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Ocular and Nasal Trigeminal Detection of Butyl Acetate and Toluene Presented Singly and in Mixtures

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Running head: Trigeminal Chemosensory Detection

ABSTRACT

To probe into the rules of trigeminal chemosensory agonism in a binary mixture of chemicals we measured, first, the detectability (i.e., psychometric) function for eye irritation and for nasal pungency of butyl acetate and toluene, singly. (To avoid olfactory biases, nasal pungency was measured in a group of anosmics, i.e., persons lacking a functional sense of smell.) Then, based on the detectability function obtained for the individual chemicals, we prepared mixtures where the two components varied in their relative proportions but, if a simple rule of complete sensory agonism (in the sense of dose-additivity) were to hold, the mixtures should be as detectable as the reference concentration of each of the single chemicals. For both trigeminal endpoints (i.e., eye irritation and nasal pungency), the results showed that stimuli of relatively low detectability did show complete sensory agonism, whereas stimuli of relatively high detectability fell short of complete sensory agonism when compared with the detectability of the single substances. Further testing of additional binary and higher order mixtures will confirm whether or not a structure-activity model of trigeminal chemosensory impact of single chemicals, based on selected physicochemical parameters of the stimuli, can also be applied to chemical mixtures.

Key words: Eye irritation - Nasal pungency - Trigeminal nerve - Butyl acetate - Toluene - Chemosensory detection - Chemical mixtures - Psychometric chemosensory functions.

INTRODUCTION

In everyday exposures, the human olfactory and trigeminal chemosensory modalities respond to mixtures of volatile organic compounds (VOCs). In the mucosae of the face, trigeminal nerve responses to airborne VOCs include nasal pungency and eye irritation. The term nasal pungency covers a variety of chemo-sensations evoked in the nasal cavity that are not properly odors. These include prickling, burning, irritation, tingling, freshness, stinging, and piquancy, among others. A suggested term for these and other sensations produced by chemicals in exposed mucosae is "chemesthesis" (Green and Lawless, 1991; Green et al., 1990). This form of chemosensitivity is also known as the "common chemical sense" (Keele, 1962; Parker, 1912).

These pungent sensations arise from the activation of receptors thought to be polymodal nociceptors that, in the mucosae of the face, are present within the free endings of the trigeminal nerve (Silver and Finger, 1991). Accordingly, chemesthesis is an aspect of the somatic sensory system. Nociceptors appear in C and A_{delta} fibers (Martin and Jessell, 1991). At least a subset of sensory C-fibers expresses a receptor particularly sensitive to capsaicin, the pungent agent in hot peppers, and to structurally related molecules known as vanilloids (Szallasi, 1994). This receptor can also be activated by noxious heat (Caterina et al., 1997). Another specific chemoreceptor present in trigeminal endings is the nicotinic acetylcholine receptor (Alimohammadi and Silver, 2000). Studies in animals and humans indicate that this receptor discriminates between S(-) and R(+)-nicotine (Thürauf et al., 1999; Walker et al., 1996).

In contrast, there is little information on the type of trigeminal chemoreceptors that might account for the responsiveness to the extremely wide chemical variety of substances collectively referred to as VOCs. These comprise alcohols, esters, ketones, carboxylic acids, aldehydes, and the like, including linear and branched, saturated and unsaturated, aliphatic and aromatic molecules. These are the kinds of substances typically encountered in indoor air

(Brown et al., 1994; Wolkoff and Wilkins, 1993) and that have been clearly shown to evoke, at high enough concentrations, the trigeminal responses of nasal pungency and eye irritation in humans (Cometto-Muñiz and Cain, 1990, 1991, 1993, 1994, 1995; Cometto-Muñiz et al., 1998a; Cometto-Muñiz et al., 1998b). Given the broad diversity of VOCs in terms of structure and properties, we reasoned that their trigeminal effectiveness would rest heavily on general physicochemical parameters that govern the transport of the stimulus from the vapor phase to the biophase where trigeminal chemoreception takes place. The success of a model based on a solvation equation to describe and predict measured human thresholds for nasal pungency (Abraham et al., 1998a) and eye irritation (Abraham et al., 1998b) confirmed this expectation. Nevertheless, the finding of a size restriction for an irritant molecule to be effective (a "cut-off" effect) (Cometto-Muñiz et al., 1998a) cannot be explained with a "transport-only" model, suggesting the existence of a receptor pocket of finite dimensions within the trigeminal chemoreception process.

In brief, the solvation equation can be stated as equation (1). Here, SP is the dependent variable such as log (1/NPT), where NPT stands for nasal pungency threshold, and the independent variables are solute (VOC) properties or descriptors as follows (Abraham et al., 2000a; Abraham et al., 2000b): **E** is the solute excess molar refractivity in units of (mol cm^{-3})/10, **S** is the solute dipolarity/polarizability, **A** and **B** are the overall or summation hydrogen bond acidity and basicity, and **L** is the logarithm of the gas-hexadecane partition coefficient.

$$SP = c + e.\mathbf{E} + s.\mathbf{S} + a.\mathbf{A} + b.\mathbf{B} + l.\mathbf{L} \quad (1)$$

Study of the trigeminal detection of mixtures of chemicals vis-à-vis that of the individual components provides a tool to gain insight into the breadth of chemical tuning in the trigeminal system and its implications for the perception of mixtures. A recent study of the olfactory and trigeminal detectability of 1-butanol and 2-heptanone singly and in binary mixtures lent support in both sensory modalities to the notion of dose additivity, as a first approximation, between

the two chemicals presented at perithreshold levels (Cometto-Muñiz et al., 1999). In the present investigation we sought to continue this line of research by selecting two chemicals that vary differently, in terms of structure and properties, from the alcohol and ketone tested previously. To this end, we selected butyl acetate and toluene. From a structural-chemical criterion, the new pair presents a sharper contrast than the previous pair: Butyl acetate is an aliphatic, lineal, relatively flexible hydrocarbon molecule with an oxygen containing chemical group (ester), whereas toluene is an aromatic, cyclic, relatively rigid hydrocarbon molecule with no oxygen. From a physicochemical criterion based on interaction, for example hydrogen-bonding capability, it is the pair 1-butanol/2-heptanone that presents the sharper contrast (see Table 1). The study aimed to probe whether the different physicochemical contrast between these two pairs would reflect itself in a different degree of agonism, i.e., dose additivity, of their mixtures. A systematic study of properly selected binary, ternary, and higher order mixtures, with the aid of an interpretable physicochemical model (such as the solvation equation described above), should be able to uncover the rules that govern the chemosensory detection of mixed VOCs.

Insert Table 1 about here

METHODS

The study protocol was approved by the Human Subjects Committee of the University of California, San Diego. All subjects gave written inform consent on forms approved by the Committee.

Subjects

Experiment 1: Eye Irritation Detectability of the Single Chemicals

We recruited a normosmic and an anosmic group of subjects. The normosmic group included 12 participants (6 females, 6 males) with an average age (±SD) of 28 (±10) years, and ranging from 19 to 51 years. One male (37 years old) was a smoker, three males (27, 40, and 51 years old) and a female (40 years old) were previous smokers, and all others were nonsmokers.

The anosmic group included 6 participants (2 females, 4 males) with an average age (±SD) of 53 (±18) years, and ranging from 34 to 74 years. All but one male (36 years old) were nonsmokers. Two (1 female, 1 male) were congenital anosmics, two (1 female, 1 male) were head-trauma anosmics, one male was anosmic most likely due to chemical exposures, and the cause of anosmia for the remaining male was not firmly established (perhaps the anosmia was secondary to Parkinson's disease).

A standardized olfactory test (Cain, 1989) served to classify participants as normosmics or anosmics.

Experiment 2: Eye Irritation Detectability of Binary Mixtures

Only normosmic participants were tested on the detection of eye irritation from binary mixtures, thus the mixtures were prepared as described below based on the detectability of the single chemicals as perceived by normosmics. A subgroup of 7 normosmics (4 females, 3 males) from the original 12 participants continued to be available for testing. Their average age (±SD) was 29 years (±12), and they ranged from 19 to 51 years. One male (37 years old) was a smoker, another one (51 years old) was a previous smoker, and all other subjects were nonsmokers.

Experiment 3: Nasal Pungency Detectability of the Single Chemicals

Five anosmics were tested, four of whom had participated in Experiment 1. The remaining subject was a 42-year-old female, congenital anosmic. This group had an average age

(±SD) of 51 (±15) years, and included three congenital anosmics (2 females, one male), one head-trauma anosmic (female), and one male whose anosmia was perhaps secondary to Parkinson's Disease. All participants were nonsmokers.

Experiment 4: Nasal Pungency Detectability of Binary Mixtures

A total of 6 anosmics (4 females, 2 males) participated. Four of them had participated in Experiment 3. The remaining two subjects were a 74-year-old male whose anosmia was probably due to chemical exposures (who had participated in Experiment 1) and a 37-year-old female, head-trauma anosmic. The group had an average age (±SD) of 49 (±16) years, and included three congenital anosmics (2 females, 1 male), two head-trauma anosmics (females) and the 74 year-old male anosmic mentioned above. All participants were nonsmokers.

Stimuli and Equipment

Experiment 1: Eye Irritation Detectability of the Single Chemicals

Stimuli included butyl acetate (99+%) and toluene (99.8%). Mineral oil (Light, Food Chemical Codex quality) served as solvent and blank. Duplicate dilution series made in two-fold dilution steps for butyl acetate and in 1.5-fold dilution steps for toluene were prepared. Each series started with undiluted chemical (100% v/v), labeled dilution step 0. In the case of butyl acetate, the series continued with 50, 25, 12.5, etc. % v/v, labeled dilution steps 1, 2, 3, etc., respectively. In the case of toluene, the series continued with 67, 44, 30, etc. % v/v, labeled dilution steps 1, 2, 3, etc., respectively. Stimuli were stored and delivered from glass vessels (1,900 ml capacity) containing 200 ml of solution. The vessels have been recently described in a study of nasal pungency thresholds (Cometto-Muñiz et al., 2000). In the present investigation the vessels were adapted for ocular testing by capping one of the two outlets for nosepieces and replacing the other nosepiece with a 25-ml, roughly conical container (of the sort used in variable volume dispensers). During testing, the subject rested one eye socket against the round rim of the container. Immediately afterwards, a tube carrying a low-flow (4 l/min) of pure

air was connected to the inlet of the bottle (see Figure 1). This connection allowed an aliquot of the headspace of the bottle to fill the conical container where the eye was being exposed. Each exposure (blank or stimulus) lasted for 3 seconds. These roughly conical eyepieces are exactly the same as those used before for ocular testing with squeeze bottles (Cometto-Muñiz and Cain, 1991).

