensity mixture interaction in supra-threshold mixtures (Ache et al., 1987; Bell et al., 1987; Laing, 1988). .

Detection threshold determination was widely used to investigate sub-threshold effects in sub-threshold mixtures. A result frequently reported is the additive effect (Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991; Patterson et al., 1993); however, in some cases, a synergistic effect was reported (Laska & Hudson, 1991; Nawar & Fagerson, 1962). Some studies, however, indicated that additive and synergistic effects are not common phenomena (Guadagni et al., 1974b; Keith & Powers, 1968).

Laska and Hudson (1991) reported both additive and synergistic effects in suprathreshold mixtures using a detection-threshold determination, but only 53% of the subjects demonstrated higher sensitivity toward the mixtures. Differences in sensitivity between subjects should cause disagreement among subjects' mixture detection thresholds. Commetto-Muniz and Cain (1997) took into account each subject's detection threshold for each component in the mixtures during the data analysis step and concluded that the effect was additive.

Perception of sub-threshold stimuli in supra-threshold background has been known as a subliminal effect in auditory system (Merikle, 1988; Merikle & Joordens, 1997). In olfaction, studies about the effect of sub-threshold odorants on the perception of supra-threshold odorants are very limited. A cognitive-level influence on detection probability of sub-threshold odorants was reported in apple-aroma mixtures when a defined concept was introduced to subjects (Bult et al., 2001). Sub-threshold suppression was reported in binary and tertiary mixtures comprising one or two sub-threshold odorants and one recognizable odorant. The odorants were different in functional groups only. The suppression was functional group dependent but not concentration dependent (Lopetcharat & McDaniel, 2002a, 2002b). Further observations revealed sub-threshold enhancement in binary mixtures comprising a sub-threshold and a recognizable aliphatic acids that were different in carbon chain-lengths, and the enhancement was carbon chain-length and concentration dependent (Lopetcharat & McDaniel, 2002c).

The present study was to investigate the effect of sub-threshold aliphatic acids with different carbon chain-lengths and concentrations on the intensity of recognizable aliphatic acids in tertiary mixtures. Tertiary mixtures used in this study were comprised of two sub-threshold aliphatic acids and one recognizable aliphatic acid. The effect of carbon chain-length was investigated by controlling other chemical features such as functional groups. Therefore acetic acid, propanoic acid, and n-butanoic acid were used in this study because they have two carbon atoms that bond together with a single-covalent bond and a carboxylic group at the

end of the carbon chain. In addition to overall intensity, two or three additional descriptors were rated in order to investigate the effect of sub-threshold odorants and their interaction on the specific qualities of recognizable odorants.

VI.3 Materials and methods

1. Odorants and odorant presentation

Food grade acetic acid, propanoic acid and n-butanoic acid (99% purity), purchased from Aldrich (Milwaukee, WI) were used as odorants in this study. Double distilled deionized water served as the solvent for all odorants. Odorant presentation and test levels for all experiments were explained in a previous study (Lopetcharat & McDaniel, 2002b).

2. Screening test and subjects

Subjects who participated in a similar previous study (Lopetcharat & McDaniel, 2002c) were used. Screening methodology, criteria, and subject information were described in previous studies (Lopetcharat & McDaniel, 2002b, 2002c).

3. Subject training

3.1 Odorants' descriptor definition

Subjects smelled standard acetic acid, propanoic acid and n-butanoic acid solutions at different concentrations and described the solutions (Table VI.1). Subjects discussed the descriptors and only descriptors where subjects agree on their definitions were used in this study (Table VI.1).

3.2 Intensity rating training

Magnitude estimation (ME) with modulus was used to rate the intensity of the odorants. Subjects were trained to use ME with modulus as described elsewhere (Lopetcharat & McDaniel, 2002b).

3.3 Individual detection threshold and recognition threshold determination

Detection threshold and recognition threshold of each subject were measured in order to ensure sub-threshold and panel-recognition levels. Determination of both detection and recognition thresholds was performed using the 3-AFC ascending concentration series method of limits (ASTM, 1999). The procedures, definitions of individual's detection and recognition thresholds, individual detection thresholds and the grouping of subjects using individual detection thresholds were reported in a previous study (Lopetcharat & McDaniel, 2002c).

3.4 Determination of panel-recognition concentration of all odorants

Panel recognition concentration of an odorant is defined as the concentration at which all subjects can 100% correctly identify the odorant. The highest individual recognition thresholds for all odorants among all subjects were used as starting concentrations. The 3-AFC technique was used to confirm the concentrations. The panel-recognition concentrations for all odorants were reported in a previous study (Lopetcharat & McDaniel, 2002c).

3.5 The effects of sub-threshold odorants on the perceived intensity of an odorant at panel-recognition concentration in tertiary odor mixtures (tertiary mixture study).

The detailed description of testing materials and protocols were explained in a previous tertiary mixture study (Lopetcharat & McDaniel, 2002a). A 4-ml vial containing a panel-concentration acid was place in a 125-ml bottle along with two other 4-ml vials containing the other acids at various selected concentrations. Two empty 4-ml vials were placed in the bottle to fill up the space. The headspace in the bottle was ~ 104 ml.

In one day, three experiments (Table VI.2) were independently conducted in three separate sessions because recognizable odorants used were different. Within each experiment, three concentrations of sub-threshold aliphatic acids (30, 50, and 70% of individual detection thresholds) were used. Nine treatment combinations were created from three sub-threshold concentrations of two aliphatic

Table VI.1 Odorants and descriptors accepted by subjects

Compound	Purity	MW	Descriptor	Concentration
Acetic acid	99%	60	Sweetness Sourness Vinegar	8.9 and 17.9 mM
Propanoic acid	99%	74	Sweetness Sourness Saltiness	273.7 and 547.4 mM
n-Butanoic acid	99%	88	Sweetness Sourness Spoiled dairy aroma	21.4 and 85.7 mM

Table VI.2 Three possible treatment combinations in the tertiary mixture study

Experiment	Recognition level odorant	Sub-threshold odorants
1	Acetic acid	Propanoic acid* n-Butanoic acid*
2	Propanoic acid	Acetic acid* n-Butanoic acid*
3	n-Butanoic acid	Acetic acid* Propanoic acid*

*9 combinations created from the factorial structures of 3 sub- threshold concentrations (30%, 50%, and 70% of each odorant's individual detection threshold) were used in order to study the interaction effect between sub-threshold odorants in each experiment.

acids using a 3x3 factorial structure (Table VI.2). Four replications for each experiment were accomplished within four days with a total of twelve testing sessions. Subjects were allowed three testing sessions per day with a mandatory of at least 5-minute rest between sessions. In each testing session, subject smelled ten sample bottles (nine treatments and a blind control), which were randomly presented. The blind control contained two 4-ml vials of double distilled deionized water instead of sub-threshold odorants. A sample-interval of at least 30 seconds was mandatory. Overall, each of the ten sample bottles was presented 16 to 17 times in each testing position across all subjects. The experiments were also randomly assigned to subjects in a balanced fashion across four replications and subjects.

4. Data analysis

Since the subjects used different scales and the data tended to follow a log-normal distribution, results from this study were normalized within each subject employing geometric mean normalization (Moskowitz & Jacobs, 1988; Stevens, 1956). Univariate analysis of variance (ANOVA) with a mixed model was employed for each descriptor response within each experiment separately. A 3^2-factorial structure within a completely randomized block design was implemented. The original general linear model of the mixed model comprised main factors: subjects (blocking factor treated as random effect) (Lundahl & McDaniel, 1988), first sub-threshold odorant (fixed effect), second sub-threshold odorant (fixed

effected), replications (random effect), all 2-way interaction factors, and a 3-way interaction factor (subjects x first sub-threshold odorant x second sub-threshold odorant). Backward elimination was used to select the most parsimonious model used in this analysis. The final model comprised all main effects, two 2-way interaction factors [(first sub-threshold odorant x second sub-threshold odorant) and (subjects x replications)]. Dunnett's and Bonferroni multiple comparison procedures were used to detect the differences from all comparisons against the blind control (0%) and general pair-wise differences between treatments, respectively. All analyses were performed on the log10 of the normalized data at α=0.05, when otherwise it will be stated. Analyses were conducted using General Linear Model in SPSS® V. 10.0 (Chicago, IL).

