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Synergistic Mixture Interactions in Detection of Perithreshold Odors by Humans

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Abstract

Laboratory demonstrations of synergistic mixture interactions in human odor perception have been rare. The current study examined perithreshold mixture interactions between maple lactone (ML) and selected carboxylic acids. An air-dilution olfactometer allowed precise stimulus control. Experimenters measured stimulus concentrations in vapor phase using a combination of solid-phase microextraction and gas chromatography/mass spectrometry. A probability of detection versus concentration, or a psychometric, functions was measured for pure ML. Psychometric functions were also measured for ML with the addition of fixed, subthreshold concentrations of carboxylic acids. Relative to statistical independence in detection, clear synergy occurred over a range of ML concentrations. To the best of our knowledge, the current results constitute the first clear demonstration of synergy in odor detection by humans from an experiment that combined precise stimulus control, vapor-phase calibration of stimuli, and a clear statistical definition of synergy.

Key words: olfaction, psychophysics, synergy

Introduction

Is the odor of a mixture the simple sum of the odors of the constituent chemicals? Some results, both at the suprathreshold and at the threshold levels, are consistent with this notion (Moskowitz and Barbe 1977; Olsson 1994; Cometto-Muñiz et al. 1999; Wise and Cain 2000). However, research on animal learning suggests that odor mixtures sometimes provide perceptual cues that single compounds lack (e.g., Giannaris et al. 2002; Wiltrout et al. 2003). Some patterns of neural response, both central and peripheral, also suggest that odor mixtures are not always simple sums of their components (Duchamp-Viret et al. 2003; Oka et al. 2004; Zou and Buck 2006).

In odor psychophysics, the rated intensity of suprathreshold mixtures generally falls below the sum of the intensities of the unmixed components, even when one accounts for the compressive nature of intensity versus concentration (psychophysical) functions (Jones and Woskow 1964; Berglund et al. 1973; Cain 1975; Laing et al. 1984; Cain et al. 1995; Laing 1995; Lawless 1997), though summation may be more complete at lower intensities (Laing et al. 1984; Cain et al. 1995). Most work at threshold level is roughly consistent with a simple form of dose addition, for example, subjects can often detect a mixture of 2 odors each at half of their individual threshold concentrations (e.g., Rosen et al. 1962; Baker 1963; Guadagni et al. 1963; Patterson et al. 1993). However, studies that have examined a range of perithreshold concentrations find that cooperation between compounds may be less complete in the high perithreshold range than the low perithreshold range (Cometto-Muñiz et al. 2003, 2005; Wise et al. 2007).

Synergy, or greater sensory impact of a mixture than one would expect based on the impacts of the unmixed components, might also occur. For example, adding subthreshold concentrations of some odorants can produce a small, but measurable increase in the perceived intensity of suprathreshold beverage aroma and the sweetness of suprathreshold sucrose solutions (Ito and Kubota 2005; Labbe et al. 2007). These studies lend credibility to anecdotal reports of professionals that adding seemingly insignificant amounts of ingredients can sometimes have a substantial impact on

aroma or flavor. However, these studies fail to qualify as definitive examples of synergy unless one assumes that the nominally subthreshold added odors had no sensory impact on their own. ''Threshold'' in psychophysics simply indicates a criterion level of detection, usually well above chance and estimated with limited precision. Without precise measurements of sensory impact and without models that make specific predictions for additivity, it is unclear whether interactions between perithreshold odors and suprathreshold stimuli exceed, equal, or fall below additivity.

