# Retronasal and Oral-Cavity-Only Responses to TRPM8 Odorants

Kathleen Elizabeth Melville

Department of Neurobiology and Behavior, Professor Bruce Halpern

Cornell University, Ithaca, NY, United States

Thesis submitted in partial fulfillment of the requirements for Bachelor of Science in Biology, with Honors.

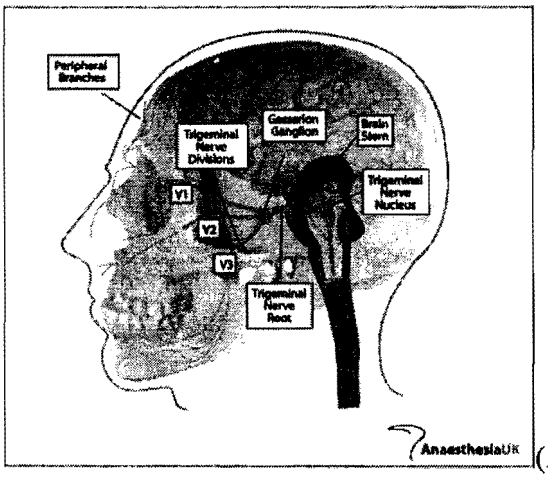
## Abstract

Retronasal (retro) and/or oral-cavity-only (OCO) smelling of vapor-phase agonists for the trigeminal chemoreceptive ion channel TRPM8 was studied. In a retro and OCO identification experiment, 1 of 6 previously practiced identifications (ID) was selected on a computer display under forced-choice conditions. Analyses of variance (ANOVA) found differences across odorants and retro and OCO, and for retro and OCO separately. Within retro smelling, median correct ID and modal ID (in parentheses) for eucalyptol was 100% (ointment), different from all other odorants. Geraniol 67% (lemon) and linalool 67% (cleaner) differed from dl-menthol, but isopulegol, dl-menthol and l-carvone did not differ. With OCO smelling, ID for geraniol fell to 0% and linalool to 33%, but no significant change for eucalyptol ID at 84%. A second, OCO-only triangle test examined discrimination of geraniol, l-carvone, and isopulegol from sunflower oil. ANOVA found overall differences and Wilcoxon tests found significant pairwise differences between all three odorants. Isopulegol was discriminated by 100% of particpants; l-carvone, by 53%; geraniol, by 13%, implying insignificant OCO geraniol discrimination, but significant discrimination of both isolpulegol and L-carvone in comparison with the non-odorant, sunflower oil. The differential OCO ID and discriminations indicate that TRPM8 channels are not the only oral cavity trigeminal mechanism for these odorants, while the retro versus OCO differences suggest either olfactory involvement or distinct oral and nasal cavity trigeminal arrays.

## Introduction

Smelling and experiencing odors can occur through a number of different ways. These different pathways have been shown to influence the perception and intensity of certain odorants. Among others, one explanation of this is that different nerves innervate the nasal and oral cavities, both of which are normally involved in a smelling experience. The trigeminal nerve has branches to the oral and nasal cavities, but the olfactory nerve only innervates the nasal cavity (Hummel et al, 2004).

*Figure 1*: The branches of the trigeminal nerve



(AnesthesiaUK, 2005).

*Orthonasal smelling* involves inhalation and exhalation through the anterior nares (nostrils). Smelling *retronasally* involves inhaling through the mouth and then exhaling through the nose. When odorants are smelled *oral-cavity-only* (OCO) the odorant is strictly contained in the oral cavity and both inhalation and exhalation occur through the mouth (Halpern, 2004).

*Trigeminal stimuli* have been categorized by their ability to be detected by anosmics (those lacking a functional olfactory system) or through successful lateralization between the nostrils (Doty et al., 1978; Cometto-Muñiz et al., 1998, 2005). In theory, trigeminal stimuli should be able to be detected and identified in the oral cavity, as there is trigeminal innervation there. It has been previously noted that peppermint extract was the only odorant out of anise, cinnamon, coffee, orange, strawberry, and peppermint extracts to be correctly identified above chance when restricted to the oral cavity (Dragich and Halpern, 2008). As a major component of peppermint extract is dL-menthol, a known trigeminal stimulant (Doty et al., 1978), a follow-up study by Parikh et al., 2009, used dL-menthol and five other known trigeminal stimuli (eugenol, heptyl alcohol, nonanal, 1-octanol, dl-menthol, valeric acid) were tested in a similar identification procedure. Again, only dL-menthol was able to be identified above chance when restricted to the oral cavity through OCO smelling (Parikh et al., 2009).

The trigeminal system is mainly involved in pain, temperature, and other tactile sensations in the face, with nerve branches ending in areas of the head and face, including both the oral and nasal cavities (Leffingwell, 2001). Temperature sensation in particular is mediated by a class of six temperature sensitive ion channels, the transient receptor potential melastatin (TRPM) channels (Bandell et al., 2007). One of these TRPM channels, the TRPM8, is activated by dl-menthol, the trigeminal odorant used in the study by Parikh et al., 2009. For this study, five other known agonists of this same membrane channel, Geraniol, L-carvone, Eucalyptol, Linalool, and Isopulegol (Bandell et al., 2007) are being used in similar identification tasks, in conjunction with dL-menthol. All six TRPM8 odorants are ten-carbon alcohols (Bandell et al., 2007).

The first phase of this study aims to discover if the TRPM8 channel plays a critical role in the detectability and ability to identify trigeminal odorants in the oral cavity. In this study the TRPM8 agonists are compared in retronasal and oral-cavity-only identification tests similar to those performed by Parikh et al., 2009. If being an agonist of the TRPM8 channel is the reason that dl-menthol has consistently been identified above chance OCO, then it is expected that the other five odorants will likewise be identified in the oral cavity. If the TRPM8 agonists all give the same retronasal or OCO identification, it could be concluded that the TRPM8 channel defines the smell of the odorants, as they are being interpreted in the same way. Identical OCO identification would indicate a common trigeminal mechanism. Differences between identifications of the odorants through retronasal and OCO presentation could indicate that there are differences between the sensory input of the oral and nasal cavities. As the olfactory nerve only branches to the nasal cavity, differences in identification retronasally and OCO suggest the involvement of the olfactory system in identifying the odorants retronasally. If all the tested TRPM8 odorants give distinctly different sensations, both retronasally and OCO, the percentage of correct identifications should be high for each odorant in each presentation condition, showing that they could be correctly discriminated and identified from one another.

