# THE OLFACTORY BUTTERFLY EFFECT: HOW PERI-THRESHOLD AND SUB-THRESHOLD ODORANTS MODIFIES ODOR PERCEPTION

A Dissertation

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by

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"Amidst the canvas of perception, a whisper unveils new perspectives."

# THE OLFACTORY BUTTERFLY EFFECT: HOW PERI-THRESHOLD AND SUB-THRESHOLD ODORANTS MODIFIES ODOR PERCEPTION

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The butterfly effect states that a small change in the original condition could trigger a tremendous effect in the end. In the human olfactory system, any change involving chemical, biological, physiological, and psychological aspects could lead to drastically different odor perception. When we inhale a mixture of odorants, these odorants can act as agonists, antagonists, or allosteric enhancers to the olfactory receptors, which means there could be enhancement, suppression, or no effect when mixing a new component with existing odorant(s). Furthermore, odor mixture perception, according to Stuart Firestein, is like "a chord with a silent note," meaning the components below the threshold are crucial in determining the overall mixture identity and quality.

In the first study, we demonstrated the temporal effects of odor recognition by showing that subjects' detection probability across different concentrations remained consistent even when the measurements were conducted three days apart. Furthermore, we showed that by using three concentrations, we could efficiently obtain threshold measurements within a 10-minute timeframe.

In the second study, we provided evidence to support the notion that the configural and elemental recognition of an odor mixture is highly influenced by

experimental conditioning. We found that when subjects underwent proper conditioning, they were able to generate well-fitted psychometric functions that accurately predicted the threshold for a configurally recognized odorant mixture. This highlights the importance of conditioning protocols in studying odor mixture perception and the possibility of adopting a top-down method to study odor mixture perception.

In the third study, we presented evidence demonstrating the masking effects of sub-threshold amounts of perfumery raw materials (PRMs) on the detection probability of a malodor, Isovaleric Acid. Our results revealed that sub-threshold concentrations of PRMs could reduce the detection probability of Isovaleric Acid by up to 72%. Moreover, when a mixture of six sub-threshold PRMs was employed, we observed the detection probability of Isovaleric Acid being masked by 98%. These findings offer promising applications in odor control and management.

In the final study, we observed diverse responses in the perception of other odorants when sub-threshold levels of IVA were present. These effects included both enhancement and suppression, but they were not consistent across different concentrations of sub-threshold IVA. These findings highlight the complex dynamics of odor interactions and emphasize the importance of considering sub-threshold odorants in odor masking research.

# BIOGRAPHICAL SKETCH

Jianbo (Dave) Huang was born and raised in Jinan, Shandong, China. He completed his bachelor's and master's degrees at the University of California, San Diego, where he conducted research on organic synthesis under the guidance of Dr. Dionicio Siegel. During this time, he co-authored five scientific papers. Dave then pursued a career as a medicinal chemist in the industry, where his interest in studying the aromas of organic compounds developed. This passion for chemistry and flavor led him to pursue a graduate degree at Cornell University, where he joined the research team of Dr. Terry Acree and focused on studying human sensory perception. Over the past three years, Dave has been actively involved in research on malodor masking with a research team from Procter & Gamble. His work has primarily focused on studying odor interactions at sub-threshold and peri-threshold levels. Alongside his research endeavors, Dave has also been actively engaged in entrepreneurship and co-founded Taoty Inc., a company dedicated to digitizing sensory evaluation and marketing research. This entrepreneurial project was selected to participate in the Cornell E-Lab 2022-2023 cohort.

This work is dedicated to my parents, 王晶 and 黄忠礼

And

My beloved significant-other, Yuxuan (Vanessa) Fu

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## LIST OF ABBREVIATIONS

Perfumery Raw Materials (PRMs)

Olfactory Receptors (ORs)

Olfactory Sensory Neurons (OSNs)

*Iso*-valeric Acid (IVA)

*2,3,5*-trimethylpyrizine (TMP)

Geraniol (Ger)

Neohivernal (Neo)

Florhydral (Flo)

Isolongifolanone (Long)

Methyl *Iso*-eugenol (Met)

*S*-Limonene (Lim)

Chapter 1

# Introduction

## 1.1 Odor Perception

Odor perception is a multifaceted process that involves chemical, biological, physiological, and psychological aspects. From a chemistry perspective, factors such as molecular weight (Rothe, 1976), polarity (Jong, 1937), functional groups (Poivet, 2016; Poivet, 2018), and concentrations (Johnson, 2000) of odorant molecules all play roles in the perceived quality of odorants. When odorants are inhaled into the human nostrils, they bind to the olfactory receptors (ORs) in the olfactory epithelium (Buck & Axel, 1991; Buck, 1996; Malnic B, 1999). The odorants can either stimulate (agonists) or inhibit Ors (antagonists) (Marc Spehr, 2003; Oka, Nakamura et al., 2004; Oka, Omura et al., 2004; Ricardo C. Araneda, 2000), thus forming a unique Ors coding (Xu, 2020; Xu, 2023) that transforms the chemical information into electrical signals in the peripheral olfactory system. The electrical signals in the olfactory sensory neurons (OSNs) are further transduced to the olfactory bulbs (Obs) in glomeruli (Buck, 2000), the piriform cortex, and other brain regions to convert the electrical signal into odor perceptions.

## 1.2 Odorant Mixture Perception

Odor mixture perception can be classified into two categories: configural, where the mixture is perceived as a unique configuration distinct from its components, and elemental, where the qualities of the individual components persist and coexist within the mixture. To illustrate this, let’s consider the famous beverage Coca Cola (Cola). In a study conducted in 2015, researchers identified 58 major aroma compounds in Cola, some of which are also found in cinnamon, lemon, orange, and other substances (Lorjaroenphon, 2015). However, when

random consumers drink Cola, they do not consciously perceive the distinct odor qualities of those 58 components. Instead, these aroma compounds work together synergistically to create a unique configuration that we recognize as “Cola.” When encountering an unfamiliar odor without prior knowledge, animals instinctively engage in configural recognition of the mixture (Livermore, 1997; Coureaud, 2009).

In 2001, Jinks described a configurational hypothesis of odor mixture perception, suggesting that “integration of characteristics of odorant mixture components can result in the identification of individual odorants, and in the production of a single percept” (Jinks, 2001). Additionally, a study by Linster and Cleland in 2004 demonstrated that the response of the olfactory bulb’s output to two perceptually similar odorants (A and B) differed from its response to the mixture of the two (AB) (Linster, 2004). Further research involving human participants, utilizing GC/MS and fMRI, has indicated that the human olfactory system employs both configural and elemental strategies to encode odor salience, with distinct brain areas supporting each of these processing modes (Howard, 2014). Whether an odor mixture is perceived configurally or elementally depends on several factors: 1) the identities of the mixture components, 2) the complexity of the mixture, 3) the concentration of the mixture components, and 4) the cognitive strategy and prior knowledge during perception.

First, the identities of the mixture components can influence perception. For instance, when both components of a binary mixture activate the rat I7 receptor, human subjects perceive the mixture configurally, whereas a mixture that suppresses each other tends to have more elemental odor properties (Kay, 2003).

Second, the complexity of the mixture plays a role in configural perception. Previous studies have found that the capacity of humans to identify the components of odor mixtures containing five to eight odorants is limited to about three (Laing, 1989; Laing, 1992; Livermore, 1998). This suggests that there is a maximum limit to the amount of elemental information one can discern in a mixture. Additionally, Jinks (1999) demonstrated that identification of mixture components decreased to chance levels when subjects were presented with sixteen odorants of equal intensity. These findings suggest that complex mixtures with equal intensity are typically perceived configurally. Weiss (2012) further supported this phenomenon by showing that random mixtures of approximately 30 or more equal-intensity components, spanning the stimulus space, had similar smells and were slightly pleasant, referred to as “Lorax” or “Olfactory White” (Weiss, 2012).

Third, changes in concentration within an AB mixture have been shown to influence perception. For example, a 30/70 ratio of ethyl isobutyrate (strawberry) and ethyl maltol (caramel) is perceived configurally as pineapple. However, a 68/32 ratio of the same odorants is perceived elementally as a mixture of strawberry and caramel (Le Berre, 2008a). This phenomenon has also been demonstrated in rabbits (Coureaud, 2011) and mice (Wilson, 2020), with Wilson further suggesting that the anterior piriform cortex in mice might be responsible for this perceptual shift (Wilson, 2020).

Finally, cognitive strategies and prior knowledge can influence whether a mixture is perceived more configurally or elementally. In animal models such as rabbits and lobsters, numerous studies have shown that experimental conditioning (Livermore, 1997; Sinding,

2011) and developmental changes (Coureaud, 2020) have a significant impact on how animals perceive mixtures. In human psychophysical experiments, individuals engaged in an analytical task rated blending mixtures as less typical compared to those engaged in a synthetic task. Furthermore, previous exposure to mixture components was found to decrease the typicality of the mixture (Le Berre, 2008b). For example, when regular people drink a glass of wine in a restaurant, they tend to perceive it configurally as simply "red wine." However, experienced wine tasters can utilize more elemental information to provide accurate descriptions regarding the wine's origin, year, and fermentation method.

### 1.3 Psychophysical Perspectives on Odorant Interaction

When we inhale a mixture of odorants, these odorants can act as agonists, antagonists, or allosteric enhancers to the Ors, which means there could be enhancement, suppression or no effect when mixing a new component with existing odorant(s). In the case of binary mixtures, William Cain introduced the concepts of odor quality masking and odor intensity counteraction. Odor quality masking aims to modify the perceived odor quality, while odor intensity counteraction aims to reduce the perceived odor intensity (Cain, 1974). In real-life scenarios, odor quality masking and intensity counteraction often coexist, meaning the addition of a second odorant changes both the odor intensity and odor quality of the first odorant (Cain, 1975).

From a quantitative perspective, the overall intensity of an odor mixture is typically greater than that of any individual odorant, but less than the sum of their intensities, indicating mutual inhibition as the most common form of interaction (Laing, 1983; Laing, 1984; Bell,

1987; Laing, 1994). From a qualitative perspective, mixtures of odorants can result in a loss of individual odor quality. Humans can typically identify up to three perceptually different components in a mixture (Jinks, 2001). During odor perception, contrast enhancement can help subjects recognize different odorants within a mixture (Cleland, 2006). However, when the components in the mixture are at equal intensity, the contrast between different odors becomes minimal. Consequently, human subjects have been shown to be unable to identify individual components in equal intensity odor mixtures containing more than 16 compounds (Jinks, 1990). Taking this further, mixtures of approximately 30 or more random equal-intensity components, spanning the stimulus space, have been found to smell similar to each other. This phenomenon is referred to as "Olfactory White" (Weiss, 2012). Overall, the interaction of odorants in a mixture can lead to complex changes in both quantitative and qualitative aspects of odor perception, affecting intensity, quality, and the ability to discriminate individual components.

### 1.4 Biological Perspectives on Odorant Interaction

Since the discovery of olfactory receptors (Ors) by Buck and Axel (1991; Buck, 1996; Malnic B, 1999), advancements have been made in the development of more accurate in-vitro testing assays (Marc Spehr, 2003; Oka, Nakamura, et al., 2004; Oka, Omura, et al., 2004; Ricardo C. Araneda, 2000), enabling scientists to collect dose-response data on different OR ligands. By utilizing OR data obtained from mice and studying binary odor mixtures, Rospars (Rospars, 2008) and Marasco (Marasco, 2016) were able to demonstrate that specific olfactory receptor responses can be mathematically predicted by manipulating mixtures. Marasco's research (2016) provided insights into the classification of five possible

outcomes for binary odor interactions. These outcomes include: i) Suppression: The response to the mixture falls between the responses to the individual components. Ii) Hypoadditivity: The response to t'e mixture is similar to the most effective component. Iii) Synergy: The response to the mixture is higher than the responses to both individual components. Iv) Inhibition: The response to the mixture is lower than the responses to both individual components. V) Overshadowing: The response to the mixture is similar to one of the components. These findings have laid a solid foundation for future research on complex odor interactions and provide a framework for understanding the diverse effects that can arise from combining odorants in mixtures.

### 1.5 Odorant Threshold

Threshold, from a neuroscience perspective, is defined as the level of nerve stimulation that just triggers a response. In human olfaction psychophysics, threshold represents the minimum concentration of odorant(s) that allows a person to sense the change 50% of the time. Two commonly used concepts that describe individuals' sensitivity to different odorants are detection threshold and recognition threshold. The odor detection threshold refers to the lowest concentration of an odorant at which an individual becomes aware of the presence of an odor. However, the odor detection threshold has been reported to be less reliable due to its low reproducibility caused by false alarms and poorly defined judgments (Leonardos, 1969; Engen, 1972). On the other hand, the odor recognition threshold is the concentration of an odorant at which an individual can correctly associate an odor with its name or a descriptor. The odor recognition threshold relies on the responses of trained subjects, and can generate more reproducible data (Dravnieks, 1986). To obtain accurate

measurements of recognition thresholds, various tools have been employed, such as odor bags (Nagata, 1980), squeeze bottles (Olsson, 2000), the ‘Sniffin’ Sticks method (Hummel, 1997; Hummel, 2007), test rooms (Leonardos, 1969), and olfactometers (Dravnieks, 1986; Schmidt & Cain, 2010; Ni, 2022). These tools provide controlled and reproducible odor stimuli to the subjects.Threshold testing involving different concentrations is typically conducted using an ascending staircase method (Leonardos, 1969; Dravnieks, 1975; Dravnieks, 1986) or a random presentation method (Ni, 2022), followed by a forced-choice question (Lawless & Heymann, 2010). The threshold is then obtained by interpolating the psychometric function at the point where the odor detection probability equals 50% (Leonardos, 1969; Dravnieks, 1986; Marin, 1991; Walker, 2003; Ni, 2022).

### 1.6 Odor Perception Around Threshold

Odor mixture perception, according to Stuart Firestein, is like “a chord with silent note”, implying that the components of the mixture that fall below the threshold of detection are still critical in determining the overall identity and quality of the mixture (Xu, 2023). Even when inhaled at peri-threshold or sub-threshold levels, odor stimuli can modify the pattern of peripheral activation and elicit responses in the human brain (Hummel, 2013). In human psychophysical studies, it has been demonstrated that peri/sub-threshold amounts of odorants can interact in synchrony with taste stimuli (Pfeiffer, 2005) and synergistically with other odorants (Miyazawa, 2008). This suggests that even at levels below conscious perception, these odorants can influence and enhance the perception of taste or other odorants, contributing to the complexity of odor mixture perception.

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Chapter 2

# Study Objectives

## 2.1 Efficient Threshold Determination Using Sniff Olfactometer

The aim of this study is to explore the minimum concentrations needed for obtaining odorant threshold using Sniff Olfactometer. This study will evaluate different methods based on their accuracy, reproducibility, and efficiency. Result of this study will provide a reference for the threshold testing method to be employed based on the purpose of future studies.

## 2.2 Threshold Determination for Configural Odor Mixtures Using Sniff Olfactometer

The aim of this study is to explore the possibility of generating threshold psychometric curve for configurally recognized odor mixtures. The result of this study will prove the recognition threshold can be obtained for any configurally perceived odorant or mixture of odorants.

## 2.3 Masking Effects on *Iso*-valeric Acid Recognition by Sub-threshold Odor Mixture

The aim of this study is to explore the possibility of masking malodor using sub-threshold amount of perfumery raw material mixture. The validation of this assumption will allow malodor control with minimal amount of chemical exposure and brings new perspective to the mixture perception.

## 2.4 Sub-threshold *Iso*-valeric Acid Modifies Odor Detection Probabilities

The aim of this study is to explore how addition of sub-threshold amount of *iso*-valeric acid will affect the detection probability of the target odorants. The result of this study will

provide insights on 1) whether if odor masking in mutual; and 2) how does odor interaction correlate with the dose of odorants.

Chapter 3

# Efficient Threshold Determination Using Sniff Olfactometer

## 3.1 Abstract

Determining the threshold concentrations at which an odor becomes perceptible and recognizable to the human nose is crucial for evaluating odor interactions at peri-threshold and sub-threshold levels. Most olfactometers used for threshold measurement have stimulus durations ranging from 1 second to several minutes, with runtime exceeding an hour, often leading to habituation. The Sniff Olfactometer (SO) was specifically designed to deliver a brief stimulus (70ms) of 15ml headspace to minimize habituation. In 2021, our laboratory accurately determined the recognition thresholds for 2,3,5-trimethyl pyrazine and hexanal within a 30-minute time frame, demonstrating the accuracy and reproducibility of this process. This article further explores the potential for simplifying the threshold determination process by using a reduced number of samples (3 samples) and a shorter duration (10 minutes).

## 3.2 Introduction

Threshold, from a neural science perspective, is defined as the degree of stimulation of a nerve that just produces a response. In human psychophysics, threshold represents the minimum change in odorant(s) concentration for a person to sense the change 50% of the time. In odor perception, there are four commonly used concepts: Detection Threshold, Recognition Threshold, Difference Threshold, and Terminal Threshold (Lawless & Heymann, 2010). The Detection Threshold refers to the concentration at which an odorant can be differentiated from a blank stimulus. On the other hand, the Recognition Threshold is the concentration at which the odorant can be associated with its label(s). The Difference Threshold, also known as the Just Noticeable Difference (JND), describes the minimum change in concentration that can be sensed

(Gescheider, 1997; Jacquot, 2010). In contrast to the Detection Threshold, the Terminal Threshold defines a concentration at which no further increase in intensity will be perceived despite an increase in concentration.

It is important to determine the subjects' recognition threshold before conducting experiments, as the threshold is necessary for calculating the Odor Activity Value (OAV) (Patton, 1957), measuring the Equal Odds Ratio (EOR) (Rochelle, 2017), and determining the appropriate concentration for studying odorant interactions at sub-threshold levels. The Sniff Olfactometer (SO) was developed to collect human psychophysical responses to controlled odor stimuli (Wyckoff, 2016; Rochelle, 2017; Ni, 2022). The SO can hold three different samples at a time in a triad configuration and deliver a stimulus with a 15mL headspace from a 250mL Teflon bottle in as short as 70ms. The SO is particularly powerful for measuring the threshold. Previous studies in our lab have shown that the threshold can be measured using six concentrations, labeled from 1 to 6, with a difference greater than the Just Noticeable Difference (JND) (> 33%) between neighboring concentrations (Rochelle, 2017; Ni, 2022). Further research demonstrated that alternating the concentration sequences at peri-threshold levels (1-3-5, 2-4-6) generated a psychometric function with a better fit (Ni, 2022).

Although the current testing method is robust and reproducible, it is time-consuming. The method involves a conditioning session (5 mins), a pre-testing session (3 mins), and a testing session with 72 puffs (25 mins) to obtain one complete measurement. Any additional repetition that requires concentration adjustment will add at least one extra session, significantly delaying the experiment progress due to subjects' limited availability. In 2022, our study on temporal and

sequential stimulus presentation demonstrated that the detection probabilities of the six concentrations were stable with different interstimulus intervals (1 day, 2 days, and 7 days) between two triads (Lin, 2022). This study opens up the possibility of acquiring the recognition threshold using fewer samples. Since the threshold is always an estimated range (Lawless & Heymann, 2010) and is interpolated from a sigmoid function fitted by six data points in our current method, we want to explore whether the thresholds interpolated from a three-point sigmoid function (an estimated sigmoid function from three data points) are statistically different from thresholds interpolated from a six-point sigmoid function (an estimated sigmoid function from six data points). Failing to reject the null hypothesis could indicate that we can use three concentrations to estimate the odor recognition threshold range fairly. In this experiment, we will test Isovaleric Acid, Geraniol, Neohivernal, and 2,3,5-trimethylpyrazine to test our hypothesis.

### 3.3 Materials and Method

**Chemicals.** Polyethylene Glycol 400 (PEG400): (CAS Registry No. 9002-88-4), Deionized Water: carbon filtered deionized water, Activated Charcoal Powder: (CAS Registry No. 7440-44-0), *2,3,5*-trimethylpyrizine (TMP): Descriptor = "Nut" (CAS No. 14667-55-1), *Iso*-valeric Acid (IVA): Descriptor = "Stink Feet" (CAS Registry No. 503-74-2), Geraniol (Ger): Descriptor = "Rosey" (CAS Registry No. 106-24-1), Neohivernal (Neo): Descriptor = "Clean Laundry" (CAS Registry No. 300371-33-9).

**Ethics Statement.** The experiment was conducted under the approval of Cornell University Institutional Review Board (IRB) and followed Declaration of Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013).

**Subjects.** 6 subjects including 4 females and 2 males, all students from Cornell University (20-27 years old), were tested. Subjects participated were asked to sign the agreement (Supplemental) and screened to make sure they 1) do not have a stuffy nose before each session; 2) do not have post-COVID anosmia; 3) could identify all the compounds presented in the session by passing the conditioning and pretesting session.

**Software.** The experiments were automated using PsychoPy® (v2021.2.3) (Peirce et al., 2019). Data analysis was executed using R (version 4.1.3 – "One Push-Up") (R-Core-Team, 2022) and Python 3.11.2 (Van Rossum, 2009). See supplemental.

**Equipment.** Sniff Olfactometry (SO)(Rochelle et al., 2017; Wyckoff & Acree, 2017).

**Stock Solution Preparation.** To prepare the **stock solution** for each odorant: 1000 PPM stock solution for each odorant in PEG 400 was prepared. 0.1g of each odorant was added to 100mL amber bottle followed by 100mL PEG 400 addition.

**10% PEG in Water Solution Preparation.** 400mL of PEG 400 was added 3600mL of DI water. The mixture solution was added 20g of Charcoal powder and vigorously mixed. The mixture was set for 2 days and performed vacuum filtration to remove the charcoal powder to obtain the deodorized 10% PEG – water solution.

**Test Sample Preparation**. For each of the odorant used for threshold testing, six concentrations were determined by bench top trials with lab members. From lowest concentration to highest

concentration were labeled 1 to 6. Concentration determination process follows the Weber's Law of Just Noticeable Difference: Mutual difference between concentrations were greater than Just Noticeable Difference (JND) ($\Delta C/C \geqq 0.33$) (Table 3.1). To make the test solution, stock solution was diluted to different concentrations to make the test solution. The solutions were prepared 1 day before the experiment, mixed on a shaker overnight, and transferred to a 250mL Teflon bottle 10mins before experiments.

| **Odorants** | **Concentrations (PPM)** | | | | | |
|---|---|---|---|---|---|---|
| ***Iso*-valeric Acid** | 0.5 | 1 | 5 | 10 | 20 | 30 |
| **Neohivernal** | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
| **Geraniol** | 0.02 | 0.05 | 0.1 | 0.3 | 1 | 2.5 |
| ***2,3,5*-trimethylpyrizine** | 0.1 | 0.5 | 1 | 5 | 10 | 20 |

*Table 3.1: Perfumery raw materials concentrations used for threshold determination.*

**Odorant Conditioning Session.** For each odorant, highest concentration (bottle 6) was used for conditioning session. During the conditioning session, a 70ms-15mL odor blast will be puffed upon subjects click the mouse. Then the monitor will prompt "This smell is XXX (descriptor)". The subjects will repeat this process for up to 6 times to familiarize themselves with the odor. Subjects who were not able to smell this concentration were discontinued for the next session.

**Threshold Pre-testing Session.** For the pre-testing session, concentration used for conditioning session and a blank sample containing 50mL 10% PEG solution was used. After clicking the mouse, subjects will receive an odor blast and a binary forced choice question "Did you smell xxx (descriptor for that odorant)?". Subjects will be asked to select "Yes" or "No". The process was repeated for 5 puffs for each bottle; subjects were required to attain 90% accuracy on this trial before moving into the threshold measurement stage of the experiment.