Other studies have suggested synergy among multiple perithreshold odorants. Laska et al. (1990) estimated thresholds for natural banana odor in 4 fruit bats. The estimated number of molecules in the headspace of the threshold-level mixture fell well below the corresponding number of molecules for threshold concentrations of many individual components. However, the authors measured concentrations and individual thresholds for only about 10 (major) constituents of the hundreds of compounds present. In a subsequent human study, Laska and colleagues measured thresholds for constructed mixtures of up to 12 compounds (Laska and Hudson, 1991). Again, for some mixtures, the estimated number of molecules in the headspace of the threshold-level mixture fell below the corresponding number of molecules for the unmixed components. Although most stimuli were not calibrated, 1 calibrated mixture conformed with the Raoult's Law predictions used to estimate mixture concentrations. However, due to the psychophysical method used, it was unclear what proportion correct threshold represented. Further, because full psychometric (proportion correct vs. concentration) functions were not measured, it was unclear how detection should change with dilution for the various compounds. Accordingly, predictions for additivity were not possible. These studies strongly suggest synergy but are not definitive demonstrations.

The current experiments avoid some methodological limitations of previous work. Stimuli included maple lactone (2-hydroxy-3-methyl-2-cyclopentene-1-one or ML), acetic acid (C_2), butyric acid (C_4), and selected binary mixtures thereof. These compounds are present in various beverages and foods and showed promise for synergistic interactions in pilot work. Predictions for additivity were based on application of a specific statistical model (see Data analysis) to measured psychometric functions for individual compounds. An automated, air-dilution olfactometer precisely controlled concentration, and stimuli were calibrated in vapor phase.

Materials and methods

Subjects

In total, 17 healthy adults (10 females) participated. Ages ranged from 23 to 47 years (average = 31.1 years). All had served in previous experiments using the same method to examine thresholds for carboxylic acids (Wise et al. 2007). In addition, subjects completed at least 1 practice session with ML (see below) to ensure that their sensitivity was not unusually low or high because we could not easily change the range of concentrations to accommodate all individuals. Subjects provided written informed consent on forms approved by the Institutional Review Board of the University of Pennsylvania. Subjects came from the Monell Chemical Senses Center and surrounding community. All were paid.

Materials

Subjects received acetic acid (C_2; CAS# 64-19-7; Nagase ChemteX Corporation, Osaka, Japan; 99.7% pure) and butyric acid (C_4; CAS# 107-92-6; Daicel Chemical Industries, Ltd, Tokyo, Japan; 99.6% pure). These compounds were placed in the olfactometer (see Olfactometer and calibration) undiluted. Subjects also received 2-hydroxy-3-methyl-2-cyclopentene-1-one (ML; CAS# 80-71-7; Toyotama International, Inc., Tokyo, Japan; 98.3% pure). ML (powder form) was dissolved in MilliQ filtered water (0.01 g/ml) before being placed in the olfactometer. Gas chromatography/mass spectrometry (GC/MS) analyses revealed that all 3 compounds met or exceeded manufacturer claims regarding purity.

The olfactometer provided a 6-step dilution series. Successive concentration steps differed by a factor of about 2.2 (Table 1). Extensive pilot work suggested that the range of concentrations would span a wide range of detection performance for most subjects, with comparable levels of detection at a given step across compounds. In addition, subjects received mixtures of ML and fatty acids. Thresholds for pure fatty acids, defined as the concentration at which detection performance fell halfway between chance level and perfect (66.7% correct or 50% chance corrected), were measured (see Procedure). For each subject, experimenters selected 2 concentration steps of each acid: one that fell at least 1 step below threshold and the next step down. These concentration steps, labeled as −1 and −2 in the rest of the manuscript, were (on average) 34% and 15% of threshold concentration for acetic acid and 30% and 14% of threshold for butyric

Table 1 Stimulus concentrations (log ppm by mass)

Concentration	Compound		
	ML	C_2	C_4
1	(−3.40)	(−3.55)	(−4.86)
2	−3.08	−3.20	−4.51
3	−2.75	−2.85	−4.15
4	−2.43	−2.50	−3.80
5	−2.10	−2.15	−3.45
6	−1.78	−1.80	−3.10

Values in parentheses are estimated by extrapolation of the calibration curves (below instrument sensitivity).

acid. In terms of detection performance, fitted psychometric functions indicated that average (across subjects), chance-corrected detection should be about 3% and 9% for −2 and −1 acetic acid and about 4% and 10% for −2 and −1 butyric acid. Thus, 4 sets of mixtures, unique to individual subjects, were created: −1 C_2 added to each concentration of ML, −2 C_2 added to each concentration of ML, −1 C_4 added to each concentration of ML, and −2 C_4 added to each concentration of ML.

