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Detection thresholds for an olfactory mixture and its three constituent compounds

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Abstract

Single sub-threshold odorants can, according to a few reports, become perceptible in concert. That is, they can exhibit mixture additivity. The present study measured thresholds for mixture of 1-butanol, 2-pentanone, and n-butyl acetate, and for each of these separately. 'Complete' additivity resulted, in that the threshold concentration of each compound in the mixture (measured by gas chromatography) turned out to be one-third of each component separately. Earlier *threshold* studies also claimed complete additivity and even hyper-additivity (synergism); in this regard they, like this study, differ from the more numerous studies of *suprathreshold* mixtures, which yield imperfect additivity (hypo-additivity). Mixtures not only enhance sensitivity, but they may also promote stability; intersubject variability was smaller for the mixture than for the components. Subjects were 20 young (18–26 years) and 20 elderly (69–91 years) persons, who gave two sets of thresholds on 2 days. Concordant with earlier findings, the elderly's thresholds averaged higher (≈ 20 times) than the young's, but complete additivity nevertheless held, even though they operated over different portions of the concentration continuum. The study affords a look at inter-test reliability of thresholds by comparing correlations between same and different compounds examined on same and different days.

Introduction

It is well recognized by the contemporary student of the chemical senses—whether physiologist, biochemist, or psychophysicist—that the studies of stimulus mixtures have a key role in understanding olfaction (Laing *et al.,* 1989). The present study addresses how mixtures can aid in detecting odors.

Studies of odor detection have, for the most part, employed single compounds and, indeed, this would seem to be an appropriate starting strategy. Eventually, however, one must address the detectability of more complex stimuli. Everyday odors are often highly complex mixtures of hundreds of compounds, and it is unknown at present how to predict their detectability from the detectabilities of their constituent compounds. The importance of mixtures became evident to some investigators when gas chromatography revealed in natural organic products, such as fruits and vegetables, the presence of large numbers of compounds, presumably sub-threshold in individual amounts, but perceptible in concert. With this in mind, Guadagni *et al.* (1963) demonstrated in a pioneer study that imperceptible quantities of various saturated aldehydes could be perceived in mixtures; detection thresholds showed impressive additivity. For example, n-heptanal and 3-methylbutanal diluted to half their threshold strength were found to be just-perceptible when mixed together.

Continuing in this vein, Laska and Hudson (1991) measured thresholds for numerous individual compounds and for 3-, 6- and 12-component mixtures of them. They concluded that:
(i) the mixtures' thresholds were less variable (more stable) than the components';
(ii) the mixtures exhibited 'hyper-additivity', i.e. the components were mutually synergistic in action.

That mixtures might prove more *stable* could help to balance a general picture of staggering variability that characterizes olfactory thresholds for most compounds, among individuals, age groups, and even among tests of the same individual over time. (For a recent review of the sources of variability of olfactory detection thresholds, see Stevens and Dadarwala, 1993.) That mixtures might be *hyper-additive* (or just simply additive) when it comes to detectability would counter a general picture of *hypo-additivity* that characterizes studies of suprathreshold mixtures (for a review of numerous studies, see Cain, 1988). Such studies typically show that the magnitude estimations of the odor intensity of a mixture fall short of the sum of the magnitude estimations of the (unmixed) components. Rather than mutually synergistic, the components appear to be somewhat mutually counteractive (for a recent example, see Berglund and Olsson, 1993).

These conclusions about mixtures are obviously important ones calling for further examination. The present study began with the conviction that fuller attention is desirable to the chemistry of the mixtures. Laska and Hudson (1991), who used a sniff-bottle technique, assume that liquid concentrations in the bottles accurately predict gas concentrations of the headspace. That is, the components of the mixture individually obey Raoult's law. This is an assumption that may or may not be true for all the compounds employed.

In their study, Laska and Hudson attempted to calibrate their odorants by first determining suprathreshold concentrations that made them all match at a given odor level, then assuming their equipotentiality at all dilutions (including thresholds) below the matched levels—on the grounds that they are all governed by the same exponent of the power function relating odor strength to concentration. In fact, there is abundant evidence that the exponent depends on the odorant—from about 0.10 to 0.80 in a review of many studies by Cain (1988). To project threshold concentrations from suprathreshold matches is risky.

We therefore decided to study a mixture of three odorants only, beginning with a gas chromatographic analysis of their gas concentrations, separately and in mixture. These particular three were chosen because previous work in our laboratory had already established their thresholds. In a given test session a subject gave thresholds to each of them separately and to the mixture of all three of them. What we sacrificed in scope here, by limiting the psychophysics to one ternary mixture, we hoped to gain by a more thorough analysis of the underlying chemistry.