**Threshold Testing Session.** In the testing round 1, bottles 1, 3, and 5 were puffed 4 times randomly each at each position in the triad, and in the testing round 2, bottles 2, 4, and 6 were puffed 4 times randomly each at each position in the triad (Figure 3.1). For each odorant, testing round 1 and round 2 were conducted on different visits that are 1 to 4 days apart. *Iso*-valeric Acid threshold measurement was repeated for three times while the threshold measurements for other odorants were only conducted once (Table 3.2).

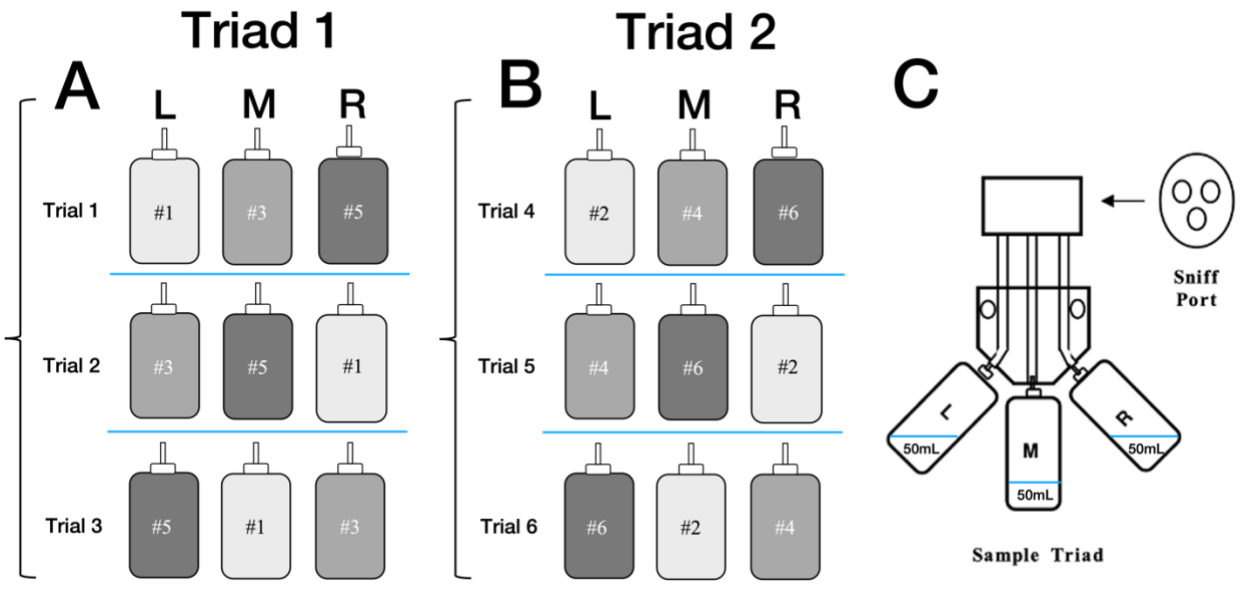


*Figure 3.1: Figure 3.1 A shows the bottle arrangement for testing trial 1 to 3. Figure 3.1B shows the bottle arrangement for testing trial 4 to 6. Figure 3.1C shows the bottle arrangement in the sample triad.*

| EXPERIMENT TIME | EXPERIMENT CONDITIONS |
|---|---|
| $1^{ST}$ VISIT | IVA triad 1 ($1^{st}$) + Geraniol triad 1 |
| $2^{ND}$ VISIT | IVA triad 2 ($1^{st}$) + Geraniol triad 2 |
| $3^{RD}$ VISIT | IVA triad 1 ($2^{nd}$) + TMP triad 1 |
| $4^{TH}$ VISIT | IVA triad 2 ($2^{nd}$) + TMP triad 2 |
| $5^{TH}$ VISIT | IVA triad 1 ($3^{rd}$) + Neohivernal triad 1 |
| $6^{TH}$ VISIT | IVA triad 2 ($3^{rd}$) + Neohivernal triad 2 |

*Table 3.2: This table shows the sequence of experiments each subject complete in six days.*

### 3.4 Results

**Threshold Measurement Reproducibility and Temporal Effect.** To ensure the reproducibility of the threshold measurements, three Isovaleric Acid (IVA) threshold measurements were compared to each other using a two-tailed paired t-test (p=0.01). The results revealed no significant differences among the three measurements ($P_{(1st\&2nd)} = 0.307$, $P_{(1st\&3rd)} = 0.065$, $P_{(2nd\&3rd)} = 0.814$) (Figure 3.2) and between each measurement and the summary ($P_{(Sum\&Test1)} = 0.14$, $P_{(Sum\&Test2)} = 0.77$, $P_{(Sum\&Test3)} = 0.38$). The goodness of fit ($R^2$) was then calculated and shown on the plot. By evaluating the P-value and $R^2$, it can be determined that if the fitted curve has an $R^2$ value greater than 0.8, repetitions will not yield significantly different results from a single measurement. Furthermore, when the concentrations were appropriately chosen, subjects were able to detect peri-threshold concentration changes by exhibiting increased or decreased detection probabilities, even when the measurements of the two triads were separated by more than 24 hours. For instance, on average, 1PPM of IVA had a lower recognition probability compared to 5PPM (Figure 3.2 & 3.3). Therefore, by combining the IVA threshold measurements from all six visits, we were able to plot a near-perfect psychometric function

(Figure 3.2), further supporting our previous finding that "the brain, instead of 'recording' the difference between adjacent pairs, is 'comparing' the difference between adjacent concentrations within a triad" (Lin, 2022).

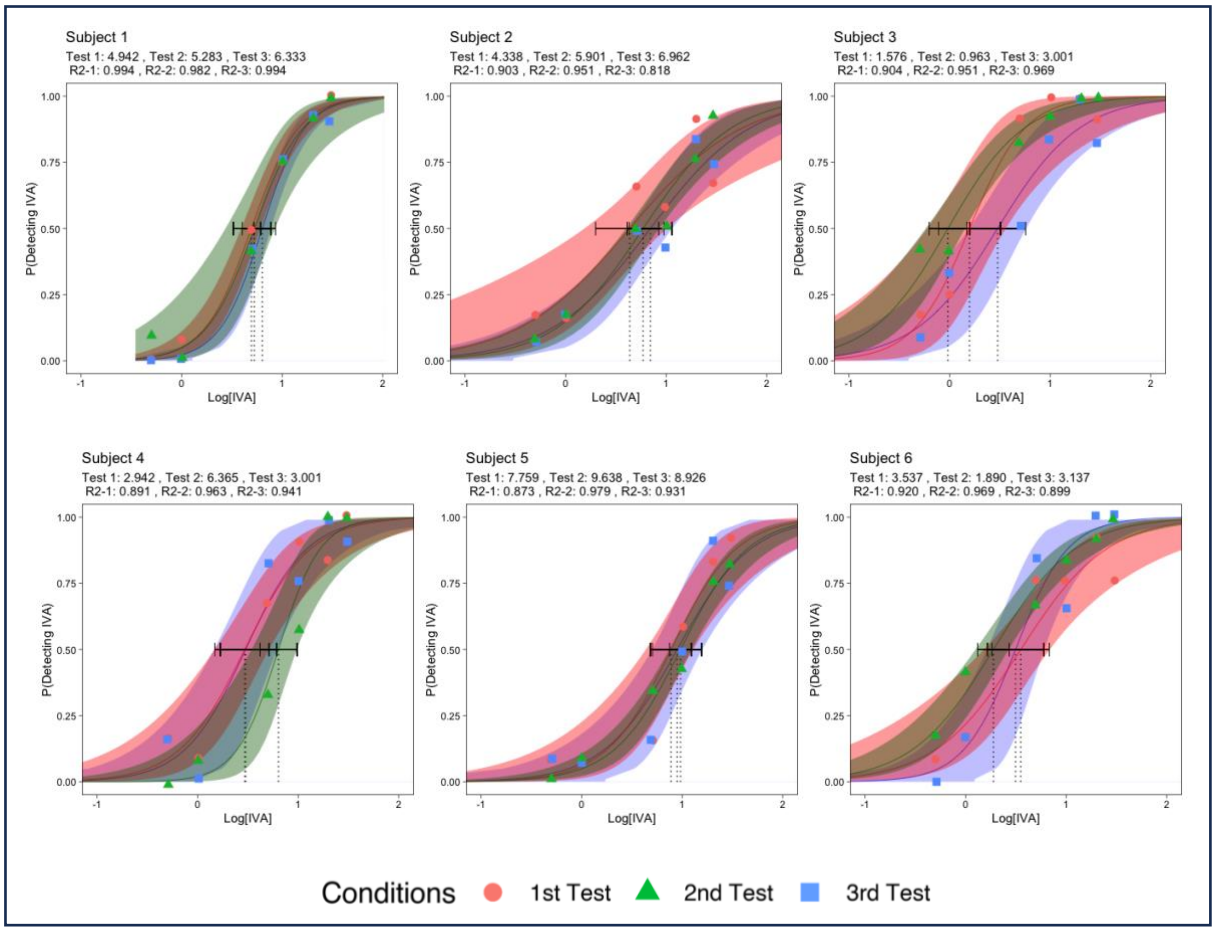


*Figure 3.2: Figure 3.2 shows the psychometric function of 3 IVA recognition threshold tests for each subject. The shaded region shows the error range, the smaller the shade, the more accurate the estimation is. The estimated thresholds were labeled as T-1,3,5, T-2,4,6 and T-All for thresholds interpolated from triad 1, triad 2 and both triads.*

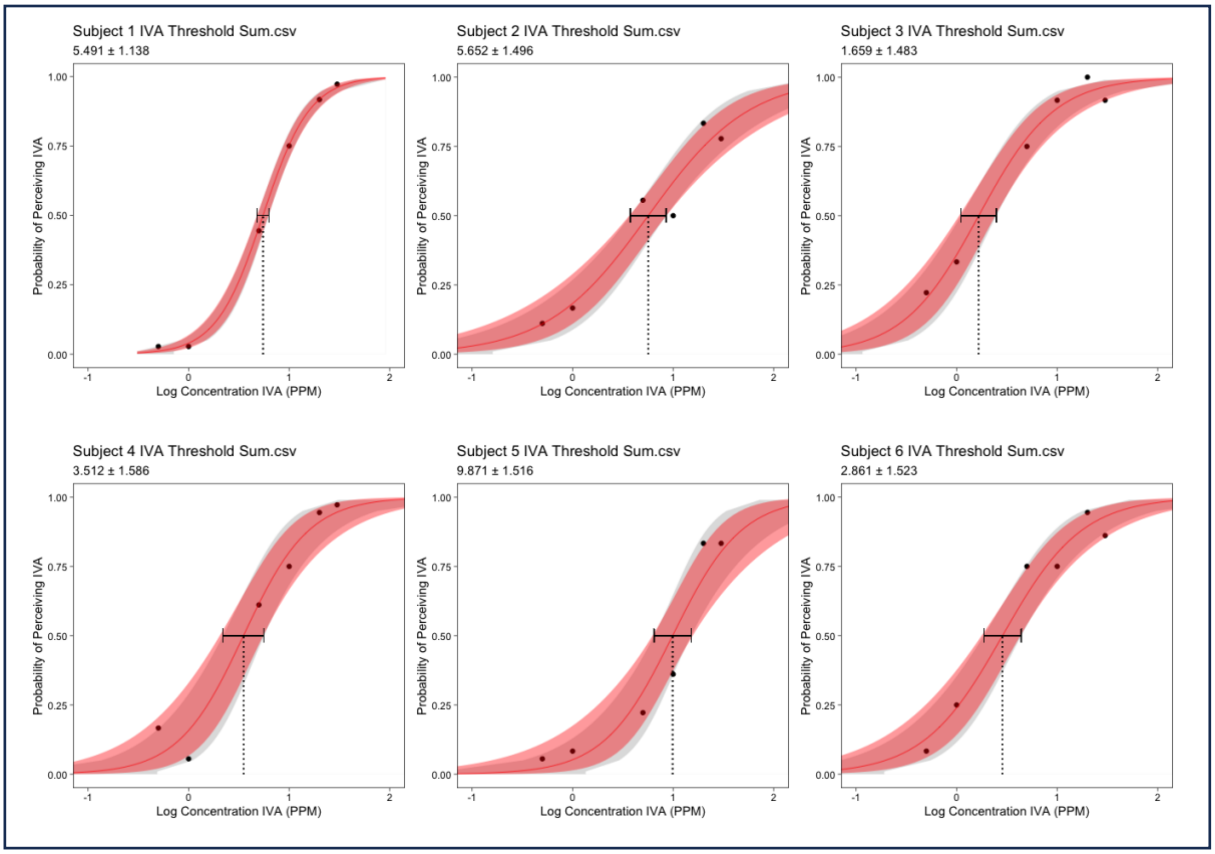


*Figure 3.3: Figure 3.3 shows the psychometric function when combining all 6 threshold measurements data for IVA.*

**Threshold Estimation Using Three Concentration.** The psychometric functions for determining the thresholds of Isovaleric Acid (IVA), Geraniol, 2,3,5-trimethylpyrazine (TMP), and Neohivernal were plotted using data from round 1 (bottles 1, 3, and 5), round 2 (bottles 2, 4, and 6), and the combination of rounds 1 and 2 (Figures 3.3-3.6). The thresholds interpolated from the psychometric curves using data from round 1, round 2, and the combination of rounds 1 and 2 were compared to each other using a two-tailed paired t-test ($p = 0.01$). The p-values from the two-tailed paired t-test indicated that the thresholds estimated from the concentrations in the second triad (bottles 2, 4, and 6) were not statistically different from the thresholds estimated

using both triads (Table 3.3). However, it should be noted that the thresholds estimated using the first triad (bottles 1, 3, and 5) underestimated the thresholds for Neohivernal and TMP compared to the thresholds calculated using both triads.

| | IVA | GER | NEO | TMP |
|---|---|---|---|---|
| SUM – 135 | **0.1074** | **0.0688** | **0.0053** | **0.0074** |
| SUM – 246 | **0.0193** | **0.0475** | **0.0464** | **0.0421** |
| 135 – 246 | **0.0253** | **0.0558** | **0.0203** | **0.0080** |

*Table 3.3: Table 3.3 shows the p-values from two-tailed paired t-test comparing thresholds calculated from triad 1 (bottle 1,3,5), triad 2 (bottle 2,4,6), and both triads. The degree of freedom is 17 for IVA, and 5 for GER, NEO and TMP.*

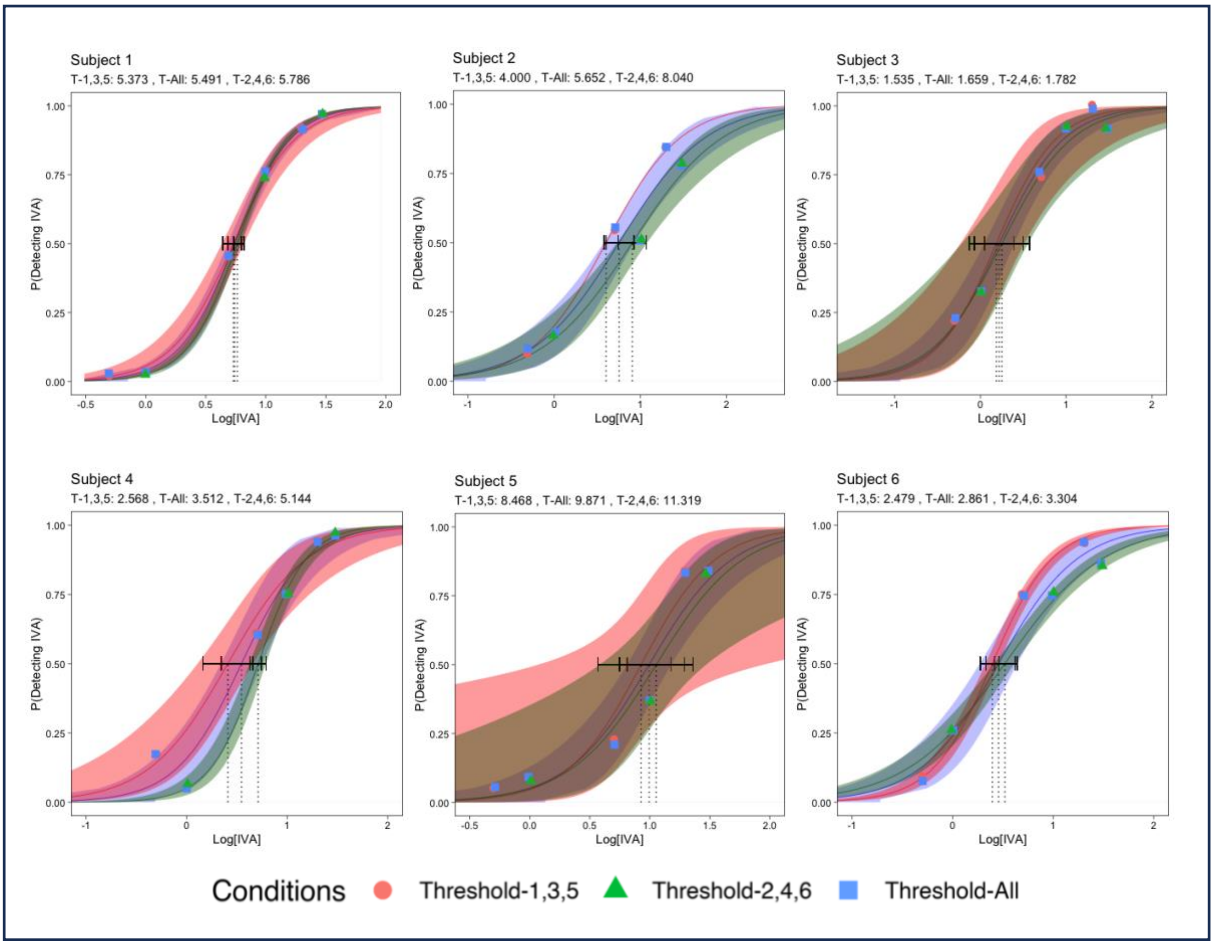


*Figure 3.4: Figure 3.4 shows the psychometric function for IVA recognition threshold plotted using three concentrations (triad 1 or 2) and six concentrations (triad 1 and 2). The shaded region shows the error range, the smaller the shade, the more accurate the estimation is. The estimated thresholds were labeled as T-1,3,5, T-2,4,6 and T-All for thresholds interpolated from triad 1, triad 2 and both triads.*

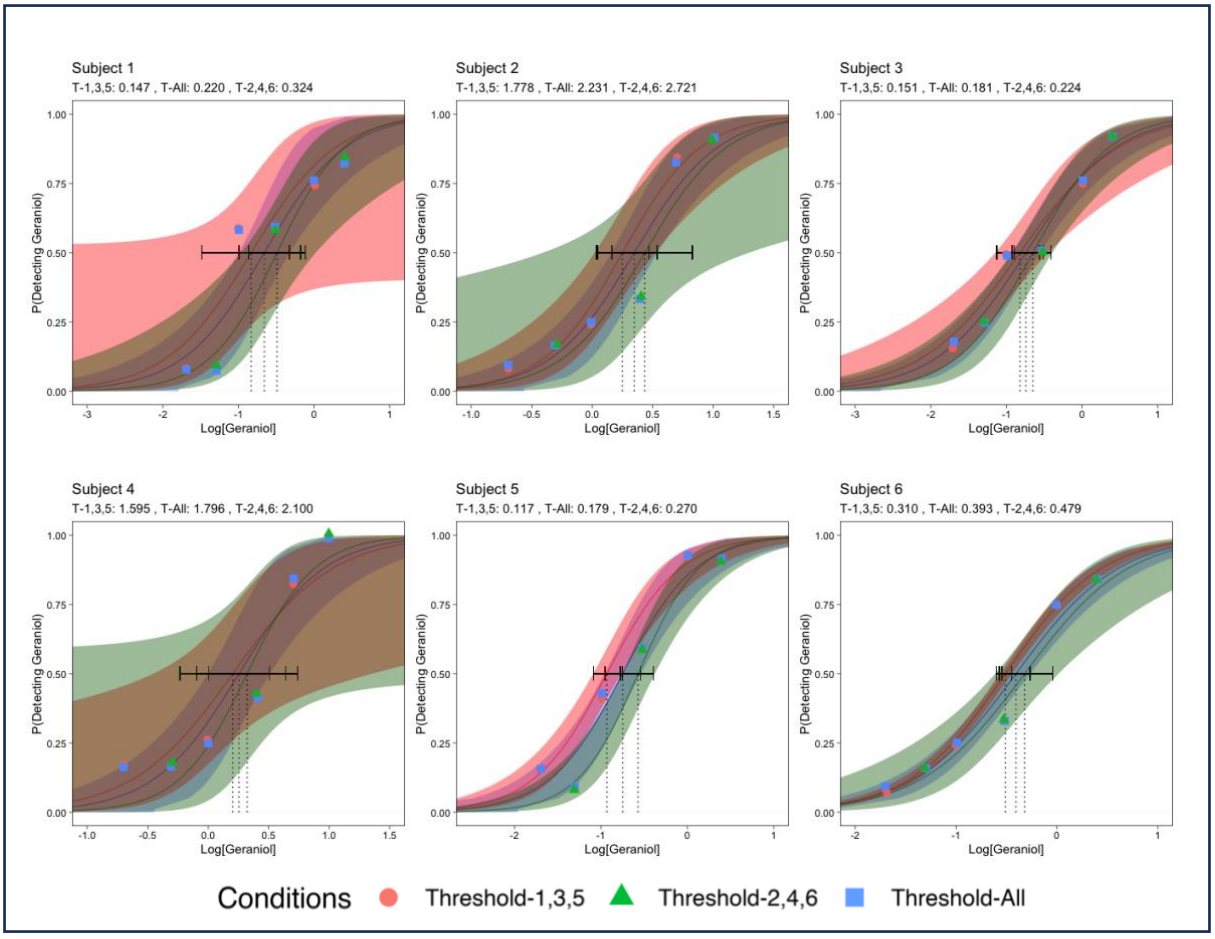


*Figure 3.5: Figure 3.5 shows the psychometric function for Geraniol recognition threshold plotted using three concentrations (triad 1 or 2) and six concentrations (triad 1 and 2). The shaded region shows the error range, the smaller the shade, the more accurate the estimation is. The estimated thresholds were labeled as T-1,3,5, T-2,4,6 and T-All for thresholds interpolated from triad 1, triad 2 and both triads.*