The second phase of this study, involving a triangle test, aims to determine if the TRPM8 odorants are discriminable OCO from a pure non-odorant, sunflower oil. If the three TRPM8 odorants used, Isopulegol, Geraniol, and L-carvone, are able to be discriminated from the sunflower oil, it would suggest that these odorants are giving enough input to the trigeminal system in the oral cavity to result in some sensation. If

they are not discriminable from sunflower oil it would suggest that the TRPM8 channel is not sufficient to produce trigeminal stimulation OCO. Furthermore, if the percentages of correct discriminations are different for these three TRPM8 odorants, it would indicate that these are providing differential input and stimulation to the trigeminal system. The TRPM8 channel would then not be the only factor in the sensory stimulation of these odorants.

## Methods

*Participants*

Participants for all phases of this study were recruited through the use of flyers posted on campus and word of mouth. Participants received $6.00 for each session they participated in, where each session lasted less than thirty minutes. Participation was limited to non-smoking, non-pregnant, non-lactating individuals over the age of 18 who could communicate in American English. Participants in the study were asked to not eat or drink anything, except water, for the hour before each session. Twenty-four participants (17 females) ranging in age from 18-55 years (median age 21, semi-interquartile range 2) were tested in the retronasal and oral-cavity only identification task, and 15 participants (9 females) aged 19-25 years (median 21, semi-interquartile range 0.5) were tested in the triangle test to detect the odorants oral-cavity only. Four of the fifteen participants who were tested in the triangle test had also participated in the identification tests.

The testing protocol was approved by the Institutional Review Board for Human Participants. Prior to each session, each participant read and signed an approved Informed Consent statement and had any questions or concerns addressed. Each participant was

given a three-digit code number, known only to the experimenter, to use in analyzing results, to protect the anonymity of the participants. In a separate record, the code numbers were linked the participants name, gender, and age.

*Odorant Delivery Containers*

In each phase of the experiment, the vapor phase of the odorants was delivered to the participants, either retronasally or oral-cavity only, through the use of *odorant delivery containers* (ODC). The ODC were constructed using 118 ml (4oz) black, oval, Ellipso Portion Cups with clear oval lids, manufactured by Newspring® Packaging and purchased from www.instawares.com (Chen and Halpern, 2008). The Portion Cups and lids were made of homopolymer polypropylene. Two holes were punched in the lids of the container; each about 3.5 cm from the ends of the container lids. The distance separating the two holes was approximately 1.8 cm. A specific amount of odorant (0.5mL or 1.0mL) was placed in the bottom of each ODC using a micropipette. The lids were placed on the ODC and a homopolymer polypropylene Jetware® 7.75″ Unwrapped Plastic drinking straw (Jet Plastica Industries, Inc., 1100 Schwab Road, Hatfield PA 1440. (215)362-1501) was cut to 6.5 cm and placed in one of the holes so that half (3.25 cm) was visible above the container. A plastic tube (polyethylene 5 mL vial with hinged cap, diameter 1-3/8cm, Fisherbrand Scientific, Inc. 2000 Park Lane, Pittsburgh, PA 15275, (877) 885-2081) was cut to 3.0 cm and inserted into the other hole so that 1.5 cm was above the lid. This tube was stoppered when testing was not in progress so that diffusion out of the container was minimal. During testing and training procedures the cap was opened to allow air flow in and out of the ODC. The experimenter was careful to not tip or tilt the ODC once the odorant added, so there could be no chance of contact between

the straw and the odorant. Participants were also instructed to hold the ODC upright during both training and testing sessions. Nose clips (Spirometrics Nose Clip #2104, Spirometrics, P.O. Box 680, 22 Shaker Rd. Gray, ME 04039; (207)657-6700) were used to accomplish correct retronasal and OCO smelling techniques. Each participant was given their own nose clip that was discarded at the end of the session.

*Figure 2*: ODC pictured with nose clip used by participants in retronasal and OCO smelling

*Odorants*

The odorants used in these studies were all ten-carbon alcohols that activated the TRPM8 membrane channel in the oral cavity (Bandell et al., 2007). They were ordered from Sigma Aldrich Chemical Company, Inc. (P.O Box 355, Milwaukee, WI 53201 (414) 273-3850). The TRPM8 odoarnts used were **Eucalyptol**, **Linalool**, **Geraniol**, **L-carvone**, and **Isopulegol**. They were approved by Cornell's Institutional Review Board for Human Participants for use in retronasal and oral-cavity only studies. **dl-menthol** had previously been approved and used in studies of retronasal and OCO smelling.

For each of L-carvone, Linalool, Geraniol, and Isopulegol, 1.0 mL of undiluted odorant was pipetted into the ODC. 0.5mL of Eucalyptol was used, due to the overwhelming sensation it had given in preliminary and benchtop testing. Since the dl-menthol used was a solid, it was dissolved in sunflower oil (Spectrum High Heat

Sunflower Oil, distributed by Spectrum Organic Products of the Hain Celestial Group, Inc. Melville, NY 11747, (800)343-7833) to yield a 0.21 molar solution; one mL of this solution was added to the ODC.

Identifier names were given to each of the six odorants based on benchtop research done to see what people associated the smell of the odorants with orthonasally. The results are as shown in the table below.

*Table 1*: **Identifier Names** for the TRPM8 Odorants used in the retronasal and OCO identification tests.

| **Odorant** | **Common Name Identifier** | **Odorant Information** |
|---|---|---|
| dl-menthol | Peppermint | CAS # 89-78-1, >99%FCC, catalog # W26650-7, batch D08240TD |
| L-carvone | Spearmint | CAS # 6485-40-1, >97% FCC, catalog W22901, batch 04309JH |
| Eucalyptol | Ointment | CAS # 470-82-6, >99% FCC, catalog # W46506, batch 36896KH |
| Isopulegol | Toothpaste | CAS # 89-79-2, >95% FCC, catalog # W296228, batch 09807JU |
| Linalool | Cleaner | CAS # 78-70-6, >97% FCC, catalog # W263508, batch 16696AJ |
| Geraniol | Lemon | CAS # 106-24-1, >97% FCC, catalog # W250708, batch 01202ME |

Participants were eventually tested using these identifier names to see if they could identify the odorants retronasally and OCO. All odorants were allowed to come to room temperature before presentation in rooms kept between 20 and 21 degrees Celsius.

*Superlab Program*

The computer software program SuperLab 4.0 (Cedrus Corporation, P.O. Box 6309, San Pedro, CA 90734) was used to track the responses of the participants in the retronasal and OCO identification tests. Slides with instructions were first presented to orient the participant and then testing screens appeared in which the participant would click on the descriptor he or she believed matched the odorant he/she was presented with. There was no time limit between the appearance of the testing screen and when the participant was required to choose one of the identifier names. The results were then interpreted from spread sheets generated by Superlab.