Olfactometer and calibration

A detailed description of the air-dilution olfactometer is available on request from the corresponding author. In brief, nitrogen that had flowed through an odor vessel that contained pure C_2, pure C_4, or ML solution was mixed with filtered air to create a 6-step dilution series. Nitrogen that had flowed through a parallel odor vessel, with subsequent air dilution, could provide a single, fixed concentration of added carboxylic acid (either C_2 or C_4). Finally, 2 additional channels provided clean air. Electronic valves determined which set of flows reached a glass cone into which subjects placed their noses to sample. Subjects might receive both clean air flows (blank), 1 of the 6 dilution steps of pure stimulus plus clean air, or 1 of the 6 dilution steps plus the fixed concentration of added carboxylic acid (mixture). The olfactometer provided a total flow of 30 l/min to support natural sniffing.

Stimulus samples were collected in Tedlar bags at the output of the olfactometer. Experimenters quantified samples using solid-phase microextraction (SPME) and GC/MS. A Thermoquest/Finnigan Voyager GC/MS with Xcalibur software (Thermo Electron Corporation, San Jose, CA) was used for all analyses. A polar, Stabilwax column, 30 m × 0.32 mm with 1.0 μ coating (Restek Corporation, Bellefonte, PA), was used for separation and analysis of the volatiles extracted from the samples. SPME fibers were used to enhance analytical sensitivity. The fibers were the 2-cm, 50/30-μm divinylbenzene/carboxen polydimethylsiloxane "StableFlex" fibers (Supelco, Inc., Bellefonte, PA). SPME fibers were extended into sample bags for 45 min. Subsequently, the compounds were desorbed in the injection port of the GC/MS system. A liquid dilution series of each compound (in chloroform) was used to convert GC area to parts per million (ppm) (by mass). These liquid standards were injected into Tedlar bags filled with nitrogen to form vapor-phase standards, and sampling procedures matched those for the vapor-phase samples from the olfactometer, that is, 45 min of SPME sampling before analysis by GC/MS.

Calibration showed that 2.2-fold air dilutions in the olfactometer produced drops of about 2.2-fold in vapor-phase concentration (ppm by mass). Calibration also showed that concentrations were stable both within and between days. Most importantly, calibration showed that concentrations for a given single compound matched concentrations of that compound when presented in a binary mixture.

Procedure

A previous report describes the general procedure in greater detail (Wise et al. 2007). In brief, subjects received five 2.5-s odor pulses separated by pauses of 3 s. Two samples were odors of identical concentration, interspersed in random order with 3 clean air blanks (subjects knew that exactly 2 stimuli were odors). After the initial presentation, subjects were allowed to resample any of the 5 stimuli if they wished. To end the trial, subjects were required to identify exactly 2 of the 5 samples as "odors," guessing if uncertain. At least 15 s elapsed between trials.

During an experimental session, which lasted about 40 min, subjects received 6 presentations each of 6 concentrations (see Table 1) of a given stimulus. The stimulus could be either a pure compound, namely, ML, C_2, or C_4, or a binary mixture, that is, a fixed concentration of C_2 or C_4 added to each of the 6 concentrations of ML. To mitigate the effects of adaptation, subjects received the lowest concentration on the first 3 trials, the next lowest in the next 3 trials, and so forth. Subjects began a 5-min break after the third presentation of the highest concentration. The sequence was repeated, again starting with the lowest concentration, after the break. In addition, subjects received each binary mixture and each single compound in 2 sessions for a total of 12 trials per condition. Psychometric functions for the fatty acids were measured first to determine subthreshold concentrations for each subject. After this determination was made, the design started with an ideal of blocked random order across sessions, that is, pure ML plus all ML mixtures once in random order, then again in random order. In practice, order of presentation was largely random, rather than purely random, because some subjects needed to reschedule sessions on occasion.