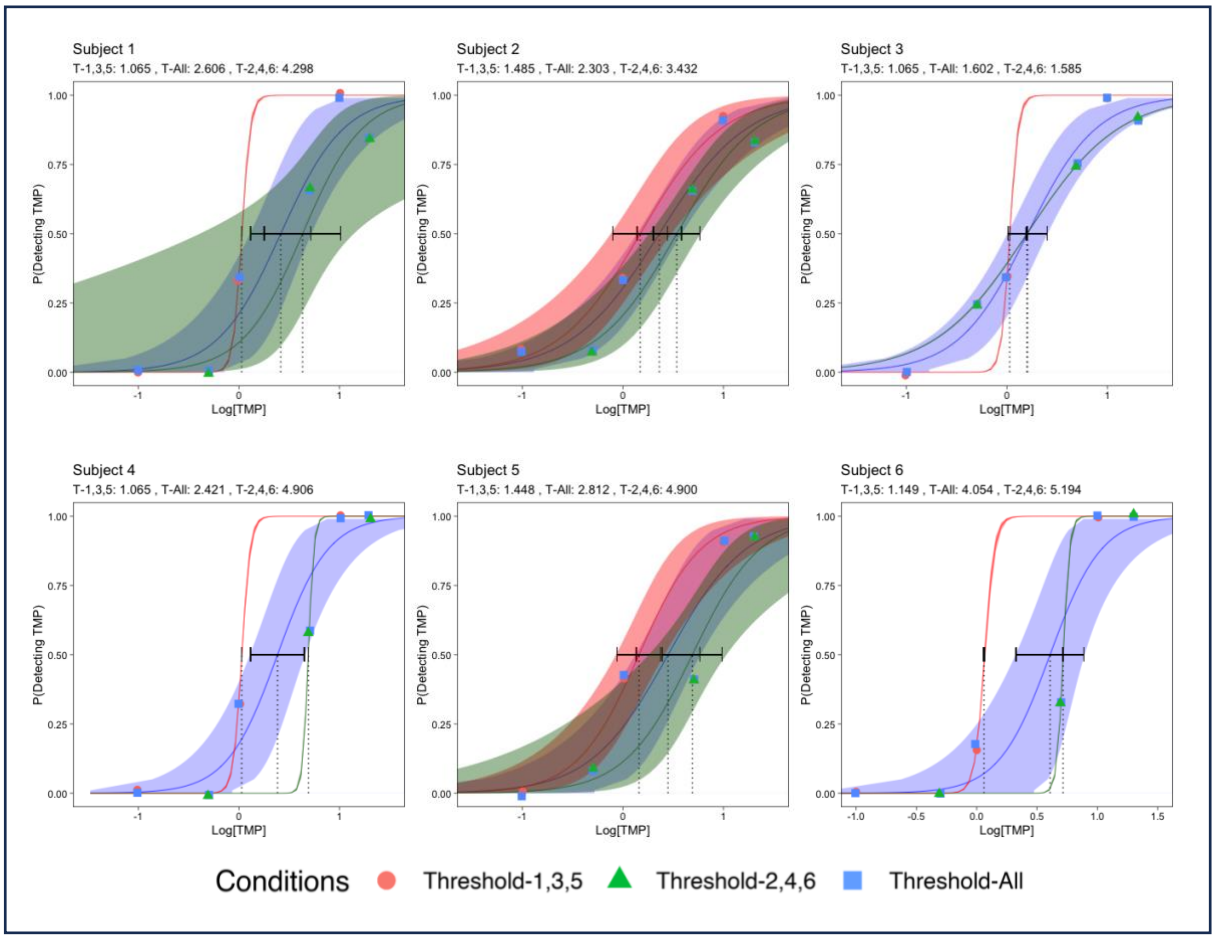


*Figure 3.6: Figure 3.6 shows the psychometric function for TMP recognition threshold plotted using three concentrations (triad 1 or 2) and six concentrations (triad 1 and 2). The shaded region shows the error range, the smaller the shade, the more accurate the estimation is. The estimated thresholds were labeled as T-1,3,5, T-2,4,6 and T-All for thresholds interpolated from triad 1, triad 2 and both triads.*

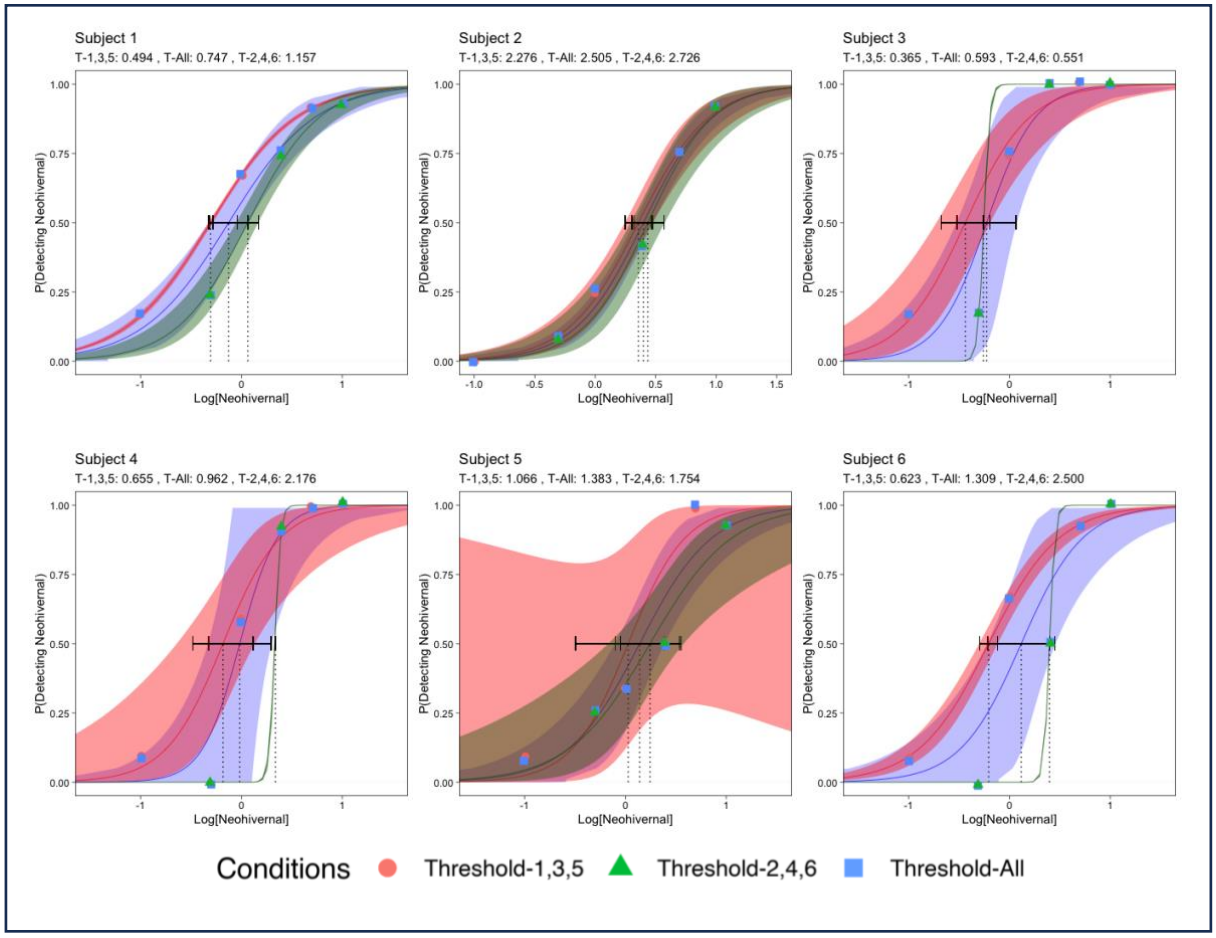


*Figure 3.7: Figure 3.7 shows the psychometric function for Neohivernal recognition threshold plotted using three concentrations (triad 1 or 2) and six concentrations (triad 1 and 2). The shaded region shows the error range, the smaller the shade, the more accurate the estimation is. The estimated thresholds were labeled as T-1,3,5, T-2,4,6 and T-All for thresholds interpolated from triad 1, triad 2 and both triads.*

### 3.5 Discussion

In the regular procedure, we typically use six concentrations and two replications to measure the threshold of an odorant. The purpose of replicating the measurement is to minimize the impact of fluctuations in sensitivity and obtain a more accurate estimation of the absolute threshold (Gescheider, 1997). Using six concentrations allows for a better-fitted psychometric function. However, there are situations where habituation (Pellegrino, 2017), limited subject availability, or the need to control odor exposure time in the actual experiment may pose challenges. Therefore, although repetition and more samples are desirable, they are not always feasible. We have shown in this experiment that, if we could fit a psychometric function with $R^2$ greater than 0.8, the interpolated threshold from each measurement does not significantly different from each other, thus proving the repetition is not always necessary.

Furthermore, we have also shown that using three concentrations to estimate the psychometric curve and threshold is an efficient approach, saving approximately 60% of the time. In most cases, it provides a fair estimation of the actual thresholds. A fair estimation requires at least two out of the three concentrations to have recognition probabilities falling between 0 and 1. When only one concentration has a recognition probability between 0 and 1, the resulting psychometric function will be highly centered around that point and exhibit more of an "I" shape rather than an "S" shape (Figure 3.5, subjects 1, 3, 4, 6; Figure 3.6, subjects 3, 4, 6). Particularly when that one point is far from P=0.5, the estimated threshold can greatly overestimate or underestimate the true threshold (Figure 3.5, subject 6).

It is crucial to understand when to estimate the threshold using three concentrations and when to obtain a more precise threshold using additional concentrations. I summarize four situations suitable for threshold estimation using three concentrations. Firstly, three concentrations are useful for estimating the suitable peri-threshold range. Once such a range is determined, it becomes easier to decide which six or nine concentrations to use for a more precise threshold measurement. This approach minimizes the chance of failing the threshold determination due to inappropriate concentration selection. Secondly, when aiming to acquire concentrations that are below the recognition threshold, three concentrations are sufficient to determine the boundary. In such cases, it is preferable to choose lower concentrations (triad 1) rather than higher concentrations (triad 2). The goal is to find the lower boundary of the psychometric curve, so underestimating the threshold is better than overestimating it. Thirdly, when conducting experiments where the goal is to ensure the concentration of the odor used is recognizable, it is sufficient to use three concentrations. For example, when testing correlation between odor and emotion/color (Kurtz, 2010), it is only necessary to ensure that the odor is detectable and recognizable.

Therefore, using a triad containing relatively higher concentrations (preferably triad 2 over triad 1) is more practical than using six or nine concentrations to estimate an upper boundary for the psychometric curve. Lastly, as subjects' thresholds towards different odorants can change over time due to habituation or learning, certain experiments may require measuring the threshold before each test session. In such cases, using three concentrations is sufficient to evaluate potential habituation and monitor subjects' sensitivities.

However, if the experiment requires a relatively precise and accurate measurement of the detection threshold, such as in calculating the Odor Activity Value (OAV) (Patton, 1957) or Equal Odds Ratio (Rochelle, 2017), more concentrations (six concentrations) are necessary to obtain a well-fitted psychometric function for interpolating the threshold.

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Chapter 4

# Threshold Determination for Configural Odor Mixtures Using Sniff Olfactometer

## 4.1 Abstract

Odor perception begins with the activation of Olfactory Receptors (ORs), which form the peripheral receptor activation pattern. In order for our central nervous system to perceive and recognize an odor, the odorant must reach a certain concentration known as the recognition threshold. In human psychophysics, the recognition threshold is defined as the concentration at which a person can recognize the odor 50% of the time. Given that Olfactory Receptors can be activated by individual odorants as well as mixtures of odorants, and that odorant mixture perception can be configural or elemental, we conducted an experiment using a Sniff Olfactometer (SO) to explore the generation of psychometric curves for configurally perceived natural flavor extracts from Rose, Jasmine, and Juniper Berry. The results of this experiment demonstrate that recognition thresholds can be measured for configurally perceived odorant mixtures, and the psychometric curves obtained are similar to those generated from single odorants.

## 4.2 Introduction

Odor perception is a complex process that involves various aspects, including chemical, biological, physiological, and psychological factors. In recent years, significant progress has been made in structure-based odor prediction for single odorants, achieving high precision and accuracy (Keller, 2017; Sanchez-Lengeling, 2019; Lee, 2022). However, odor mixture perception remains a fascinating area of research, as complex odor mixtures often elicit a nonlinear response across their components (Singh, 2019).

Odorant mixture perception can be characterized as either configural or elemental. Configural perception refers to the perception of a mixture as a unique configuration that differs from its individual components, while elemental perception suggests that the qualities of the components persist and coexist in the mixture (Jinks, 2001). Early studies on odor mixture perception primarily adopted a bottom-up approach, focusing on the odor quality and intensity of single odorants and their simple mixtures (Laing, 1983; Laing, 1984; Bell, 1987; Laing, 1989; Laing, 1992; Livermore, 1998; Jinks, 1999; Jinks, 2001). Due to the nature of these bottom-up studies, the focus was primarily on elemental mixture detection.

However, when encountering an odor for the first time without prior knowledge, animals tend to naturally perceive the mixture in a configural manner (Livermore, 1997; Coureaud, 2009). Further research in humans, utilizing techniques such as GC/MS and fMRI, has indicated that the human olfactory system employs both configural and elemental strategies to encode odor salience, with distinct brain areas supporting each of these processing modes (Howard, 2014; Romagny, 2016). These findings suggest that adopting a top-down approach, starting with the characterization of familiar odor mixtures, could provide valuable insights into odor mixture perception. Natural flavor extracts present promising opportunities for conducting top-down studies on odor mixtures (Howard, 2014), as these extracts are typically familiar to subjects and their compositions have been well-studied.

Previous psychophysical studies in the field of flavor have often focused on relating odorant compositions to semantic labels through methods such as scaling (Lawless and Heymann, 2010).

However, cross-modal matching procedures like scaling can produce subjective and vague data that is challenging to map against mixture components. Detection and recognition, on the other hand, have demonstrated the ability to generate reproducible psychometric curves at the peri-threshold level using the Sniff Olfactometer (Ni, 2022). In order to conduct further studies on mixtures using a top-down approach, it is crucial to investigate whether configurally recognized natural flavor extracts behave similarly to single odorants at the peri-threshold level. This paper aims to address two key questions: 1) Can we generate a psychometric function for a configurally recognized mixture? and 2) Are there any elemental characteristics that stand out at specific concentrations? The experiment utilizes natural extracts of Rose, Jasmine, Juniper Berry, and the single odorant Floracetate to explore these questions.

### 4.3 Materials and Method

**Chemicals.** Polyethylene Glycol 400 (PEG400): (CAS Registry No. 9002-88-4), Deionized Water: carbon filtered deionized water, Activated Charcoal Powder: (CAS Registry No. 7440-44-0), Rose Oil Bulgaria (Rose): CAS No. 90106-38-0 (International Flavors & Fragrances), Juniper Berry Oil Balkans Rect BHT (Juniper Berry): CAS No. 8002-68-4 (International Flavors & Fragrances), Jasmin Absolute Egypt (Jasmin): CAS No. 8022-96-6 (International Flavors & Fragrances), Floracetate: CAS No. 17511-60-3 (Descriptor = “Flower”).

**Ethics Statement.** The experiment was conducted under the approval of Cornell University Institutional Review Board (IRB) and followed Declaration of Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013).

**Subjects.** 4 subjects including 3 females and 1 male, all students from Cornell University (21-26 years old), were tested. Subjects participated were asked to sign the agreement (Supplemental) and screened to make sure they 1) do not have a stuffy nose before each session; 2) do not have post-COVID anosmia; 3) could identify all the compounds presented in the session by passing the conditioning and pretesting session.

**Software.** The experiments were automated using PsychoPy® (v2021.2.3) (Peirce et al., 2019). Data analysis was executed using R (version 4.1.3 – "One Push-Up") (R-Core-Team, 2022) and Python 3.11.2 (Van Rossum, 2009). See supplemental.

**Equipment.** Sniff Olfactometry (SO)(Rochelle et al., 2017; Wyckoff & Acree, 2017) was used for threshold measurement.

**Stock Solution Preparation.** To prepare the **stock solution**: 10000 PPM stock solution for each flavor in PEG 400 was prepared. 1g of each odorant was added to 100mL amber bottle followed by 100mL PEG 400 addition.

**10% PEG in Water Solution Preparation.** 400mL of PEG 400 was added 3600mL of DI water. The mixture solution was added 20g of Charcoal powder and vigorously mixed. The mixture was set for 2 days and performed vacuum filtration to remove the charcoal powder to obtain the deodorized 10% PEG – water solution.

**Test Sample Preparation**. For each of the odorant used for threshold testing, six concentrations were determined by bench top trials with lab members. From lowest concentration to highest

concentration were labeled 1 to 6. Concentration determination process follows the Weber's Law of Just Noticeable Difference: Mutual difference between concentrations were greater than Just Noticeable Difference (JND) ($\Delta C/C \geqq 0.33$) (Table 4.1). To make the test solution, stock solution was diluted to different concentrations to make the test solution. The solutions were prepared 1 day before the experiment, mixed on a shaker overnight, and transferred to a 250mL Teflon bottle 10mins before experiments.

| **Odorants** | **Concentrations (PPM)** | | | | | |
|---|---|---|---|---|---|---|
| ***Rose Extract*** | 0.2 | 0.5 | 1 | 3 | 10 | 20 |
| ***Juniper Berry*** | 1 | 5 | 10 | 15 | 20 | 25 |
| ***Jasmine*** | 0.5 | 1 | 2.5 | 5 | 10 | 20 |
| ***Floracetate*** | 0.1 | 0.5 | 1 | 2 | 4 | 10 |

*Table 4.1: Perfumery raw materials concentrations used for threshold determination.*

**Conditioning Session.** For each flavor, highest concentration (bottle 6) was used for conditioning session. During the conditioning session, a 70ms-15mL odor blast will be puffed upon subjects click the mouse. Then the monitor will prompt "This smell is XXX (descriptor)". The subjects will repeat this process for up to 6 times to familiarize themselves with the odor. Subjects who were not able to smell this concentration were discontinued for the next session.

**Threshold Pre-testing Session.** For the pre-testing session, concentration used for conditioning session and a blank sample containing 50mL 10% PEG solution was used. After clicking the mouse, subjects will receive an odor blast and a binary forced choice question "Did you smell xxx (descriptor for that odorant)?". Subjects will be asked to select "Yes" or "No". The process was repeated for 5 puffs for each bottle; subjects were required to attain 90% accuracy on this trial before moving into the threshold measurement stage of the experiment.

**Experiment Session and Planning.** At each visit, subjects are asked to complete the threshold testing using both binary forced choice and tertiary forced choice. For the **binary forced choice** test, after clicking the mouse, subjects will receive an odor blast and be presented with a question: "Did you smell xxx (name/descriptor for that odor)". Subjects will then be asked to select "Yes" or "No". For the **tertiary forced choice** test, after clicking the mouse, subjects will receive an odor blast and be presented with a question: "What did you smell?". Subjects will then be asked to select among three choices: "XXX (name of the odor)", "something but not XXX (name of the odor)" or "Nothing". Subjects have to complete a binary-forced choice test and a tertiary forced choice test to complete one session. Under each test condition, there are 2 test rounds. In test round 1, bottles 1, 3, and 5 were puffed 4 times randomly each at each position in the triad, and in the testing round 2, bottles 2, 4, and 6 were puffed 4 times randomly each at each position in the triad (Figure 4.1).

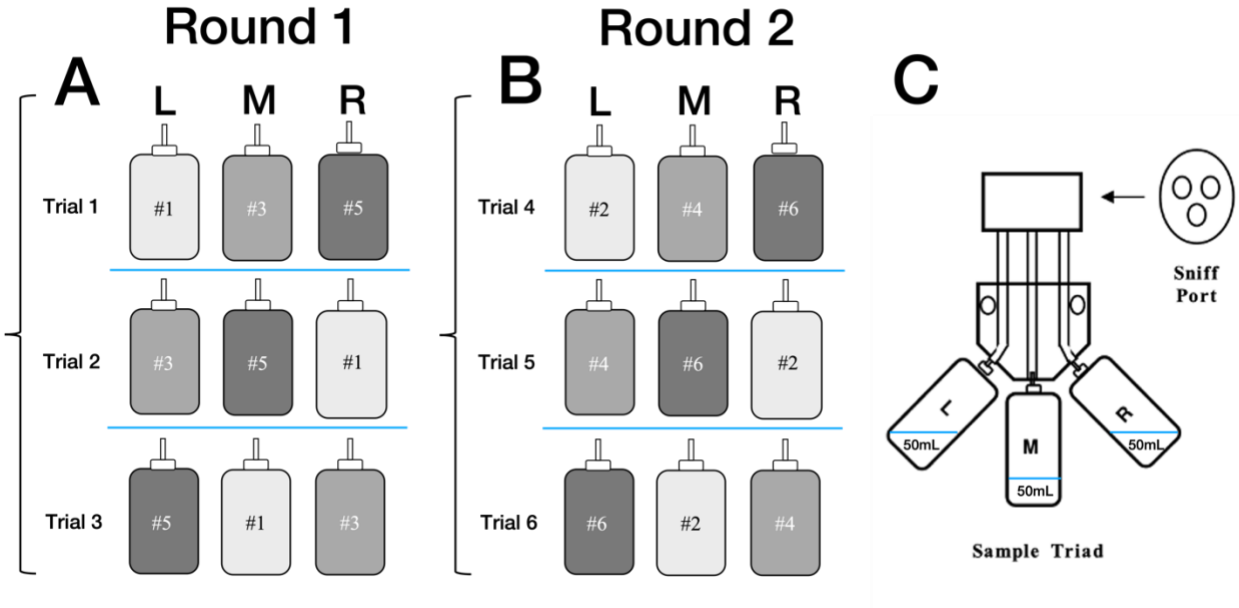


*Figure 4.1: Figure 4.1A shows the bottle arrangement for testing trial 1 to 3. Figure 4.1B shows the bottle arrangement for testing trial 4 to 6. Figure 4.1C shows the bottle arrangement in the sample triad.*

**Experiment Session and Planning.** During each of the first four visits, subjects were instructed to undergo two threshold measurements. The first measurement involved a binary forced choice test, while the second measurement involved a tertiary forced choice test. Over the course of the initial three days, thresholds for the three natural extracts (Rose, Jasmine, and Juniper Berry) were assessed (Table 4.2). During the fourth visit, the threshold for the single odorant Floracetate was tested. For the final visit, an equal-intensity mixture of Rose, Jasmine, Juniper Berry, and Floracetate was blended together to create a new odor referred to as "Bubble" (Table 4.3). Subjects were reconditioned to this new odor and subsequently tested for its threshold using both the binary forced choice and tertiary forced choice methods.

| EXPERIMENT TIME | EXPERIMENT CONDITIONS |
|---|---|
| 1ST VISIT | Rose Binary / Tertiary |
| 2ND VISIT | Jasmin / Tertiary |
| 3RD VISIT | Juniper Berry Binary / Tertiary |
| 4TH VISIT | Floracetate Binary / Tertiary |
| 5TH VISIT | Bubble Binary / Tertiary |

*Table 4.2: Table 4.2 shows the sequence of experiments each subjects have to complete five days.*

| | *Rose* | *Jasmin* | *Juniper Berry* | *Floracetate* | *Total* |
|---|---|---|---|---|---|
| **Bubble Bottle 1** | 0.2 PPM | 0.5 PPM | 1 PPM | 0.1 PPM | 1.8 PPM |
| **Bubble Bottle 2** | 0.5 PPM | 1 PPM | 5 PPM | 0.5 PPM | 7 PPM |
| **Bubble Bottle 3** | 1 PPM | 2.5 PPM | 10 PPM | 1 PPM | 14.5 PPM |
| **Bubble Bottle 4** | 3 PPM | 5 PPM | 15 PPM | 2 PPM | 25 PPM |
| **Bubble Bottle 5** | 10 PPM | 10 PPM | 20 PPM | 4 PPM | 44 PPM |
| **Bubble Bottle 6** | 20 PPM | 20 PPM | 25 PPM | 10 PPM | 75 PPM |

*Table 4.3: Table 4.3 shows the components and their concentrations in each bottle of Bubble.*

### 4.4 Results

**Threshold for Natural Extract.** The psychometric functions were generated by plotting the detection probabilities of odors against the logarithm of their concentrations. For the binary forced choice test, the recognition threshold was determined by interpolating the psychometric function at a detection probability of 0.5. For the tertiary forced choice test, the recognition threshold was obtained by interpolating the concentration at which the detection probability equals 0.33. The psychometric functions for Floracetate are depicted in Figure 4.2, while Figures 3.3-3.5 display the psychometric functions for Rose, Jasmine, and Juniper Berry. In these figures, the shaded regions represent the 95% confidence interval, and the $R^2$ values are calculated and displayed next to the predicted thresholds. In our experiment, an $R^2$ value greater than 0.7 indicates a good fit of the psychometric function, thus validating its reliability. With the exception of subject 1's Juniper Berry tertiary forced choice test, subject 3's Jasmine binary forced choice test, and subject 4's Floracetate tertiary forced choice test, all other psychometric curves for the natural extracts exhibited an $R^2$ value greater than 0.7. Furthermore, a paired two-tailed t-test comparing the single odorant Floracetate to the three odor mixtures yielded non-

significant differences (pRose=0.35, pJasmin=0.98, pJuniper Berry=0.96), confirming our ability to generate psychometric functions from configurally recognized odor mixtures.