**Experimental Procedure**

*Retronasal Identification Testing Procedure*

To smell retronasally, one inhales through the mouth and exhales through the nose. The participants were shown how to accomplish this using the ODC and a nose clip. To smell retronasally, the participant puts the nose clip on, inhales through the straw in the ODC (being careful not to tip the container), removes the nose clip and exhales through the nose, keeping his or her lips together. This procedure was demonstrated and the participant had a chance to practice the procedure on an empty ODC. The tube was unstoppered for the training and testing procedures to allow airflow into the container as the participant drew air through the straw. It was stoppered between the time of preparation and testing to diminish the possibility of losing vapor phase odorant to diffusion out of the hole in the container.

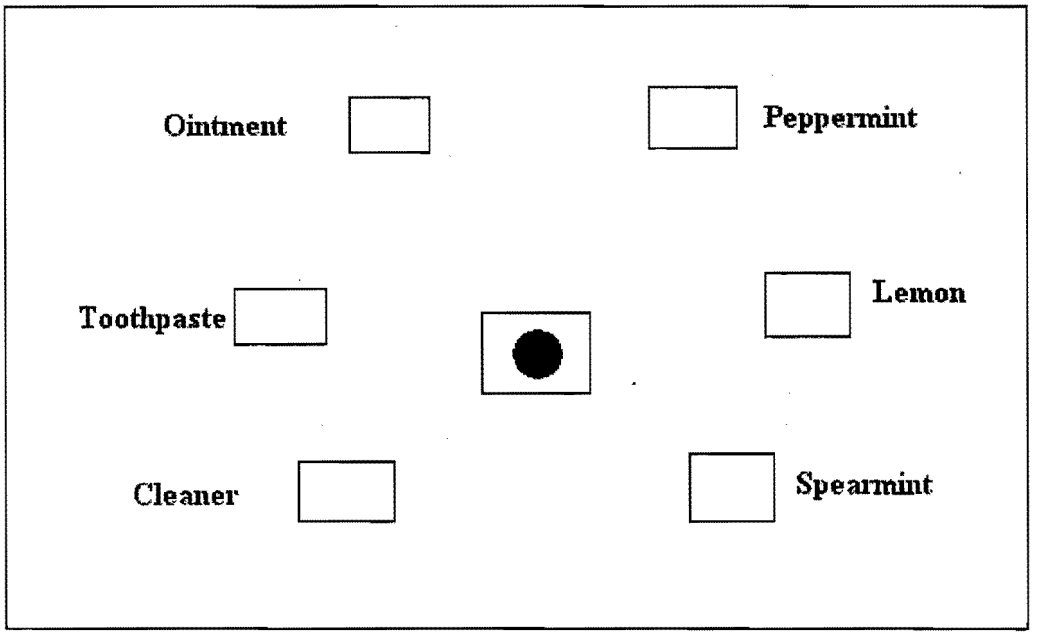
A *training session* was then conducted in order to familiarize the participants with the identifier names chosen for the odorants. The participant was presented with each

ODC separately, told what the identifier name was, and given time to retronasally smell the odorant as many times as he/she wanted. This procedure was repeated for the other five odorants. The odorants were presented in the following order for both retronasal and OCO training: dl-menthol, L-carvone, Eucalyptol, Isopulegol, Linalool, Geraniol. At the end of the training session the participant was given the opportunity to re-smell any or all of the odorants.

The participant was then directed to sit in front of the computer. The SuperLab program reiterated the instructions for smelling retronasally and outlined the procedure of the experiment. The participant was presented with the six odorants, three times each in random order. The odorants were presented in three blocks of six, with 30 seconds between each presentation in a block, and 1minute breaks between blocks.

Before an odorant was presented, the participant would be seated at the computer with the odorant in front of them and their nose clip on. The computer screen would display only a centered rectangle with a filled circle within it. The participant would inhale through the straw, hold the odorant in their mouth, and then take off the nose clip and exhale through the nose. The participant was instructed that, upon exhalation, they were to click the filled circle within the centered rectangle. A screen would then appear with the six identifier names equidistant from the filled circle within the centered rectangle. (Figure 3).

*Figure 3* shows the **six-choice display** that was presented to each of 24 participants on each retronasal or OCO identification test. Participants would click the mouse in the square they believed correctly identified the odorant presented.




Using the mouse, the participant would click whichever identifier name he/she believed corresponded to the odorant just presented. That screen would remain until the participant clicked in one of the six boxes. This was a forced-choice procedure.

The participant was informed that he/she could take a break, remove the nose clip, or get a drink of water at any time in between the odorant presentations.

Responses were recorded by the SuperLab program and then compared to correct identifications for analysis.

*Oral-Cavity-Only Identification Testing Procedure*

OCO testing was very similar to the retronasal testing procedure. The procedure for smelling odorants OCO is to put the nose clip on, inhale through the straw in the ODC, and then exhale through the mouth, keeping the nose clip on the entire time. This was

demonstrated and practiced by the participant with an empty ODC. Participants were informed that they may not receive the same degree of sensory input while smelling OCO as they had in the retronasal presentations, so to use any sensations, especially cooling or tingling, that they felt to help make their identifications. The same training procedure was then conducted, in which the participant smelled each odorant OCO after being told the identifier name for that odorant. The participants had the opportunity to re-smell any of the odorants as many times as needed before they felt comfortable moving on to the testing.

Again, a SuperLab program was set up to display instructions and record the responses of the participants. The odorant was presented to the participant, who sat at the computer with the nose clip on. The participant would inhale through the straw, hold the odorant in his/her mouth and then exhale through the mouth, leaving the nose clip on the entire time. Upon exhalation and concurrent click of the mouse, the same six-choice display screen was presented, allowing participants to choose the identifier. This was repeated eighteen times (six odorants three times each in random order in blocks of six).

*Triangle Test OCO Detectability Procedure*

A triangle test refers to an experiment in which three stimuli are presented, two identical and one different. The participant's task is to determine which is different (Introduction to Statistics Glossary, Triangle Test, 2009). In this case, three ODCs were presented OCO to the participants. Two of these contained pure non-odorant (sunflower oil), and one contained 1 mL of either Geraniol, Isopulegol, or L-carvone. Only these three odorants were selected for the triangle test because Eucalyptol and Linalool were both identifiable during the Identification experiment, and thus detectable in the oral

cavity, and dL-menthol was shown to be identified OCO in an earlier study (Parikh et al., 2009). These three odorants were thus left out of the Triangle Test, which aimed to determine if the odorants were discriminable from non-odorant OCO.

The participants' task was to identify the container that contained the odorant. Each container was labeled with a three-digit code generated by a random-number generator. Each odorant was presented three times, for a total of nine presentations. The three containers for each presentation were presented at one time, lined up horizontally on the table in front of the participant. After the participant had made his or her selection the containers were removed and the next three were set in front of him/her. There was at least 30 seconds between each presentation and participants were informed that they could take a break, remove their nose clip, or get a drink of water at any time between the presentations.