VI.4 Results

1. Tertiary mixture study

1.1 The effects of propanoic acid (P) and n-butanoic acid (B) at sub-threshold concentrations on the perceived intensity of acetic acid

The sub-threshold mixtures of propanoic acid and n-butanoic acid did not affect the intensities of acetic acid descriptors compared to the acetic acid blind control (Figure VI.1) as indicated by non-significant interaction terms for all descriptors: [$F(4, 603)=0.74$, $p = 0.57$ for overall intensity], [$F(4, 603)=0.51$, $p = 0.73$ for sourness], [$F(4, 603)=0.88$, $p = 0.48$ for sweetness] and [$F(4, 654)=0.26$, p

= 0.90 for vinegar]. Propanoic alone did not significantly affect the intensities of acetic acid descriptors. The F-values, F(2, 603)s, of propanoic acid main effects for overall intensity, sourness, sweetness and vinegar were 0.37, 0.39, 0.29 and 0.77 with p-values 0.69, 0.68, 0.75 and 0.46, respectively. In the same way, n-butanoic acid main effects were not significant, which indicated no significant difference detected among samples for all descriptors caused by n-butanoic acid alone. The F(2, 603)s of overall intensity, sourness, sweetness and vinegar for the n-butanoic main effects were 1.09, 1.15, 2.51 and 0.51 with p-values 0.33, 0.32, 0.08 and 0.60, respectively.

The median of magnitudes estimated ranged from 89 (the blind control and 70%P/30%B) to 105 (70%P/70%B) (18% increase) for overall intensity and 88 (the blind control) to 109 (70%P/70%B) (24% increase) for sourness. Sweetness rating ranged from 87 (50%P/30%B and 70%P/30%B) to 107 (70%P/50%B) (23% difference) and vinegar rating ranged from 88 (the blind control) to 104 (70%P/70%B) (13% increase) (Figure VI.1).

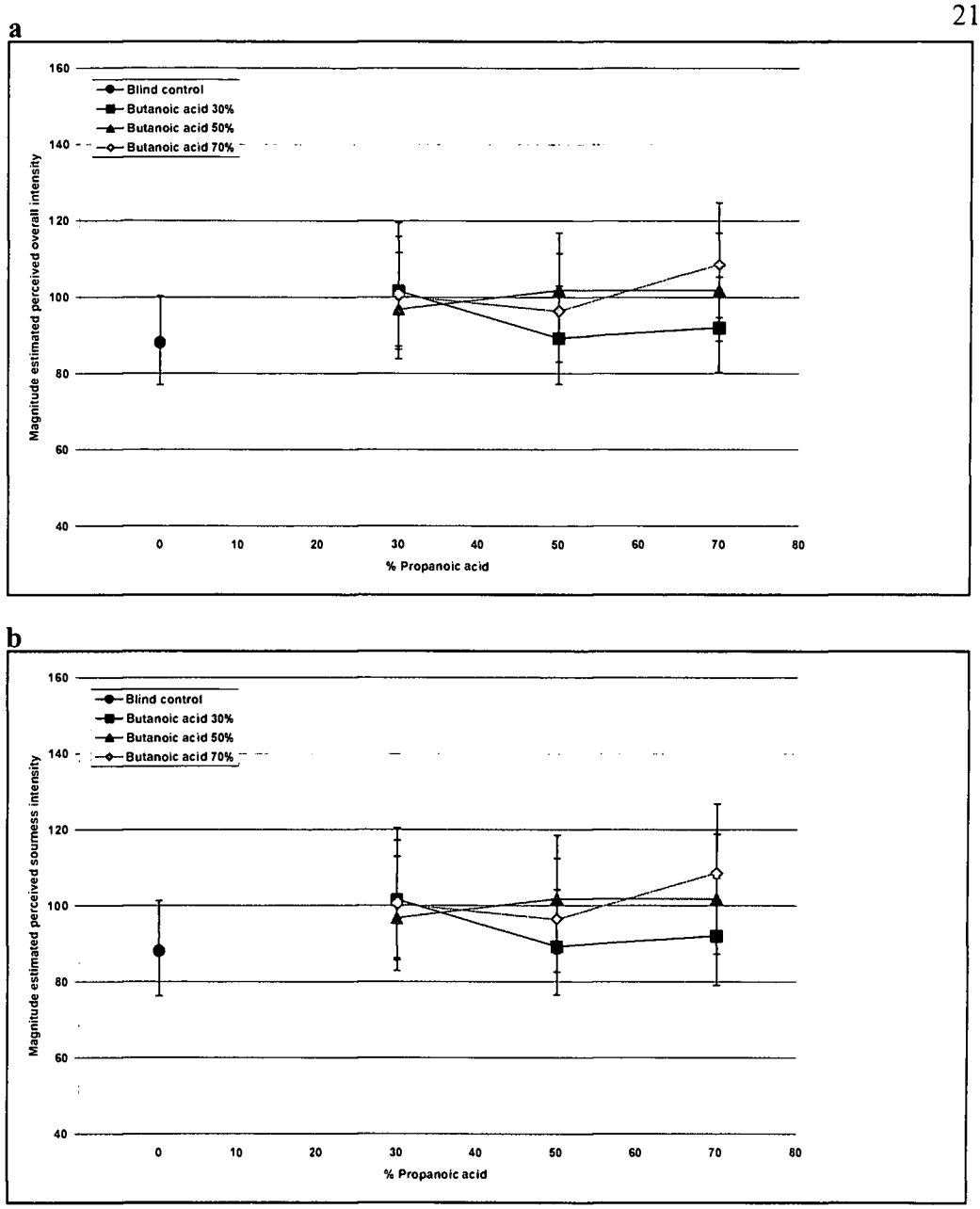

Figure VI.1 Estimated medians of the perceived overall intensity (a), sourness (b), sweetness (c), and vinegar (d) of acetic acid at panel-recognition concentration in the presence of sub-threshold mixtures of propanoic acid and n-butanoic acid. Propanoic acid and n-butanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

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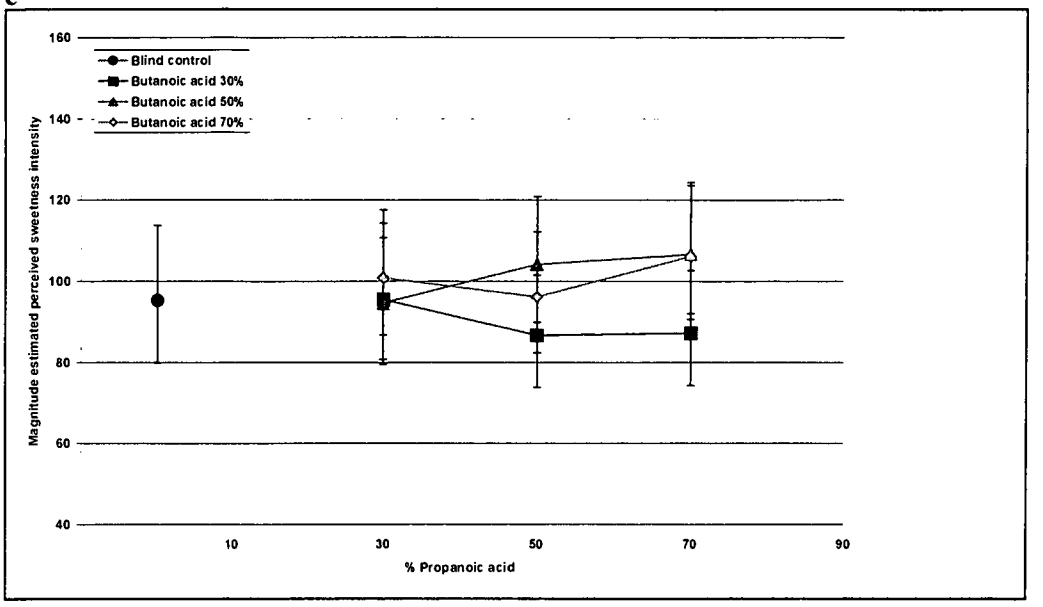