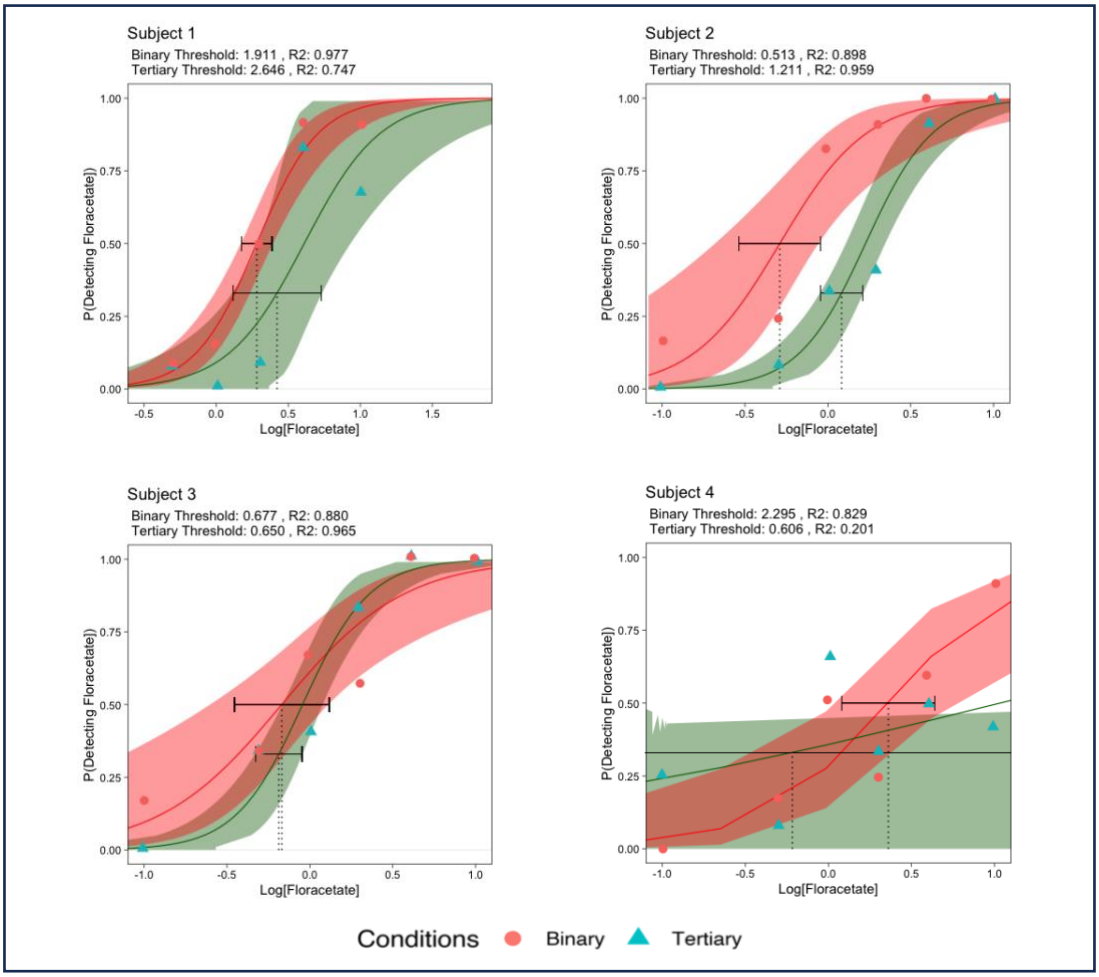


*Figure 4.2 : Figure 4.2 shows the fitted psychometric function for Floracetate under both binary and tertiary forced choice conditions. The 95% confidence interval were shown on the figure in shade. Thresholds interpolated from both functions and $R^2$ were shown and labeled on the figure.*

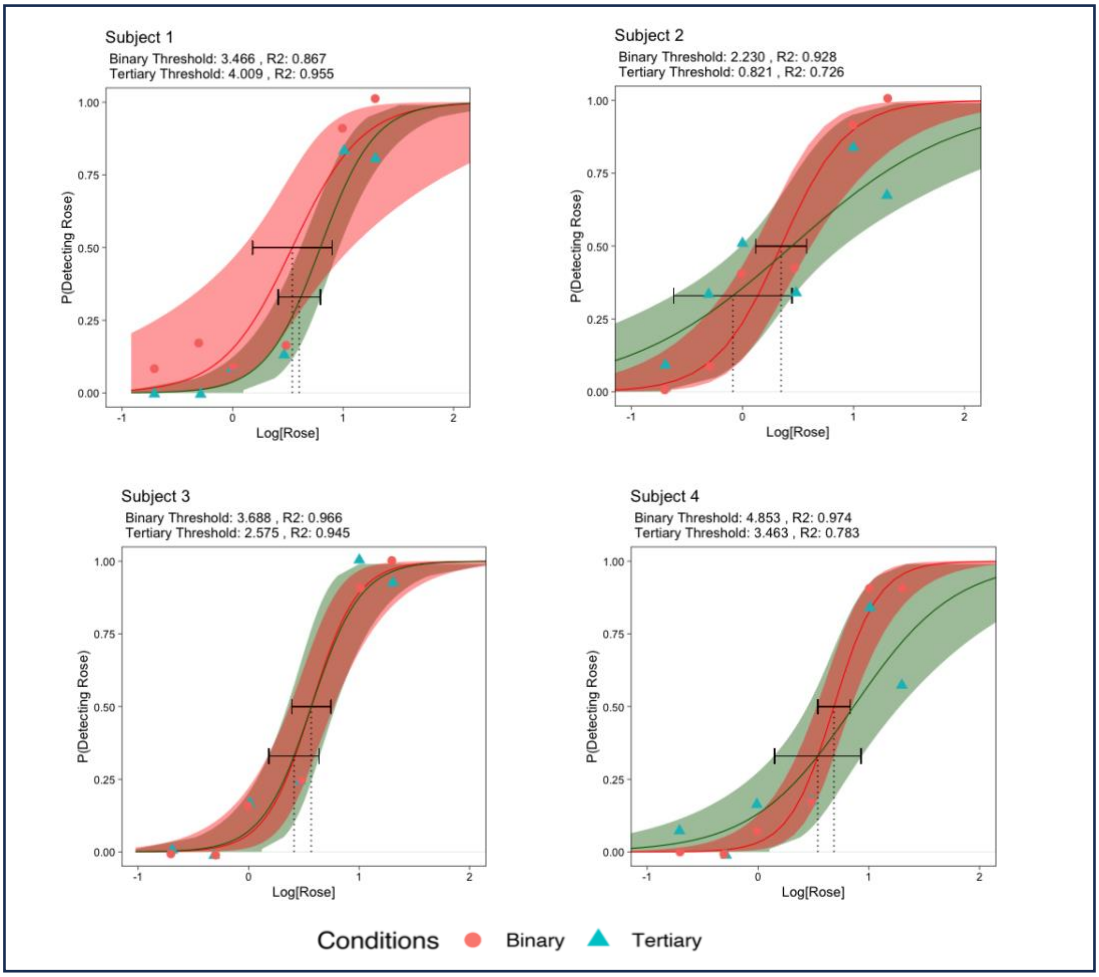


*Figure 4.3: Figure 4.3 shows the fitted psychometric function for Rose extract under both binary and tertiary forced choice conditions. The 95% confidence interval were shown on the figure in shade. Thresholds interpolated from both functions and $R^2$ were shown and labeled on the figure.*

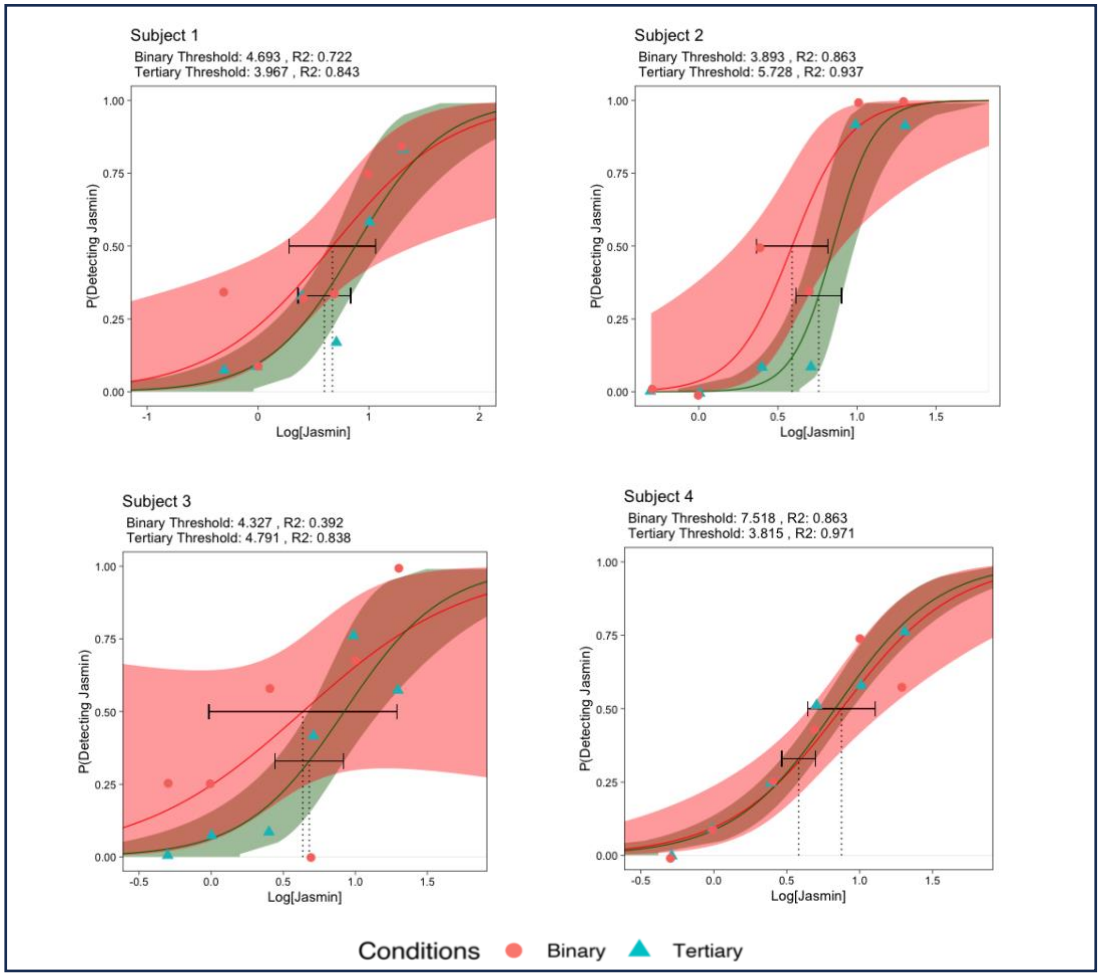


*Figure 4.4: Figure 4.4 shows the fitted psychometric function for Jasmin extract under both binary and tertiary forced choice conditions. The 95% confidence interval were shown on the figure in shade. Thresholds interpolated from both functions and $R^2$ were shown and labeled on the figure.*

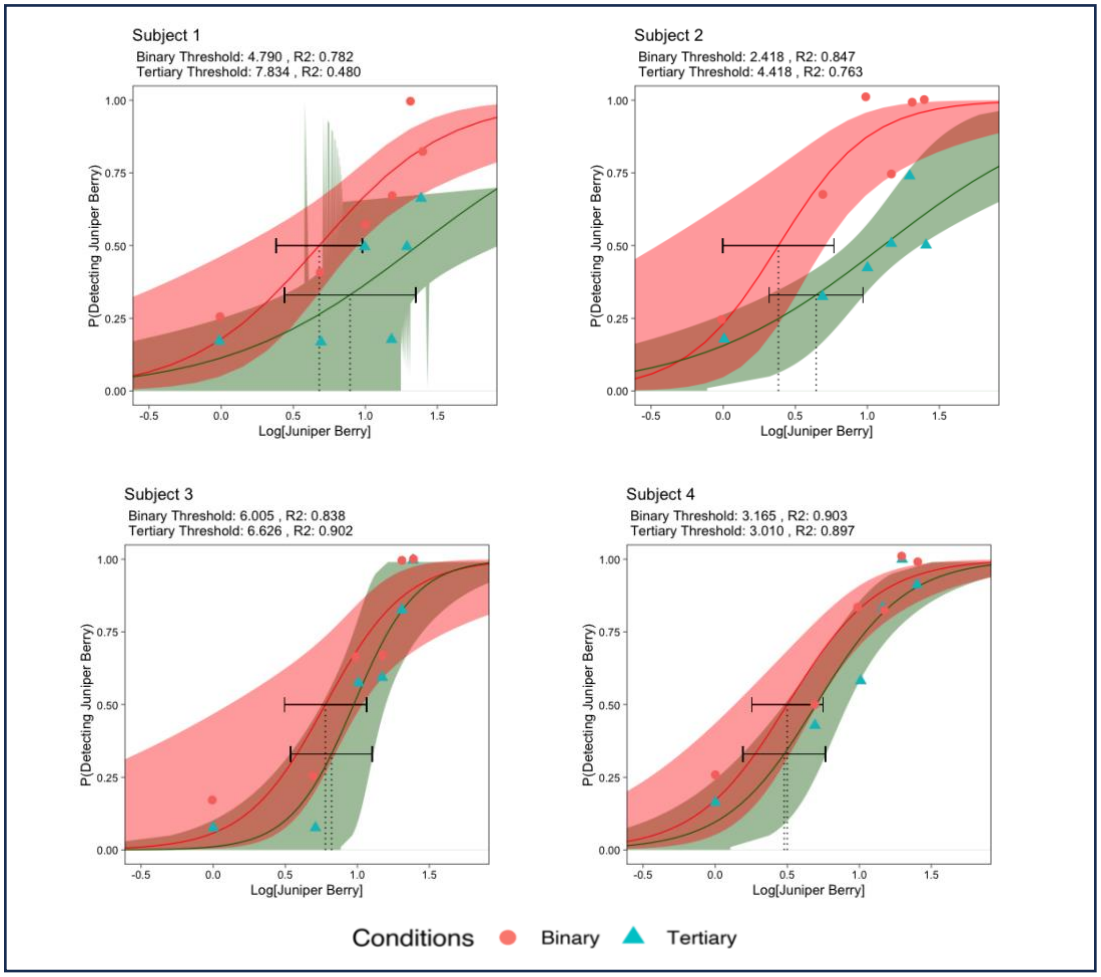


*Figure 4.5: Figure 4.5 shows the fitted psychometric function for Juniper Berry extract under both binary and. tertiary forced choice conditions. The 95% confidence interval were shown on the figure in shade. Thresholds interpolated from both functions and $R^2$ were shown and labeled on the figure.*

**Binary and Tertiary Forced Choice.** To assess if the binary and tertiary forced choice methods yield different thresholds, we conducted a two-tailed paired t-test (p=0.05) between the thresholds obtained from two methods. The results indicated that the odor recognition thresholds derived from the two methods were not significantly different from each other (Table 4.4).

| | FLORACETATE | ROSE | JASMIN | JUNIPER BERRY |
|---|---|---|---|---|
| **P-VALUE** | 0.90 | 0.16 | 0.61 | 0.15 |

*Table 4.4: This table shows the p-value for two-tailed paired t-test comparing the thresholds derived from binary and tertiary forced choice tests.*

To evaluate whether the tertiary forced choice method provides us with more information, we plotted the number of "Something Not XXX (Odor name)" selections across all concentrations for the three natural extracts and Floracetate (Figure 4.6). The p-value of the one-way ANOVA conducted on the combined data for the number of "Something Not XXX (Odor name)" selections for the three natural extracts and Floracetate is 0.01. The Tukey HSD test indicates that there is a significant difference in the number of "Something Not XXX (Odor name)" selections between concentration 4 and concentration 5. Additionally, concentrations 3 and 4 on average have the highest number of "Something" selections. The average detection probabilities of concentration 3 and 4 under both binary and tertiary forced choice conditions were calculated and are shown in Table 4.5. The detection probabilities under all conditions are close to the theoretical detection probability for the threshold (0.5 for binary, 0.33 for tertiary).

| | B-3 | B-4 | T-3 | T-4 |
|---|---|---|---|---|
| **P**DETECTION | 0.48 | 0.45 | 0.32 | 0.37 |

*Table 4.5: Table 4.4 shows average detection probability at concentration 3 and 4 under both binary and tertiary forced choice conditions. B-3/B4 stands for binary forced choice concentration 3 and 4. T-3/T4 stands for tertiary forced choice concentration 3 and 4.*

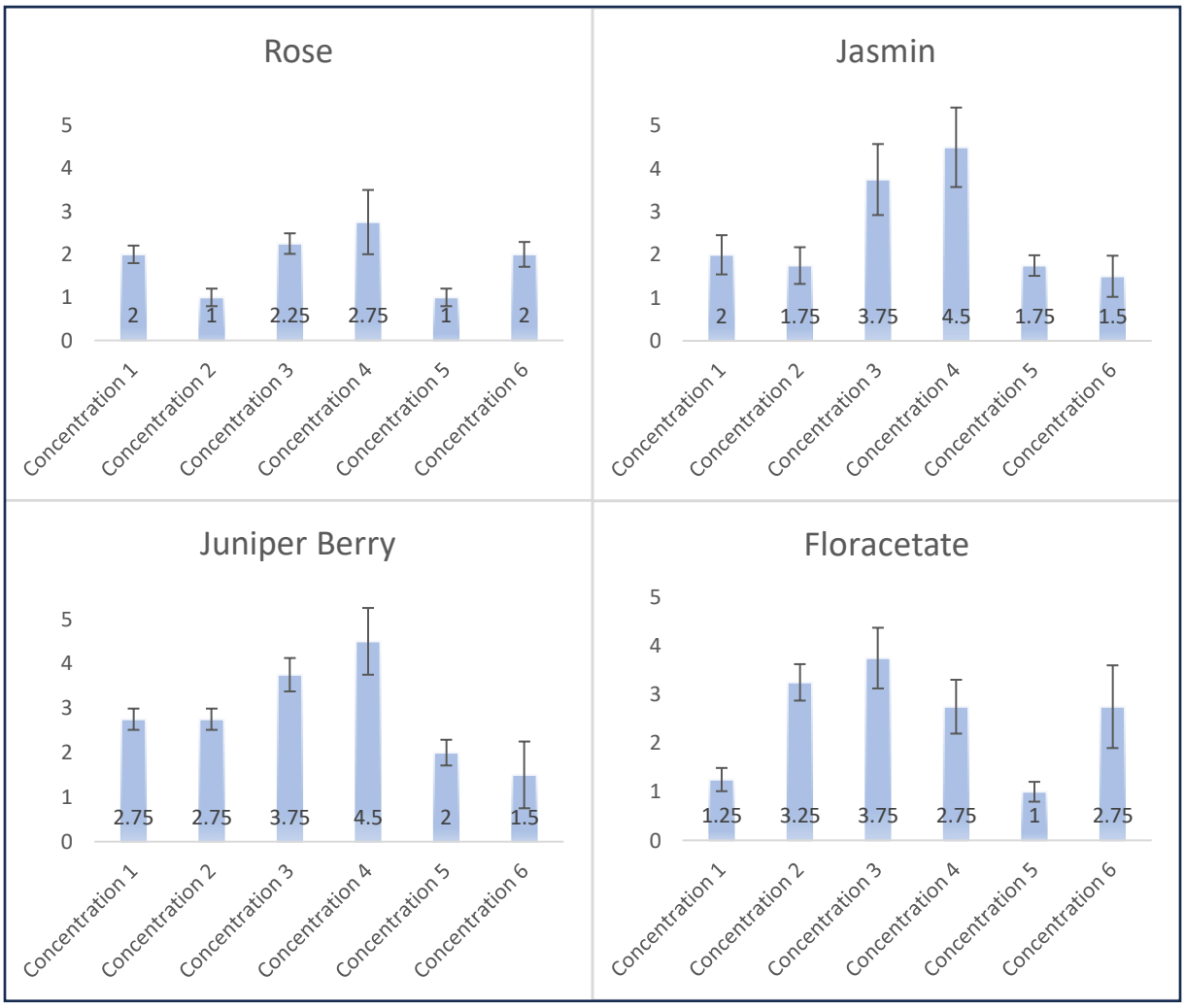


*Figure 4.6: Figure 4.6 shows the number of "Something Not XXX (Odor name)" subjects selected across all concentrations for 3 natural extracts and Floracetate.*

**Threshold for Conditioned New Mixture.** After conditioning the subjects to recognize the mixture of the four components as a new odor called "Bubble," we obtained the recognition probabilities using both binary and tertiary forced choice methods. The recognition probabilities were plotted against the log of the concentration (the sum of the individual component concentrations) (Figure 4. 7). The interpolated thresholds and $R^2$ values were shown in the plot. The paired two-tailed t-test showed no significant difference between the thresholds derived from the binary and tertiary forced choice tests ($p = 0.31$). Except for subject 4, all other subjects had $R^2$ values greater than 0.87, indicating that they were able to generate psychometric curves after being conditioned to the new odor mixture.

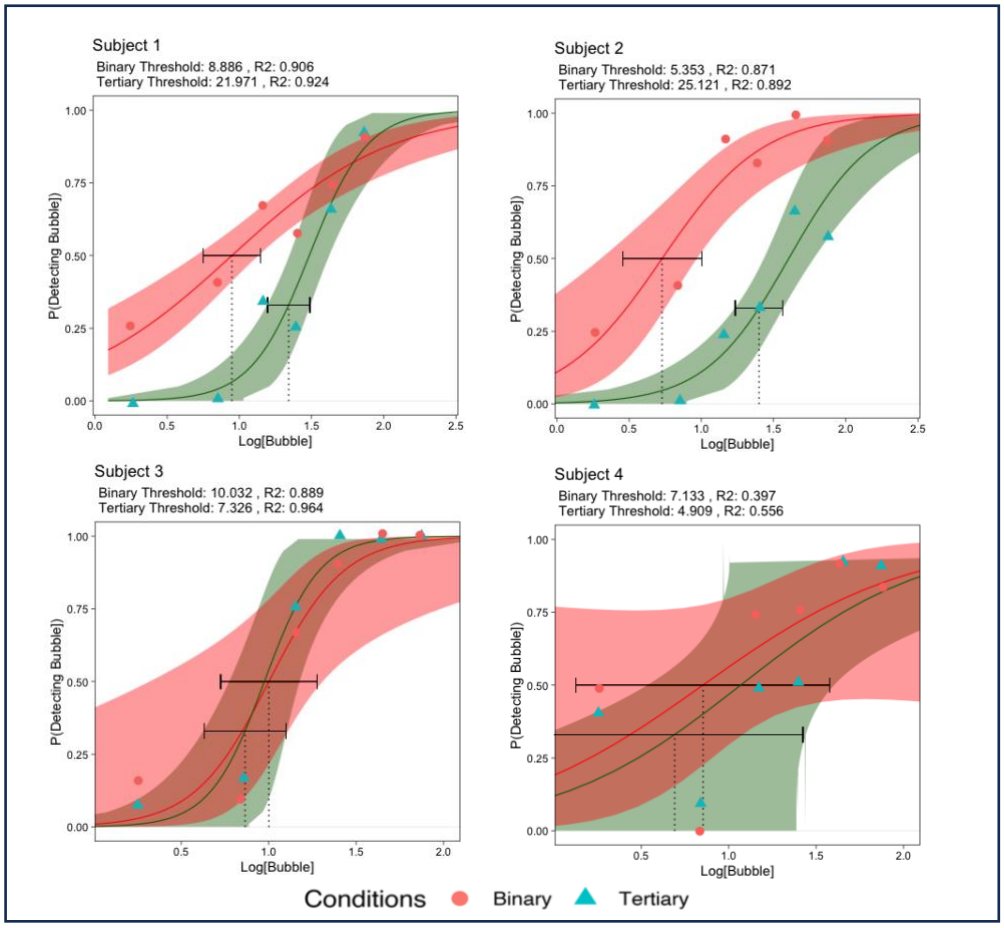


*Figure 4.7: Figure 4.7 shows the fitted psychometric function "Bubble" under both binary and tertiary forced choice conditions. The 95% confidence interval were shown on the figure in shade. Thresholds interpolated from both functions and $R^2$ were shown and labeled on the figure.*

Furthermore, the number of "Something Not Bubble" selections at each concentration was counted and plotted in figure 4.8. The pattern observed in figure 4.8 is consistent with the components of "Bubble", and the One-way Anova shows no significant difference (p-value = 0.58), which indicates that the configurality of this new mixture was maintained throughout the threshold measurement.