Participants were trained on the proper method of OCO smelling (inhaling through the straw and exhaling through the mouth, while keeping the nose clip on the entire time) and given the opportunity to practice with an empty ODC. Reponses were recorded on a participant response form that was divided into the nine presentations with the corresponding code numbers that were in each presentation. The participants were instructed to circle the code of the container they believed contained the odorant. Again, participants were instructed to be aware of any cooling or tingling sensations in the oral cavity to help with this task.

*Statistical Analyses*

Results were analyzed using non-parametric statistics because of the small sample size of both experiments. Non-parametric statistics also allowed us to avoid assumptions,

such as a normal distribution of responses in the population. These tests focus on ranks, which worked well with the pair-wise comparisons that were calculated. An $\alpha$ level was set to 0.05, so probability values (p-values) $<0.05$ were considered statistically significant.

For the Retronasal and Oral Cavity Only Identification tests, results were analyzed as percentages of correct identifications. In the Triangle Test Discrimination experiment, results were analyzed based on the number of correct discriminations for each odorant. Medians, 1st and 3rd quartiles (Q1 and Q3 respectively) and semi-interquartile ranges (SIRs) were determined for both data sets to be used for inferential statistics. A non-parametric one-way Analysis of Variance test, the Friedman ANOVA, was used to determine if there were statistically significant differences between participant responses. All pair-wise comparisons (across presentation condition or odorants) were done using the Wilcoxon signed rank test for comparisons. As the number of comparisons increases, the probability that a difference that is simply due to chance appears significant increases. To correct for this possibility, Bonferroni corrections of the p-values were made. The adjustments are done by multiplying the smallest, most significant p-value, by the number of comparisons, the second smallest by the number of comparisons minus one, and so on, until the largest p-value is multiplied by one (Introduction to Statistics Glossary, Bonferroni Adjustment, 2009).

## Results

### *Retronasal and Oral Cavity Only Identification Test*

Overall, the ANOVA analyses of the results illustrated that there were significant differences in the percentage of correct identifications ($p<0.001$) across all six TRPM8 odorants and both presentation conditions (Retronasal and OCO), as well as across odorants for just OCO or just Retronasal presentation.

*Table 2* presents raw data on the **percentage of correct IDs each participant had retronasally**. 24 participants were presented with each of six odorants 3 times in a random order. Participants could thus have obtained 0 (0%), 1 (33%), 2(67%) or 3(100%) correct IDs. Numbers in bold show instances when a participant chose the correct identifier 2/3 or 3/3 times.

| *Participant* | *dl-menthol* | *L-carvone* | *Eucalyptol* | *Isopulegol* | *Linalool* | *Geraniol* |
|---|---|---|---|---|---|---|
| *1* | **67** | **67** | **67** | 33 | **100** | **100** |
| *2* | 33 | **67** | **100** | 0 | **67** | **67** |
| *3* | **67** | 0 | **100** | 0 | **100** | **67** |
| *4* | 33 | 0 | **100** | **100** | 33 | 33 |
| *5* | 33 | **67** | **100** | **100** | **100** | 33 |
| *6* | 0 | 0 | **100** | 33 | 0 | **67** |
| *7* | 33 | **67** | **100** | 0 | **100** | **100** |
| *8* | 0 | 33 | **67** | 33 | **100** | 0 |
| *9* | 33 | 33 | **100** | **67** | **100** | 0 |
| *10* | 0 | **67** | **67** | 33 | 33 | **100** |
| *11* | 33 | **100** | **100** | 33 | **100** | **67** |
| *12* | 0 | **67** | **100** | **67** | **100** | **100** |
| *13* | **100** | 33 | **100** | 33 | **100** | 33 |
| *14* | 0 | 0 | **67** | 33 | **67** | 33 |
| *15* | **100** | 33 | **67** | 33 | 33 | **67** |
| *16* | 0 | 33 | **67** | **100** | 33 | **67** |
| *17* | 33 | 33 | **100** | **67** | 33 | **100** |
| *18* | **67** | **67** | **100** | 33 | **67** | **100** |
| *19* | 0 | **67** | **100** | 33 | **67** | **100** |
| *20* | 0 | 0 | **100** | 33 | 0 | 0 |
| *21* | 0 | **67** | **100** | 33 | **67** | **100** |
| *22* | 0 | **67** | **100** | 33 | **67** | **100** |
| *23* | 0 | **100** | **67** | 0 | **67** | **67** |
| *24* | 0 | **100** | **100** | **67** | **100** | **67** |

*Table 3* presents raw data on the **percentage of correct IDs each participant had oral cavity only**. 24 participants were presented with each of six odorants 3 times in a random order. Participants could thus have obtained 0 (0%), 1 (33%), 2(67%) or 3(100%) correct IDs. Numbers in bold show instances when a participant chose the correct identifier 2/3 or 3/3 times.

| *Participant* | *dL-menthol* | *L-carvone* | *Eucalyptol* | *Isopulegol* | *Linalool* | *Geraniol* |
|---|---|---|---|---|---|---|
| *1* | **67** | 33 | **67** | 33 | **67** | **67** |
| *2* | 0 | 0 | **100** | 0 | 0 | 0 |
| *3* | **67** | **67** | **100** | **100** | 33 | 33 |
| *4* | 0 | 0 | **100** | **67** | 0 | 33 |
| *5* | **67** | 0 | **100** | 33 | 33 | **67** |
| *6* | 0 | 0 | **67** | **67** | 0 | 0 |
| *7* | 33 | 0 | **100** | 0 | **67** | 33 |
| *8* | 0 | **67** | **67** | 33 | 0 | 33 |
| *9* | 0 | 33 | **100** | 0 | **67** | 0 |
| *10* | 0 | **67** | 33 | 33 | 33 | 0 |
| *11* | 0 | 0 | **67** | 33 | **67** | 0 |
| *12* | 33 | 0 | **100** | 0 | **100** | 0 |
| *13* | 0 | 0 | **100** | 0 | 0 | **67** |
| *14* | 33 | 33 | **67** | 33 | **67** | 33 |
| *15* | **67** | 0 | **100** | **67** | **67** | 33 |
| *16* | 0 | 33 | **67** | 0 | 33 | 0 |
| *17* | 33 | 33 | **67** | 0 | 33 | 0 |
| *18* | **67** | **67** | **67** | 33 | 33 | **100** |
| *19* | 33 | **67** | **100** | **67** | 33 | 0 |
| *20* | 0 | 33 | **100** | **67** | 33 | 0 |
| *21* | 0 | 33 | **67** | 0 | 33 | 0 |
| *22* | 0 | 33 | **100** | 33 | 33 | 0 |
| *23* | **100** | 0 | **67** | **67** | 0 | 0 |
| *24* | **100** | 0 | **67** | **67** | 0 | 0 |