Insert Figure 1 about here

Vapor concentrations in the headspace of every vessel were measured off-line by gas chromatography (flame ionization detector, FID) via direct sampling with a gas-tight syringe. Measurements were done right after preparation of the stimuli and weekly thereafter to confirm stability. Figure 2 shows the average vapor-phase concentration (±SD) corresponding to each liquid dilution step of butyl acetate and toluene (Figure 2). The coefficient of variation across dilution steps for the gas chromatographic measurements performed weekly averaged (±SD) 12.2% (±9.8) for butyl acetate and 8.7% (±5.6) for toluene. Repetitive liquid injections of known masses of the respective compounds allowed conversion of the chromatographic readings to concentration in ppm.

Insert Figure 2 about here

Experiment 2: Eye Irritation Detectability of Binary Mixtures

Butyl acetate, toluene, and the solvent mineral oil were identical to those used in Experiment 1. The glass vessels adapted for ocular testing were also identical to those used for the single chemicals (Figure 1). In this experiment, though, the stimuli comprised both binary mixtures of the two substances and the single substances.

In order to prepare the binary mixtures we relied on the linear range of the psychometric function (in normosmics) for each compound as stressed in Figure 5. We selected three levels

of detection probability: 0.50, 0.75, and 1.00, and applied the linear equations to calculate the corresponding concentrations of each chemical producing those detection probabilities (concentrations labeled $BA_{0.50}$, $BA_{0.75}$, and $BA_{1.00}$ for butyl acetate and $T_{0.50}$, $T_{0.75}$, and $T_{1.00}$ for toluene, respectively). Next, again using the equations, we calculated the concentrations necessary to produce detection probabilities equal to 3/4, 1/2, and 1/4 of the three target detection probabilities selected and mentioned above. In other words, we calculated the concentration of each chemical producing detection probabilities of: 0.375, 0.250, and 0.125 (that is, 3/4, 1/2, and 1/4 of 0.5); 0.562, 0.375, and 0.188 (that is, 3/4, 1/2, and 1/4 of 0.75); and 0.750, 0.500, and 0.250 (that is, 3/4, 1/2, and 1/4 of 1.00). Once these concentrations were calculated, we prepared the three sets of stimuli listed in Table 2 where concentrations are labeled following the same notation as above.

Insert Table 2 about here

It can be seen in Table 2 that each set of five stimuli contains two single chemicals and three binary mixtures. The three mixtures on each set contain varying concentrations of the individual compounds such that the sum of the detection probabilities produced by each component by itself produces the same total for the three mixtures. For example, in the third set of five stimuli, numbered 11 through 15, the concentration of BA in mixture 12 was chosen to produce 0.562 detection probability and that of T to produce 0.188 detection probability, which, when added, produce 0.75 detection probability. For stimulus 13 we have 0.375 + 0.375 = 0.75, and the same result for stimulus 14 (0.188 + 0.562 = 0.75). Note that each five-stimulus set is completed with two stimuli that are single chemicals (one BA, the other T) instead of mixtures, and that the concentration at which they are present was chosen to produce the same total as the mixtures, in our example 0.75. In summary, assuming simple dose additivity in the detection of mixtures, the second set of five stimuli is expected to produce 0.50 detection probability, the third set to produce 0.75, and the fourth set to produce 1.00.

In order to avoid depletion of the headspace of the vessels containing these 15 stimuli (numbers 6 through 20 in Table 2) as subjects were tested, we prepared each in quintuplicate.

As before, vapor concentrations in the headspace of every vessel (including all quintuplicates) were measured off-line by gas chromatography (flame ionization detector, FID) via direct sampling through a gas-tight syringe. Measurements were done right after preparation of the stimuli and weekly thereafter to confirm stability. Figure 3 shows the average vapor concentration (±SD) for each of the 15 stimuli tested in this experiment.

Insert Figure 3 about here

Experiment 3: Nasal Pungency Detectability of the Single Chemicals

Stimuli and blank were the same as described for Experiment 1. The same glass vessels were used but adapted with two nosepieces as described in a recent paper (Cometto-Muñiz et al., 2000), instead of the eyepiece. Considerations about measurement and calibration of vapor concentrations in the headspace of every bottle by gas chromatography are identical to those in Experiment 1.

Experiment 4: Nasal Pungency Detectability of Binary Mixtures

The two chemicals, butyl acetate and toluene, and the solvent, mineral oil, were identical to those used in Experiment 3. Stimuli were stored and delivered from glass vessels adapted with two nosepieces, as in Experiment 3. In Experiment 4, though, the stimuli included binary mixtures of the two substances and the single substances.

Preparation of all stimuli (single and mixtures) to test for nasal pungency detectability followed the same logic as that used in Experiment 2 for eye irritation. To prepare the stimuli, we relied on the linear range of the psychometric function for each compound as shown in Figure 7, but, in this experiment, we selected four levels of detection probability: 0.25, 0.50,

0.75, and 1.00. Table 2 lists all 20 stimuli tested for nasal pungency (four sets of five stimuli). (For a detailed explanation of nomenclature and symbols refer to subsection "Stimuli and Equipment", Experiment 2.) Assuming simple dose additivity in the detection of mixtures, the first set of five stimuli in Table 2 is expected to produce 0.25 detection probability, the second set to produce 0.50, the third set to produce 0.75 and the fourth set to produce 1.00. As in Experiment 2, in order to avoid depletion of the headspace of the vessels containing these 20 stimuli (numbers 1 through 20 in Table 2) as subjects were tested, we prepared each in quintuplicate.

Considerations about measurement and calibration of vapor concentrations in the headspace of every bottle by gas chromatography are identical to those in Experiment 2. The corresponding results for Experiment 4 are shown in Figure 4.

Insert Figure 4 about here

Procedure

Experiment 1: Eye Irritation Detectability of the Single Chemicals

To obtain stimulus-response (psychometric) functions for eye irritation from the single chemicals we employed a two-alternative, forced-choice procedure with presentation of ascending concentrations. Briefly, the method required the subject to chose, on each trial, the stronger of two stimuli. Unknown to the participant, one stimulus was always a blank (mineral oil) and the other a dilution step of the chemical (starting with a step clearly below detection). Over the course of a session, and in ascending order of concentration, each step was presented paired with a blank to a selected eye four times. Testing ended when the subject chose the chemical over the blank eight times in a row, four for each of two consecutive dilution steps. This performance was considered 100% detection. Next, the other eye was tested in an identical way.

Each subject participated in 2 to 4 sessions as the one described above. In each session, the subject provided at least one complete psychometric functions for a chemical. The order of testing of eyes and chemicals was irregular across sessions for the same subject and across subjects. The data from all sessions for each chemical were averaged within individuals and across individuals from the same group, i.e., normosmic or anosmic.

Experiment 2: Eye Irritation Detectability of Binary Mixtures

We used a two-alternative forced-choice procedure. In a session, subjects were tested with one of the three sets of stimuli listed in Table 2 for eye irritation. In irregular order, one of the five stimuli of the set was presented to one eye for 3 sec at 4 l/min, paired with a blank (i.e., mineral oil) five times (since we had prepared them in quintuplicate, no bottle was used twice up to that moment). Next, the same stimulus was tested another five times but using the other eye (here is where the quintuplicates are used for a second and final time for the session). Then, this procedure was repeated with the other four stimuli of the set. Participants plugged their noses during trials (to avoid odor cues) until after they chose the vessel producing the stronger sensation. Order of testing of the five stimuli in the set, order of testing of eyes, and order of testing for blank or stimulus on each trial were all irregular.

For each one of the three sets of stimuli listed in Table 2 for eye irritation, sessions as just described were repeated for every subject at least once and as many as three more times, depending on the available time of the participant.

Experiment 3: Nasal Pungency Detectability of the Single Chemicals

We used a procedure analogous to that described in Experiment 1 only that the stimulus was delivered to the nose and both nostrils were tested simultaneously on each trial. Anosmics participated in 2 to 5 sessions with the same characteristics as those in Experiment 1.

Experiment 4: Nasal Pungency Detectability of Binary Mixtures

Again, we used a two-alternative forced-choice procedure. In each session anosmics were tested with one of the four sets of stimuli listed in Table 2 for nasal pungency. In irregular order, each of the five stimuli of the set was presented birhinally, paired with a blank (i.e., mineral oil), ten times (since we had prepared each stimulus in quintuplicate, each bottle was used only twice). Order of presentation for blank or stimulus on each trial was randomized.

For each one of the four sets of stimuli listed in Table 2 for nasal pungency, sessions as just described were typically run with every anosmic for a total of 4 to 8 times, depending on the available time of the participant.

Data Analysis.

Plots of detection probability as a function of stimulus concentration (in ppm by volume) summarized the outcome. Detection probability was corrected for chance (Macmillan and Creelman, 1991) and ranged from 0.0, that is, chance detection, to 1.0, that is, perfect detection.

RESULTS

Experiment 1: Eye Irritation Detectability of the Single Chemicals

For both substances, butyl acetate and toluene, normosmics tended to be more sensitive than anosmics across a range of concentrations covering detection probabilities from slightly above chance up to about 0.8 (Figure 5). (Not a surprising result in view of the age difference betweent the groups, cf. Stevens and Cain, 1986; Stevens et al., 1982) From the value 0.8 on, and for both substances, normosmics and anosmics converged in detection sensitivity.