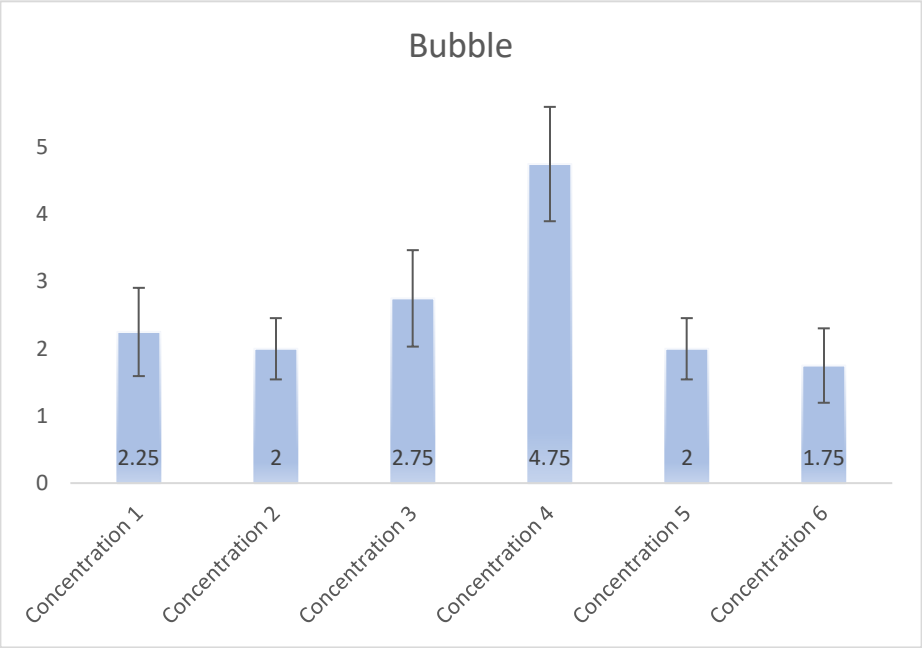


*Figure 4.8: Figure 4.8 shows the number of "Something Not Bubble" subjects selected across all concentrations.*

**4.5 Discussion**

A single odorous molecule (odorant) is the smallest unit in odor perception. It requires at least one odorant to be present above its threshold concentration to be perceived as an odor. When multiple odorants are mixed, they can interact with each other in various ways, including suppression, hypoadditivity, synergy, inhibition, and overshadowing (Rospars, 2008; Marasco, 2016). These interactions become more pronounced as the mixture becomes more complex, resulting in the emergence of new perceivable properties that encompass both elemental and configural aspects. In our previous studies, we demonstrated that with appropriate conditioning, the thresholds of elemental properties in binary and tertiary odor mixtures can be measured using psychometric functions (Rochelle, 2017). In this experiment, by successfully generating psychometric functions using natural extracts, we have further shown that the configural

property of odor mixtures can also be measured psychophysically. This finding supports the notion that whether a mixture is perceived more elementally or configurally is partially dependent on personal experience and conditioning (Livermore, 1997; Coureaud, 2009; Sinding, 2011).

**Configural Odor Mixture Perception.** When we combined Rose, Jasmin, Juniper Berry, and Floracetate at six concentration levels to create a new odor called "Bubble" (Table 4.3), the detection probabilities of "Bubble" in a binary forced choice test were not significantly different from its individual components (Figure 4.9). In the tertiary forced choice threshold testing, we conditioned subjects using the highest concentration (6th concentration), and we instructed them to select "Something not Bubble" if they detected an odor that was different from what they were conditioned to. The number of "Something not XXX" selected for the “Bubble” were significantly different from its components. These results demonstrate that the response to odor mixtures is not a linear process from the perspective of recognition probabilities (Singh, 2019). Based on this logical reasoning, future studies can focus on exploring odor interactions using a top-down approach. For example, we can generate a psychometric function for the rose extract using six concentrations. Then, after measuring the individual concentrations of its components, we can use six concentrations of the components to generate another psychometric function. By comparing the detection probabilities and the slope and shift of the psychometric functions, we can understand how the individual odor components interact to create a common odor, such as the combination of rose and jasmin. This approach allows us to quantify both the qualitative and quantitative aspects of odor interactions.

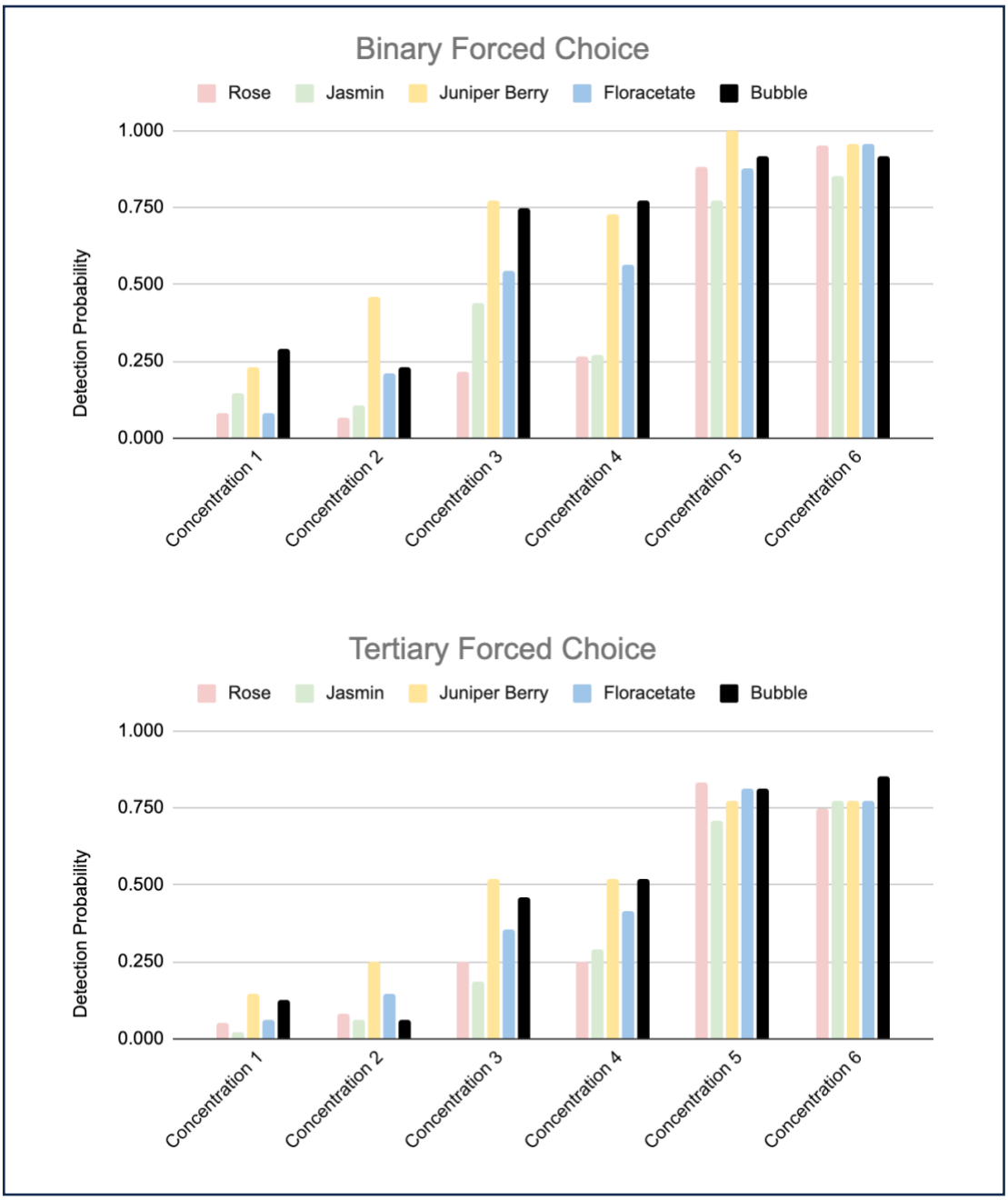


*Figure 4.9: Figure 4.9 detection probability of Rose, Jasmin, Juniper Berry, Floracetate and Bubble at each concentration level.*

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Chapter 5

# Masking Effects on *Iso*-valeric Acid Recognition by Sub-threshold Odor Mixture

## 5.1 Abstract

Masking unpleasant odors with pleasant-smelling odorants has a long history and is utilized in various industries, including perfumery and consumer products. However, the effectiveness of odor masking is idiosyncratic and temporary. In this study, we employed Sniff Olfactometry (SO) to investigate the psychophysics of masking using brief 70ms stimulations with mixtures of the mal-odorant iso-valeric Acid (IVA) and different masking agents. IVA is a component of human sweat that can overpower its smell and is often associated with unpleasant descriptors such as "gym locker," "smelly feet," "dirty clothes," and so on. Traditionally, high concentrations of pleasant-smelling odorants are used to mitigate the unpleasantness of IVA in situations involving clothing or environments contaminated with IVA. To examine the masking effects of sub-threshold levels of various masking agents (neohivernal, geraniol, florhydral, decanal, iso-longifolanone, methyl iso-eugenol, and s-limonene) on IVA, we conducted experiments using SO to measure the probability of recognizing IVA after 70ms stimulations with headspaces containing mixtures of super-threshold concentrations of IVA and sub-threshold concentrations of IVA-suppressors. The study involved nine subjects, and on average, a single masking agent was found to decrease IVA recognition probability by 14% to 72%. Moreover, a sub-threshold odor mixture consisting of six masking agents demonstrated a substantial decrease in IVA recognition, with a reduction of 96%.

## 5.2 Introduction

Consumers often choose scented products over unscented versions to combat the presence of malodors commonly associated with consumer products (Herz et al., 2022). The use of pleasant odors to mask malodors has been shown to reduce the negative impact on well-being caused by malodors (Dalton et al., 2020). Traditionally, strong pleasant odors such as citronellal, limonene, and citral have been employed to mask unpleasant odors like dimethyl sulfide (Osada et al., 2013). However, exposure to high levels of fragrance has been linked to various health hazards, including sensory irritation, respiratory symptoms, and lung dysfunction (Kim, 2015; Steinemann, 2016).

In order to minimize consumers' exposure to excessive amounts of odorants, we investigated the feasibility of using peri- and/or sub-threshold concentrations of masking agents to mask the malodor caused by iso-valeric Acid (IVA), a compound found in human sweat known for its unpleasant odor and associated descriptors such as "gym locker," "smelly feet," and "dirty clothes." While the definitions of odor masking (modifying perceived odor quality to enhance acceptability) and odor counteraction (reducing perceived intensity) are well-established in the literature (Cain, 1974; Hoffmann, 1986; Bell, 1987; Jones, 1964; Laing & Francis, 1989; Laing, 1984; Oka, Omura, et al., 2004; Osada et al., 2013), limited research has focused on the specific concentrations of masking agents required to effectively mask malodors.

In this study, we define "**Odor Covering**" as using supra-threshold amount of strong pleasant odorants to cover the malodor (counteraction and masking), while "**Odor Masking**" refers to the

use of barely detectable amounts (sub-threshold or peri-threshold concentrations) of pleasant odorants to make malodors undetectable or unrecognizable.

**Experiment Design Rationale.** Odor perception involves the binding of odorants to specific olfactory receptors (ORs) in the olfactory epithelium, resulting in unique coding patterns (Buck, 1996; Kajiya et al., 2001; Malnic B, 1999). These odorants can either stimulate (agonists) or inhibit (antagonists) certain ORs, leading to distinct receptor activations (Marc Spehr, 2003; Oka, Nakamura, et al., 2004; Oka, Omura, et al., 2004; Ricardo C. Araneda, 2000). The encoded signals are then transmitted to the olfactory bulbs (OBs), where they are further processed and decoded in the central nervous system (Strutz et al., 2014). It is well-established that human odor perception is influenced by specific receptor activations and inhibitions (Keller et al., 2007; Menashe et al., 2007; Pfister et al., 2020; Reddy et al., 2018). Preliminary research has indicated that odorants that act as antagonists to malodors can reduce their intensity when mixed (Aya Kato, 2015; KAO et al., 2019). Iso-valeric Acid (IVA) has been identified as an agonist for the OR51E1 receptor.

In this study, seven perfume raw materials (PRMs) were selected as potential masking agents for IVA. These include Neohivernal (Neo), which has been reported to reduce IVA intensity (Stacy Renee Hertenstein et al., 2017), and Florhydral (Flo), which has been identified as an antagonist to OR51E1 (Aya Kato, 2015). Additionally, Methyl Iso-eugenol (Met), Decanal (Dec), Iso-longifolanone (Long), Geraniol (Ger), and s-Limonene, PRMs traditionally used to reduce malodor but not interacting with OR51E1 (Bushdid et al., 2018; Halperin Kuhns et al., 2019; Saito et al., 2009; Sean M. Wetterer, 2015), were included. Since odor coding is concentration-

dependent for both single odorants and mixtures (Kajiya et al., 2001; Xu et al., 2020), and humans brain can respond to sub-threshold odorants (Hummel et al., 2013), our hypothesis is that sub-threshold concentrations of IVA odor-suppressors can partially mask human perception of IVA. To investigate the effects of sub-threshold IVA odor-suppressors, we used Sniff Olfactometry (SO) to compare the detection probability of IVA after 70ms stimulations with headspaces containing mixtures of super-threshold concentrations of IVA with and without sub-threshold concentrations of IVA-suppressors.

## 5.3 Materials and Methods

### Chemicals.

Polyethylene Glycol 400 (PEG400): CAS Registry No. 9002-88-4, 90% Deionized Water: carbon filtered deionized water, Charcoal powder: CAS Registry No. 7440-44-0, *Iso*-valeric Acid (IVA): CAS Registry No. 503-74-2, Geraniol (Ger): CAS Registry No. 106-24-1, Neohivernal (Neo): CAS Registry No. 300371-33-9, Florhydral (Flo): CAS Registry No.125109-85-5, Decanal (Dec): CAS Registry No. 112-31-2, Isolongifolanone (Long): CAS Registry No. 14727-47-0, Methyl *Iso*-eugenol (Met): CAS Registry No. 6379-72-2, *S*-Limonene (Lim): CAS Registry No. 5989-54-8

**Ethics Statement.** The experiment was conducted under the approval of Cornell University Institutional Review Board (IRB) and followed Declaration of Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013).

**Subjects.** 9 subjects including 7 females and 2 males, all students from Cornell University (22-27 years old), were tested. Subjects participated were asked to sign the agreement (Supplemental) and screened to make sure they 1) do not have a stuffy nose before each session; 2) do not have post-COVID anosmia; 3) could identify all the compounds presented in the session by passing the conditioning and pretesting session.

**Software.** The experiments were automated using PsychoPy® (v2021.2.3) (Peirce et al., 2019). Data analysis was executed using R (version 4.1.3 – "One Push-Up") (R-Core-Team, 2022) and Python 3.11.2 (Van Rossum, 2009). See supplemental.

**Equipment.** Sniff Olfactometry (SO)(Rochelle et al., 2017; Wyckoff & Acree, 2017) was used to investigate the psychophysics of masking during 70ms-stimulations with mixtures of the mal-odorant *iso*-valeric Acid (IVA) and different masking agents.

**Sample Preparation.**

**Stock Solution Preparation.** 1000 PPM stock solution for each odorant in PEG 400 was prepared. 0.1g of each odorant was added to 100mL amber bottle followed by 100mL PEG 400 addition.

**10% PEG – Water Solution Deodorization.** 400mL of PEG 400 was added 3600mL of DI water. The mixture solution was added 20g of Charcoal powder and vigorously mixed. The mixture was set for 2 days and performed vacuum filtration to remove the charcoal powder to obtain the deodorized 10% PEG – water solution.

**Test Sample Solution Preparation**. Each odorant was diluted to different concentrations to make 50mL 10% PEG water solution. These solutions were prepared one day prior to the experiment and mixed on a shaker overnight to ensure uniform distribution. Ten minutes before the experiments, the solutions were transferred to a 250mL Teflon bottle.

**Threshold Determination.**

**Descriptor Determination**. Each odorant used in the study was assigned a consensus descriptor based on bench-top sniffing conducted by lab members to capture their odor characteristics. The assigned descriptors for the odorants are as follows: Iso-valeric Acid (IVA) - "Stinky Feet", Neohivernal - "Clean Laundry", Geraniol - "Rosey", Florhydral - "Floral", Iso-longifolanone - "Woody", Decanal - "Soapy", Methyl Iso-eugenol - "Clove", and S-limonene - "Citrus".

**Concentrations Determination.** The concentrations were determined based on the following rules: Firstly, the mutual difference between concentrations was set to be greater than the Just Noticeable Difference (JND), with the criterion $\Delta C/C \geqq 0.33$. This ensures that the concentrations are distinct enough to be perceptually discriminated. Subsequently, two test runs were conducted on lab members to verify that the chosen concentrations can generate a logistic function that accurately predicts the range for odor recognition threshold. This step ensures that the selected concentrations effectively cover the desired concentration range for the threshold measurements.

**Conditioning and Training**. During the conditioning and training phase, the highest concentration of the odorant was used. Each subject took a seat and adjusted the chair to the appropriate height, positioning their nose above the sniffing port before the PsychoPy® session began (Ni et al., 2022). The subjects initiated the first trial and followed the instructions and cues

displayed on the screen until the trial concluded, typically lasting around 8 seconds. The prompts included instructions such as "Click when you are ready," "When you are ready to inhale, Click again," "Inhale," and "Exhale." 700ms after the cue to inhale was presented, the Sniff Olfactometer (SO) released a 15ml burst of headspace gas for 70ms. Following this, the monitor displayed the prompt "This smell is XXX (descriptor)," indicating the odor being presented. This process was repeated up to six times to familiarize the subjects with the odor. Subjects who were unable to detect the odor at this concentration were excluded from the subsequent sessions. All nine subjects successfully passed this session, indicating their ability to detect the odor.

**Pre-testing Session.** During the threshold measurement stage, the bottles containing the odorant solution and the blank sample (10% PEG and water) were used. The procedure was the same as in the training session, with the addition of a binary forced choice question displayed on the monitor after the puff: "Did you smell xxx (descriptor for that odorant)?" The subjects were required to choose either "Yes" or "No" to proceed. Subjects were informed that they might perceive the target odor, something different from the target odor, or nothing at all. In cases where they were unsure about what they had detected, they were instructed to randomly choose either "Yes" or "No". This process was repeated for five puffs from each bottle. Subjects needed to achieve 90% accuracy on this trial before proceeding to the subsequent threshold measurement phase of the experiment.

**Threshold Measurement.** 6 bottles containing ascending concentrations of an odorant was labeled 1 to 6 (supplemental table 1). In the first round, bottles 1, 3, and 5 were randomly puffed four times at each position in the triad, and in the second round, bottles 2, 4, and 6 were randomly puffed four times at each position in the triad (Ni et al., 2022). The probability of detecting the odor at each designated concentration was plotted against the log concentration and

fitted to a binary logistic model to generate a psychometric function. The recognition threshold was determined by calculating the concentration at which the detection probability equals 0.5 (Ni et al., 2022; Wichmann, 2018). The thresholds for both the and the seven masking agents were measured for all nine subjects. The threshold measurement was performed one to three times for each subject. Based on their sensitivity to IVA, the subjects were divided into two groups. Group 1 consisted of subjects who were sensitive to IVA (recognition threshold < 4PPM), and Group 2 consisted of subjects who were moderately sensitive to IVA (recognition threshold > 4PPM). Subjects who were hyposensitive to IVA were not included in the experiments.

**Masking Effect Determination.**

**Masking IVA by subthreshold single odor mixture.** In each of the three bottles of the Sniff Olfactometer (SO) triad, the following were added: 50mL of a supra-threshold amount of IVA in Bottle 1, 50mL of a mixture containing a supra-threshold amount of IVA and a sub-threshold level of the first masking agent in Bottle 2, and 50mL of a mixture containing a supra-threshold amount of IVA and a sub-threshold level of another masking agent in Bottle 3. Each experiment session consisted of three trials, where each bottle was puffed four times at each position on the triad (Figure 5.1). After each puff, subjects were asked to respond with a "Yes" or "No" to the question: "Did you smell Stinky Feet (IVA)?" Each subject completed two sessions during each visit. The purpose of this step was to determine if the addition of sub-threshold/peri-threshold odor masking agents could decrease the detection probability of IVA.

**Masking IVA using sub-threshold amount of masking agent mixture.** In the experiment, three bottles were prepared on the Sniff Olfactometer (SO) triad. Bottle 1 contained a 50mL solution with a supra-threshold amount of IVA. Bottle 2 contained a 50mL mixture with

a supra-threshold amount of IVA combined with a sub-threshold level of a masking agent mixture. Bottle 3 contained a blank sample consisting of a 10% PEG in water solution. The concentration of IVA used depended on the subject's sensitivity: 10 PPM for IVA-sensitive subjects and 15 PPM for subjects with moderate sensitivity to IVA. Each experiment session consisted of three trials, with each bottle being randomly puffed four times at each position on the triad (as shown in Figure 5.1). After each puff, subjects were asked to indicate whether they smelled "Stinky Feet" (IVA) by answering "Yes" or "No" to the question. Subjects were instructed to respond "Yes" if they detected even a trace amount of IVA. Each subject completed two sessions during each visit. Following the experiment session, a verbal interview was conducted with each subject. The interview aimed to gather information on whether the subjects detected any odor other than IVA and, if so, whether the odor was perceived as pleasant or unpleasant.

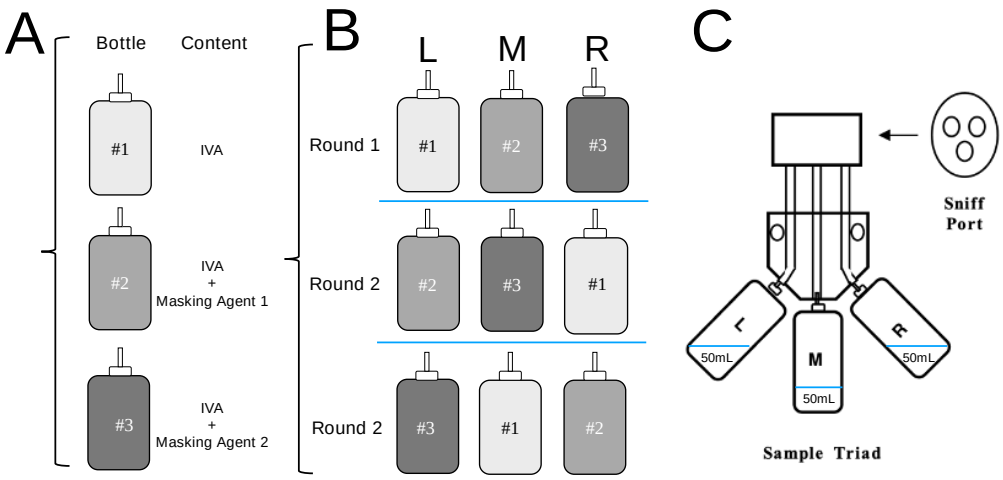


*Figure 5.1: Figure 5.1A shows the content of the solution in each bottle. Figure 5.1B shows 3 bottles was puffed 4 times randomly in one trial. For each experiment session, total of 3 trials were conducted, so that each bottle was puffed 4 times at each position on the triad. Figure 5.1C displays the arrangement of bottles relative to the sniff port.*

## 5.4 Results

**Thresholds Measurement.** The thresholds for IVA and the Masking Agents were measured for each subject using the Sniff Olfactometer (SO), and the values can be found in the supplement. Based on the obtained threshold for IVA, the subjects were divided into two subgroups: Group 1 and Group 2. Group 1 consists of subjects 1, 2, 3, 4, and 5, who were found to be sensitive to IVA (threshold < 4PPM). Group 2 includes subjects 6, 7, 8, and 9, who were moderately sensitive to IVA (threshold > 4PPM).