Although the main goal was to understand the variability and nature of the threshold for an odor mixture (i.e. whether hypo-, hyper-, or simple-additive), there were two secondary issues of considerable interest. One was *aging,* and for this reason we studied a group of young and a group of older subjects. We already know from numerous studies that advancing age drives thresholds up by one or two orders of magnitude (Cain and Stevens, 1989; Cain and Gent, 1991; Stevens and Dadarwala, 1993); the issue here is whether the rules of mixture additivity and variability are the same regardless of age.

The other secondary goal was to examine test-retest reliability of thresholds, a subject of current attention in this laboratory. Hence, each subject was tested on two separate days, providing interesting matrices of same-day and different-day correlations relating the same and different compounds. The hope was to derive from them some insight into the nature of olfactory sensitivity.

Materials and methods

Subjects

Twenty young (18-26 years) and 20 elderly (69-91 years) subjects, matched for gender, took part. Each served in two sessions of a little less than 1 h apiece, separated by at least 1 day, but usually less than a week. The young were students or workers at Yale University; the elderly, members of three local senior day-centers. Subjects gave informed consent verbally and were paid $16.

Stimuli

As in many previous studies of thresholds, the odorants were made up in liquid dilution steps and stored in polypropylene, 'shampoo' squeeze-bottles (260 ml capacity). For stimulation the subject squeezed the bottle and thereby ejected its gas headspace through the spout at the top, just below the nostrils. Blank stimuli for forced-choice comparison contained the solvent (mineral oil) only.

The selection of the three odorants used in this study (1-butanol, 2-pentanone and n-butyl acetate) was based on:
(i) the availability of data from a battery of odorants previously examined in this laboratory for odor thresholds;
(ii) the fact that the odorants belong to distinct chemical series (alcohols, ketones, acetates);
(iii) the fact that each series has a characteristic odor, or perceptual theme (for 1-butanol, the odor character may be termed 'woody alcohol'; for 2-pentanone, 'ethereal solvent'; for n-butyl acetate, 'fruity-artificial banana').

On the basis of previous measurements (Cometto-Muñiz and Cain, 1990, 1991, 1993), we started the dilution series for each chemical at a 'stock' concentration (labeled dilution step 0) that, when diluted in successive three-fold steps, would reach the threshold for each substance at the same numerical step. Such stock solutions in mineral oil were 1.11% v/v for butanol and n-butyl acetate, and 11.11% v/v for 2-pentanone. This stimulus arrangement in terms of equal dilution steps at threshold offers a convenience, not a necessity, for evaluating additivity of mixtures. The expectation was, accordingly, that our subjects' average threshold, measured in dilution steps, should be the same for each of the three odorants (we shall see that this was approximately the outcome). Our chemical analysis showed that the gas concentrations of each component in the mixture were unaffected by the other components. That is, the p.p.m. was the same for each odorant whether measured alone or in mixture.

Depending on the degree of additivity, the average threshold of the mixture, in dilution steps, is predicted to be as follows:

(i) if there is *no stimulus additivity,* the same dilution step as the components;
(ii) if there is *complete additivity,* one dilution step weaker than the components (i.e. the mixture threshold would contain one-third the threshold p.p.m. values of the components tested separately);
(iii) if there is *hypo-additivity,* somewhat less than one dilution step weaker than the components;
(iv) if there is *hyper-additivity* (synergism), more than one dilution step weaker than the components;

(v) if there is *counteraction,* one or more dilution steps stronger than the components.

As will be seen, our results favored (ii).