Insert Figure 5 about here

These psychometric functions show the typical sigmoidal shape with an approximately linear trend in the middle of the range. Figure 5 focuses on that middle range and presents the corresponding linear equation for each compound. Functions for the anosmics are steeper and displaced to the right — more markedly for butyl acetate than for toluene — along the concentration range that produces up to 0.8 detection probability. Given these differences between the two groups we decided to test the binary mixtures only on normosmics (the largest group) and to prepare such mixtures based on the results for single chemicals obtained only with normosmics (Experiment 2). The strategy aimed at minimizing the variability of the sensory responses by making the subject group more homogeneous.

Experiment 2: Eye Irritation Detectability of Binary Mixtures

Figure 6 illustrates the results on the comparative detectability of the single chemicals and the binary mixtures for the three levels of expected detectability: 0.50, 0.75, and 1.00. The obtained detectability for each of the two single compounds (which were the internal standards against which to compare the detectability of the mixtures) was lower than expected for all three levels: around 0.15 (compared to 0.50) for both substances at the lowest level, 0.57 (compared to 0.75) for both substances at the middle level, and 0.59 and 0.83 (compared to 1.00) for butyl acetate and toluene, respectively, at the highest level.

Insert Figure 6 about here

In an analysis of variance (ANOVA) of all data, we considered two factors: expected detectability and type-of-stimulus. Expected detectability consisted of three levels: 0.50 (stimuli 6 through 10 in Table 2), 0.75 (stimuli 11 through 15 in Table 2), and 1.00 (stimuli 16 through 20 in Table 2). Type-of-stimulus consisted of five levels: BA alone (stimuli 6, 11, and 16 in Table 2), BA at 3/4 + T at 1/4 (stimuli 7, 12, and 17 in Table 2), BA at 1/2 + T at 1/2

(stimuli 8, 13, and 18 in Table 2), BA at 1/4 + T at 3/4 (stimuli 9, 14, and 19 in Table 2), and T alone (stimuli 10, 15, and 20 in Table 2). The outcome showed a significant difference for expected detectability [$F(2,12)=13.835$, $p<0.001$] and for type of stimulus [$F(4,24)=3.785$, $p<0.02$] but no significance for their interaction. This confirmed two trends seen in Figure 6: First, the obtained detectabilities for the three sets of stimuli were in the expected relative order. Second, at least for the two sets with the highest detectability, the mixtures were less detectable than the single compounds. Specific statistical comparisons within the expected detectability factor showed the lowest level (expected detectability=0.50) significantly lower than the middle level (expected detectability=0.75) and than the highest level (expected detectability=1.00) ($p=0.005$ and $p=0.0002$, respectively) with the difference between the middle and the highest levels approaching significance ($0.05<p<0.10$). Among the specific statistical comparisons within the type-of-stimulus factor, we found that the average detectability of the single substances was significantly higher than the average detectability of the various mixtures ($p=0.002$).

Experiment 3: Nasal Pungency Detectability of the Single Chemicals

Figure 7 shows the outcome of nasal pungency detectability for butyl acetate and toluene. Again, the typical sigmoidal functions were obtained with a close-to-linear section in the middle of the range. Figure 7 focuses on that range and presents the linear equations characterizing each chemical.

Insert Figure 7 about here

A comparison of nasal pungency and eye irritation detectability functions within the same anosmic group shows that the two trigeminal responses tend to fall closely into register (Figure 8). Functions for toluene are characterized by steeper slopes than those for butyl acetate. Nevertheless, within each chemical, the slopes obtained for eye irritation and for pungency in the same subjects are comparable.

Insert Figure 8 about here

Experiment 4: Nasal Pungency Detectability of Binary Mixtures

Figure 9 shows the outcome for nasal pungency on the comparative detectability of the two single chemicals and the three mixtures presented at the four levels of expected detectability: 0.25, 0.50, 0.75, and 1.00. The obtained detectability for each single chemical at these four levels was: 0.29, 0.38, 0.64 and 0.75 for butyl acetate, and 0.37, 0.51, 0.67, and 0.68 for toluene, values relatively close to the expected ones. The trend for detectability of the mixtures varied between those mixtures producing low detection (i.e., expected 0.25 and 0.50) and those producing high detection (i.e., expected 0.75 and 1.00). The mixtures producing low detection tended to be roughly as detectable as the equivalent single substances (Figure 10), suggesting an outcome of detection agonism based on dose additivity. In contrast, the mixtures producing high detection tended to be less detectable than the equivalent single substances (Figure 10), suggesting an outcome of partial agonism as a result of incomplete dose additivity.

Insert Figures 9 and 10 about here

An ANOVA analogous to that performed on the data for eye irritation of mixtures (Experiment 2) was performed on the data for nasal pungency of mixtures. In this case, the expected detectability factor had four levels: 0.25, 0.50, 0.75, and 1.00 whereas the type-of-stimulus factor had five levels: BA alone, BA at 3/4 + T at 1/4, BA at 1/2 + T at 1/2, BA at 1/4 + T at 3/4, and T alone. The outcome showed a significant difference for expected detectability [$F(3,15)=5.474$, $p<0.01$], no significant difference for type-of-stimulus, but a significant interaction between the two factors [$F(12,60)=1.884$, $p=0.05$]. The significant interaction indicates that the trend seen in type-of-stimulus is not uniform across the four expected detectabilities. Figure 10 illustrates this point showing that the function representing the trend across type-of-stimulus is relatively flat for the low detection stimuli (i.e., expected

p=0.25 and 0.50) but it is shaped like a "U" for the high detection stimuli (i.e., expected p=0.75 and 1.00). A specific statistical comparison within the interaction term showed that, taken across all expected detectabilities, the single compounds were detected at a significantly higher level than the mixtures (p=0.02).

DISCUSSION

As mentioned in the Introduction, research done on the chemesthetic impact of pungent substances has focused on compounds of narrow chemical diversity (e.g., the vanilloids or the enantiomers of nicotine) that stimulate receptors finely tuned to such molecules. It is also important to understand the basis for the sensory irritation produced by a chemically-broad variety of relatively unreactive airborne substances collectively referred to as VOCs. A number of studies (Hodgson et al., 1994; Kostiainen, 1995; Mølhave, 1991; Rothweiler and Schlatter, 1993) have implicated VOCs in the production of neurogenic symptoms (Kjærgaard et al., 1991) as a result of exposures to polluted environments. An example of such situations is the sick building syndrome (Apter et al., 1994; Kostiainen, 1995). Among the wide range of symptoms evoked, sensory irritation of the eyes, nose, and throat figure prominently (Cometto-Muñiz and Cain, 1992; Hudnell et al., 1992; Kjærgaard et al., 1992; Mølhave et al., 1991).

In a number of investigations (see reviews in Cometto-Muñiz, 2001; Cometto-Muñiz and Cain, 1996) we have shown that most nonreactive VOCs (for definition of reactive/nonreactive VOCs see Alarie et al., 1998a; Alarie et al., 1995; Alarie et al., 1996, 1998b) can evoke the trigeminally-mediated sensations of nasal pungency and eye irritation at high-enough concentrations. These concentrations are far above what is encountered in realistic indoor exposures. Even when correction factors (see Cometto-Muñiz et al., 2000) are introduced to account for mode of stimulus presentation (nose-only or eye-only in our experiments vs. whole body in the field) and for time of stimulation (1 to 3 sec in our experiments vs. days or months in the field), the outcome will likely produce values still higher than those found in the indoor

environments generating complaints of sensory irritation. At least two lines of research are looking into this matter. The first looks into the possibility that sensory irritation might be brought about by short-lived, reactive, strong irritants that could be formed as a result of chemical reactions between unsaturated VOCs (e.g., terpenes) and oxidants (e.g., ozone) (Wolkoff et al., 1999; Wolkoff et al., 2000). Such putative compounds would produce irritation by mucosal tissue damage via chemical reaction, and might not necessarily need to interact directly with any particular receptor: It is possible that endogenous chemicals released from damaged cells (e.g., ATP, H^+, bradykinin, see Cesare and McNaughton, 1997; McCleskey and Gold, 1999) are the ones that act specifically upon ion channels to produce the neural response. Reactive irritants can also induce their effects directly as shown in animal studies (Cassee et al., 1996; Kane and Alarie, 1978; Kasanen et al., 1999; Nielsen, 1991; Nielsen et al., 1988). The second line of research looks into the possibility that perceptible sensory irritation could result from the combined action of dozens (or even hundreds) of nonreactive VOCs, each at a level well below its individual threshold, impinging upon a common, broadly-tuned reception process (Cometto-Muñiz et al., 1999; Cometto-Muñiz et al., 1997). Both lines of research are not exclusive of one another and in combination might help to explain the symptoms of sensory irritation reported in indoor environments.

In an early study looking at mixtures of three, six, and nine components, we observed various degrees of stimulus agonism that increased with number of components and with the lipophilicity of such components (Cometto-Muñiz et al., 1997). This work did not include complete detectability (i.e., psychometric) functions for olfactory and trigeminal detection and, thus, only allowed for a restricted interpretation of the results. Our present approach calls for a systematic testing of selected VOCs, representative of particular structural and physicochemical properties, starting with simple binary mixtures and building up to more complex ones as the role of the various physicochemical parameters begins to be better understood. As a start we chose representatives of aliphatic and aromatic VOCs. We chose VOCs that were hydrogen bond

acids (1-butanol) and VOCs that were not; we chose VOCs that were reasonably strong hydrogen bond bases and VOCs that were weak hydrogen bond bases (toluene) (see Table 1). It was not practical to work with VOCs that had no hydrogen bond basicity at all (i.e., alkanes). The first mixture studied under this comprehensive and detailed approach (that included measuring detectability functions) was the mixture of 1-butanol and 2-heptanone (Cometto-Muñiz et al., 1999). As a first approximation, the results indicated chemosensory agonism, in the sense of dose additivity, for both olfaction and chemesthesis.