**Experiment Concentration Determination.** In Group 1, a concentration of 5PPM of IVA was used for the subjects, while in Group 2, a concentration of 10PPM of IVA was used. These concentrations were selected to ensure that IVA was detectable but not excessively strong for the subjects. Additionally, concentrations were chosen for the masking agents that were approximately half of their individual detection thresholds (as shown in table 5.1). However, since the concentrations of Lim1 (limonene) were recognized by all subjects, the experiment was repeated using a lower concentration of 1PPM of limonene for all subjects.

**Masking Effect Measurement 1.** The IVA detection probability was calculated using the formula: $P_{(IVA\ Detection)}$ = Number of IVA stimuli detected / total number of IVA stimuli (Supplemental). The results were plotted in a whisker plot, showing the IVA detection probability for different experiment conditions (Figure 5.2). A one-tailed paired t-test with a significance level of 0.05 was conducted on the IVA detection probability with and without masking agents (Supplemental Table 3), revealing a significant decrease in IVA detection probability under all conditions. Following the SO experiment, subjects were asked if they were able to detect any other smells. Out of the 9 subjects, 7 were able to detect limonene at 1 PPM.

Consequently, additional tests were conducted using lower concentrations of limonene (0.02 PPM and 0.05 PPM) to assess their masking effects against IVA. Additionally, to minimize chemical exposure to the subjects, a lower concentration of methyl iso-eugenol (0.5 PPM) was also tested (Table 5.2).

**Masking Effect Measurement 2.** The IVA detection probability was calculated with lowered concentrations of s-limonene, methyl iso-eugenol, and iso-longifolanone (Supplemental). The results were presented in a whisker plot shown in Figure 5.3. A one-tailed paired t-test with a significance level of 0.05 was conducted on the IVA detection probability with and without masking agents (Supplemental Table 4), revealing a significant decrease in IVA detection probability when mixed with 0.05 PPM Limonene, 0.3 PPM Iso-longifolanone, and 0.5 PPM Methyl Isoeugenol. IVA habituation was observed during sessions 5 and 6, where the IVA detection probability for pure IVA dropped to around 50%. A one-tailed paired t-test with a significance level of 0.01 was conducted between the IVA detection probability during the last two visits and the first four visits (Supplemental Table 5 & 6). The results showed a significant decrease in IVA detection probability starting at the 5th visit. However, there was no significant difference observed among the first four visits or between the 5th and 6th visits (p-value = 0.65).

| | IVA | Neo | Flo | Dec | Long 1 | Ger | Lim 1 | Met 1 | Lim 2 |
|---|---|---|---|---|---|---|---|---|---|
| **Subject 1** | 5 | 0.5 | 0.4 | 0.3 | 2 | 0.2 | 3 | 40 | 1 |
| **Subject 2** | 5 | 0.5 | 0.01 | 0.3 | 4 | 0.2 | 3 | 3 | 1 |
| **Subject 3** | 5 | 0.1 | 0.01 | 0.3 | 2 | 0.2 | 0.15 | 15 | 1 |
| **Subject 4** | 5 | 1 | 0.04 | 0.3 | 0.5 | 0.2 | 3 | 15 | 1 |
| **Subject 5** | 5 | 0.5 | 0.04 | 0.6 | 2 | 0.7 | 10 | 40 | 1 |
| **Subject 6** | 10 | 0.5 | 0.4 | 0.3 | 4 | 0.3 | 3 | 15 | 1 |
| **Subject 7** | 10 | 0.5 | 0.01 | 0.3 | 2 | 0.7 | 3 | 10 | 1 |
| **Subject 8** | 10 | 0.1 | 0.01 | 0.01 | 2 | 0.3 | 1.2 | 15 | 1 |
| **Subject 9** | 10 | 0.5 | 0.04 | 0.6 | 4 | 0.1 | 3 | 15 | 1 |

*Table 5.1: Table displays the concentrations of each PRM used in the masking study, measured in parts per million (PPM).*

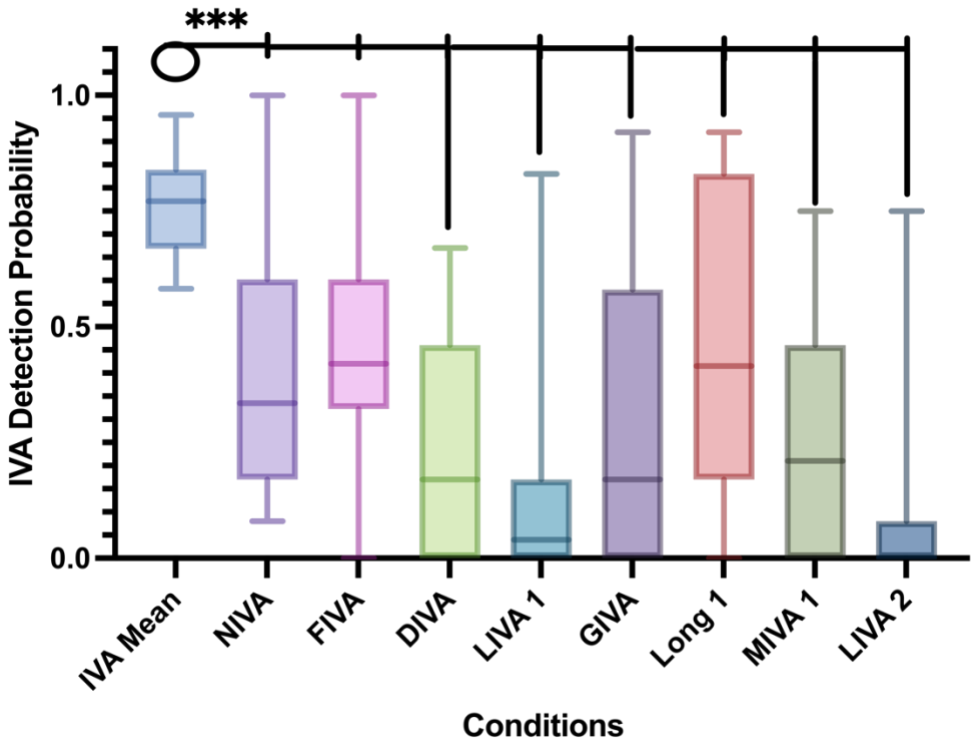


*Figure 5.2: Figure 5.2 presents a box plot of the IVA detection probability under all experimental conditions. The data for the IVA mean were obtained by averaging the IVA detection probabilities across all trials. The abbreviations NIVA, FIVA, DIVA, GIVA, Long, MIVA, and Lim represent the combinations of neohivernal + IVA, florhydral + IVA, decanal + IVA, geraniol + IVA, iso-longifolanone + IVA, methyl iso-eugenol + IVA, and s-limonene + IVA, respectively. The results of the one-tailed paired t-test are indicated in the figure with significance labels ("***" = 0.001, "**" = 0.01, "*" = 0.05).*

| | IVA | Lim 3 | Lim 4 | Met 2 | Long 2 |
|---|---|---|---|---|---|
| **Subject 1** | 5 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 2** | 5 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 3** | 5 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 4** | 5 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 5** | 5 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 6** | 10 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 7** | 10 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 8** | 10 | 0.02 | 0.05 | 0.5 | 0.3 |
| **Subject 9** | 10 | 0.02 | 0.05 | 0.5 | 0.3 |

*Table 5.2: Table illustrates the concentrations of each odorant used in the second round of IVA masking analysis.*

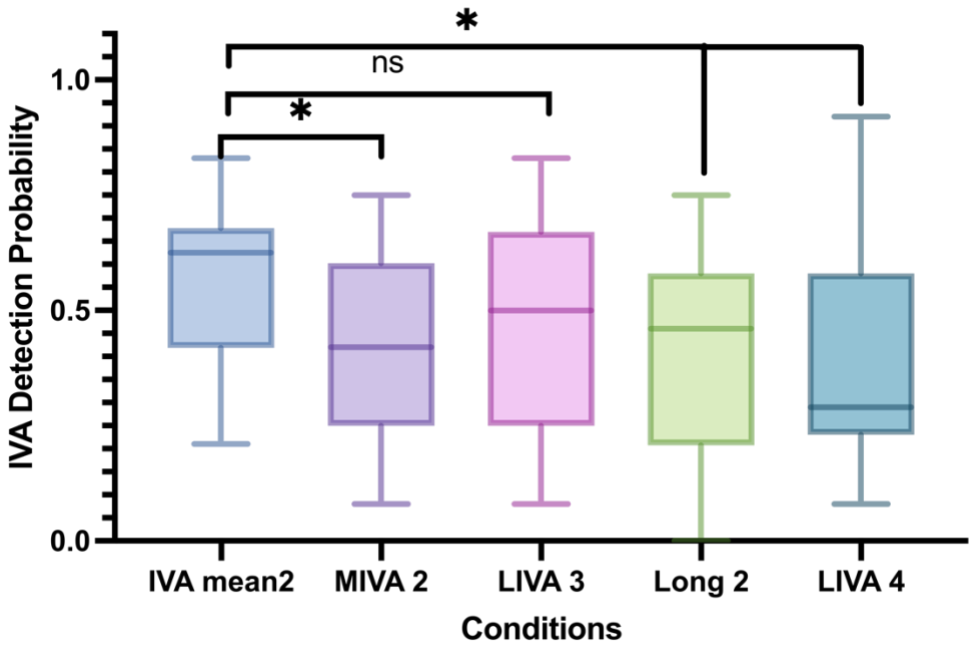


*Figure 5.3: Figure 5.3 presents the IVA detection probability with lowered concentrations of masking agents for methyl iso-eugenol, limonene, and iso-longifolanone. The results of the one-tailed paired t-test are labeled in the figure using the significance labels described above.*

**Dose Dependence of Odor Masking.** To examine the relationship between the dose of a masking agent and its masking effect, we calculated the masking capacity using the equation: Masking Capacity = $(P_{IVA} - P_{Masked\ IVA})/ P_{IVA}$, where $P_{IVA}$ represents the IVA detection probability without masking, and $P_{Masked\ IVA}$ represents the IVA detection probability with masking agent. In this study, we focused on the dose-masking relationship using s-limonene. Figure 5.4 illustrates the overall masking capacity, which includes both masking and covering effects, plotted against the concentration of s-limonene used. From 0.02 PPM to 0.05 PPM, there is an 16% increase in the average Limonene masking capacity. However, when comparing the masking capacities for IVA with 1PPM limonene and 2.65PPM limonene, there was no difference observed. The average IVA masking capacity was 0.88 for 1PPM limonene and 0.87 for 2.65PPM limonene. These findings suggest that the masking capacity has the most significant increase at sub-threshold and peri-threshold levels of s-limonene. Beyond a certain concentration, further increases in dose do not significantly enhance the masking capacity. These results support the assumption that the best masking effects can be achieved by using sub-threshold levels of masking agent mixtures.

**Masking Agents Mixture Determination.** To address the issue of IVA habituation, subjects in Group 1 were exposed to 10PPM IVA, while subjects in Group 2 were exposed to 15PPM IVA. In each triad, three bottles were used: one containing a blank solution (10% PEG), one containing 10PPM or 15PPM IVA, and one containing 10PPM or 15PPM IVA mixed with a 1.2PPM masking agent mixture. The masking agent mixture consisted of all masking agents except for methyl iso-eugenol (Table 5.3).

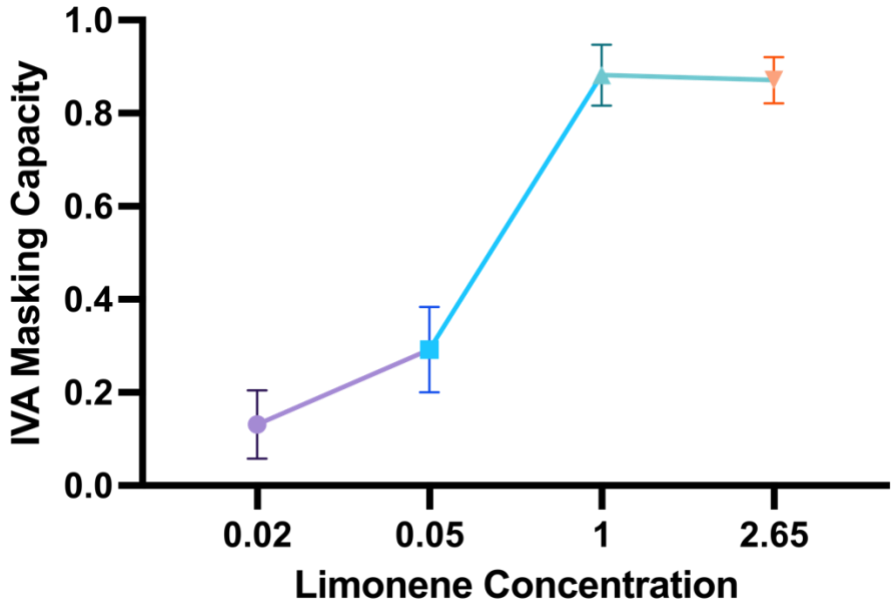


*Figure 5.4: A line graph depicting the relationship between dose and masking capacity was plotted for s-limonene at different concentrations: 0.02 PPM, 0.05 PPM, 1 PPM, and 2.65 PPM. This plot illustrates that there is a dose-dependence relationship between the concentration of s-limonene and its masking capacity. As the concentration of s-limonene increases, the masking capacity also increases. However, this relationship reaches a critical point where further increases in the concentration of s-limonene do not lead to a corresponding increase in masking capacity. This indicates that there is an upper limit to the masking effect of s-limonene, beyond which additional concentration does not result in a significant enhancement of masking capacity.*

**Masking Agents Mixture Determination.** To address the issue of IVA habituation, subjects in Group 1 were exposed to 10PPM IVA, while subjects in Group 2 were exposed to 15PPM IVA. In each triad, three bottles were used: one containing a blank solution (10% PEG), one containing 10PPM or 15PPM IVA, and one containing 10PPM or 15PPM IVA mixed with a 1.2PPM masking agent mixture. The masking agent mixture consisted of all masking agents except for methyl iso-eugenol (Table 5.3).

**Masking Agent Mixture Masking Effects.** The IVA detection probabilities for pure IVA, IVA with the masking agents' mixture, and the blank were measured and plotted (Figure 5.5). The IVA detection probabilities under the three conditions were 73% for pure IVA, 2% for the blank, and 3% for the masked IVA. A one-tailed paired t-test (p=0.05) showed no significant difference in IVA detection probability between the blank and masked IVA conditions (p-value = 0.06). According to the post-experiment interviews, 8 out of 9 subjects were unable to perceive IVA when it was masked by the masking agent mixture. Additionally, one subject showed a significant decrease in IVA detection probability (72% decrease). Four subjects reported not being able to detect any smell with the mixture, while the remaining 5 subjects described a slight pleasant smell with hints of cleanliness and citrus notes. None of the specific odorants in the masking agent mixture could be identified by the subjects.

| Component | Concentration |
|---|---|
| **Neohivernal** | 0.5 PPM |
| **Florhydral** | 0.05 PPM |
| **Geraniol** | 0.1 PPM |
| **Decanal** | 0.2 PPM |
| **Iso-longifolanone** | 0.3 PPM |
| **S-Limonene** | 0.05 PPM |

*Table 5.3: Table displays the components of the masking agent mixture and their corresponding concentrations.*

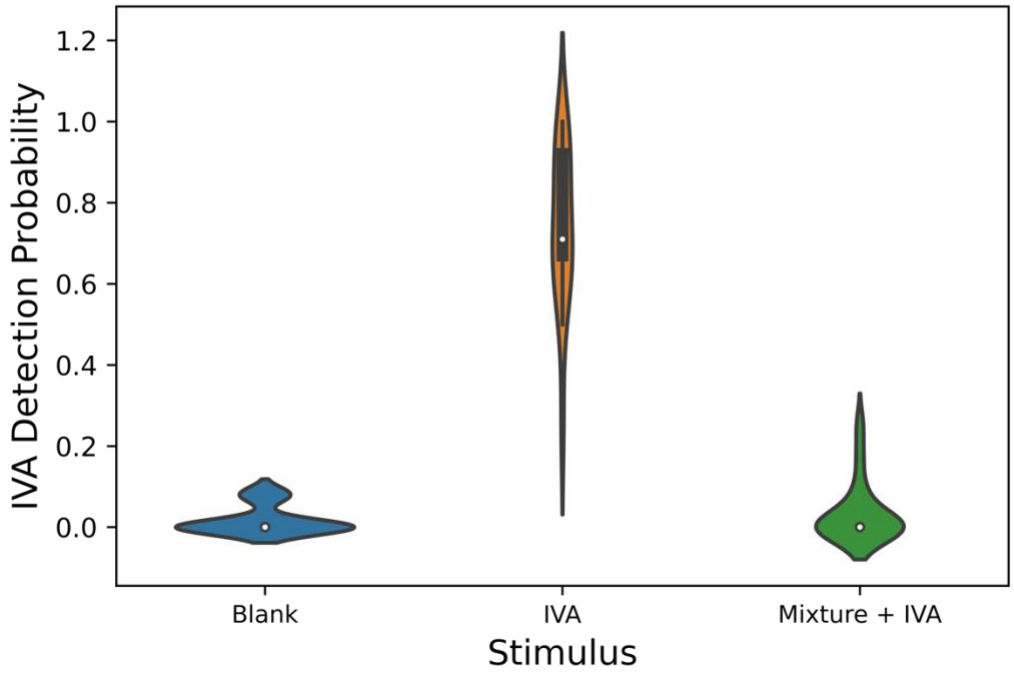


*Figure 5.5: This figure presents a violin plot illustrating the IVA detection probability for the blank sample, 10PPM or 15PPM IVA, and 10PPM or 15PPM IVA with 1.2 PPM masking agent mixture. The plot clearly demonstrates that the IVA was undetectable when mixed with the masking agent mixture, as indicated by the significantly lower detection probability compared to the blank and pure IVA conditions.*

**5.5 Discussion**

The results of this study provide evidence that single masking agents are effective in masking IVA, and that a sub-threshold masking agent mixture can completely mask the IVA, resulting in a significantly reduced detection probability of only 3%. In the discussion section, we will delve into the differences between odor masking and odor covering, examine the phenomena of short-term and long-term habituation, explore potential explanations for the functioning mechanism of the odor masking agent mixture, and discuss the limitations of the study.

**Odor Masking and Odor Covering.** In this experiment, we distinguished between "Odor Covering" and "Odor Masking" based on the detectability of masking agents used. Odor Covering involved using supra-threshold amounts of strong pleasant odorants to cover the malodor, while Odor Masking used sub-threshold or peri-threshold amounts of pleasant odorants to make the malodor unrecognizable or slightly pleasant. Previous studies have shown that the total intensity of a mixture is often less than the sum of the intensities of its components, indicating mutual suppression when both odors are detectable (Bell, 1987; Jones, 1964; Laing & Francis, 1989; Laing, 1984). In the case of Odor Covering, where the masking agents were detectable, both counteraction and odor masking effects likely contributed to the decrease in IVA detection probability.

To further differentiate between odor covering and odor masking, we compared the IVA detectability when subjects could and could not detect the masking agents (Figure 5.6). When subjects were able to detect the masking agents, the IVA detection probability dropped to 0.2 (a 74% decrease), whereas when subjects couldn't detect the masking agents, the IVA detection probability remained at 0.48 (a 37% decrease). Additionally, we compared the masking capacity of odor masking and odor covering for all masking agents (Figure 5.7). The results showed that, except for decanal and iso-longifolanone, all other masking agents had stronger masking capacity when detectable.

To explain these findings, we speculate that when masking agents are undetectable, the central nervous system processes the IVA and masking agents together in a configural recognition. However, when the masking agents are detectable, the mixture is perceived elementally, likely

triggering different neural responses in the central nervous system. Configural and elemental recognition of AB mixtures with varying concentrations have been shown to elicit distinct neural responses in animal models (Linster, 2004; Le Berre, 2008; Wilson, 2020). Therefore, the larger drop in IVA detection probability observed when the masking agents are detectable may be attributed to the activity in the central nervous system.

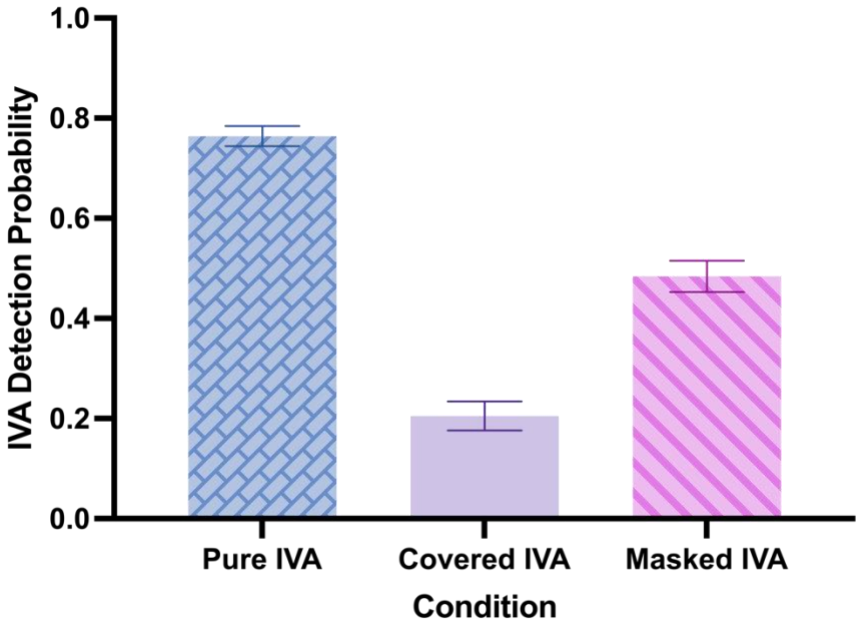


*Figure 5.6: Figure shows of bar graph of IVA detection probability when IVA is pure, covered (detectable amount of masking agents) and masked (undetectable amount of masking agents), which demonstrates that, in general, the odor covering effect contributes to more IVA detection probability drop than does odor masking effect.*

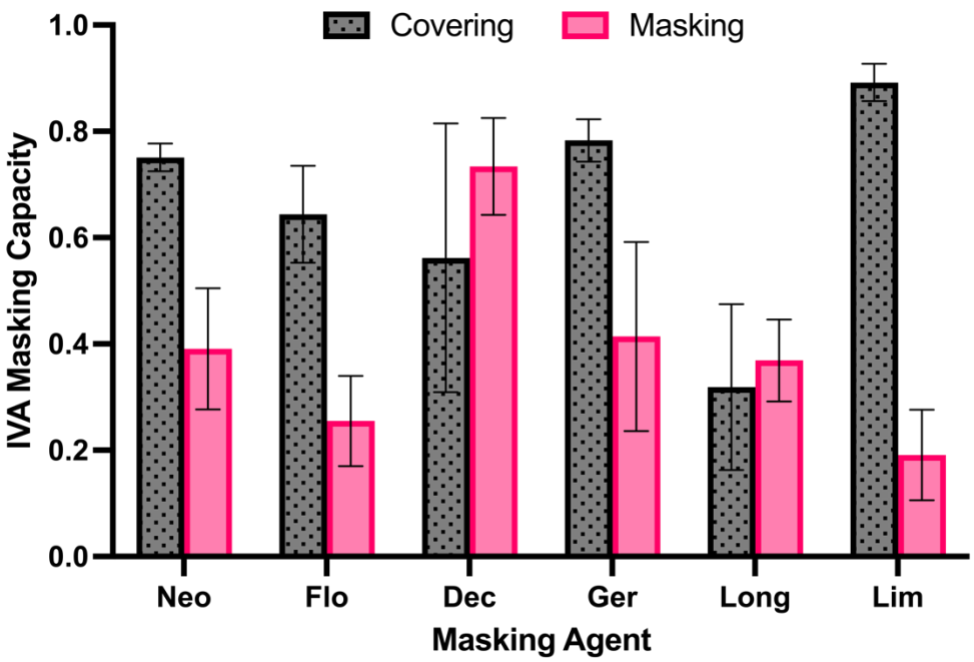


*Figure 5.7: Figure shows a Comparison of the IVA masking capacity for odor covering and odor masking of each masking agent across all concentrations, which shows except for decanal and Iso-longifolanone, all other making agents possess stronger masking capacity when detectable.*

**Short-term Habituation VS Long-term Habituation.** Habituation has been defined as a decreased behavioral response to odors after repeated exposure to those odors (Pellegrino et al., 2017). In a recent review (Rankin et al., 2009), habituation was redefined as short-term habituation and long-term habituation based on the duration of habituation. We define short-term habituation as response decrement that could be restored in minutes (Philpott et al., 2008), while Long-term habituation as response decrement that "lasts hours, days or weeks" (Dalton, 1996).