**Table 4.** Each of 6 odorants was presented 3 times, in random order in blocks of 6, to each of 6 participants, retronasal and oral cavity only. Participants selected 1 of 6 possible identifications (ID) under forced-choice conditions on each trial.

| | RETRONASAL | | ORAL-CAVITY-ONLY | |
|---|---|---|---|---|
| ODORANT and Correct ID | Median % Correct ID[a] | % of Participants Who Selected[b] the Correct ID $p \leq 0.05$ | Median % Correct ID[a] | % of Participants Who Selected[b] the Correct ID $p \leq 0.05$ |
| Eucalyptol "ointment" | **100%** | **100%** | **84%** | **96%** |
| Geraniol "lemon" | **67%** | **71%** | 0%* | 17% |
| Linalool "cleaner" | **67%** | **71%** | 33%* | 29% |
| L-carvone "spearment" | **67%** | **54%** | 33% | 21% |
| Isopulegol "toothpaste" | 33% | 29% | 33% | 29% |
| DL-menthol "peppermint" | 17% | 31% | 17% | 29% |

Percentages above 50% are in bold and underlined.
a = Median calculated from % of correct ID selections across the 24 participants, where each participant's % could be 0%, 33%, 67%, or 100% (i.e., correct on 0, 1, 2, or 3 trials).
b = Probability of a participant selecting the correct ID on each trial was 1/6, i.e., 0.17; for two trials, 0.03, and therefore < 0.05.
* Significantly different from retronasal % correct ID.

## Oral Cavity Only

Oral cavity only median % correct ID and modal ID were: dL-menthol 17% (peppermint), L-carvone 33% (peppermint), Eucalyptol 84% (ointment), Isopulegol 33% (toothpaste), Linalool 33% (cleaner), Geraniol 0% (peppermint). Identifications for Eucalyptol and Linalool differed significantly from all other OCO ID except for Geraniol. The modal, median, Q1, and Q3 ID for Eucalyptol were all ointment (median % correct: 84%). OCO Linalool had a modal and Q1 ID of cleaner, which no other stimulus had. This data supports OCO ID of these two stimuli as distinct from any other stimuli presented. By contrast, there were not significant differences between the IDs of dL-menthol, L-carvone,

and Geraniol. The modal and median IDs for all three of these odorants were all peppermint (Tables 3,4).

***Table 5*: P-values for the number of Correct IDs for the six odorants presented OCO** Odorants were presented 3 times each in a randomized order, in blocks of six, to 24 participants. Uncorrected p-values were derived from Wilcoxon Signed Rank Tests

| **Pair of Odorants Compared for Number of Correct IDs** | **Uncorrected p-value** | **Bonferroni corrected p-value** | **Bonferroni adjustment** |
|---|---|---|---|
| dL-menthol versus L-carvone | 0.1608 | 0.6432 | 4 |
| **dL-menthol versus Eucalyptol** | **< 0.0001** | **0.001** | 11 |
| dL-menthol versus Isopulegol | 0.425 | 0.85 | 2 |
| **dL-menthol versus Linalool** | **<0.0001** | **0.001** | 11 |
| **dL-menthol versus Geraniol** | **<0.0001** | **0.001** | 11 |
| | | | |
| **L-carvone versus Eucalyptol** | **<0.0001** | **0.001** | 11 |
| L-carvone versus Isopulegol | 0.2387 | 0.7161 | 3 |
| **L-carvone versus Linalool** | **<0.0001** | **0.001** | 11 |
| **L-carvone versus Geraniol** | **<0.0001** | **0.001** | 11 |
| | | | |
| **Eucalyptol versus Isopulegol** | **<0.0001** | **0.001** | 11 |
| **Eucalyptol versus Linalool** | **<0.0001** | **0.001** | 11 |
| Eucalyptol versus Geraniol | 0.02223 | 0.13338 | 6 |
| | | | |
| **Isopulegol versus Linalool** | **<0.0001** | **0.001** | 11 |
| Isopulegol versus Geraniol | 0.8587 | 0.8587 | 1 |
| | | | |
| Linalool versus Geraniol | 0.02477 | 0.12385 | 5 |

Table 5 values in bold represent statistically significant ($p<0.05$) differences. For all comparisons n (number of participants) = 24, df (degrees of freedom) = 11, 253.

## Retronasal Identifications

Retronasal median %correct identifications and modal ID, in parentheses, for the six stimuli were Eucalyptol 100% (ointment), dL-menthol 17% (toothpaste), L-carvone 67% (spearmint), Isopulegol 33% (toothpaste), Linalool 67% (cleaner), Geraniol 67% (lemon). Identifications for each presented stimulus were significantly different from the ID selected for the other retronasally presented stimuli ($p<0.05$, Bonferroni corrected) except for dL-menthol and L-carvone when compared with Isopulegol ($p>0.2$, corrected).

***Table 6:*** **P-values for the number of Correct IDs for the six odorants presented Retronasally**

Odorants were presented 3 times each in a randomized order, in blocks of six, to 24 participants. P-values were derived from Wilcoxon Signed Rank Tests