Data analysis

A previous report provides details on basic analyses (Wise et al. 2007). In brief, proportion correct for each subject and each condition was first corrected for chance (to range from 0 to 1) and then logit transformed to render psychometric functions (logit of proportion correct vs. log stimulus concentration) approximately linear (Figure 1). The formula for the logit, or log odds ratio, transform follows: logit = ln[pcorr/(1 − pcorr)], where pcorr represents chance-corrected proportion correct and ln indicates natural log. Finally, the log odds ratio was averaged across subjects for each compound and concentration. Repeated-measures analysis of variance (ANOVA) was used to assess differences in dose–response trends between single and mixed stimuli.

In addition, linear functions were fit to detection data for pure compounds, using least squares regression, for individual subjects. The resulting functions were used to generate individual predictions of response addition for the mixtures. According to response addition, which assumes statistical independence, the probability of detecting a binary mixture

equals the probability of detecting at least 1 of the components. The formula follows: $P(AB) = P(A) + P(B) - P(A)P(B)$, where $P(AB)$ represents the probability of detecting the mixture and $P(A)$ and $P(B)$ represent the probability of detecting components A and B, respectively. This equation could calculate, for example, the probability of rolling at least one "4" with a toss of a pair of fair dice (Feller 1968). According to the model, if detection performance for the mixture matches response addition, then little or no mixture interaction has occurred, that is, independence of detection. If performance falls below response addition, some degree of suppression has occurred. If performance falls above response addition, then some form of mutual enhancement, or synergy, has occurred. Other models exist, including the dose addition model discussed in detail in other reports (Cometto-Muñiz et al. 2003, 2005). A dose addition model was also applied to the current data, but these results are not discussed because they support the same conclusions as response addition. Under the conditions of this experiment, the 2 models of additivity made virtually identical predictions (for further discussion, see Wise et al. 2007).

Results

Psychometric functions for individual components

Functions of detection performance versus concentration showed an orderly dose–response relationship (Figure 1). Threshold values, that is, the concentrations corresponding to detection halfway between chance level and perfect, were as follows: 2.08×10^{-3} ppm by mass (2.71×10^{-3} µg/l) for C_2, 1.18×10^{-4} ppm (1.53×10^{-4} µg/l) for C_4, and 2.53×10^{-3} ppm (3.29×10^{-3} µg/l) for ML. Slopes were similar across stimuli. A 3 (odorant) × 6 (concentration step) ANOVA confirmed these impressions. Concentration step reached significance, $F(5,80) = 275.66$, $P \ll 0.001$, but the effect of odorant and the odorant × concentration interaction failed to reach significance ($P > 0.40$). In short, slopes of the functions for the 3 single compounds were quite similar and spanned a similar range of performance across the 6 concentration steps.