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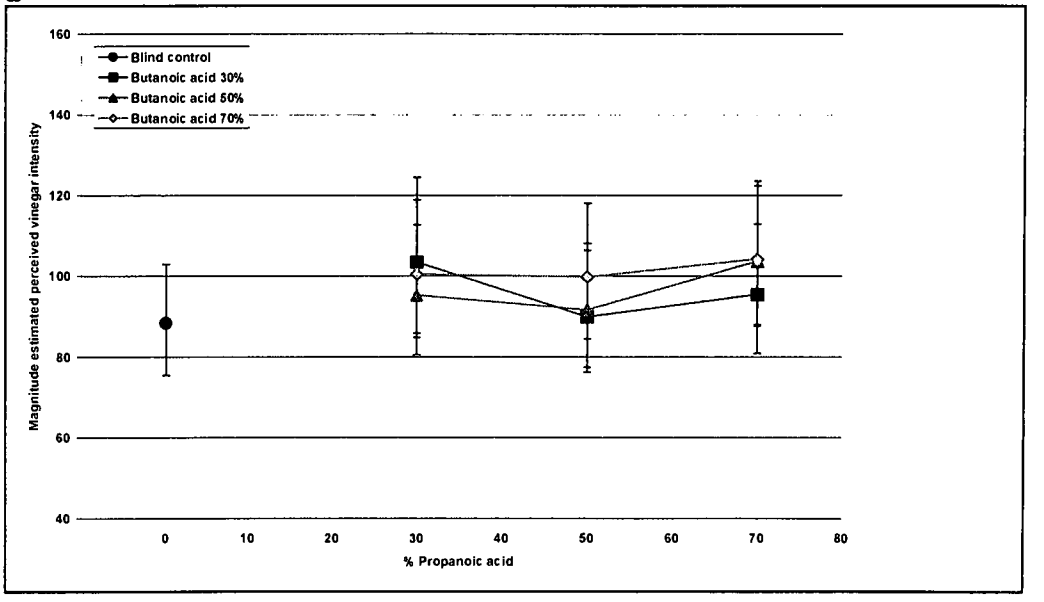

Figure VI.1 (continued) Estimated medians of the perceived overall intensity (a), sourness (b), sweetness (c), and vinegar (d) of acetic acid at panel-recognition concentration in the presence of sub-threshold mixtures of propanoic acid and n-butanoic acid. Propanoic acid and n-butanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

1.2 The effects of acetic acid (A) and n-butanoic acid (B) at sub-threshold concentration of the perceived intensity of propanoic acid

The intensities of propanoic acid descriptors was not altered in the presence of sub-threshold mixtures of propanoic acid and n-butanoic acid (Figure VI.2) as indicated by non-significant interaction terms for all descriptors: [$F(4, 603)=1.18$, $p = 0.32$ for overall intensity], [$F(4, 603)=1.81$, $p = 0.12$ for sourness], [$F(4, 603)=0.75$, $p = 0.56$] and [$F(4, 603)=1.67$, $p = 0.15$ for saltiness]. Acetic acid and n-butanoic acid alone did not significantly affect the intensities of acetic acid descriptors as shown by non-significant main effects in ANOVA. The F-values, $F(2, 603)$s, of acetic acid main effects for overall intensity, sourness, sweetness and saltiness were 2.55, 0.48, 0.49 and 0.60 with p-values 0.08, 0.62, 0.62 and 0.55, respectively. Meanwhile, the F-values, $F(2, 603)$ of n-butanoic acid main effects for overall intensity, sourness, sweetness and saltiness were 0.58, 0.57, 0.29 and 1.38 with p-values 0.56, 0.57, 0.75 and 0.25, respectively.

The median of magnitudes estimated ranged from 88 (50%A/50%B) to 108 (30%A/70%B) (23% difference) for overall intensity and 88 (50%A/70%B) to 110 (70%A/70%B) (29% difference) for sourness. Sweetness rating ranged from 93 (50%A/70%B) to 111 (the propanoic acid blind control) (19% increase) and saltiness rating ranged from 92 (50%A/70%B) to 110 (30%A/70%B) (20% difference) (Figure VI.2).

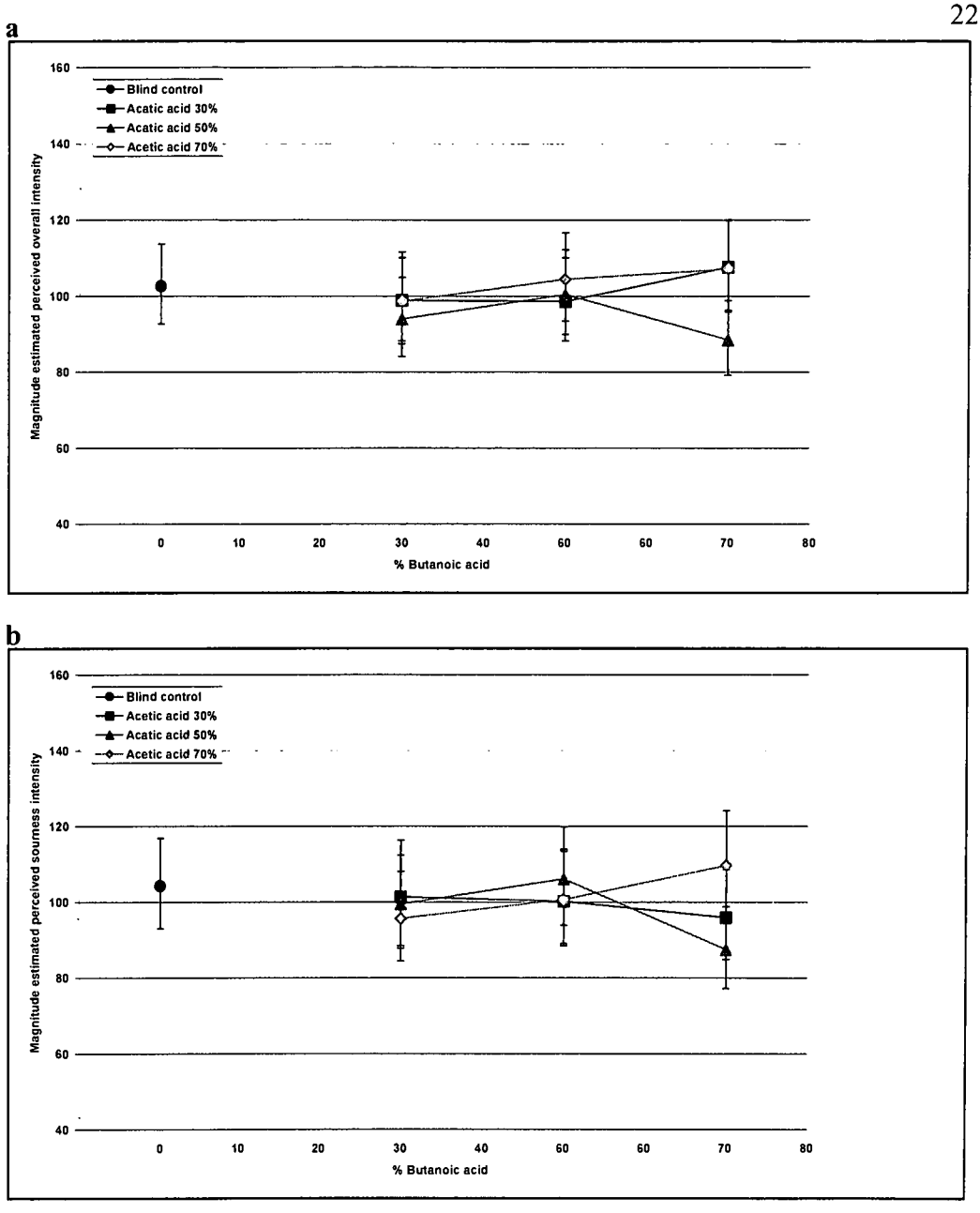

Figure VI.2 Estimated medians of the perceived overall intensity (a), sourness (b), sweetness (c), and saltiness (d) of propanoic aid at panel-recognition concentration in the presence of sub-threshold mixtures of acetic acid and n-butanoic acid. Acetic acid and n-butanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

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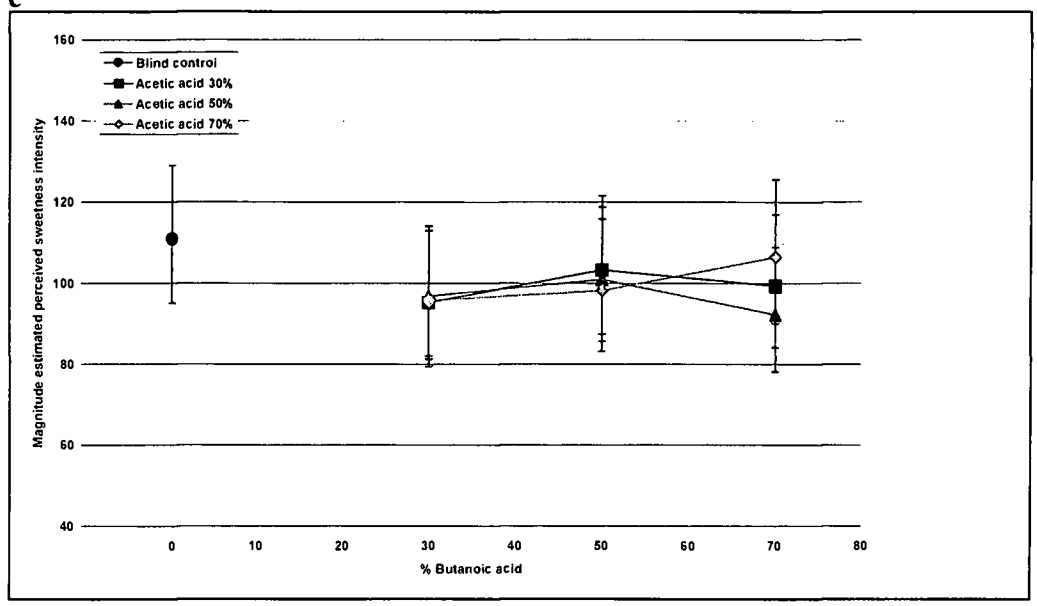