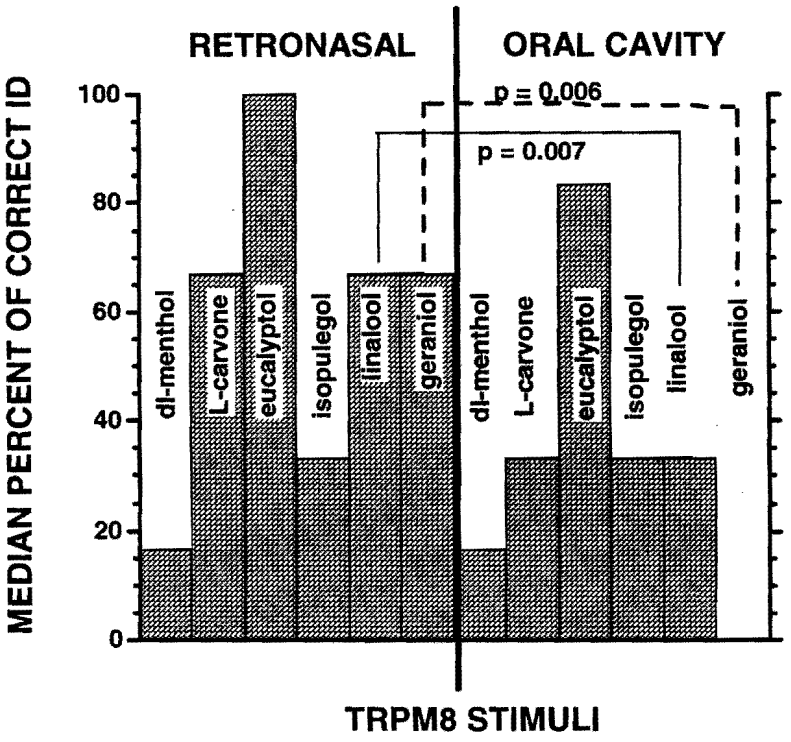
| Pair of Odorants Compared for Number of Correct IDs | Uncorrected p-value | Bonferroni corrected p-value | Bonferroni adjustment |
|---|---|---|---|
| **dL-menthol versus L-carvone** | **0.01346** | **0.05** | 4 |
| **dL-menthol versus Eucalyptol** | **<0.0001** | **0.001** | 10.5 |
| dL-menthol versus Isopulegol | 0.425 | 0.425 | 1 |
| **dL-menthol versus Linalool** | **<0.0001** | **0.001** | 10.5 |
| **dL-menthol versus Geraniol** | **<0.0001** | **0.001** | 10.5 |
| | | | |
| **L-carvone versus Eucalyptol** | **<0.0001** | **0.001** | 10.5 |
| L-carvone versus Isopulegol | 0.2387 | 0.4774 | 2 |
| **L-carvone versus Linalool** | **<0.0001** | **0.001** | 10.5 |
| **L-carvone versus Geraniol** | **<0.0001** | **0.001** | 10.5 |
| | | | |
| **Eucalyptol versus Isopulegol** | **<0.0001** | **0.001** | 10.5 |
| **Eucalyptol versus Linalool** | **<0.0001** | **0.001** | 10.5 |
| **Eucalyptol versus Geraniol** | **0.0177** | **0.05** | 3 |
| | | | |
| **Isopulegol versus Linalool** | **<0.0001** | **0.001** | 10.5 |
| **Isopulegol versus Geraniol** | **<0.0001** | **0.001** | 10.5 |

| | | | |
|---|---|---|---|
| **Linalool versus Geraniol** | **0.002812** | **0.02** | 5 |

Table 6 values in bold represent statistically significant ($p<0.05$) differences. For all comparisons $n = 24$, $df = 11, 253$.

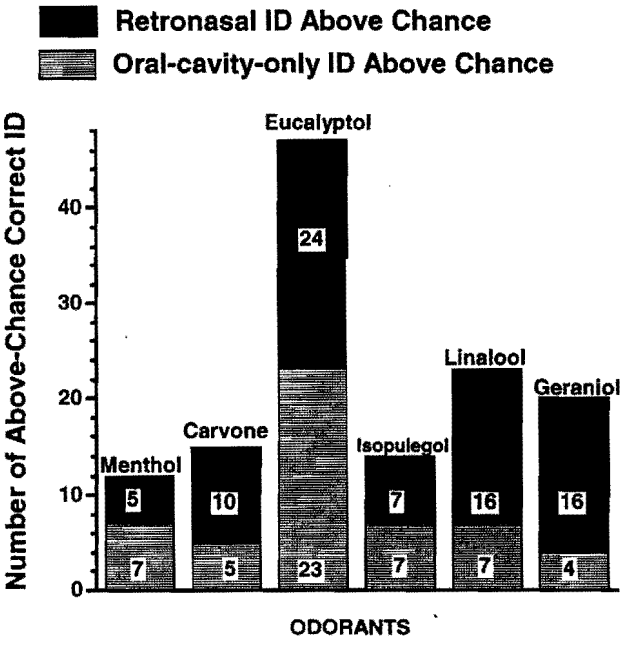
**Comparing Retronasal and OCO Identifications** of individual odorants shows that there were significant differences between the median correct identifications of Linalool (67% retro and 33% OCO, n=24, p=0.007 Bonferroni corrected) and Geraniol (67% retro and 0% OCO, n=24, p=0.006 Bonferroni corrected). For the other four stimuli presented there were not statistically significant differences between correct IDs made retronasally or OCO (Bonferroni corrected p-values are 0.725 for dL-menthol, 0.07 for L-carvone, 0.05 for Eucalyptol, and 0.374 for Isopulegol). The p-values were obtained from Wilcoxon Signed Rank Tests for paired comparisons.

*Figure 4*: **Comparisons of median percent correct identifications of odorants retronasally and OCO.** P-values are given for the differences between retronasal and OCO IDs for Linalool and Geraniol, as they were statistically significant. Bonferroni corrected P-values were determined using Wilcoxon Signed Rank Tests. 24 Participants were tested both retronasally and OCO for IDs of six TRPM8 odorants, presented 3 times each in random order in blocks of six.




For four of the six odorants (excluding dL-menthol and Isopulegol), retronasal correct identifications were higher than OCO identifications. dL-menthol was consistently hardest to identify, both retronasally and OCO. Eucalyptol had the highest rates of correct identifications across both presentations (Figures 4,5).

*Figure 5*: **Comparison of Above-Chance Correct IDs between retronasal and OCO presentations.** Comparison values based on Wilcoxon Signed Rank Tests, n=24, df=11,253. Six TRPM8 odorants delivered both retronasally and OCO three times each, random order, in blocks of six. Significant differences between correct ID across presentation condition exist for Linalool (p=0.007) and Geraniol (p=0.006).



## *Triangle Test Results*

The triangle-test discrimination aimed to determine if Geraniol, L-carvone, and Isopulegol could be discriminated from pure non-odorant (sunflower oil) OCO. Each odorant was presented three times, in random order, simultaneously with two containers of sunflower oil. Responses were recorded for each participant and each odorant.

***Table 7***: Number of **correct discriminations made by each of 15 participants** in the triangle test. All instances where a participant obtained 2/3 or 3/3 correct discriminations are shown in bold. The three TRPM8 odorants were presented three times each in random order, concurrently with two presentations of sunflower oil. A correct discrimination is deemed one in which the participant chooses the container that contains the TRPM8 odorant.

| *Participant* | *Geraniol* | *L-carvone* | *Isopulegol* |
|---|---|---|---|
| *T1* | 0 | 0 | **3** |
| *T2* | **3** | **3** | **3** |
| *T3* | 0 | 1 | **3** |
| *T4* | 1 | **2** | **3** |
| *T5* | 1 | 1 | **3** |
| *T6* | 1 | **2** | **3** |
| *T7* | 1 | **2** | **3** |
| *T8* | 1 | **2** | **3** |
| *T9* | 1 | **3** | **3** |
| *T10* | 1 | **3** | **3** |
| *T11* | **2** | **3** | **3** |
| *T12* | **2** | **3** | **3** |
| *T13* | **2** | **3** | **3** |
| *T14* | **2** | **3** | **3** |
| *T15* | **3** | **3** | **3** |

The probability that a participant would obtain 1/3 correct discriminations by chance alone is 0.33, the probability that a participant would obtain 2/3 by chance alone is 0.11, and the probability that a participant would obtain 3/3 correct by chance is 0.037.