In the present study, we have focused on chemesthetic responses, i.e., eye irritation and nasal pungency, and have probed into whether agonism would still hold for a pair of compounds with a different physicochemical contrast than the previous pair tested. In addition, the experimental methodology employed here allowed for an analysis of the results with a finer detail. The outcome from both trigeminal endpoints supports similar conclusions: At relatively low levels of detectability of the single compounds, though still above chance (i.e., $0.00<p\leq0.50$, approximately), the mixtures show complete sensory agonism (see results for expected $p=0.50$ in Figure 6 and for expected $p=0.25$ and 0.50 in Figure 9). This means that the detectability of mixtures of the two substances prepared in varying complementary proportions, all adding to a unit level (and created based on the detectability of the single compounds) do not deviate systematically from the detectability of each substance presented by itself at the same unit level. In contrast, at relatively high levels of detectability (i.e., $0.50<p\leq1.00$, approximately), the mixtures show partial (or incomplete) sensory agonism (see results for expected $p=0.75$ and 1.00 in Figure 6 and for expected $p=0.75$ and 1.00 in Figure 9). Here, the detectability of the mixtures, prepared as described, is significantly lower than that of each single substance by itself.

The above mentioned results in humans agree with those obtained in mice and rats via measurement of the decrease in respiratory rate that occurs from exposure to irritants (Alarie, 1966). As noted recently (Kasanen et al., 1999), sensory irritation evoked in mice from binary

mixtures of acrolein and formaldehyde (Kane and Alarie, 1978) and of cumene and n-propanol (Nielsen et al., 1988), and in rats from ternary mixtures of formaldehyde, acrolein, and acetaldehyde (Cassee et al., 1996) showed additivity (here called complete agonism) at low concentrations that changed to competitive agonism (here called partial agonism) at higher concentrations. The sensory irritation properties of turpentine, a mixture of monoterpenes, also showed a shift from additivity at low concentrations to competitive agonism at higher concentrations (Kasanen et al., 1999). A number of alternative models of stimulus-receptor interactions in the trigeminal chemesthetic system have been proposed to account for these observations (Nielsen, 1991).

We have explored two chemical reasons as a possible explanation for lack of complete agonism. The first one involves hydrogen-bonding. If the two components show a high percentage of interaction in the gas-phase or at the receptor, the response to the mixture could fall short of agonism. For complexation of two molecules (A and B) to give a hydrogen bond complex (C), the equilibrium constant, K, is given by the equilibrium concentration equation

$$K = [C]/[A]\ [B] \qquad (2)$$

The solution to equation (2) is a quadratic expression. For a given pair of compounds and a given equilibrium constant, the percentage of complex formed is lower the lower are the initial concentrations of A and B. In the gaseous mixtures, concentrations are so low as to preclude any hydrogen bond interaction between components of the mixtures we have studied (see Marco et al., 1994). We have determined (unpublished work) hydrogen bond complexation constants in 1-octanol (a likely model for the receptor phase, cf. Abraham et al., 2000) and from the complexation constants we calculate that for the pair 1-butanol/2-heptanone there could be a small amount of hydrogen-bond associated species at the receptor area, amounting to no more than 10%. For the pair butyl acetate/toluene there would be no associated species at all. So from this perspective we do not find an explanation for the lack of complete additivity at the higher detection levels of the butyl acetate/toluene mixtures.

The second chemical reason regards molecular length which can be a descriptor in the solvation equation (Abraham et al., 2001). There are two lines of evidence, one trigeminal the other olfactory, that suggests that molecular size is an important factor in chemosensory effectiveness. We have already mentioned the presence of "cut-offs" in the chemesthetic potency of homologous chemical series (Cometto-Muñiz et al., 1998a). In addition, attempts to correlate odor thresholds with physicochemical properties via a solvation equation have shown that a significant improvement in correlation can be obtained with the introduction of a size parameter (Abraham et al., 2001). For olfaction the best length is around 12 Å. If this were also the case for chemesthesis, then, in the study of mixtures, if the two components where to have different lengths, one component might be excluded from the receptor. The maximum lengths for 1-butanol and 2-heptanone are 8.88 and 11.61 Å, respectively, and for butyl acetate and toluene, 8.08 and 11.34 Å, respectively. Thus, if there were indeed a "maximum length" effect in the mixtures, it would not differ for the two pairs of compounds.

A psychophysical analysis of the basis for agonism or partial agonism in the detection of binary mixtures can be performed using the slopes on the linear range of the stimulus-response (i.e., psychometric) function of the individual substances. 1-Butanol and 2-heptanone had very similar slopes for trigeminal detection, with values between 0.7 and 0.8 (Cometto-Muñiz et al., 1999). In contrast, butyl acetate and toluene differed greatly in their slopes for trigeminal detection, with the slopes for toluene always being steeper by a factor of approximately 3, within the same group of subjects (i.e., normosmics or anosmics) (Figures 5 and 7). The results suggest that compounds presenting similar psychometric slopes will be closer to complete agonism in binary mixtures than those presenting dissimilar slopes. These slopes indicate rate of increase in detection with vapor-phase concentration and reflect the integrated outcome of the complete trigeminal chemosensory channel: from the periphery all the way to perception at the highest levels of the CNS. In this sense, the psychophysical analysis captures the process in its entirety, with a better chance to offer robust predictive parameters for the overall response (i.e., detection of mixtures), although, for this very same reason, might offer less precise

information than a chemical analysis on the details of the peripheral chemico-biological interaction. It should also be borne in mind that the experimental methodology used in the present study allows for a more detailed analysis of the sensory effects of the mixtures vis-à-vis those of the single components than that used previously (Cometto-Muñiz et al., 1999). It is possible that a small decrease in complete agonism in some of the mixtures of 1-butanol/2-heptanone (i.e., those of relatively higher detectability) could have gone undetected since the strategy looked at the broad trend for all mixtures as a whole. In any case, an important fact to stress is that there are two dimensions to be considered: degree of agonism and concentration dependence of this agonism.

At this early stage, there is not enough data to obtain a clear, unified picture combining the psychophysical and the chemical approaches. It is risky to generalize from results obtained with only a couple of binary mixtures. Still, the outcome provides a starting point for an orderly and progressive testing of the chemosensory detectability of mixtures of increasing complexity versus that of their individual components. The strategy will also incorporate the analysis of the role that similar or dissimilar values of physicochemical descriptors (see Abraham et al., 1996) for the tested chemicals could play on the sensory results obtained. The applicability of such descriptors to describe and predict human trigeminal responses in the nasal (Abraham et al., 1998a) and ocular (Abraham et al., 1998b) mucosae to airborne chemicals has already been shown for single VOCs, and our previous (Cometto-Muñiz et al., 1997) and present work suggest that they will have relevance for mixtures as well.

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Figure legends

Figure 1. Top. Picture of the glass vessels with the eyepiece adapted. Bottom. Picture of a subject being tested for eye irritation with the glass vessels.

Figure 2. Gas chromatography of the single stimuli used in Experiments 1 (eye irritation) and 3 (nasal pungency). Average (±SD) of vapor-phase concentrations measured for each dilution step of butyl acetate and toluene (filled circles), including the undiluted chemicals (i.e., 2.0 log %v/v or 100% v/v).

Figure 3. Gas chromatography of the stimuli used in Experiment 2 (Eye irritation detectability of binary mixtures). Average (±SD) vapor-phase concentrations of the 15 stimuli (6 single-chemical and 9 binary mixtures) listed in Table 2. BA stands for butyl acetate. T stands for toluene. The binary mixtures are composed of the two substances at the proportions 3/4-1/4, 1/2-1/2, and 1/4-3/4 of the concentrations producing expected probabilities of detection (p) of 0.50, 0.75, and 1.00, according to the results of Experiment 1, normosmic group (depicted in Figure 5) (see text). Bars, sometimes hidden by the symbol, indicate standard deviations (SD).

Figure 4. Gas chromatography of the stimuli used in Experiment 4 (Nasal pungency detectability of binary mixtures). Average (±SD) vapor-phase concentrations of the 20 stimuli (8 single-chemical and 12 binary mixtures) listed in Table 2. BA stands for butyl acetate. T stands for toluene. The binary mixtures are composed of the two substances at the proportions 3/4-1/4, 1/2-1/2, and 1/4-3/4 of the concentrations producing expected probabilities of detection (p) of 0.25, 0.50, 0.75, and 1.00, according to the results of Experiment 3 (depicted in Figure 7) (see text). Bars, sometimes hidden by the symbol, indicate standard deviations (SD).

Figure 5. Detectability of the eye irritation evoked by butyl acetate and toluene in normosmics and anosmics as a function of vapor-phase concentration in log ppm by volume. Each plotted symbol represent the average of 176 responses (half with each eye) from the group of 12 normosmics and of 56 responses (half with each eye) from the group of 5 anosmics. Bars indicate standard errors (SE). The filled symbols stress the linear range of the function and the corresponding linear equation for each chemical (butyl acetate and toluene) and group (normosmic and anosmic) is shown to the side.

Figure 6. On the left Y-axis we show detection probability for eye irritation of five stimuli (BA, BA3/4 + T1/4, BA1/2 + T1/2, BA1/4 + T3/4, and T) at each of the three expected probabilities (p=0.50, 0.75, 1.00). Bars indicate standard errors (SE). On the right Y-axis we show, for the same five stimuli and same three expected probabilities, the ratio of the measured vapor-phase concentration when butyl acetate (open circles) and toluene (diamonds) were presented singly versus when they were presented at the same concentration but mixed with the other chemical. All ratios are tightly distributed around 1.00 indicating: 1) that the presence of a second compound did not modify the vapor-phase concentration of the first compound, and 2) that the observed changes in detection probability of pungency cannot be attributed to variability of presented vapor-phase concentrations.

Figure 7. Detectability of nasal pungency evoked by butyl acetate and toluene in anosmics as a function of vapor-phase concentration in log ppm by volume. Each plotted symbol represents the average of 96 responses from the group of 5 anosmics. Bars indicate standard errors (SE). The filled symbols stress the linear range of the function and the corresponding linear equation for each chemical is shown to the side.

Figure 8. Detectability functions for eye irritation and nasal pungency from butyl acetate and toluene obtained from the same group of 5 anosmics. Each point on the nasal pungency

functions is the average of 96 responses and each point on the eye irritation functions is the average of 56 responses. Bars indicate standard errors (SE).