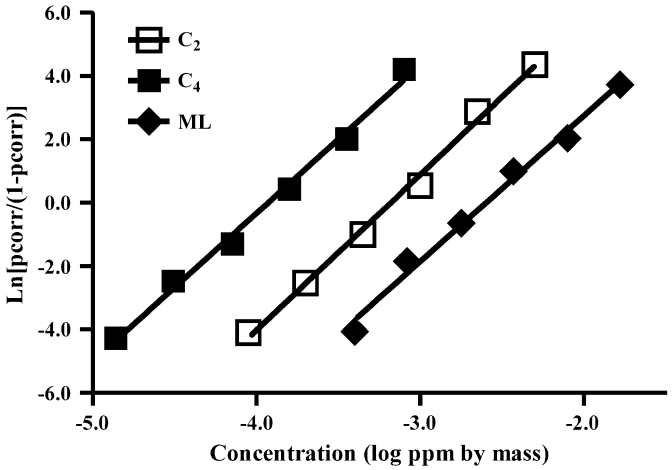

Figure 1 Detection functions for pure compounds. *X* axis: concentration in log ppm. Note that the curve for C_2 is shifted 0.5 log units to the left for clarity (see Table 1). *Y* axis: logit proportion correct. Lines represent best-fit (least squares) linear functions. Equations follow—C_2: logit(pcorr) = 4.91log(C) + 15.63, $R^2 > 0.99$; C_4: logit(pcorr) = 4.67log(C) + 18.34, $R^2 > 0.99$; and ML: logit(pcorr) = 4.59log(C) + 11.92, $R^2 = 0.99$.

Psychometric functions for mixtures

Psychometric functions for binary mixtures also demonstrated an orderly dose–response relationship (for the effect of concentration, $P \ll 0.001$). Further, functions with added fatty acids show significant differences from functions for pure ML. For each acid, a 3 × 6 repeated-measures ANOVA was conducted: added acid (pure ML and ML with each of the 2 added concentrations of acid) × ML concentration (6 concentrations) (Figure 2). For C_2, the effect of added acid reached significance, $F(2,32) = 22.19$, $P \ll 0.001$. On average, added carboxylic acids increased detection performance. A follow-up ANOVA found no significant difference between the 2 levels of added C_2. The interaction approached significance, $F(10,160) = 2.86$, $P = 0.07$. Inspection of Figure 2 suggests that the addition of carboxylic acids had a relatively small effect on detection of the highest concentration of ML. For C_4, the effect of added acid also reached significance, $F(2,32) = 106.41$, $P \ll 0.001$. On average, added carboxylic acids increased detection performance. Again, a follow-up ANOVA found no significant difference between the 2 levels of added C_4. Unlike the ANOVA on C_2, the interaction did not approach significance ($P > 0.80$). In short, the addition of relatively low concentrations of short-chain carboxylic acids significantly enhanced detection relative to that of pure ML. However, for the chosen stimuli, the concentration of added odorant did not seem to matter very much.

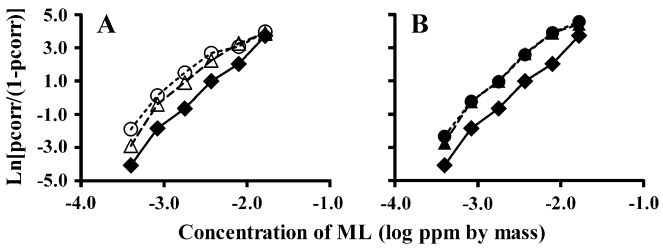

Figure 2 Detection functions for ML (replotted from Figure 1, filled diamonds) together with detection functions for mixtures of ML and carboxylic acids. **(A)** Data for added acetic acid, at least 1 step (see Table 1) below threshold (open triangles) and at least 2 steps below threshold (open circles). **(B)** Data for added butyric acid (C_4), at least 1 step below threshold (filled triangles) and at least 2 steps below threshold (filled circles). For all but the lowest concentration in (B), the filled triangles and filled circles overlap.

Mixture interaction with respect to additivity

Figure 3 illustrates additivity predictions together with mixture data replotted from Figure 2. Data for each added fatty acid were submitted to a 3-way ANOVA: model comparison (mixture detection vs. response addition) × level added (−1 and −2) × concentration step (6 levels). The main effect of model comparison reached significance for both C_2, $F(1,16) = 17.38$, $P \ll 0.01$, and C_4, $F(1,16) = 43.87$, $P \ll 0.01$. Overall, mixture detection exceeded additivity. Further, the interaction between model comparison and concentration reached significance for both C_2, $F(5,80) = 3.89$, $P < 0.01$, and C_4, $F(5,80) = 3.53$, $P < 0.01$. Inspection of Figure 3 shows that mixture detection failed to exceed additivity at the lowest concentration and exceeded additivity to a greater extent for intermediate concentrations than for the highest concentration. In short, though there was some evidence of concentration dependence, the overall picture is detection that exceeds independence, that is, synergy.