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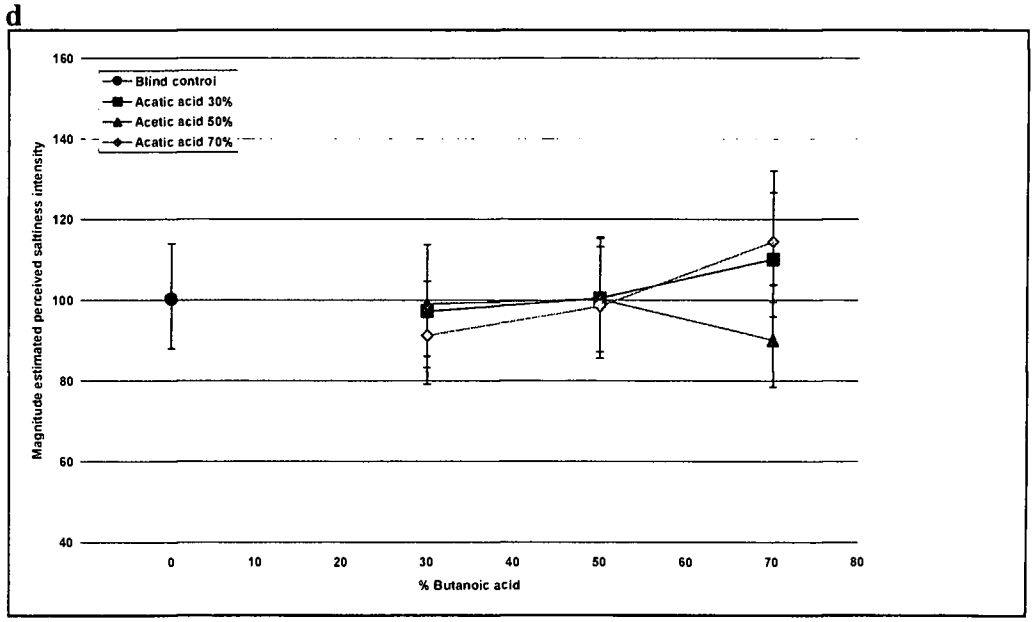

Figure VI.2 (continued) Estimated median of the perceived overall intensity (a), sourness (b), sweetness (c), and saltiness (d) of propanoic aid at panel-recognition concentration in the presence of sub-threshold mixtures of acetic acid and n-butanoic acid. Acetic acid and n-butanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

1.3 Effect of acetic acid (A) and propanoic acid (P) at sub-threshold concentration of the perceived intensity of n-butanoic acid

Acetic acid and propanoic acid at sub-threshold concentrations affected the intensities of n-butanoic acid descriptors differently depending on the descriptors (Figure VI.3). For overall intensity, the sub-threshold effect depended on both acetic acid and propanoic acid concentrations [$F(4, 602)=3.36$, $p = 0.01$] (Figure VI.3a). Subjects rated 72 for the overall intensity of n-butanoic acid in the sample 30%A/30%P, which was significantly less intense than the samples 30%A/50%P, 50%A/50%P, 50%A/30%P, 70%A/30%P and the n-butanoic blind control with ratings of 124, 118, 106, 95 and 96, respectively.

Sourness intensity of n-butanoic acid depended on the propanoic concentration at sub-threshold level only [$F(2,02)=7.22$, $p = 0.00$] (Figure VI.3b). The intensity of sourness increased, peaked, and decreased when the sub-threshold concentration of propanoic acid increased. The samples with 50% propanoic acid (rated 114) were significantly more sour than the samples with 30% propanoic acid (93) and the samples with 70% propanoic acid (94) regardless of acetic acid concentrations. Subject did not rate the blind control (94) differently from the other levels.

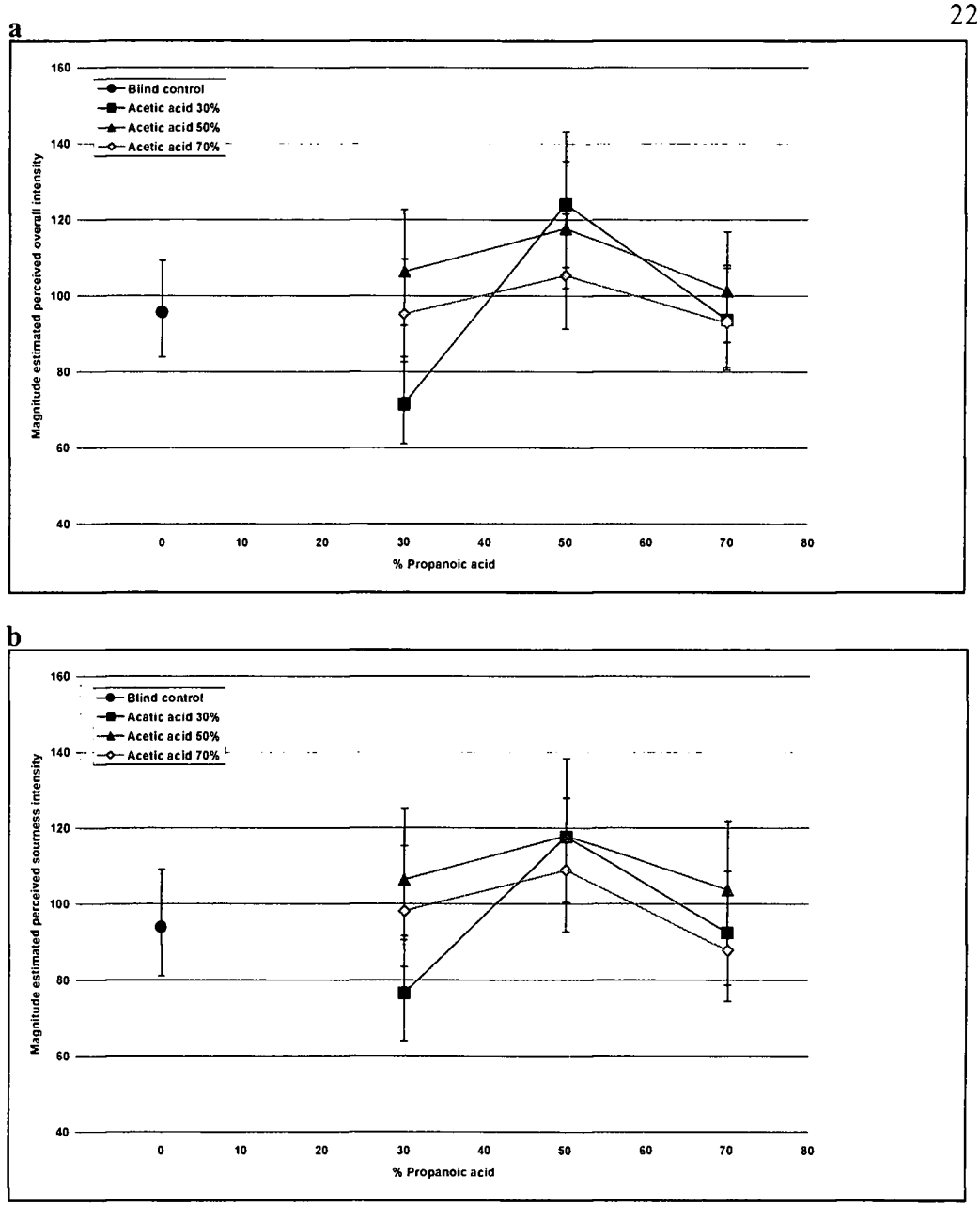

Figure 3 Estimated medians of the perceived overall intensity (a), sourness (b), sweetness (c), and spoiled-dairy aroma (d) of n-butanoic acid at panel recognition concentration in the presence of sub-threshold mixtures of acetic acid and propanoic acid. Acetic acid and propanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Asterisk indicates significant different from the blind control. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