Figure 9. On the left Y-axis we show detection probability for nasal pungency of five stimuli (BA, BA3/4 + T1/4, BA1/2 + T1/2, BA1/4 + T3/4, and T) at each of the four expected probabilities (p=0.25, 0.50, 0.75, 1.00). Bars indicate standard errors (SE). On the right Y-axis we show, for the same five stimuli and same four expected probabilities, the ratio of the measured vapor-phase concentration when butyl acetate (open circles) and toluene (diamonds) were presented singly versus when they were presented at the same concentration but mixed with the other chemical. All ratios are tightly distributed around 1.00 indicating: 1) that the presence of a second compound did not modify the vapor-phase concentration of the first compound, and 2) that the observed changes in detection probability of pungency cannot be attributed to variability of presented vapor-phase concentrations.

Figure 10. Same data as in Figure 9 but where the values for expected p=0.25 and p=0.50 have been averaged and labeled "lower detection stimuli", and the values for expected p=0.50 and p=1.00 have also been averaged and labeled "higher detection stimuli". Bars indicate standard errors (SE).

Table 1. Value of descriptors on solvation equation (1) for the components of the binary mixture studied in the present investigation (butyl acetate/toluene) and those studied in a previous investigation (1-butanol/2-heptanone) (Cometto-Muñiz et al., 1999).

VOC	E	S	A	B	L
1-Butanol	0.224	0.42	0.37	0.48	2.601
2-Heptanone	0.123	0.68	0.00	0.51	3.760
Butyl acetate	0.071	0.60	0.00	0.45	3.353
Toluene	0.601	0.52	0.00	0.14	3.325

Table 2. List of all 15 stimuli tested for eye irritation (stimuli number 6 through 20) and of all 20 stimuli tested for nasal pungency (stimuli 1 through 20), including single substances and mixtures. The stimuli for eye irritation include three sets of five stimuli (i.e., 6–10, 11–15, and 16–20). The stimuli for nasal pungency include four sets of five stimuli (i.e., 1–5, 6–10, 11–15, and 16–20). The letters refer to the particular substance (BA for butyl acetate and T for toluene), and the subindices refer to the particular detection probability for that substance. For example, $BA_{0.50}$ represents the concentration of butyl acetate producing 0.50 detection probability, $BA_{0.375}+T_{0.125}$ represents the mixture of the concentration of butyl acetate producing 0.375 detection probability plus the concentration of toluene producing 0.125 detection probability, and so on.

1) $BA_{0.25}$
2) $BA_{0.188} + T_{0.063}$
3) $BA_{0.125} + T_{0.125}$
4) $BA_{0.063} + T_{0.188}$
5) $T_{0.25}$

6) $BA_{0.50}$
7) $BA_{0.375} + T_{0.125}$
8) $BA_{0.250} + T_{0.250}$
9) $BA_{0.125} + T_{0.375}$
10) $T_{0.50}$

11) $BA_{0.75}$
12) $BA_{0.562} + T_{0.188}$
13) $BA_{0.375} + T_{0.375}$
14) $BA_{0.188} + T_{0.562}$
15) $T_{0.75}$

16) $BA_{1.00}$
17) $BA_{0.750} + T_{0.250}$
18) $BA_{0.500} + T_{0.500}$
19) $BA_{0.250} + T_{0.750}$
20) $T_{1.00}$

FIGURE 1

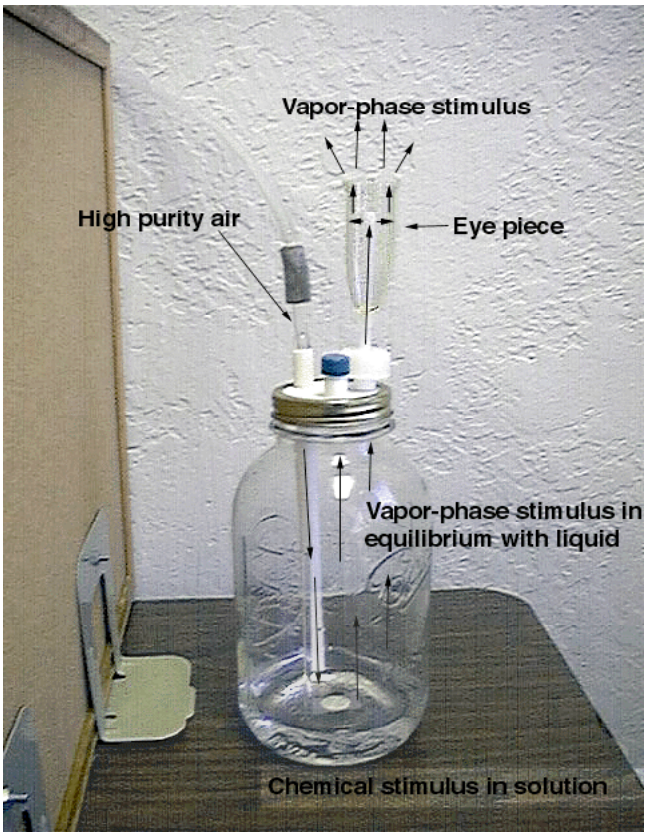

Vapor-phase stimulus
High purity air
Eye piece
Vapor-phase stimulus in
equilibrium with liquid
Chemical stimulus in solution

FIGURE 2

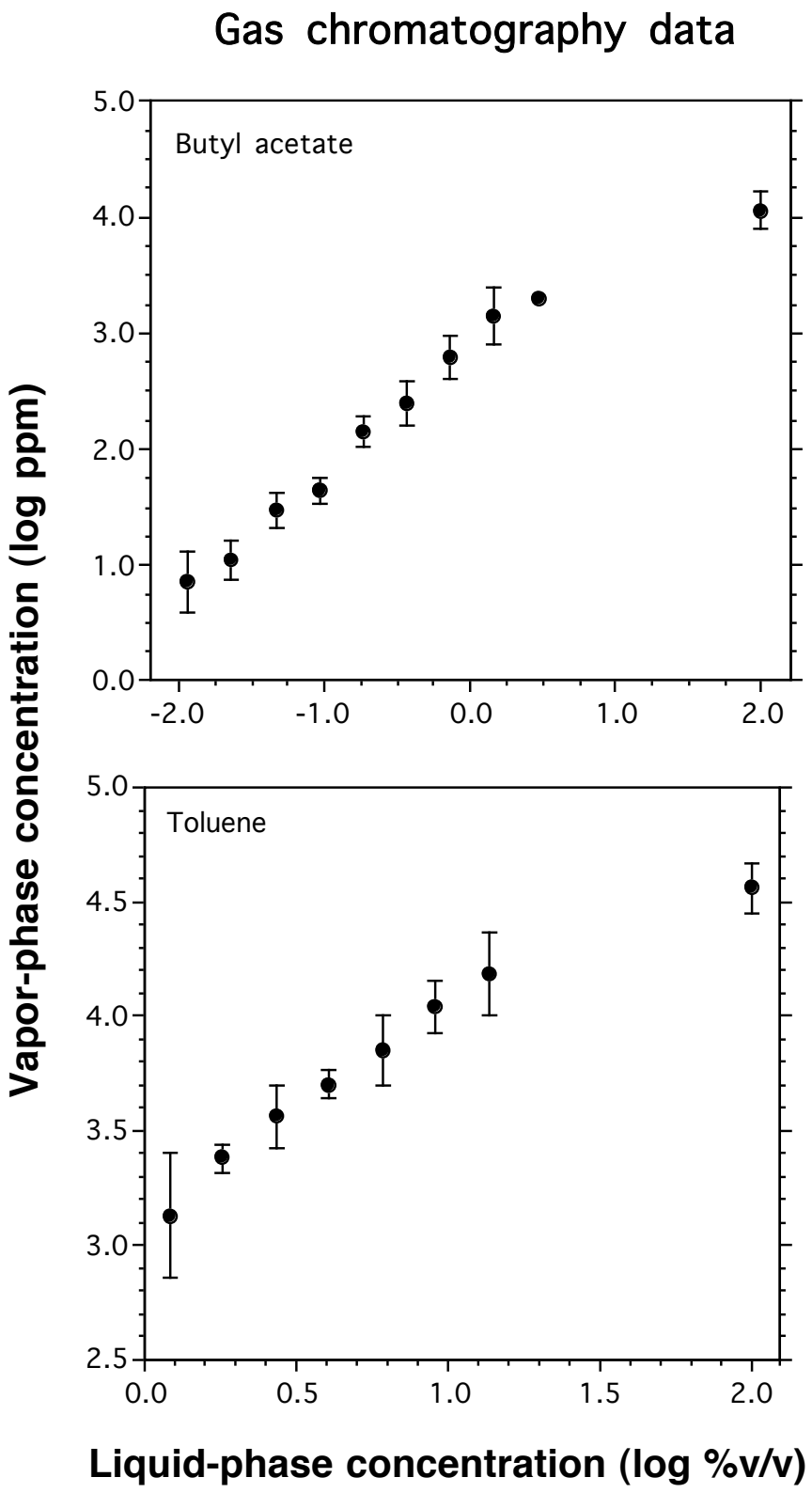

Gas chromatography data
Butyl acetate
Toluene
Vapor-phase concentration (log ppm)
Liquid-phase concentration (log %v/v)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
4.5
3.5
2.5
0.5
1.5