Discussion

The addition of weak concentrations of acetic and butyric acids, that is, concentrations that give rise to chance-corrected detection probabilities of 0.10 or less, made mixtures significantly easier to detect across a range of concentrations. Further analysis showed that the perceptual impact of added carboxylic acids was not only measurable but also greater than additive. Detection failed to exceed additivity for some concentrations of ML. The fact that detection fell closer to additivity at the highest concentrations is perhaps unsurprising because detection performance was already quite high. That mixture detection failed to exceed additivity for the lowest concentration of ML seems more difficult to explain. It is difficult to accurately measure proportion correct for low concentrations, but the result was consistent across conditions (Figure 3). To the best of our knowledge, the overall results constitute the first demonstration of synergy for which a range of concentrations was studied, stimuli were tightly controlled via air-dilution olfactometry, vapor-phase concentrations were verified using physical measurements, and a clear statistical definition of synergy was applied.

Limitations

Within practical limits, the experiments were conducted with stimuli of the highest obtainable purity. Still, it is possible (though unlikely) that some trace compounds, present in concentrations below instrument sensitivity, might have influenced the results. However, synergy was defined according to response addition. Thus, the possibility that our nominally pure chemicals might actually be chemical mixtures would not necessarily invalidate the results. In addition, the sample of test compounds is quite limited. Acetic and butyric acids, chosen based on pilot work and practical considerations, served well to demonstrate clear synergy. However, only similar tests on additional compounds can determine how frequently synergy occurs.

Basic significance

In broad terms, the current findings agree with a growing body of literature showing cooperation among perithreshold odorants: concentrations of individual chemicals in a threshold-level mixture tend to fall below individual threshold concentrations (e.g., Rosen et al. 1962; Baker 1963; Guadagni

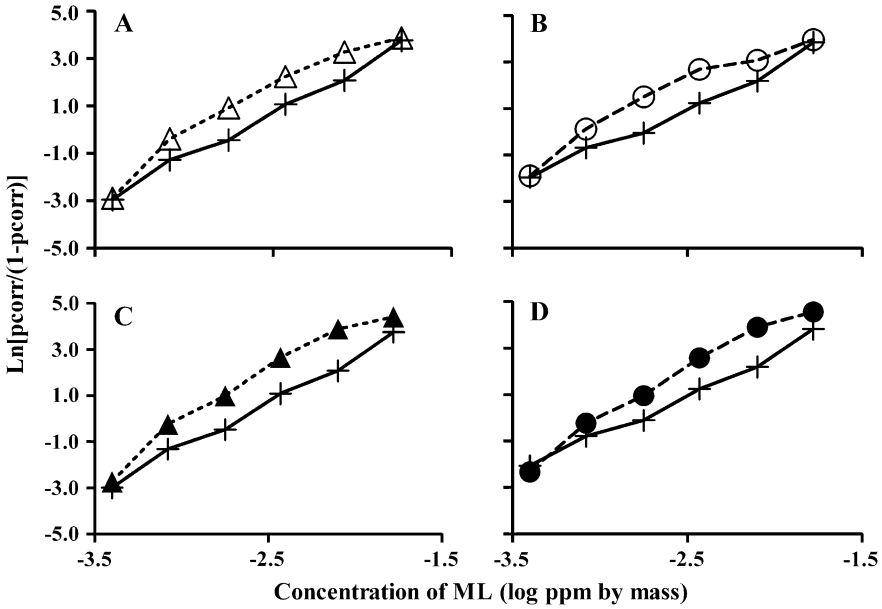

Figure 3 Detection functions for mixtures (replotted from Figure 2) compared with the predictions of response addition (+symbols connected by solid lines). **(A)** ML plus acetic acid at least 1 step below threshold. **(B)** ML plus acetic acid at least 2 steps below threshold. **(C)** ML plus butyric acid at least 1 step below threshold. **(D)** ML plus butyric acid at least 2 steps below threshold.