*Table 8* presents the data from the triangle test arranged by **how many participants were able to correctly discriminate the TRPM8 odorants from sunflower oil** 0,1,2, or 3 times. 15 participants were presented with the 3 odorants three times each in random order.

| *Number of correct discriminations* | *Geraniol* | *L-carvone* | *Isopulegol* |
|---|---|---|---|
| *0 correct* | 2 | 1 | 0 |
| *1 correct* | 7 | 2 | 0 |
| *2 correct* | 4 | 4 | 0 |
| *3 correct* | 2 | 8 | 15 |

***Table 9*: Descriptive Statistics for the number of correct discriminations for the three odorants.** Q1, Median, and Q3 correct discriminations are listed.

| | **Q1** | **Median** | **Q3** |
|---|---|---|---|
| *Geraniol* | 1 | 1 | 2 |
| *L-carvone* | 2 | 3 | 3 |
| *Isopulegol* | 3 | 3 | 3 |

*Figure 6*: **Median Number of correct Discriminations and SIR** – Triangle-test discrimination results for Geraniol, L-carvone, and Isopulegol versus sunflower oil. Each odorant was presented 3 times, in random order, to 15 participants.

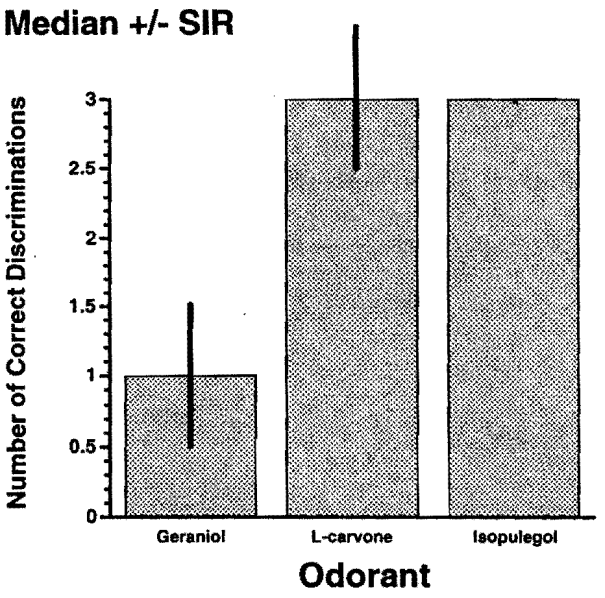



*Table 10:* **P-values for the number of Correct Median Discriminations for the three odorants presented in the triangle test**

Odorants were presented 3 times each in a randomized order with sunflower oil as the non-odorant. P-values were derived from Wilcoxon Signed Rank Tests. n=15, df= 2, 28. Significant differences were found in the median number of correct discriminations across all three odorants ($p<0.05$).

| Pair of Odorants Compared for Number of Correct Discriminations | Uncorrected p-value | Bonferroni corrected p-value |
|---|---|---|
| Geraniol versus L-carvone | 0.002 | 0.006 |
| Geraniol versus Isopulegol | 0.001 | 0.003 |
| L-carvone versus Isopulegol | 0.02 | 0.02 |

*Table 11:* **P-values of single comparisons between discriminations of the odorants in the triangle test and one correct discrimination.** Odorants were presented 3 times each in a randomized order with sunflower oil as the non-odorant. P-values were derived from Wilcoxon Signed Rank Tests, with Bonferroni adjustments. Significant differences (in bold) were found for Isopulegol and L-carvone, but not for Geraniol.

| **Odorant discrimination compared with one correct discrimination** | **Uncorrected p-value** | **Bonferroni corrected p-value** |
|---|---|---|
| Isopulegol | **0.0001** | **0.0004** |
| L-carvone | **0.002** | **0.005** |
| Geraniol | 0.12 | 0.12 |

## Discussion

These studies have helped to further elucidate the relationship between trigeminal stimulation in the oral cavity and the TRPM8 channel. The differences in the percentage of correct identifications between the six odorants when presented oral-cavity-only (OCO) suggest that the TRPM8 channel does not provide the same stimulation for all of the odorants that activate it and there is, thus one or more other trigeminal mechanisms acting in conjunction to moderate the sensation of these trigeminal odorants.

The *Retronasal and OCO Identification tests* provided useful information regarding the ability to identify these odorants and discriminate them from one another. There were significant differences in the percentage of correct identifications across all six TRPM8 odorants and both presentation conditions (Retronasal and OCO), as well as across odorants for just OCO or just Retronasal presentation. The participants were experiencing the odors in ways different enough that their responses to them were significantly different.

Given that the modal, median, Q1, and Q3 ID for Eucalyptol were all ointment and Linalool had a modal and Q1 ID of cleaner, it seems that these stimuli were identified as distinctly different from any other stimulus when presented OCO (Table 3). The sensory information provided by the oral cavity trigeminal system for eucalyptol is both similar to that provided by the combined trigeminal and olfactory systems of the nasal cavities and is sufficient to provide a very consistent identification. The fact that participants consistently chose the same ID for Eucalyptol does not, however, demonstrate that the retronasal and OCO responses to Eucalyptol are identical.

The OCO identifications of dL-menthol, L-carvone, Isopulegol, and Geraniol did not give the same level of certainty that they were being interpreted in distinctly different ways by the trigeminal system. There was a lot of overlap in identifications, with 'peppermint' being the ID most often given, even though it was only the correct ID for dL-menthol. Although in previous studies ID of dl-menthol as 'peppermint' has been high and distinct (Parikh et al., 2009), these new results may be attributable to the similarity of the other stimuli to dl-menthol, in that they all activate the same transmembrane channel. It is possible that these four odorants do not deliver sufficiently different sensations to the trigeminal system to be discriminated from one another and matched up to their correct identifier name. An additional consideration is the difference in concentrations of dL-menthol in the two studies. Parikh et al., 2009 used 0.15 grams of solid dl-menthol in each ODC for both retronasal and OCO testing (Parikh et al, 2009). This identification test used a 0.21 M solution of dl-menthol dissolved in sunflower oil. It is possible that the dissolved dl-menthol had a lower vapor phase headspace concentration than that of the solid dl-menthol. In the ODC with the dissolved dl-menthol, vapor phase of the sunflower oil would have also been present in the headspace of the container. This could have contributed to a lower percentage of correct identifications in both retronasal and OCO testing.