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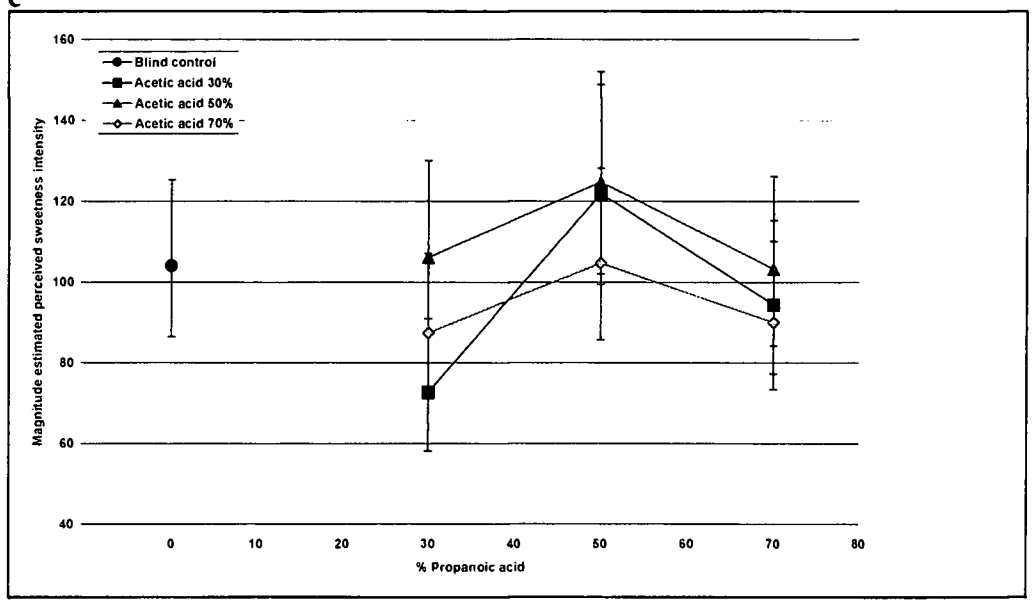

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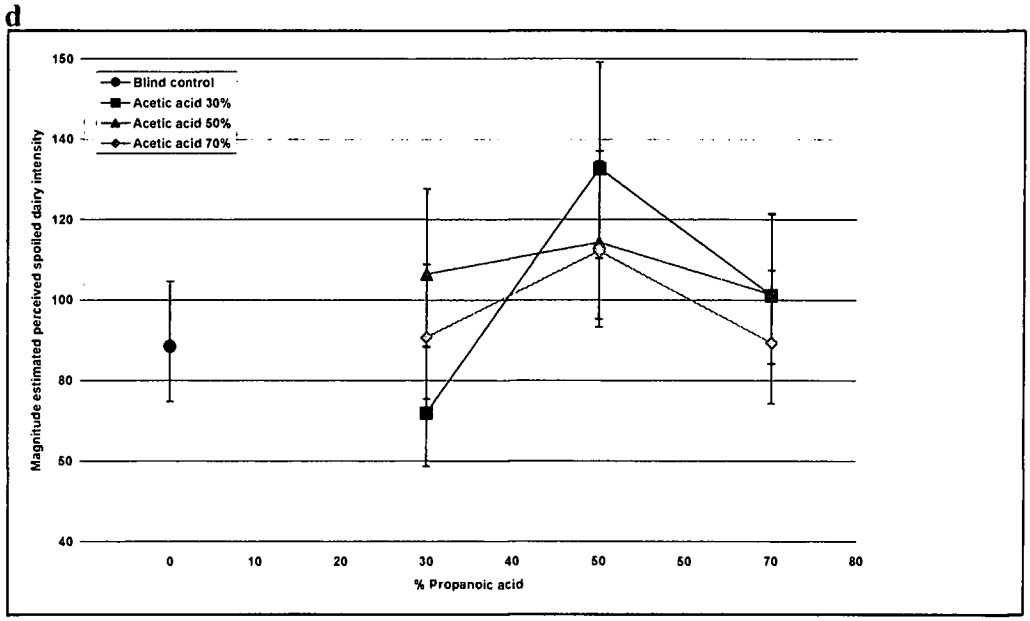

Figure VI.3 (continued) Estimated medians of the perceived overall intensity (a), sourness (b), sweetness (c) and spoiled-dairy aroma (d) of n-butanoic acid at panel-recognition concentration in the presence of sub-threshold mixtures of acetic acid and propanoic acid. Acetic acid and propanoic acid were at 30%, 50%, and 70% of subjects' individual detection thresholds. Asterisk indicates significant different from the blind control. Upper bars and lower bars indicate the upper and the lower 95% confidence interval of the median. Sixteen subjects with four replications provided the data for the median estimation.

Propanoic acid at sub-threshold concentrations caused the changes of the sweetness intensity of n-butanoic acid [$F(2, 602)=9.70$, $p = 0.00$] (Figure VI.3c). Sweetness increased, peaked, and decreased when propanoic acid increased in concentrations. The samples with 30% propanoic acid (89) and 70% propanoic acid (97) were less sweet than the samples with 50% propanoic acid (119) regardless of acetic acid concentrations. The n-butanoic blind control (104) was not significantly different from other samples. Acetic acid at sub-threshold concentration influenced the sweetness intensity of butanoic acid [$F(2,602)=4.30$, $p=0.01$]. The samples with 30% acetic acid (94) and 70% acetic acid (96) were less sweet than the samples with 50% acetic acid (111) and the differences (~15%) were significant. The blind control (101) was not significantly different from the other samples.

The ratings for the intensity of spoiled-dairy aroma changed with the combination of acetic acid and propanoic acid at sub-threshold concentrations [$F(4, 602)=2.55$, $p = 0.04$] (Figure VI.3d). The median of the spoiled-dairy intensity of the samples with 50% propanoic acid was most likely driven by a sample that comprised 30% acetic acid and 50% propanoic acid (Figure VI.3d). The 30%A/50%P sample (133) was 49% and 85% higher in spoiled-dairy aroma than the blind control (89) and the 30%A/30%P sample (72), respectively. ANOVA result indicated that propanoic acid was more influential than acetic acid at sub-threshold concentrations [$F(2,602)=9.01$, $p=0.00$] on alteration of spoiled-dairy aroma intensity. The spoiled-dairy aroma intensity increased, peaked, and

decreased when the concentrations of propanoic acid increased. The samples with 50% propanoic acid (117) were more intense in spoiled-dairy aroma than the samples with 30% propanoic acid (88) (33% less intense) and 70% propanoic acid (96) (22% less intense). The n-butanoic acid blind control (89) was significantly less intense in spoiled-dairy aroma than the samples with 50% propanoic acid (32% less intense); otherwise, the blind control was not different from the other samples.

VI.5 Discussion

1. The effects of carbon chain-length on the sub-threshold effect in tertiary mixtures

With different functional groups in binary and tertiary mixtures comprising sub-threshold odorant(s) and a recognizable odorant, the sub-threshold effects on the intensity of the recognizable odorant depended on functional groups and suppression was common (Lopetcharat & McDaniel, 2002a, 2002b). In binary mixtures that comprised acetic acid at a sub-threshold level and propanoic acid at panel-recognition concentration, sub-threshold enhancement was dependent on carbon chain-length and concentration (Lopetcharat & McDaniel, 2002c). Relative polarity, which is governed by both functional groups and carbon chain-length, between components in mixtures was proposed as a unified molecular property that governs intensity mixture interaction in supra-threshold mixtures (Cometto-Muniz et al., 1997; Laing, 1988, 1989). This hypothesisl could be extended to sub-threshold/recognizable concentration mixtures as well (Lopetcharat & McDaniel, 2002a, 2002b, 2002c).

As anticipated, the sub-threshold mixtures of propanoic acid and n-butanoic acid did not significantly alter the intensities of the descriptors of acetic acid at recognition concentration in tertiary mixtures (Figure VI.1). In binary mixtures, propanoic acid or n-butanoic acid at sub-threshold concentrations did not significantly affect the intensities of acetic acid descriptors; however, acetic acid at 50% and 70% of individual detection thresholds significantly enhanced the intensities of propanoic acid descriptors (Lopetcharat & McDaniel, 2002c). The directional sub-threshold effect of acetic acid on other acids (Lopetcharat & McDaniel, 2002a, 2002b, 2002c) could be explained by the findings of overlapping but distinct chemotopic maps in the glomerular layer (Johnson & Leon, 1996; Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999). Acetic acid, propanoic acid and n-butanoic acid, in general, stimulated the same areas in the glomerular layer called modules or fields (field 1, 2, 3 and 4) in rat; however, acetic acid acted the most differently (Johnson et al., 1999). Acetic acid stimulate two receptive fields (field 3 and 4) much less than propanoic acid and n-butanoic acid (Johnson et al., 1999). Therefore, propanoic and n-butanoic acids at sub-threshold concentrations should not affect the fields stimulated by acetic acid (field 1 and 2), which, in turn, are not affected by sub-threshold concentrations of propanoic acid and/or n-butanoic acid.