FIGURE 3

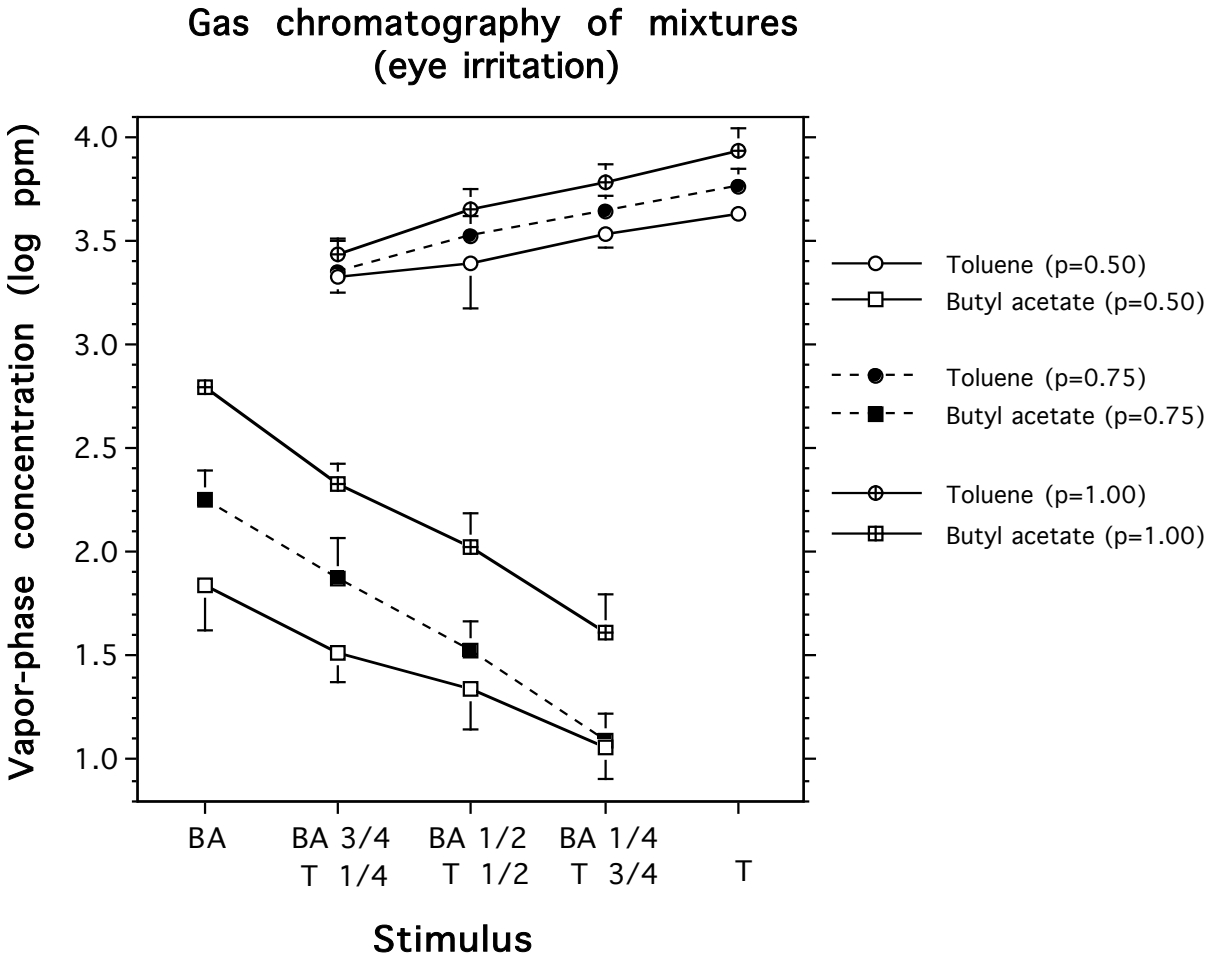

Gas chromatography of mixtures
(eye irritation)
Vapor-phase concentration (log ppm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
BA
BA 3/4
T 1/4
BA 1/2
T 1/2
BA 1/4
T 3/4
T
Stimulus
Toluene (p=0.50)
Butyl acetate (p=0.50)
Toluene (p=0.75)
Butyl acetate (p=0.75)
Toluene (p=1.00)
Butyl acetate (p=1.00)

FIGURE 4

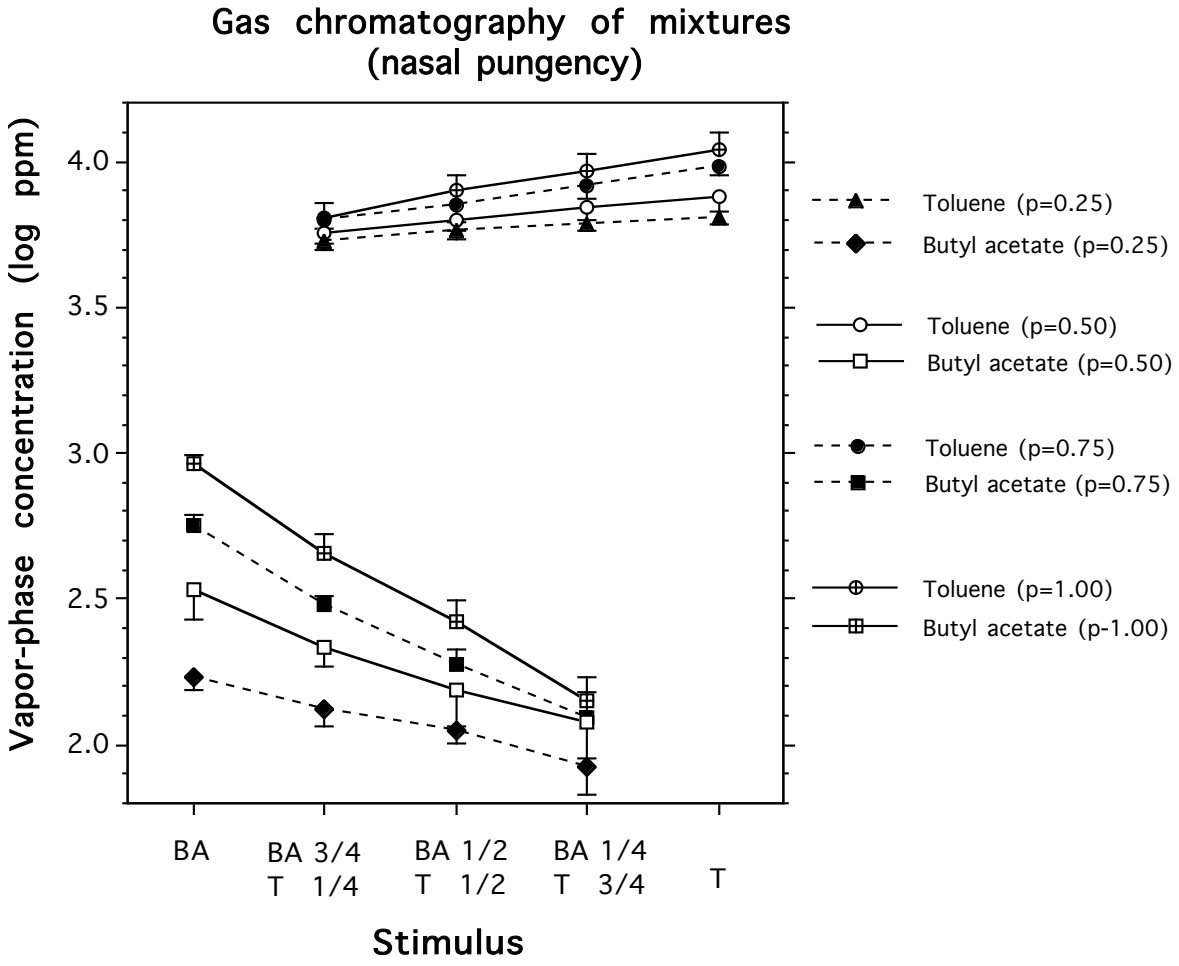

Gas chromatography of mixtures
(nasal pungency)
Vapor-phase concentration (log ppm)
4.0
3.5
3.0
2.5
2.0
BA
BA 3/4 T 1/4
BA 1/2 T 1/2
BA 1/4 T 3/4
T
Stimulus
Toluene (p=0.25)
Butyl acetate (p=0.25)
Toluene (p=0.50)
Butyl acetate (p=0.50)
Toluene (p=0.75)
Butyl acetate (p=0.75)
Toluene (p=1.00)
Butyl acetate (p-1.00)

FIGURE 5

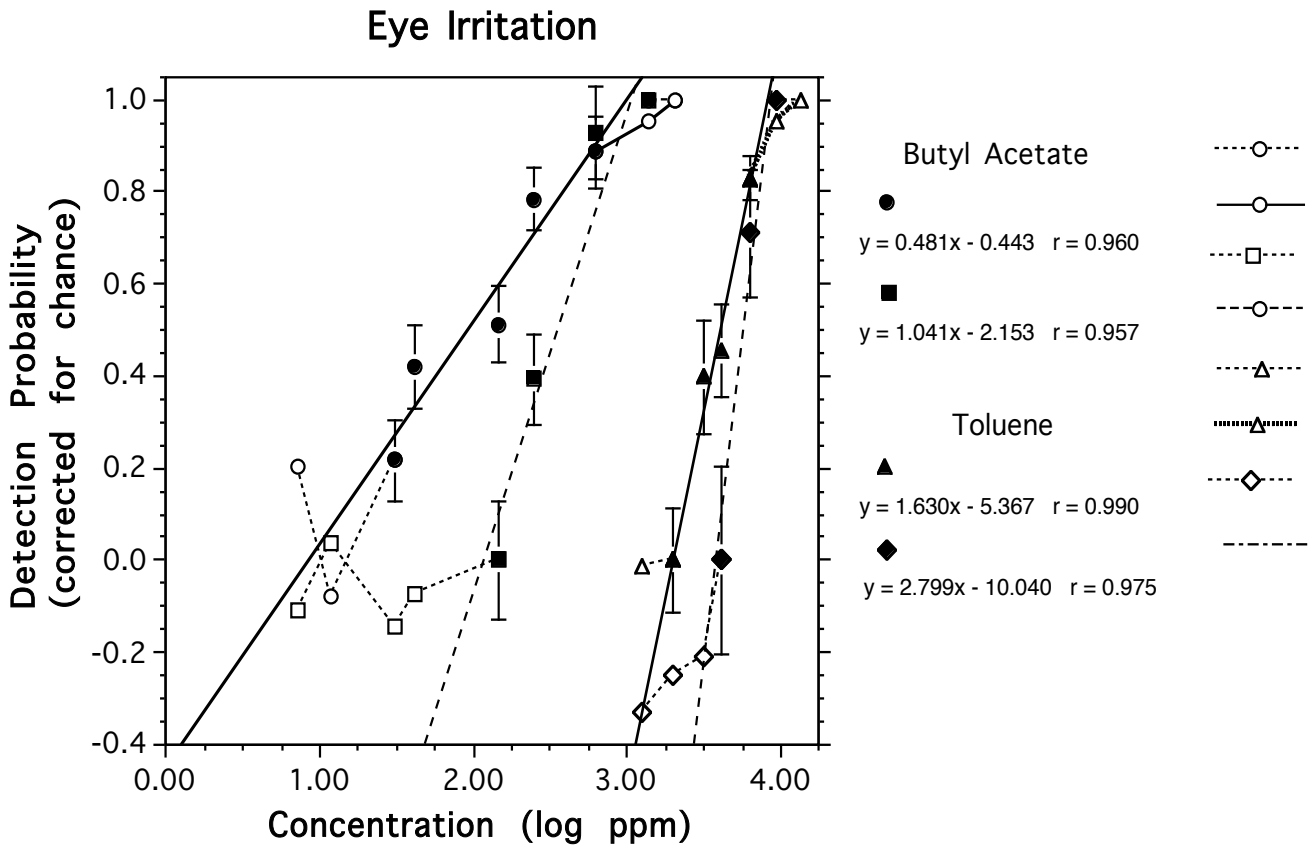

Eye Irritation
Detection Probability (corrected for chance)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0.00
1.00
2.00
3.00
4.00
Concentration (log ppm)
Butyl Acetate
y = 0.481x - 0.443 r = 0.960
y = 1.041x - 2.153 r = 0.957
Toluene
y = 1.630x - 5.367 r = 0.990
y = 2.799x - 10.040 r = 0.975