The Triangle Test was done to determine whether or not the results from the overlapping identifications of these odorants were because they were not being detected in the oral cavity or because Isopulegol, L-carvone, and Geraniol were delivering similar sensations to each other and dL-menthol. By comparing the TRPM8 odorants to pure non-odorant it was possible to make this distinction. DL-menthol was not included in the

triangle test because it has been shown to be discriminable from solvent (Parikh et al, 2009).

All three odorants (Isopulegol, Geraniol, and L-carvone) had median discrimination values that were statistically different from one another (Table 10). These odorants were being interpreted in a uniquely different way from one another. Geraniol did not seem to stimulate the oral cavity trigeminal system and could not be smelled, but the L-carvone could be smelled somewhat, and the Isopulegol could be easily smelled. Only two out of the fifteen participants were able to discriminate Geraniol from sunflower oil on all three presentations (Table 8). There is, thus, little evidence that Geraniol is being detected OCO. For the triangle test, on a single trial selecting the one ODC with the odorant from the three ODC would have a probability of 0.33; on two trials selecting the one ODC with the odorant from the three ODC would have a probability of 0.11, but on all three trials, a probability of 0.04. Therefore, discrimination by a participant was recognized only if the ODC with odorant was selected on all three presentations, because that gave a probability $\leq 0.05$. A Wilcoxon signed rank test was done between the actual discrimination data for the three odorants and a scenario in which one out of three correct discriminations were obtained. There were significant differences between the two cases for both Isopulegol and L-carvone but not for Geraniol (Table 11). It does not seem like there is evidence that Geraniol is an OCO stimulus, which coincides with the identification experiment, in which the median percent correct OCO identifications for Geraniol was 0%.

Individual differences across participants were observed for both the identification and triangle tests (Tables 3, 7). Some participants were consistently better at choosing the correct identifier for the stimulus presented. This may be a result of better concentration or memory during the training procedure, or heighted sensitivity in the oral cavity. A hierarchy of ease of odorant discrimination and identification point to the conclusion that different people have varying thresholds for these stimuli. In the triangle test, no one performed better on discriminations of Geraniol than they did on L-carvone. Therefore, for each participant, Isopulegol was always the most readily discriminated, followed or matched by L-carvone, and then followed or matched by Geraniol. The sensory experience of Geraniol seems to be less intense than that of L-carvone, which is less intense than that of Isopulegol (Tables 7).

The six TRPM8 odorants are all ten-carbon alcohols with molecular weights ranging from 150.22g/mol to 156.3 g/mol (Median 154.25g/mol, SIR 0). All odorants were presented at room temperature, between 20 and 21 degrees Celsius. It, therefore, seems unlikely that the vapor pressures, and thus the amount of vapor phase odorant in the headspace of the ODC had significant influence on the participant responses.

It is possible that participants did not perform as well as they could have on the identification experiment simply because they could not remember which ID corresponded to which smell. It may be useful to conduct a similar experiment in which participants are allowed to pick identifier names for the odorants after smelling them retronasally and OCO. These names would be more meaningful to the participant and may yield higher percentages of correct identifications.

Additionally, it may be useful to look at experiments in which these odorants are used in isointense concentrations. Preliminary testing would have to be done to determine concentrations that gave similar sensations to Geraniol, which appears to give the least OCO sensation. This could make identifications more reliable and eliminate the possibility that IDs or discriminations were only being made because of differences in concentration, as opposed to OCO smell.

---

## Conclusion

It appears as though the TRPM8 channel may be necessary for detectability of trigeminal stimuli in the oral cavity, but it is neither sufficient, nor the only factor. There must be another trigeminal mechanism in the oral cavity that aids in mediating the sensation of trigeminal odorants since there were differences in the extent that the six TRPM8 odorants were able to be identified and discriminated.

The results from the identification and triangle discrimination experiments illustrate that the six TRPM8 odorants are interpreted in different ways and able to be correctly identified to different extents. Eucalyptol and Linalool were able to be identified OCO, as dl-menthol had been in a previous study. Isopulegol and L-carvone, which were not able to be identified OCO were able to be discriminated from sunflower oil, whereas Geraniol was not.

**References**


AnesthesiaUK (2005). Anatomy of the trigeminal nerve. Available at: http://www.frca.co.uk/article.aspx?articleid=100533. Cited March 31, 2009.

Bandell, M., Macpherson L.J., and Patapoutian, A. From chills to chilis: mechanisms for thermosensation and chemesthesis via thermoTRPs. *Current Opinion in Neurobiology.*, 17, 490-7.

Chen, V. and Halpern, B. P. (2008). Retronasal but not oral-cavity identifications of non-trigeminal odorants. *Chem. Senses*, 33, 107-118.

Cometto-Muñiz, J. E., Cain, W. S., and Abraham, M. H. (1998). Nasal pungency and odor of homologous aldehydes and carboxylic acids. *Exp. Brain Res.*, 118, 180-188.

Cometto-Muñiz, J. E., Cain, W. S., and Abraham, M. H. (2005). Determinants for nasal trigeminal detection of volatile organic compounds. *Chem. Senses*, 30, 627-642.

Dragich A. M. and Halpern B. P. (2008). An oral-cavity component in retronasal smelling of natural extracts. *Physiol. Behav.*, 93, 521-528.

Doty, R, L., Cometto-Muñiz, J. E., Brugger, W. E., Jurs, P. C., Orndorff, M. A., Snyder, P. J., and Lowry, L. D. (1978). Intranasal trigeminal stimulation from odorous volatiles: psychometric responses from anosmic and normal humans. *Physiol. Behav.*, 20, 175-185.

Halpern, B. P. (2004). Retronasal and orthonasal smelling. *Chemo Sense*. 6 (3), 1-7.

Hummel, T. and Livermore, A. (2004). Intranasal chemosensory function of the trigeminal nerve and aspects of its relation to olfaction. *Int Arch Occu Environ Health.* 75, 305-13.

Introduction to Statistics Glossary. Bonferroni Adjustment. Available at http://www.statistics.com/resources/glossary/b/bonfadj.php. Cited May 2, 2009.

Introduction to Statistics Glossary. Triangle Test. Available at: http://www.statbasics.com/difftest/glossary.htm#triangle%20test. Cited March 31, 2009.

Leffingwell, J. C. (2001). Olfaction. Available at http://www.leffingwell.com/olfaction.htm. Cited March 31, 2009.

Parikh, V., Lee-Lim A.P., and Halpern B.P. (2009). Retronasal and oral-cavity only identification of air-phase trigeminal stimuli. *Chem. Percept.* 2, 9-24.