Surprisingly, propanoic acid, which was significantly enhanced by acetic acid at 50% and 70% of individual detection threshold and non-significantly enhanced in the presence of n-butanoic acid (Lopetcharat & McDaniel, 2002c), was

not affected by the sub-threshold mixtures of acetic acid and n-butanoic acid (Figure VI.2). The effect tended to be suppression, even though it was not significant. The absence of the sub-threshold effect of acetic acid on propanoic acid in the presence of n-butanoic acid at sub-threshold levels demonstrates sub-threshold suppression. Sub-threshold suppression was common in binary mixtures of odorants that were different in functional groups (Lopetcharat & McDaniel, 2002a, 2002b). The sub-threshold suppression of n-butanoic acid at sub-threshold levels on the enhancing ability of acetic acid at sub-threshold levels was believed to be a result of peripheral mechanisms. Peripheral mechanisms were more likely explanation than central mechanisms because of no lateral inhibition within a field, and the inability to activate lateral inhibition network in the glomerular layer when exposed to low concentrations (both acetic acid and n-butanoic acid were at sub-threshold levels).

Peripheral mechanisms could be involved because of the competition between acetic acid and n-butanoic acid to reach the propanoic receptor sites. Results from binary mixture experiments indicated that both acetic acid and n-butanoic acid at sub-threshold levels affected propanoic acid, however, acetic acid were more effective than n-butanoic acid (Lopetcharat & McDaniel, 2002c). Besides competition, other intracellular mechanisms such as inhibition signaling (Ache, 1994; Daniel et al., 1996; Gentilcore & Derby, 1998; Olson & Derby, 1995), binding inhibition (Ache et al., 1987; Laing & Livermore, 1992; Simon & Derby, 1995), suppressive effect on ion channels or second messenger metabolisms

(Kurahashi et al., 1994; Miller, 1971), and "cross-talk" between two excitatory transduction pathways (non-competitive) (Anholt & Rivers, 1990) are possible. The contribution of central mechanisms and peripheral mechanisms to mixture interaction is still unknown and needs further investigation.

The influence of carbon chain-lengths on the sub-threshold effect was obvious in tertiary mixtures. The effects were suppression and enhancement and depended on sub-threshold odorants and their concentrations. The significant effect of sub-threshold mixtures on different descriptors suggested the contribution of central mechanisms. Different odor quality and intensity were suggested to be represented in olfactory bulb as different chemotopic maps, and carbon chain-length was an aspect governing the complexity of the maps (Johnson & Leon, 2000a; Johnson & Leon, 2000b; Johnson et al., 1999); therefore adding different acids with different carbon chain-lengths affected descriptors of recognizable odorants differently. In addition, the significant effects observed on different descriptors were caused by different variations of the ratings for the descriptors. This phenomenon suggests that sub-threshold acids affect partial areas of mixture chemotopic map and that area are associated with specific descriptors.

2. The effect of concentration on sub-threshold effect in tertiary mixtures

In binary mixtures of aliphatic acids, the sub-threshold effect depended on the concentrations of sub-threshold odorants; moreover, the intensities of n-butanoic acid descriptors were enhanced the most by propanoic acid at 50% of

individual detection thresholds but the enhancement was not significant (Lopetcharat & McDaniel, 2002c). In this study, the concentration-dependent sub-threshold effect was observed in the tertiary mixtures comprising n-butanoic acid at panel-recognition concentration, propanoic acid at 50% individual detection threshold, and acetic aid at sub-threshold levels especially at 30% level (Figure VI.3).

Increasing concentration of odorants results in recruitment of nearby olfactory neurons (Hildebrand & Shepherd, 1997). Consequently, chemotopic maps in the glomerular layer changed with concentration and resulted in changes in intensity and/or quality of odorants (Johnson & Leon, 2000a). Adding sub-threshold odorants with close molecular features (same functional group with different carbon chain-lengths) as we performed in this study could mimic minimum increase in concentration of recognizable odorants. Thus the activity of the glomeruli, composing the chemotopic map in the olfactory bulb, increased without triggering inhibition processes that caused the alteration of the map (Johnson & Leon, 2000a).

The role of inhibition was observed as the decrease of the intensities of n-butanoic descriptors when the concentrations of both propanoic acid and acetic acid were increased to 70% (Figure VI.3). Even though, the 70% level was a sub-threshold level for an individual odorant; but, collectively, it could be at suprathreshold level in sub-threshold mixtures as commonly reported (Cometto-Muniz et al., 1997; Guadagni et al., 1963b; Laska & Hudson, 1991; Patterson et al.,

1993). Therefore, odorants at 70% of individual thresholds could additively activate lateral inhibition processes in the olfactory bulb and cause a common intensity mixture interaction, suppression, observed in supra-threshold mixtures (Cain, 1975; Carr et al., 1989; Derby et al., 1985; Miller, 1971).

The explanation for the sub-threshold effect observed is based on the presumption that the olfactory neurons for recognizable acids (in this case n-butanoic acid) were stimulated by both acetic and propanoic acids, and there were no significant inhibition processes such as intracellular inhibition, competition between acids, etc. If inhibition and/or suppression and/or enhancement occurred simultaneously, the explanation for the sub-threshold enhancement observed in aliphatic acid mixtures was that the degree of enhancement overcame the total degree of inhibition and/or suppression. Moreover, the decrease in the enhancement when sub-threshold concentrations increased was caused by an increasing degree of inhibition and/or suppression.

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Chapter 7

VII. THESIS SUMMARY

This research successfully developed a novel methodology to demonstrate the effect of sub-threshold odorants on the perceived intensity of odorants at panel-recognition concentrations. The method allowed researchers to maintain sub-threshold level by expressing sub-threshold concentrations as percentages of subjects' individual detection thresholds. Ratings of the intensities of the descriptors of recognizable odorants were collected by magnitude estimation.

Sub-threshold suppression was common in binary and tertiary mixtures of a panel-recognition concentration odorant and sub-threshold odorant(s) with different functional groups. Differences in the polarities of odorants in mixtures were suggested to govern the directions of sub-threshold suppression, as high polarity odorants suppressed low polarity odorants in the mixtures but not *vice versa*. The sub-threshold suppression observed in the binary and the tertiary mixtures comprising odorants with different functional groups did not depend on the concentrations of sub-threshold odorants in the mixtures. Sub-threshold enhancement was observed in tertiary mixtures comprising acetaldehyde at panel-recognition concentration, acetic acid at sub-threshold levels and ethanol at sub-threshold levels. The enhancement depended on both acetic acid and ethanol concentrations. Sub-threshold odorants affected the intensities of the descriptors of

recognizable odorants differently, and this phenomenon suggested the contribution of central mechanisms.

A difference in carbon chain-lengths as large as two carbon atoms eliminated the sub-threshold effects in binary and tertiary mixtures. In addition, the intensities of descriptors increased (from 10% to 30%), peaked (at 50% or 70%) and decreased (from 70% to 100%) when sub-threshold concentrations increased. By comparison to blind controls, sub-threshold suppression and sub-threshold enhancement occurred at low concentrations (~10% to 30%), and medium to high concentration (~50%-70%), respectively. The increase-peak-decrease pattern suggested the simultaneous existence of enhancement and suppression caused by sub-threshold odorants. The outcome depended on the ratio of the degrees of enhancement and suppression. Peripheral sub-threshold suppression was suggested at low concentrations (10% to 30%) and lateral inhibition was suggested at near detection threshold concentrations (70% and up).