et al. 1963; Laska and Hudson 1992; Patterson et al. 1993; Cometto-Muñiz et al. 1997, 2003, 2005; Wise et al. 2007). In addition, the current results provide more definitive support for previous hints that synergy might occur (e.g., Laska et al. 1990; Laska and Hudson 1991; Ito and Kubota 2005). Clear synergy has been demonstrated in taste, namely, synergy between the savory (umami) taste of monosodium glutamate and certain 5′-ribonucleotides and between some sweet compounds (Yamaguchi 1967; Rivkin and Bartoshuk 1980; Lawless 1998). Synergy may be uncommon in the chemical senses, but we can now say with greater confidence that it occurs in olfaction, as well as taste.

The physiological underpinnings of the observed synergy, and mixture interactions more generally, are not completely clear. In animal models, odor–odor interactions, including summation and synergy, can take place at the level of individual receptor neurons (see O′Connell and Grant 1987; Derby 2000; Duchamp-Viret et al. 2003). Interactions may occur at central levels as well. One study found that the response of some cortical neurons to mixtures exceeded the sum of the responses to individual components (Lei et al. 2006). Another study showed that binary mixtures activated some cortical neurons that neither individual component activated (Zou and Buck 2006). These results, consistent with some psychophysical evidence (Cain 1975; Laing and Willcox 1987), suggest that mixture interactions might occur at both peripheral and central levels.

In addition, transformations of the stimulus might play a role in the observed synergy. Both the ML and the carboxylic acids are very polar molecules, and the carboxylic acids at least are capable of dimerization through hydrogen bonding (see Vawdrey et al. 2004). Interactions might yield new vapor-phase species that might interact with different receptors. Alternatively, new vapor-phase species could differ with respect to enzymatic metabolism in the nasal mucosa (Zhang et al. 2005). Although the low concentrations involved in these experiments make molecular interactions less likely, the possible role of interactions in the vapor phase and perireceptor environment is worth considering as research on the perception of odor mixtures continues.

Practical significance

It can prove difficult to identify unpleasant contaminants in products using standard analytical techniques, in part, because some compounds smell strong at very low concentrations. Gas chromatography–olfactometry (GC/O), in which a human panelist samples the output from a GC column as compounds elute, can help to identify such problem odors (Dravnieks and O'Donnell 1971; Grosch 1993; Acree 1997). However, GC/O would tend to miss chemicals that have little or no perceptual impact on their own. Yet, the current results suggest that chemicals with little individual impact might combine with other compounds to have a measurable impact (for some discussion, see Bult et al. 2001). This observation also has potential relevance for indoor air quality, where analytical studies sometimes fail to find problematic concentrations of any single chemical in air samples taken from a clearly problematic environment.

Future directions

Studies of cross-modal integration in detection of perithreshold mixtures imply that higher level neural processes play a role in which tastes and smells will cooperate (Dalton et al. 2000; also see Small et al. 2004; McCabe and Rolls 2007). Perceptually congruent stimuli, like sucrose and cherry odor, may cooperate, whereas incongruent stimuli may not. Congruence may come from learned associations between stimuli from repeated coexposure (Diamond et al. 2005). This idea is consistent with the suggestion that synergy might be more likely in naturally occurring, biologically relevant odor mixtures (Laska et al. 1990). ML is found together with short-chain fatty acids in coffee beverages and tropical fruit (particularly passion fruit). Future studies could compare mixtures of odors that naturally co-occur to mixtures of odors that do not. Future studies could also determine if repeated pairing between odors in a laboratory setting increases the probability that synergy will occur.

In addition to possible cognitive (top-down) influences, future studies could investigate stimulus-driven (bottom-up) influences. Recent evidence suggests that similarity of molecular structure can influence mixture interactions (Wise et al. 2007). Gradual and systematic manipulations of molecular parameters might lead to hypotheses regarding the relationship between similarity of molecular parameters and tendency to form synergistic interactions.

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