FIGURE 6

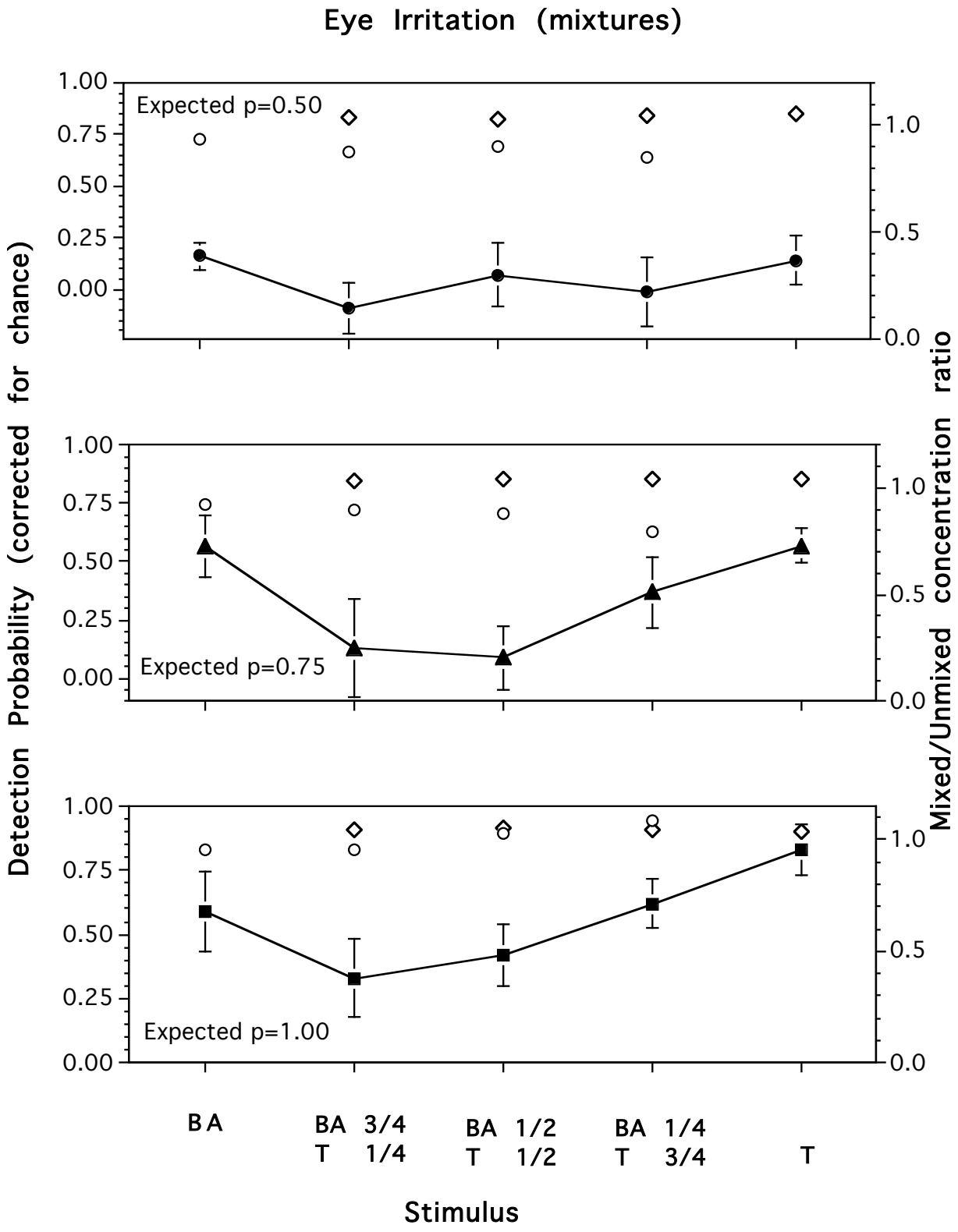

Eye Irritation (mixtures)
Expected p=0.50
Expected p=0.75
Expected p=1.00
Detection Probability (corrected for chance)
Mixed/Unmixed concentration ratio
1.00
0.75
0.50
0.25
0.00
1.0
0.5
0.0
B A
BA 3/4
T 1/4
BA 1/2
T 1/2
BA 1/4
T 3/4
T
Stimulus

FIGURE 7

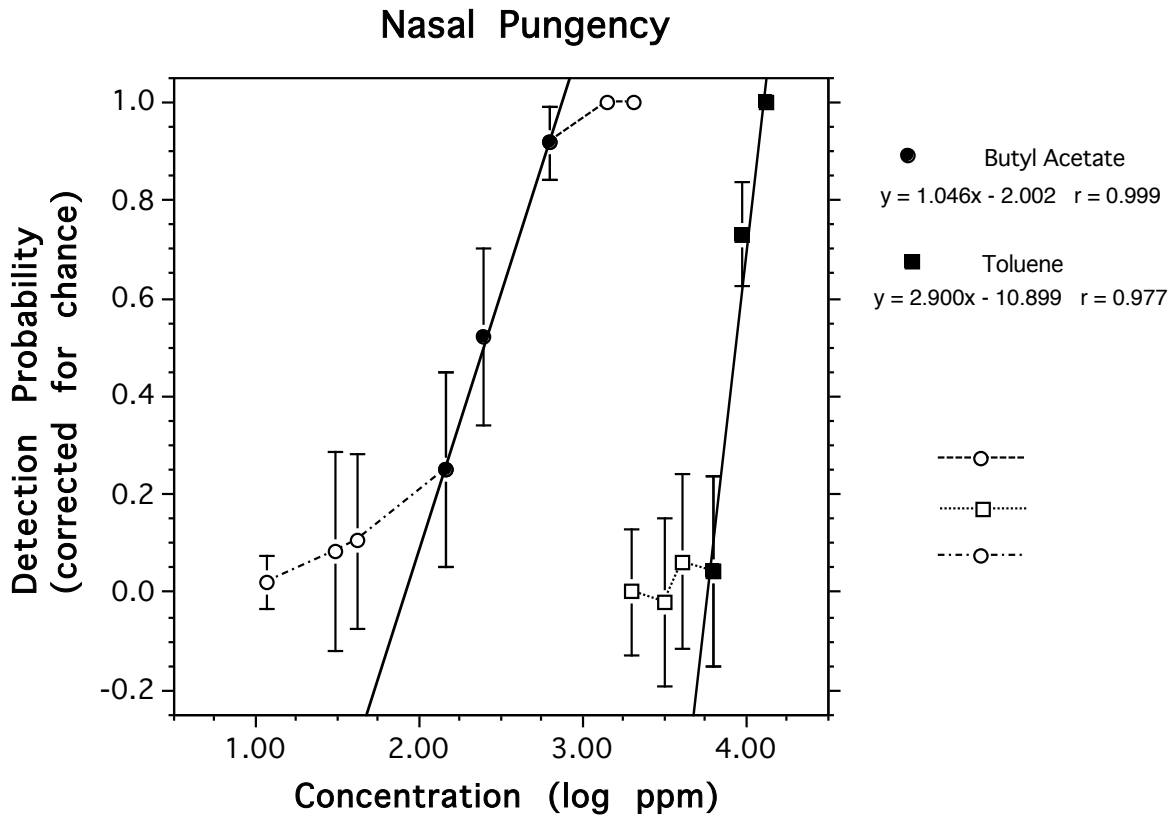

Nasal Pungency
Detection Probability (corrected for chance)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
1.00
2.00
3.00
4.00
Concentration (log ppm)
Butyl Acetate
y = 1.046x - 2.002 r = 0.999
Toluene
y = 2.900x - 10.899 r = 0.977