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APPENDICES

Appendix 1: The example of model selection for general linear models of binary-mixture systems of different functional group study: Recognizable odorant (Acetaldehyde), sub-threshold odorants (Acetic acid (AA))

First model

Source		SS*	Df	MS**	F-value	p-value
Concentration (Conc)	Hypothesis	0.52	7	0.07	1.9	0.09
	Error	2.22	56.4	0.04		
Replications (Rep)	Hypothesis	0.11	3	0.04	1.4	0.26
	Error	0.78	28.8	0.03		
Subjects	Hypothesis	3.20	15	0.21	4.4	0.00
	Error	4.09	83.3	0.05		
Conc x Rep	Hypothesis	0.33	20	0.02	1.1	0.36
	Error	3.58	238	0.02		
Conc x S	Hypothesis	3.94	100	0.04	2.6	0.00
	Error	3.58	238	0.02		
Rep x S	Hypothesis	1.21	45	0.03	1.8	0.00
	Error	3.58	238	0.02		

"*" = Type III sum of squares
"**" = Mean Square

Second model

Source		SS*	Df	MS**	F-value	p-value
Concentration (Conc)	Hypothesis	0.61	7	0.09	2.3	0.04
	Error	3.95	102	0.04		
Replications (Rep)	Hypothesis	0.13	3	0.04	1.5	0.22
	Error	1.31	47.2	0.03		
Subjects	Hypothesis	3.28	15	0.22	4.3	0.00
	Error	4.18	83.0	0.05		
Conc x S	Hypothesis	3.93	100	0.04	2.6	0.00
	Error	3.90	258	0.02		
Rep x S	Hypothesis	1.27	45	0.03	1.9	0.00
	Error	3.90	258	0.02		

"*" = Type III sum of squares
"**" = Mean Square

Final model

Source		SS*	Df	MS**	F-value	p-value
Concentration (Conc)	Hypothesis	0.61	7	0.09	2.3	0.04
	Error	3.95	102	0.04		
Subjects	Hypothesis	3.28	15	0.22	4.2	0.00
	Error	4.50	86.2	0.05		
Conc x S	Hypothesis	3.93	100	0.04	2.6	0.00
	Error	3.90	258	0.02		
Rep x S	Hypothesis	1.46	48	0.03	2.0	0.00
	Error	3.90	258	0.02		

"*" = Type III sum of squares
"**" = Mean Square

Appendix 2: The example of model selection for general linear models of tertiary-mixture systems of different functional group study: Recognizable odorant (Ethanol), sub-threshold odorants (Acetic acid (AA) and Acetaldehyde (AC))

First model

Source		SS*	Df	MS**	F-value	p-value
AC	Hypothesis	0.05	2	0.02	0.6	0.60
	Error	0.40	9.4	0.04		
AA	Hypothesis	0.05	2	0.02	0.2	0.83
	Error	1.62	12.2	0.11		
Replications (Rep)	Hypothesis	0.03	3	0.01	0.1	0.97
	Error	3.60	28.8	0.12		
Subjects	Hypothesis	3.30	16	0.21	1.2	0.29
	Error	9.09	53.4	0.17		
AC x AA	Hypothesis	0.53	4	0.13	2.1	0.09
	Error	4.05	64.0	0.06		
AC x Rep	Hypothesis	0.15	6	0.02	0.7	0.68
	Error	16.24	442	0.04		
AC x S	Hypothesis	1.78	32	0.06	0.9	0.65
	Error	4.05	64.0	0.06		
AA x Rep	Hypothesis	0.32	6	0.05	1.5	0.19
	Error	16.24	442	0.04		
AA x S	Hypothesis	2.84	32	0.09	1.4	0.12
	Error	4.05	64.0	0.06		
Rep x S	Hypothesis	6.82	48	0.14	3.9	0.00
	Error	16.24	442	0.04		
AC x AA x S	Hypothesis	4.05	64	0.06	1.7	0.00
	Error	16.24	442	0.04		

"*" = Type III sum of squares

"**" = Mean Square

Second model

Source		SS*	Df	MS**	F-value	p-value
AC	Hypothesis	0.05	2	0.02	0.6	0.66
	Error	1.77	32.0	0.05		
AA	Hypothesis	0.04	2	0.02	0.2	0.84
	Error	1.88	15.8	0.12		
Replications (Rep)	Hypothesis	0.03	3	0.01	0.1	0.97
	Error	5.04	35.8	0.14		
Subjects	Hypothesis	3.30	16	0.21	1.2	0.29
	Error	9.07	53.4	0.17		
AC x AA	Hypothesis	0.53	4	0.13	2.1	0.09
	Error	4.03	64.0	0.06		
AC x S	Hypothesis	1.77	32	0.06	0.9	0.65
	Error	4.03	64.0	0.06		
AA x Rep	Hypothesis	0.55	6	0.06	1.6	0.10
	Error	16.39	448	0.04		
AA x S	Hypothesis	2.83	32	0.09	1.4	0.12
	Error	4.03	64.0	0.06		
Rep x S	Hypothesis	6.80	48	0.14	3.9	0.00
	Error	16.39	448	0.04		
AC x AA x S	Hypothesis	4.03	64	0.06	1.7	0.00
	Error	16.39	448	0.04		

"*" = Type III sum of squares
"**" = Mean Square

Third model

Source		SS*	Df	MS**	F-value	p-value
AC	Hypothesis	0.05	2	0.02	0.4	0.68
	Error	5.73	96.1	0.06		
AA	Hypothesis	0.04	2	0.02	0.2	0.82
	Error	1.39	13.4	0.10		
Replications (Rep)	Hypothesis	0.03	3	0.01	0.1	0.97
	Error	5.04	35.8	0.14		
Subjects	Hypothesis	3.49	16	0.22	1.3	0.23
	Error	10.26	60.8	0.17		
AC x AA	Hypothesis	0.53	4	0.13	2.2	0.07
	Error	5.73	96.1	0.06		
AA x Rep	Hypothesis	0.55	9	0.06	1.7	0.10
	Error	16.39	448	0.04		
AA x S	Hypothesis	3.41	48	0.07	1.2	0.23
	Error	5.72	95.9	0.06		
Rep x S	Hypothesis	6.80	48	0.14	3.9	0.00
	Error	16.39	448	0.04		
AC x AA x S	Hypothesis	5.73	96	0.06	1.6	0.00
	Error	16.39	448	0.04		

"*" = Type III sum of squares
"**" = Mean Square

Forth model

Source		SS*	Df	MS**	F-value	p-value
AC	Hypothesis	0.05	2	0.02	0.4	0.69
	Error	9.05	144	0.06		
AA	Hypothesis	0.04	2	0.02	0.2	0.81
	Error	1.19	12.9	0.10		
Replications (Rep)	Hypothesis	0.03	3	0.01	0.1	0.97
	Error	5.04	35.8	0.14		
Subjects	Hypothesis	3.49	16	0.24	1.4	0.15
	Error	10.54	62.8	0.17		
AC x AA	Hypothesis	0.53	4	0.13	2.1	0.08
	Error	9.05	144	0.06		
AA x Rep	Hypothesis	0.55	9	0.06	1.7	0.10
	Error	16.39	448	0.04		
Rep x S	Hypothesis	6.80	48	0.14	3.9	0.00
	Error	16.39	448	0.04		
AC x AA x S	Hypothesis	9.04	144	0.06	1.7	0.00
	Error	16.39	448	0.04		

"*" = Type III sum of squares

"**" = Mean Square

Final model

Source		SS*	Df	MS**	F-value	p-value
AC	Hypothesis	0.04	2	0.02	0.4	0.69
	Error	9.06	144	0.06		
AA	Hypothesis	0.04	2	0.02	0.3	0.73
	Error	9.06	144	0.06		
Replications (Rep)	Hypothesis	0.06	3	0.02	0.2	0.92
	Error	6.81	48.0	0.14		
Subjects	Hypothesis	3.49	16	0.24	1.4	0.15
	Error	10.48	62.5	0.17		
AC x AA	Hypothesis	0.53	4	0.13	2.1	0.08
	Error	9.06	144	0.06		
Rep x S	Hypothesis	6.80	48	0.14	3.8	0.00
	Error	16.93	457	0.04		
AC x AA x S	Hypothesis	9.05	144	0.06	1.7	0.00
	Error	16.93	457	0.04		

"*" = Type III sum of squares
"**" = Mean Square

Appendix 3: The example of model selection for general linear models of binary-mixture systems of different carbon chain-length study: Recognizable odorant (Propanoic acid), sub-threshold odorants (Butanoic acid)

First model

Source		SS*	Df	MS**	F-value	p-value
Concentration (C)	Hypothesis	0.50	7	0.04	2.0	0.11
	Error	0.63	17.9	0.03		
Subjects (S)	Hypothesis	0.24	12	0.02	0.5	0.92
	Error	1.21	27.5	0.04		
Replications (Rep)	Hypothesis	0.19	3	0.06	1.8	0.20
	Error	0.49	13.4	0.04		
C x S	Hypothesis	3.28	84	0.04	1.1	0.26
	Error	8.34	238	0.04		
C x Rep	Hypothesis	0.66	21	0.03	0.9	0.60
	Error	8.35	238	0.04		
S x Rep	Hypothesis	1.37	34	0.04	1.1	0.23
	Error	23.8	238	0.04		