In our experiment, each visit includes 2 trials of the same set of odor stimulus. The IVA detection probabilities under all conditions between these 2 trials was plotted against each other (Figure 5.8), Two-tailed Paired t-test ($p=0.05$) showed no significant difference between IVA detection probabilities for the two trials, meaning there is no significant short-term habituation

observed. The bar chart of IVA detection probability for the pure IVA at each visit was shown in Figure 5.9, and Two-tailed Paired t-test (p=0.05) was conducted on each pair to see if there is any significant difference. Results showed there wasn't any significant difference in IVA detection probability for the first 4 visits. While at the 5th visit, the IVA detection dropped significantly and maintained at the same level at the 6th visit. IVA 5 and IVA 6 were at least 48 hours apart from each other, meaning that the decrement in response was not restored to the previous level after the 5th session, suggesting long-term habituation. The same sample preparation procedure was used to prep the odorants during the experiments, suggesting the difference is not likely caused by the sample concentration difference or experimental error. When adjusting the IVA concentration to 10 PPM and 15 PPM respectively for Group 1 and Group 2, the IVA detection probability was tested to be 70% (Figure 5.5), further suggesting that it was the habituation that caused the steep drop of IVA detection probability in the 5th session. It is still unclear when the habituation happened and why it happened that way.

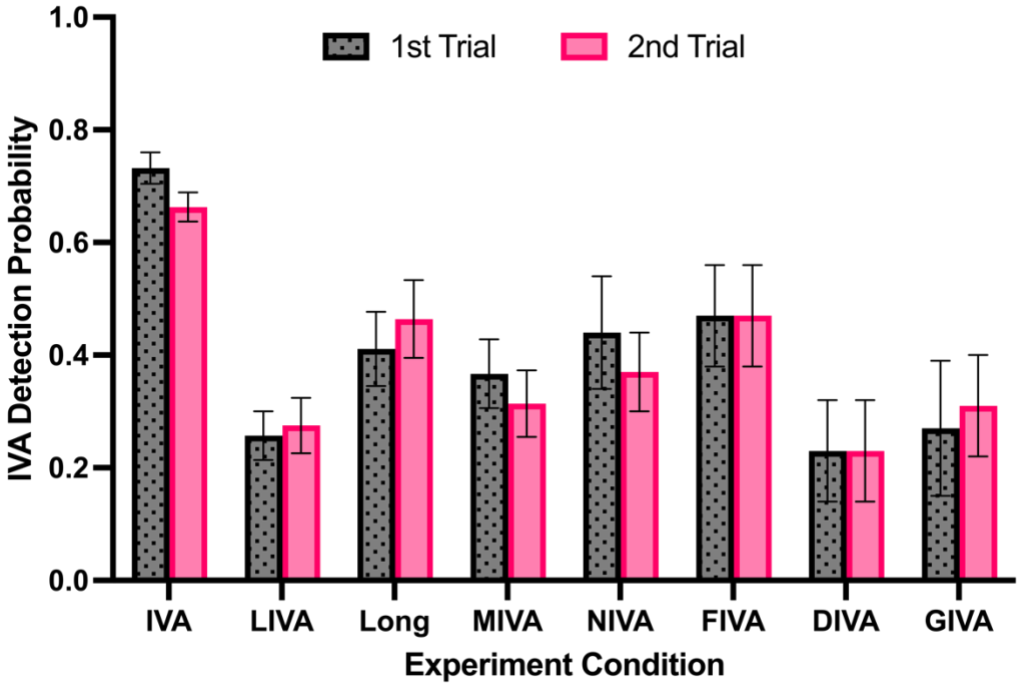


Figure 5.8: In this Figure, IVA Detection probability for the 1st trial and 2nd trial that are 1 min apart from 1st trial under each condition, which shows no significance short-term habituation happened between 2 experiment sessions.

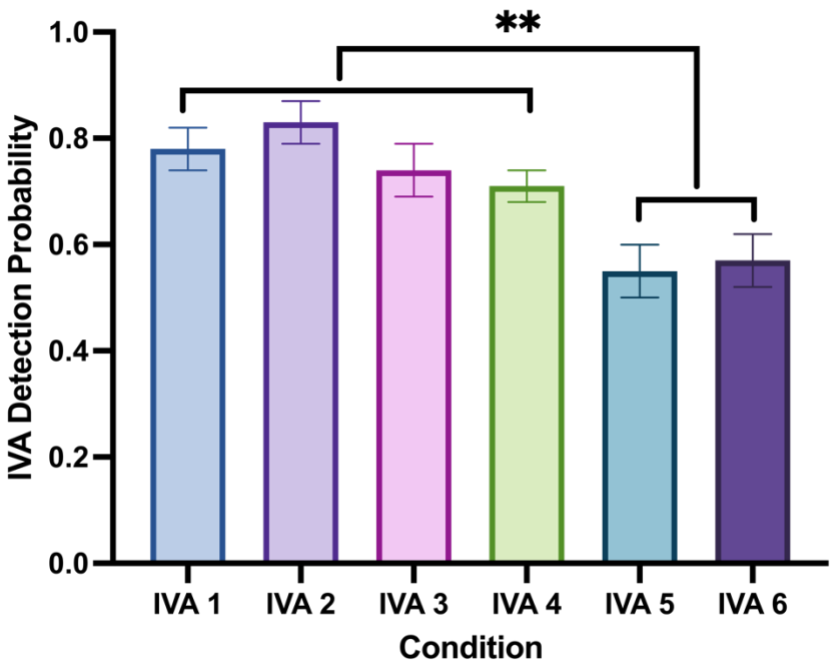


*Figure 5.9: In this Figure, a Bar chart of IVA 1-4 was plotted. Two-tailed paired t-test shows a significant difference for both IVA 5 & IVA 6 against IVA 1-4, and a paired T-test showed no significant difference between IVA 5 and IVA 6 (P = 0.33), which demonstrates the IVA habituation started to happen at 5th visit*

**Masking Effect of Masking Agents Mixture.** With single masking agents, the IVA detection probability could be reduced to a level where it was unrecognizable ($P_{IVA} < 0.5$) (Figure 5.2, Figure 5.3), but complete undetectability ($P_{IVA} = 0$) was rare. However, when using a 1.2PPM scentless masking agent mixture, the IVA detection probability dropped significantly to an average of 0.03. Two theories can help explain this remarkable masking effect by mixtures.

Firstly, masking could occur through competitive binding, as demonstrated in in-vitro studies on olfactory receptors (ORs) (Oka, Nakamura, et al., 2004; Oka, Omura, et al., 2004; Sanz et al., 2005) and in models that predict odor mixture quality (Rospars et al., 2008; Singh et al., 2019). The peri-threshold level of the masking agent mixture may competitively bind to the OR51E1

receptor, thereby minimizing its activation. Consistent with previous studies, the antagonist florhydral, which targets OR51E1, demonstrated odor masking effects against IVA (KAO et al., 2019). However, the competitive binding theory based on a single OR may not fully explain the potent masking effects observed with s-limonene, decanal, and geraniol. These odorants do not bind to OR51E1, nor do we have any evidence to prove they are allosteric modulators for OR51E1. Yet they still exhibit strong masking effects against IVA. Possible explanations for this phenomenon include: 1) the in-vitro OR experimental procedure may not have had the resolution to identify these masking agents as substrates, while they may act as antagonists or partial agonists to OR51E1; 2) OR51E1 may not be the only major receptor for IVA, and these odorants could be antagonistic to other unknown major receptors for IVA; 3) These odorants could be the allosteric modulators for OR51E1.

Another possible explanation comes from the "odor primacy code" theory, which suggests that odor identification primarily relies on the activation pattern of a small set of receptors that are activated earliest (< 100ms) (Chong et al., 2020; Cleland et al., 2012; Wilson et al., 2017). In extreme cases, when equal intensities of 30 odorants spanning the olfactory space are presented, resulting in an "olfactory white" smell, the individual characteristics of the odorants are not perceived (Weiss et al., 2012a). In our study, disrupting the primacy code by using sub-threshold or peri-threshold amounts of odorants may render the malodor, iso-valeric acid in this case, unrecognizable. These theories provide potential explanations for the powerful masking effects observed with masking agent mixtures, but further research is needed to fully understand the mechanisms underlying these phenomena.

**Future Directions.** Firstly, our study has demonstrated the successful masking of IVA using a sub-threshold masking agent mixture. In the future, we aim to expand our participant pool to include individuals from diverse demographic backgrounds in order to further investigate odor masking effects. Secondly, the presence of IVA habituation poses a challenge in testing different concentrations of masking agents to explore the dose dependence. However, we are planning to overcome this limitation and investigate the relationship between masking agent dose and masking capacity. Thirdly, our future research endeavors will focus on exploring the effectiveness of this masking agent mixture in masking other malodors such as butyric acid or methyl mercaptan. By expanding our investigation to different malodors, we can gain a better understanding of the broad applicability and efficacy of the masking agent mixture. By addressing these areas, we aim to advance our understanding of odor masking and develop effective strategies for odor control and management.

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Chapter 6

# Sub-threshold *Iso*-valeric Acid Modifies Odor Detection Probabilities

## 6.1 Abstract

The perception of odors is influenced by the interactions between different odorants, which can impact the overall odor quality and the perception of individual components. Our previous research demonstrated the successful masking of the malodor Iso-valeric Acid (IVA) using sub-threshold and peri-threshold mixtures of Perfumery Raw Materials (PRMs). To further investigate the nature of odor interaction and the influence of sub-threshold levels of IVA on the perception of other odorants, we conducted experiments using a Sniff Olfactometer (SO). Specifically, we examined the impact of sub-threshold levels of IVA on the overall perception of Lemonene, Florhydral, and Methyl Iso-eugenol in binary mixtures. The results of our study confirmed that Lemonene, Florhydral, and Methyl Iso-eugenol exhibited masking effects, inhibiting the perception of IVA. Interestingly, the presence of sub-threshold levels of IVA elicited diverse responses in the perception of other odorants, including both enhancement and suppression effects. Importantly, these effects were not consistently observed across different concentrations of sub-threshold IVA. These findings provide valuable insights into the complex dynamics of odor interactions and underscore the significance of considering sub-threshold odorants in the context of odor masking. Further research is necessary to unravel the underlying mechanisms and explore the practical implications of these findings.

## 6.2 Introduction

**Odor Perception.** The process of odor perception begins with odorants activating Olfactory Receptors (ORs) in the olfactory epithelium (Buck & Axel, 1991), which generates a peripheral

activation pattern (Buck, 1996; Malnic, 1999; Xu, 2020) and converts chemical information into electrical signals. These electrical signals are then transmitted by olfactory sensory neurons (OSNs) to the olfactory bulbs (OBs) where they are further processed in glomeruli (Buck, 2000). Ultimately, the signals are transmitted to the piriform cortex and other brain regions to be transformed into perceptual experiences of odor. When multiple odorants are inhaled, they can interact with each other either at the peripheral level (receptors) or at the central level (central nervous system).

**Odor Interaction.** Odor interactions in binary mixtures have primarily been studied at supra-threshold levels (Laing, 1983; Laing, 1984; Laing, 1994), and the common finding is that odorants tend to inhibit each other in a mixture (Jones, 1964; Laing, 1983). In most reported cases, although the odor intensities are mutually inhibited, individuals can still recognize the individual odor components in the binary mixture, indicating that the patterns associated with each component remain intact. Therefore, mutual suppression at supra-threshold levels is likely to occur at the central nervous system level. However, very few studies have focused on odor interaction below the recognition threshold, mainly due to the challenges in capturing odor quality and intensity at such low levels. Yet, the perception of odor mixtures, as described by Stuart Firestein, is akin to "a chord with a silent note" (Xu, 2023), suggesting that components below the threshold play a crucial role in determining the overall identity and quality of the mixture. Functional Magnetic Resonance Imaging (fMRI) data collected from humans has demonstrated that sub-threshold amounts of ambroxan can still elicit brain responses (Hummel, 2013), indicating that in a binary odorant mixture, if one component is below the recognition threshold (sub-threshold), it may still modify the perception of the supra-threshold component by

either masking (suppression) or synergizing (enhancement) it. Recent psychophysical studies in humans have shown that peri/sub-threshold amounts of odorants can work in harmony with taste stimuli (Pfeiffer, 2005), mask malodor (Huang, 2022), and synergize with other odorants (Miyazawa, 2008). In this study, we aim to explore whether sub-threshold amounts of IVA can affect the recognition of other odorants. Using the Sniff Olfactometer (SO) (Wyckoff & Acree, 2017), we investigate how sub-threshold amounts of IVA influence the overall perception of Lemonene, Florhydral, and Methyl Iso-eugenol in a binary mixture.

### 6.3 Material and Method

**Chemicals.** Polyethylene Glycol 400 (PEG400): CAS Registry No. 9002-88-4, Deionized Water: carbon filtered deionized water, Charcoal powder: CAS Registry No. 7440-44-0, *Iso*-valeric Acid (IVA): CAS Registry No. 503-74-2, Neohivernal (Neo): CAS Registry No. 300371-33-9, Florhydral (Flo): CAS Registry No.125109-85-5, Methyl *Iso*-eugenol (Met): CAS Registry No. 6379-72-2, *S*-Limonene (Lim): CAS Registry No. 5989-54-8.

**Ethics Statement.** The experiment was conducted under the approval of Cornell University Institutional Review Board (IRB) and followed Declaration of Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013).

**Subjects.** In the first part of the experiment, 8 subjects including 5 females and 3 males, all students from Cornell University (19-31 years old), were recruited. In the second part of the experiment, 4 female, all students from Cornell University (22-26 years old), were recruited.

Subjects participated were asked to sign the agreement (Supplemental) and screened to make sure they 1) do not have a stuffy nose before each session; 2) do not have anosmia; 3) could identify all the compounds presented in the session by passing the conditioning and pretesting session.

**Software.** The experiments were automated using PsychoPy® (v2021.2.3) (Peirce et al., 2019). Data analysis was executed using R (version 4.1.3 – "One Push-Up") (R-Core-Team, 2022) and Python 3.11.2 (Van Rossum, 2009). See supplemental.

**Equipment.** Sniff Olfactometry (SO)(Wyckoff & Acree, 2017) was used to investigate measure the recognition threshold and odorants detection probabilities.

**Sample Preparation.** The odorant stock solution, 10% PEG solution, and test solution were prepared following the procedure described in our previous experiment (Huang, 2022). Prior to this experiment, we conducted threshold measurements and benchtop trials with lab members to determine the appropriate concentrations for each odorant. Four concentrations were selected for each odorant, labeled as 1 to 4, with increasing concentration from lowest to highest. The concentration determination process follow the Weber's Law of Just Noticeable Difference, ensuring that the mutual difference between concentrations was greater than the Just Noticeable Difference (JND) ($\Delta C/C \geqq 0.33$) (Table 6.1).

| Odorants | Concentrations (PPM) | | | |
|---|---|---|---|---|
| ***Iso*-valeric Acid** | 0.1 | 0.5 | 5 | 10 |
| **Neohivernal** | 0.1 | 0.5 | 1 | 2.5 |
| **Florhydral** | 0.02 | 0.06 | 0.3 | 0.6 |
| ***Methyl Iso-eugenol*** | 5 | 10 | 30 | 80 |
| ***s-Limonene*** | 0.03 | 0.03 | 3 | 10 |

*Table 6.1: Perfumery raw materials concentrations used for threshold determination.*

**Threshold Determination.** In this experiment, we followed the same conditioning and pre-testing procedure as described in our previous study (Huang, 22). The highest concentration (Bottle 4) was used during the conditioning session and pre-testing session to ensure that subjects could associate the odorants with their respective descriptors. The threshold determination process consisted of two rounds of measurements. In round 1 (Figure 6.1A), bottles 1, 2, and 3 were randomly puffed four times at each position within the triad (Figure 6.1C). In round 2, bottles 2, 3, and 4 were randomly puffed four times at each position within the triad.

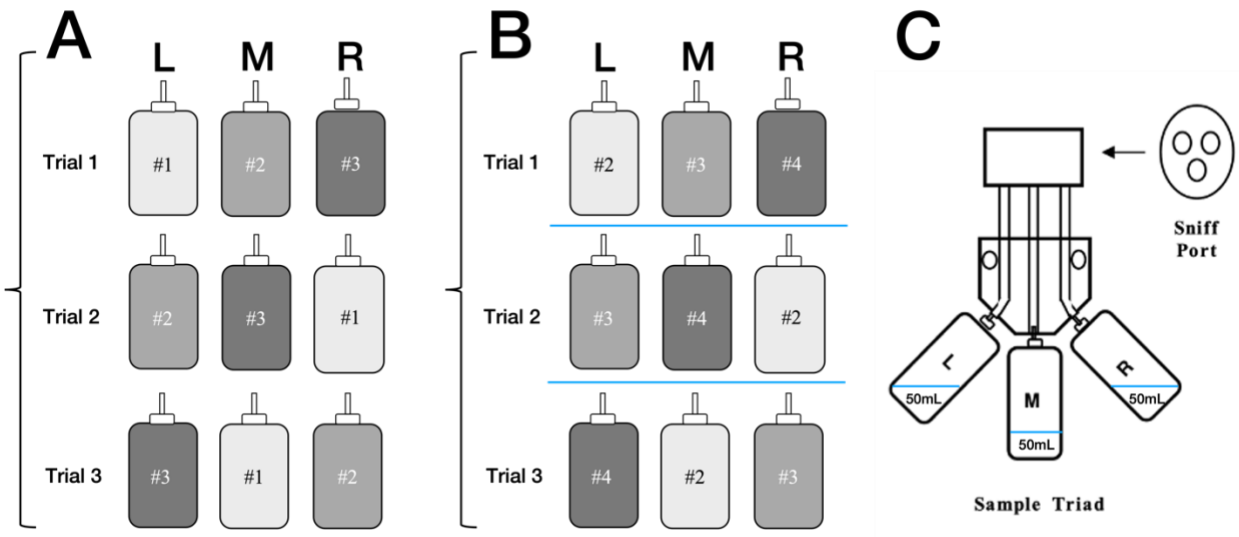


*Figure 6.1: Figure 6.1A shows the bottle arrangement for round 1 of the threshold testing. Figure 6.1B shows the bottle arrangement for round 2 of the threshold testing. Figure 6.1C shows the triad arrangement.*

**Experiment Procedure.** The experiment will be conducted in two stages. **Stage 1** will focus on evaluating the detection probability of IVA after adding ¼ threshold level of PRMs, and the detection probability of PRMs after adding ¼ threshold level of IVA (Figure 6.2). **Stage 2** will examine how different doses of IVA affect the detection probability of IVA.

In Stage 1, the forward interaction between PRMs and IVA will be investigated. Three bottles containing the threshold amount of IVA, half the threshold amount of IVA, and a mixture of ¼ threshold amount of PRMs and the threshold amount of IVA will be puffed four times at three positions on the triad. The detection probabilities will be recorded with and without the addition of PRMs. This comparison will determine whether there is odor masking (suppression) or synergy (enhancement) between the PRMs and IVA (Figure 6.2). Following the forward interaction, the reverse interaction between IVA and PRMs will be examined. Three bottles containing the threshold amount of PRMs, half the threshold amount of PRMs, and a mixture of ¼ threshold amount of IVA and the threshold amount of PRMs will be puffed four times at three positions on the triad. The detection probabilities will be measured with and without the addition of IVA. This analysis will help determine if there is odor masking (suppression) or synergy (enhancement) between IVA and the PRMs (Figure 6.2).

In Stage 2, four concentrations of IVA ($^1/_8$ threshold, $^1/_4$ threshold, $^1/_2$ threshold, threshold) will be added to the threshold amount of PRMs. The detection probabilities of PRMs with and without the addition of IVA will be compared to determine if there is odor masking (suppression) or synergy (enhancement).