FIGURE 8

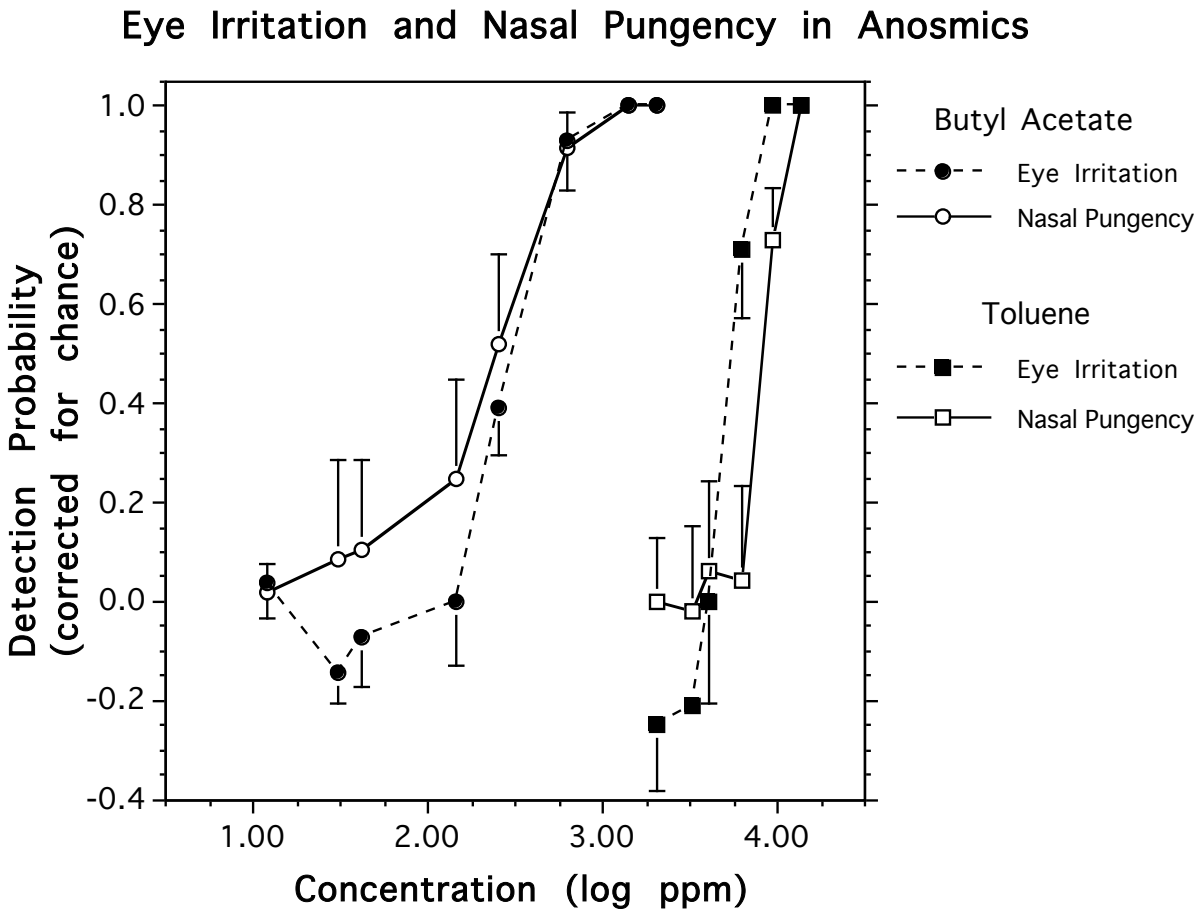

Eye Irritation and Nasal Pungency in Anosmics
Detection Probability (corrected for chance)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
1.00
2.00
3.00
4.00
Concentration (log ppm)
Butyl Acetate
Eye Irritation
Nasal Pungency
Toluene
Eye Irritation
Nasal Pungency

FIGURE 9

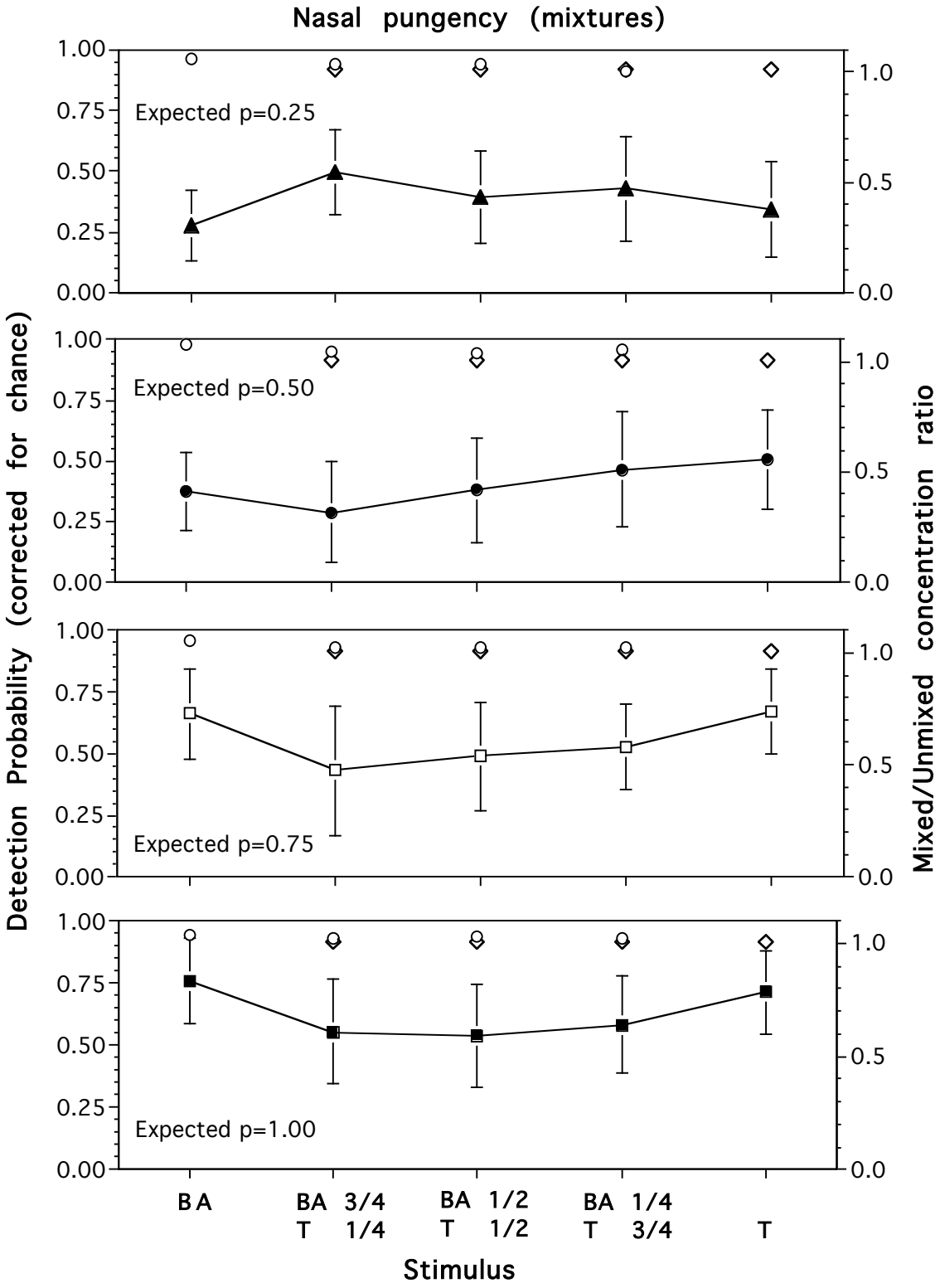
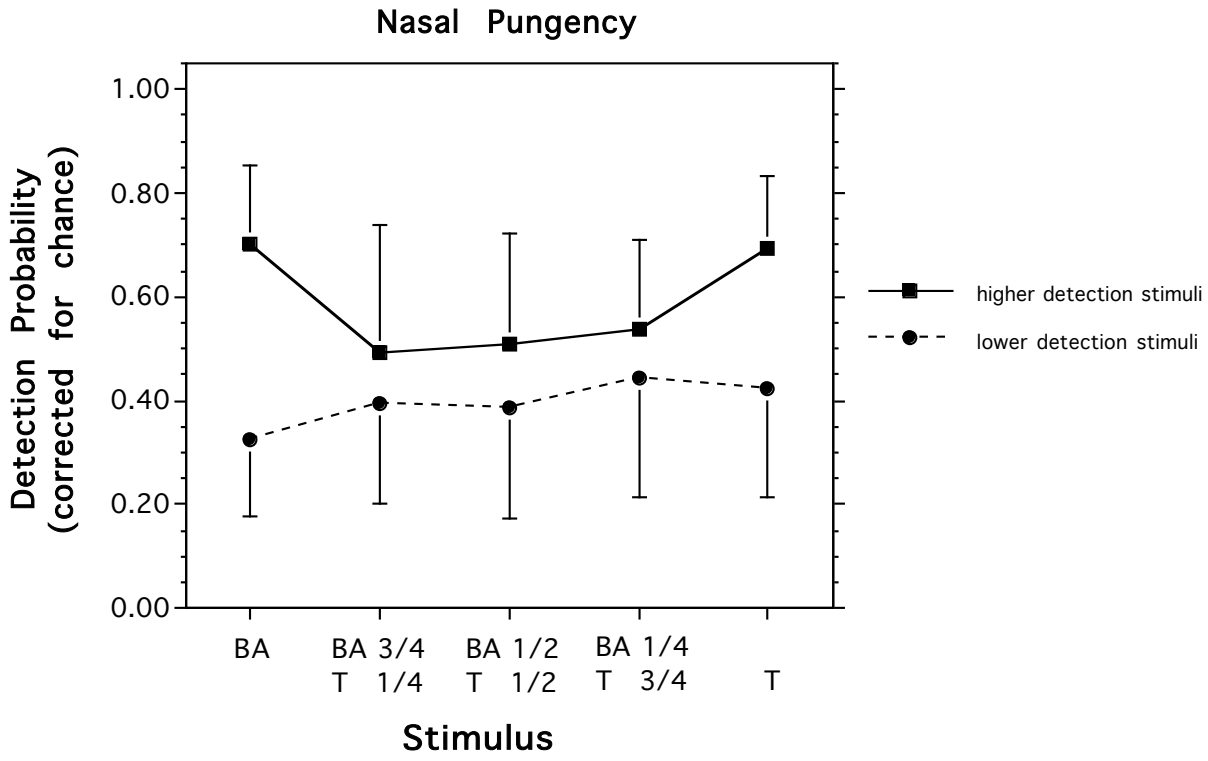

Nasal pungency (mixtures)
Expected p=0.25
Expected p=0.50
Expected p=0.75
Expected p=1.00
Detection Probability (corrected for chance)
Mixed/Unmixed concentration ratio
1.00
0.75
0.50
0.25
0.00
1.0
0.5
0.0
B A
BA 3/4 T 1/4
BA 1/2 T 1/2
BA 1/4 T 3/4
T
Stimulus

FIGURE 10


Nasal Pungency
Detection Probability (corrected for chance)
1.00
0.80
0.60
0.40
0.20
0.00
BA
BA 3/4 T 1/4
BA 1/2 T 1/2
BA 1/4 T 3/4
T
Stimulus
higher detection stimuli
lower detection stimuli