"*" = Type III sum of squares
"**" = Mean Square

Second model

Source		SS*	Df	MS**	F-value	p-value
Concentration (C)	Hypothesis	0.51	7	0.07	1.8	0.10
	Error	3.44	85.6	0.04		
Subjects (S)	Hypothesis	0.25	12	0.02	0.4	0.93
	Error	1.33	29.2	0.04		
Replications (Rep)	Hypothesis	0.19	3	0.06	1.6	0.21
	Error	1.36	34.0	0.04		
C x S	Hypothesis	3.38	84	0.04	1.2	0.20
	Error	9.01	259	0.03		
S x Rep	Hypothesis	1.36	34	0.04	1.2	0.27
	Error	9.01	259	0.03		

"*" = Type III sum of squares
"**" = Mean Square

Third model

Source		SS*	Df	MS**	F-value	p-value
Concentration (C)	Hypothesis	0.51	7	0.07	1.8	0.10
	Error	3.47	85.6	0.04		
Subjects (S)	Hypothesis	0.25	12	0.02	0.5	0.90
	Error	3.43	84.5	0.04		
Replications (Rep)	Hypothesis	0.19	3	0.06	1.8	0.15
	Error	10.37	293	0.04		
C x S***	Hypothesis	3.41	84	0.04	1.1	0.21
	Error	10.37	293	0.04		

"*" = Type III sum of squares
"**" = Mean Square
"***" = This factor remained in the analysis because it was used to test the significant of Concentration factor

Final model

Source		SS*	Df	MS**	F-value	p-value
Concentration	Hypothesis	0.51	7	0.07	1.8	0.10
(C)	Error	3.49	85.6	0.04		
Subjects (S)	Hypothesis	0.23	12	0.02	0.5	0.93
	Error	3.43	84.1	0.04		
C x S	Hypothesis	3.43	84	0.04	1.1	0.21
	Error	10.56	296	0.04		

"*" = Type III sum of squares
"**" = Mean Square

Appendix 4: The example of model selection for general linear models of tertiary-mixture systems of different carbon chain-length study: Recognizable odorant (Butanoic acid), sub-threshold odorants (Acetic acid (A) and Propanoic acid (P))

First model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.37	2	0.19	3.3	0.09
	Error	0.45	8.2	0.05		
P	Hypothesis	1.22	2	0.61	5.2	0.03
	Error	1.03	8.7	0.12		
Subjects (S)	Hypothesis	0.17	16	0.01	0.1	1.00
	Error	11.36	52.4	0.22		
Replications (Rep)	Hypothesis	0.39	3	0.13	0.7	0.56
	Error	4.36	23.9	0.18		
A x P	Hypothesis	0.72	4	0.18	3.3	0.01
	Error	3.52	64.6	0.05		
A x S	Hypothesis	2.40	32	0.07	1.4	0.14
	Error	3.51	64.6	0.05		
A x Rep	Hypothesis	0.20	6	0.03	0.6	0.70
	Error	23.8	443	0.05		
P x S	Hypothesis	2.57	32	0.08	1.5	0.09
	Error	3.51	64.6	0.05		
P x Rep	Hypothesis	0.55	6	0.09	1.7	0.11
	Error	23.8	443	0.05		
S x Rep	Hypothesis	9.09	48	0.19	3.5	0.00
	Error	23.81	443	0.05		
A x P x S	Hypothesis	3.48	64	0.05	1.0	0.46
	Error	23.81	443	0.05		

"*" = Type III sum of squares

"**" = Mean Square

Second model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.40	2	0.20	3.7	0.07
	Error	0.42	7.9	0.05		
P	Hypothesis	1.28	2	0.64	5.5	0.03
	Error	1.00	8.5	0.12		
Subjects (S)	Hypothesis	0.27	16	0.02	0.1	1.00
	Error	11.20	55.0	0.20		
Replications (Rep)	Hypothesis	0.41	3	0.14	0.7	0.54
	Error	4.45	24.2	0.18		
A x P	Hypothesis	0.80	4	0.20	3.7	0.005
	Error	27.30	507	0.05		
A x S	Hypothesis	2.40	32	0.07	1.4	0.09
	Error	27.30	507	0.05		
A x Rep	Hypothesis	0.20	6	0.03	0.6	0.71
	Error	27.30	507	0.05		
P x S	Hypothesis	2.57	32	0.08	1.5	0.05
	Error	27.30	507	0.05		
P x Rep	Hypothesis	0.55	6	0.09	1.7	0.12
	Error	27.30	507	0.05		
S x Rep	Hypothesis	9.09	48	0.19	3.6	0.00
	Error	27.30	507	0.05		

"*" = Type III sum of squares

"**" = Mean Square

Third model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.40	2	0.20	2.7	0.08
	Error	2.37	32.2	0.07		
P	Hypothesis	1.27	2	0.64	5.0	0.03
	Error	1.27	10	0.13		
Subjects (S)	Hypothesis	0.27	16	0.02	0.1	1.00
	Error	11.30	55.3	0.20		
Replications (Rep)	Hypothesis	0.41	3	0.14	0.7	0.58
	Error	6.68	32.7	0.20		
A x P	Hypothesis	0.79	4	0.20	3.7	0.006
	Error	27.50	513	0.05		
A x S	Hypothesis	2.36	32	0.07	1.4	0.09
	Error	27.50	513	0.05		
P x S	Hypothesis	2.54	32	0.08	1.5	0.05
	Error	27.50	513	0.05		
P x Rep	Hypothesis	0.83	9	0.09	1.7	0.08
	Error	27.50	513	0.05		
S x Rep	Hypothesis	9.20	48	0.19	3.6	0.00
	Error	27.50	513	0.05		

"*" = Type III sum of squares
"**" = Mean Square

Forth model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.38	2	0.20	2.6	0.09
	Error	2.38	32.2	0.07		
P	Hypothesis	1.24	2	0.62	7.8	0.00
	Error	2.56	32.2	0.07		
Subjects (S)	Hypothesis	0.25	16	0.02	0.1	1.00
	Error	11.30	54.9	0.20		
Replications (Rep)	Hypothesis	0.27	3	0.08	0.5	0.71
	Error	9.28	48.0	0.19		
A x P	Hypothesis	0.74	4	0.19	3.4	0.01
	Error	28.3	522	0.05		
A x S	Hypothesis	2.36	32	0.07	1.4	0.09
	Error	28.3	522	0.05		
P x S	Hypothesis	2.54	32	0.07	1.5	0.05
	Error	28.3	522	0.05		
S x Rep	Hypothesis	9.28	48	0.19	3.6	0.00
	Error	28.3	522	0.05		

"*" = Type III sum of squares

"**" = Mean Square

Fifth model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.38	2	0.19	3.4	0.03
	Error	30.7	554	0.05		
P	Hypothesis	1.24	2	0.62	7.4	0.00
	Error	3.12	37.0	0.08		
Subjects (S)	Hypothesis	0.24	16	0.01	0.1	1.00
	Error	10.87	56.2	0.19		
Replications (Rep)	Hypothesis	0.30	3	0.09	0.5	0.68
	Error	9.25	48.0	0.19		
A x P	Hypothesis	0.74	4	0.18	3.3	0.01
	Error	30.7	554	0.05		
P x S***	Hypothesis	3.81	32	0.07	1.4	0.03
	Error	30.7	554	0.05		
S x Rep	Hypothesis	9.25	48	0.19	3.5	0.00
	Error	30.7	554	0.05		

"*" = Type III sum of squares
"**" = Mean Square
"***" = This term was deleted in the next model because it did not aid interpretation of the results and its counterpart term (A x S) was deleted in the forth model

Final model

Source		SS*	Df	MS**	F-value	p-value
A	Hypothesis	0.39	2	0.20	3.4	0.03
	Error	34.5	602	0.05		
P	Hypothesis	1.26	2	0.63	11.0	0.00
	Error	34.5	602	0.05		
Subjects (S)	Hypothesis	0.003	16	0.0002	0.0	1.00
	Error	9.44	48.0	0.20		
Replications (Rep)	Hypothesis	0.30	3	0.09	0.5	0.68
	Error	9.44	48.0	0.20		
A x P	Hypothesis	0.77	4	0.19	3.4	0.01
	Error	34.5	602	0.05		
S x Rep	Hypothesis	9.45	48	0.19	3.4	0.00
	Error	34.5	602	0.05		

"*" = Type III sum of squares
"**" = Mean Square