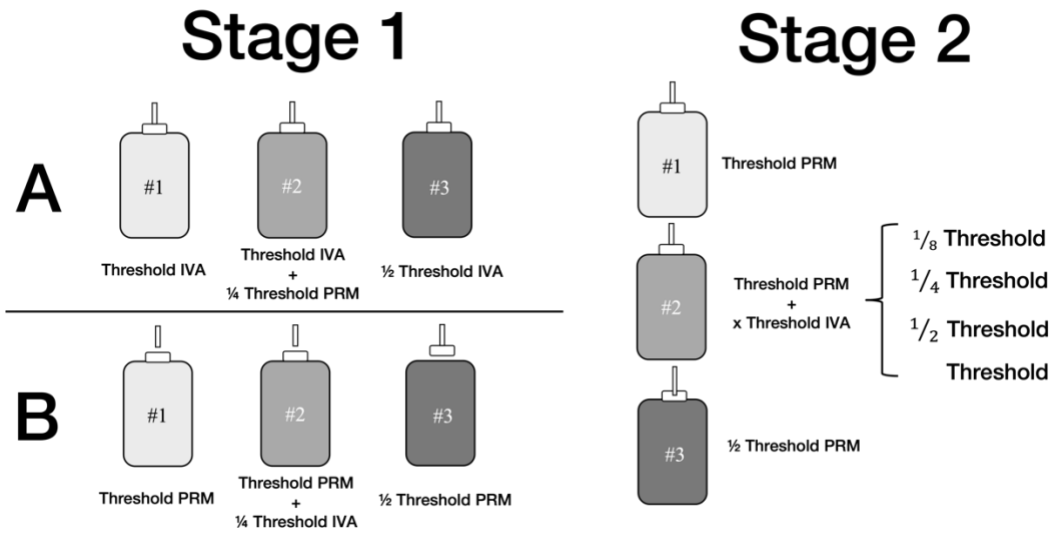


*Figure 6.2: Figure 6.2 shows the experimental conditions for both stages of the experiment.*

### 6.4 Results

**Threshold Determination.** After obtaining the detection probabilities for each odorant at the four concentrations, the data was plotted to generate psychometric curves. The detection probabilities were plotted against the logarithm of the concentration, allowing for the interpolation of thresholds at $P_{\text{detecting Odor}}=0.5$. The calculated thresholds for subjects in Stage 1 are presented in Table 6.2, and the calculated thresholds for subjects in Stage 2 are displayed in Table 6.3.

| | IVA (ppm) | Lim (ppm) | Neo (ppm) | Flo (ppm) | Met (ppm) |
|---|---|---|---|---|---|
| Subject 1 | 1.36 | 0.33 | 1.70 | X | 12.70 |
| Subject 2 | 1.53 | 1.03 | 2.30 | 0.62 | 66.21 |
| Subject 3 | 0.76 | 0.30 | 1.20 | 0.10 | 26.20 |
| Subject 4 | 0.71 | 0.89 | 0.26 | 0.09 | 23.10 |
| Subject 5 | 0.49 | 0.18 | 0.60 | 0.21 | 24.80 |
| Subject 6 | 0.43 | 0.56 | 3.40 | X | 21.40 |
| Subject 7 | 0.44 | 0.29 | 0.65 | 0.07 | 12.50 |
| Subject 8 | 0.17 | 0.38 | 0.60 | 0.05 | 8.50 |

*Table 6.2: Table 6.2 shows the thresholds measured for 8 subjects participated in the experiment stage 1. The X in the table stands for subject is anosmic to that odorant.*

| | IVA (ppm) | Lim (ppm) | Neo (ppm) | Flo (ppm) | Met (ppm) |
|---|---|---|---|---|---|
| Subject 1 | 0.34 | 0.84 | 1.19 | 0.24 | 11.99 |
| Subject 2 | 1.35 | 0.92 | 0.25 | 0.05 | 20.55 |
| Subject 3 | 1.13 | 1.45 | 1.72 | 0.25 | 20.18 |
| Subject 4 | 0.89 | 1.76 | 1.30 | 0.34 | 23.27 |

*Table 6.3: Table 6.3 shows the thresholds measured for 4 subjects participated in the experiment stage 2.*

**Stage One.** In the forward interaction experiment (PRMs' effect on IVA detection probability) conducted in Stage 1, the IVA detection probabilities under the three conditions were plotted together (Figure 6.3). Paired two-tailed t-tests were performed among the three conditions to determine if the observed effects were significant (Figure 6.3). It is worth noting that there were two subjects who were anosmic to florhydral, resulting in a total of six subjects being tested for any condition involving florhydral in this stage. The results showed that the threshold amount of IVA had a significantly higher detection probability compared to half the threshold amount of IVA, indicating that subjects were able to distinguish the difference in intensity between the two (Figure 6.3). Additionally, Limonene, Methyl Isoeugenol, and Neohivernal exhibited a significant masking effect on IVA, consistent with our previous findings. However, the IVA masking effect demonstrated by florhydral was not found to be significant.

In the reverse interaction experiment (IVA's effect on PRM detection probability) in Stage 1, the PRM detection probabilities under the three conditions were plotted together (Figure 6.4). Paired two-tailed t-tests were performed among the three conditions to determine if the observed effects were significant. The results showed that the threshold amount of PRM had a significantly higher detection probability compared to half the threshold amount of PRM (Figure 6.4). On average, the addition of ¼ threshold amount of IVA did not significantly affect PRM detection probability. However, when examining the individual responses (Figure 6.5), the forward interaction consistently showed suppression, while the reverse interaction did not exhibit a visible pattern. Upon closer examination of the individual data (supplemental), it became apparent that the non-significant effect observed in Figure 6.4 was due to averaging subjects who experienced masking effects and subjects who experienced synergy effects when adding ¼ threshold amount of IVA.

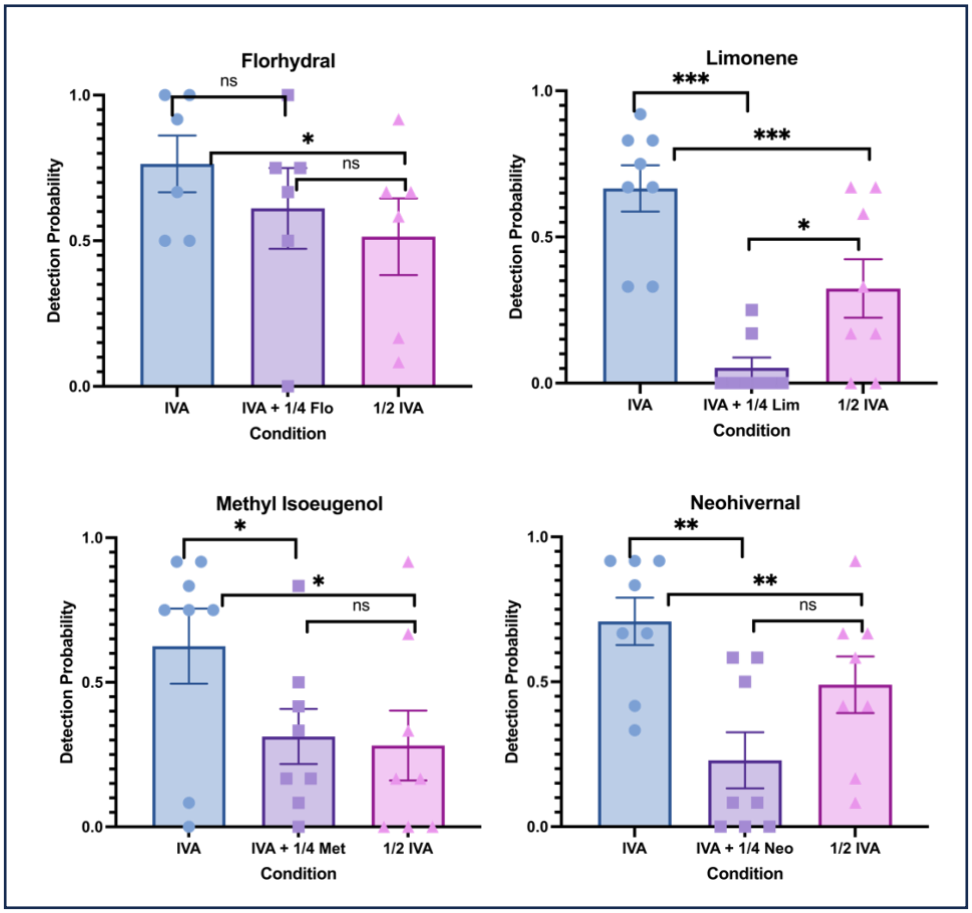


*Figure 6.3: Figure 6.3 shows the IVA detection probabilities under different conditions (Forward). X-axis shows the stimulus conditions and Y-axis shows the IVA detection probabilities. The paired two-way t-test result is labeled on the figure: "***" = 0.001, "**" = 0.01, "*" = 0.05, "ns" = Not Significant. The standard error was labeled on the plot as the error bar.*

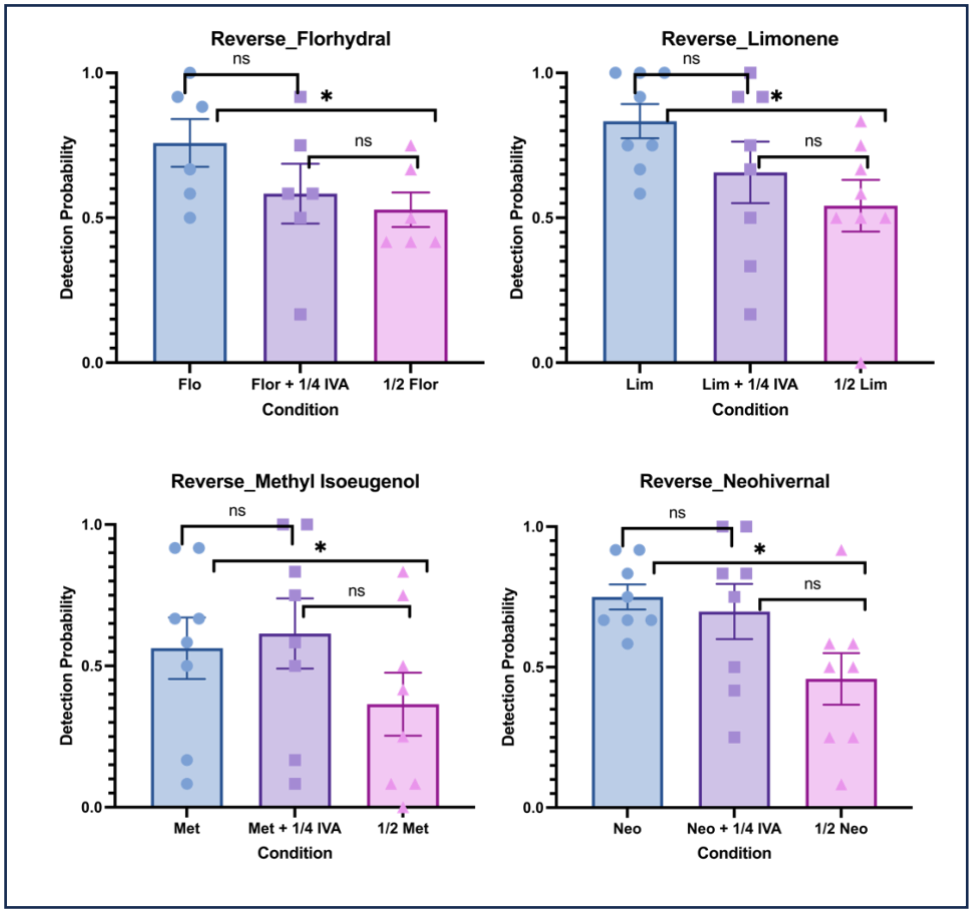


*Figure 6.4: Figure 6.4 shows the PRMs detection probabilities under different conditions (Reverse). X-axis shows the stimulus condition, and Y-axis shows the PRMs detection Probabilities. The paired two-way t-test result is labeled on the figure: "***" = 0.001, "**" = 0.01, "*" = 0.05, "ns" = Not Significant. The standard error was labeled on the plot as the error bar.*

| FORWARD | | | | | REVERSE | | | |
|---|---|---|---|---|---|---|---|---|
| *Lim* | *Met* | *Flo* | *Neo* | Subject | *Neo* | *Flo* | *Met* | *Lim* |
| Suppression | Suppression | Suppression | Suppression | 1 | Enhancement | Suppression | Enhancement | Enhancement |
| Suppression | Suppression | Suppression | Suppression | 2 | Enhancement | Suppression | Enhancement | Enhancement |
| Suppression | Suppression | Suppression | Enhancement | 3 | Enhancement | Suppression | Enhancement | Suppression |
| Suppression | Suppression | Suppression | Suppression | 4 | Suppression | No Func | Enhancement | Enhancement |
| Suppression | Suppression | Suppression | Suppression | 5 | No Func | Enhancement | Suppression | Enhancement |
| Suppression | Suppression | Suppression | No Func | 6 | Suppression | Enhancement | Suppression | Suppression |
| Suppression | Suppression | Suppression | Suppression | 7 | Suppression | No Func | No Func | Suppression |
| Suppression | Suppression | Suppression | No Func | 8 | Suppression | Suppression | Suppression | Suppression |

*Figure 6.5: Figure shows the individual response pattern under both forward and reverse conditions. Suppression means the detection probability decreased after adding in the second component. Enhancement means the detection probability increased after adding in the second component. No function means adding in the second component does not alter the detection probability.*

**Stage Two.** After obtaining the PRMs (Lim, Neo, Flo, Met) detection probabilities when adding different sub-threshold concentrations of IVA, the PRM detection probabilities at the threshold level and half the threshold level were compared to ensure that subjects were able to respond to the concentration differences accordingly. All subjects consistently showed higher detection probabilities for the threshold level of PRMs (supplemental data). Next, the net detection probability difference was calculated using the equation: Net Difference = $P_{PRM+IVA} - P_{PRM}$. A positive net difference value indicates a synergy (enhancement) after adding IVA, while a negative net difference suggests a masking (suppression) after adding IVA. A violin plot was generated to illustrate the net differences under each condition (Figure 6.6).

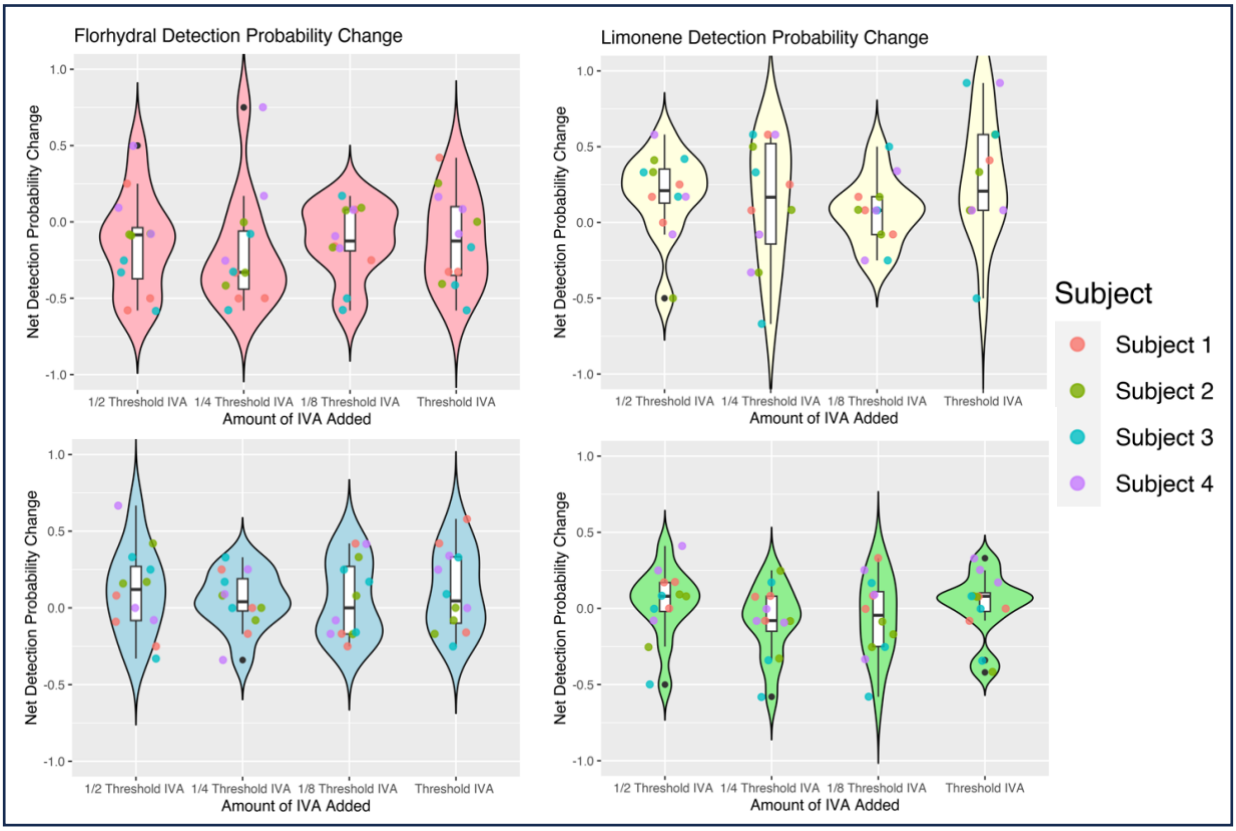


*Figure 6.6: Figure shows the net detection probability change of PRMs when different concentrations of IVA were added. The individual data points are shown in different colors based on subjects, and the mean value was shown in the boxplot overlapping the violin plot.*

A two-tailed t-test (P=0.05) was performed to compare the net differences under each condition to a value of 0, in order to determine if adding different concentrations of IVA significantly changed the PRMs' detection probability (Table 6.4). Based on the combination of the boxplot and t-test results, it was found that only when more than half the threshold amount of IVA was added to the threshold amount of Limonene, subjects started to show a significant synergy (enhancement) effect. However, no other PRMs showed a significant change in detection probability when different sub-threshold concentrations of IVA were added.

| | *Flo* | *Lim* | *Met* | *Neo* |
|---|---|---|---|---|
| *1/8 Threshold IVA added* | 0.11 | 0.29 | 0.46 | 0.43 |
| *1/4 Threshold IVA added* | 0.09 | 0.29 | 0.2 | 0.25 |
| *1/2 Threshold IVA added* | 0.16 | 0.04 | 0.21 | 0.62 |
| *Threshold IVA added* | 0.21 | 0.02 | 0.17 | 0.77 |

*Table 6.4: Table 6.4 shows p-value for the Two-tailed paired t-test comparing the net differences under each condition and 0.*

### 6.5 Discussion

In this study, we have provided evidence to support the validity of the IVA masking effect and have demonstrated that odor masking is unidirectional rather than mutual. Additionally, by comparing the detection probabilities of threshold amounts of PRMs with those of half the threshold amounts, we have shown that subjects are able to perceive and distinguish concentration differences. In our previous experiment, we observed consistent and reproducible masking effects when mixing the masking agents with IVA (Huang, 2022). However, when IVA was added to the PRMs, we observed significant fluctuations in detection probabilities among subjects, with both positive and negative net differences observed under the same conditions. For example, subject 3 exhibited a net difference of -67 during the first ¼ IVA + Limonene session, but a net difference of +0.58 during the second session (Figure 6.6). These fluctuations suggest that instead of simply masking or synergizing with other odorants, the sub-threshold amount of IVA is modifying the odor qualities of the other components in the mixture, thus causing confusion for the subjects. Regarding dose dependence, we plotted a line chart illustrating the net detection probability change as a function of IVA dose for each subject (Figure 6.7). While

individual subjects exhibited fluctuations, the average net change values remained relatively steady, indicating a lack of strong dose-dependent tendencies.

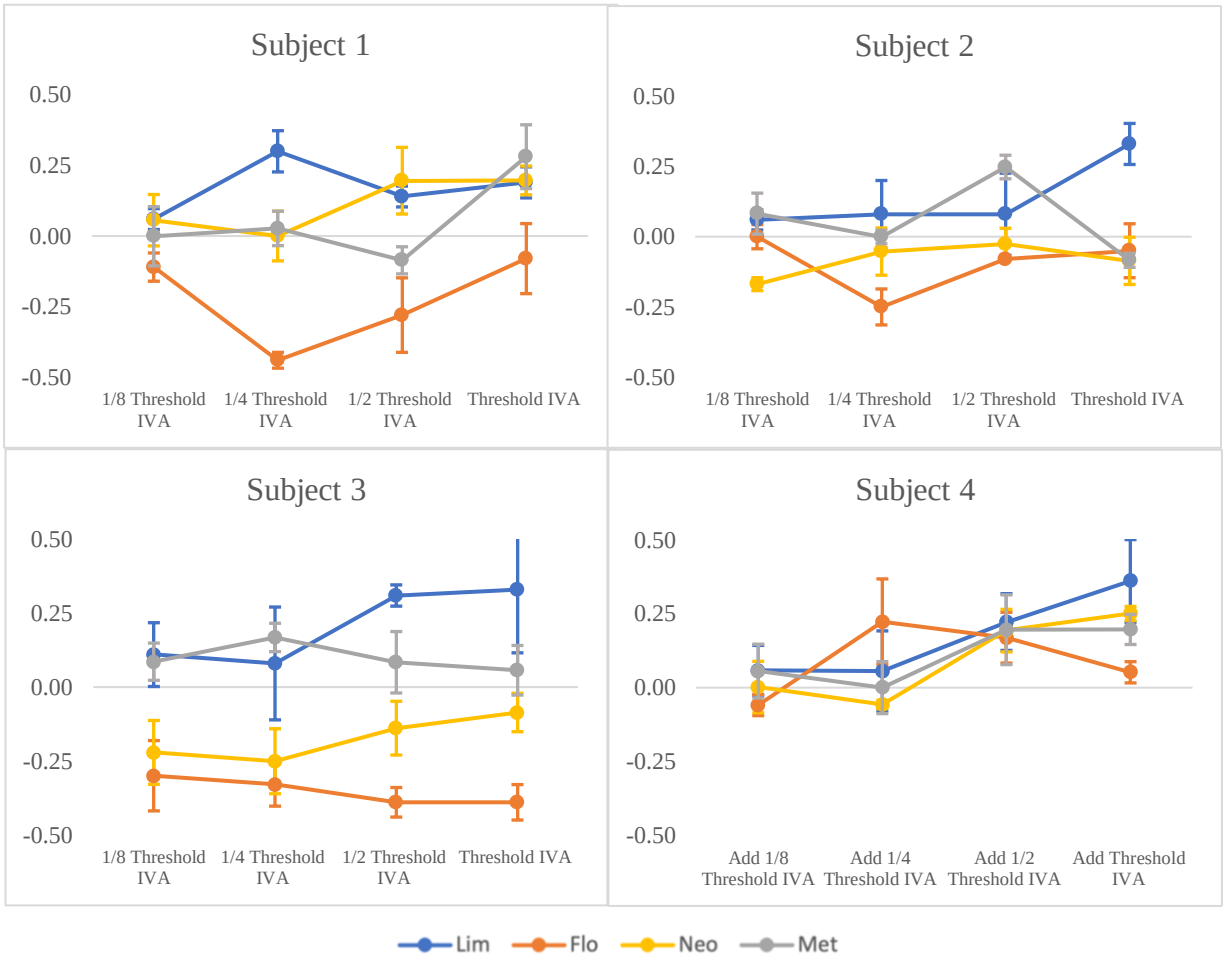


*Figure 6.7: Figure 6.7 displays individual line charts representing each subject's response to changes in the amount of IVA added to the sample. The x-axis represents the amount of IVA added, and the y-axis represents the net change in detection probability. Each line corresponds to one PRM (Limonene, Florhydral, Neohivernal, and Methyl Isoeugenol). The error bars represent the standard errors associated with the measurements. The line charts provide an overview of how each subject's detection probability changed with increasing amounts of IVA for each PRM.*

**Odor Interaction.** In our previous experiment, we observed that when mixed with IVA, Limonene could be detected at concentrations 10 times below its measured recognition

threshold. The results from the current experiment align with our previous observations and further confirm that such synergistic effects can occur with sub-threshold amounts of IVA. Another interesting finding is the commonality between this synergistic effect and the reported synergistic effects involving organic acids, such as Co at equal concentrations (Miyazawa, 2008). This suggests that the presence of organic acids may play a role in odor synergy.

One possible explanation for odor synergy is the interaction between trigeminal neurons and olfactory sensory neurons (OSNs). Acids are known to activate trigeminal receptors (Welch, 2000; Silver, 2006), and there is evidence of interaction between the trigeminal and olfactory systems at both the peripheral and central levels (Tremblay, 2018; Boyle, 2007). Psychophysical experiments have demonstrated that supra-threshold trigeminal stimuli can mask olfactory stimuli (Hummel, 2005) and be synergized by olfactory stimuli (Cain, 1980; Livermore, 1992). Therefore, it is reasonable to speculate that sub-threshold to supra-threshold levels of trigeminal stimuli could synergize certain odorants. However, it should be noted that the trigeminal stimuli typically have higher detection thresholds than olfactory stimuli (Cometto-Muñiz, 1998 & 2016), which raises the possibility of an alternative explanation involving allosteric interaction.

Allosteric modulation has been shown to alter the binding activities of olfactory receptors (Tsitoura, 2016), and recent research has indicated that the activation of OR5AN1 likely contributes to human sensitivity to nitromusks and macrocyclic ketones (Trimmer, 2023). Therefore, it is plausible that the organic acids are allosterically modulating the olfactory receptors for Limonene and 2-hydroxy-3-methyl-2-cyclopentene-1-one, leading to the observed synergistic effects.

In conclusion, our study provides evidence for the unidirectional odor masking effect of IVA and the potential for synergistic interactions between IVA and certain odorants, particularly Limonene. The involvement of organic acids and the possible roles of trigeminal-neuronal interaction and allosteric modulation in odor synergy warrant further investigation. Understanding the mechanisms underlying odor interactions can deepen our understanding of olfaction and contribute to the development of innovative odor-masking and odor-synergy strategies in various applications.

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