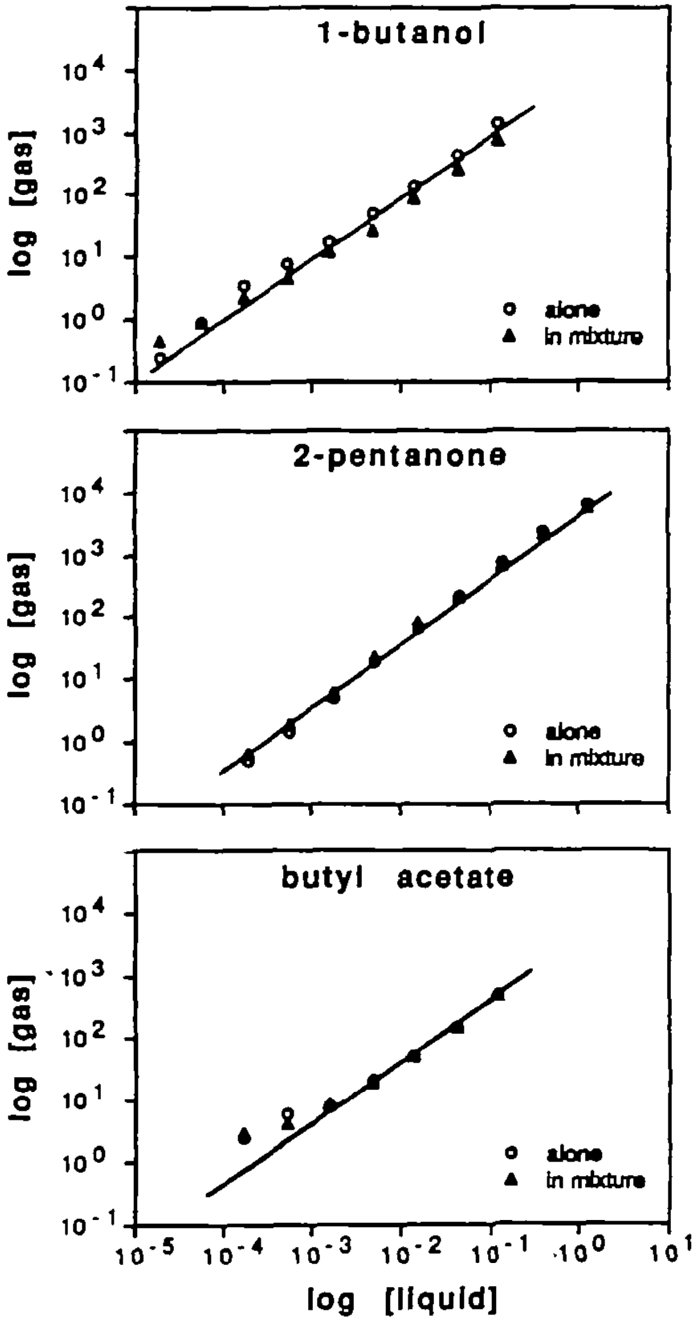

Figure 1. Log-log plot of liquid concentration (in p.p.m.) versus gaseous headspace concentration (in %v/v) for the three mixture components calibrated alone (circles) and in the mixture (triangles). Solid lines are drawn in with a slope of 1.0 to show proportionality, i.e. conformity to Raoult's law. Apparent deviations at low concentrations are attributable to noise in the chromatographic readings.

Headspace concentrations were measured using a Hewlett-Packard 5890A gas chromatograph (photoionization detector) equipped with a gas sampling valve. Repeated chromatograph readings were taken from each bottle in the four dilution series, including a measurement of saturated vapor (at 23°C and ≈50% humidity—the same conditions as those for psychophysical testing). The exact concentration of saturated vapor was known from handbooks or databases on physical properties. A simple conversion factor between the known saturated vapor and the saturated vapor reading from the chromatograph produced a calibration curve for each odorant. Figure 1 is a log-log plot of the gas concentration as a function of the liquid concentration of each odorant. If proportionality (Raoult's law) prevails, these results must conform to a straight line having a slope of 1.0. For comparison, the lines drawn in the figure have been given a slope of 1.0. Except for the lowest points (especially for n-butyl acetate), which are affected by the 'noise level' in the chromatographic readings, the data conform well. Also note that the circles (measurements made of the components separately) and the triangles (measurements made of each component in mixture) are essentially the same, indicating that the presence of the other components did not alter the headspace concentrations of any one substance. We deem it essential to know this if the nature of additivity is at stake.

Each of the four dilution series was made up in duplicate, and, in a test, bottles were selected alternately from the two series to allow the headspace to return to equilibrium before a bottle was used again (see Cain *et al.,* 1992). Deliberate swirling of the liquid in the bottles further ensured that equilibrium was restored before presentation (Dravnieks, 1975).

Psychophysical method

On each trial the subject had to choose which of two bottles, one being a blank, smelled stronger, guessing when necessary. A relatively new tracking procedure called the Step Method (Simpson, 1989) was used. One of the great advantages in using this method is that it gives the experimenter the opportunity to see what happens to the track over time. This method has been used in at least two studies (Cain *et al.,* under revision; Stevens and Dadarwala, 1993) of olfactory thresholds of young and old subjects, and is more fully described in those studies and in Simpson's (1989) article. The Step Method resembles conventional up-down tracking methods (Wetherill and Levitt, 1965), but differs from them in that it permits multi-step changes in stimulus level in addition to single-step changes. Multi-step changes typify the start of a track and rather quickly give way to single-step and zero-step changes as the track takes shape. This feature is designed to reduce the bias that can occur when a starting stimulus level is far removed from a subject's real threshold. Our starting point was always step nine for young subjects and step seven for elderly subjects. This initial difference is on the order of the young/elderly threshold difference (Stevens and Dadarwala, 1993; Cain and Stevens, 1989; Cain and Gent, 1991). The initial multi-step changes in the Step Method, however, lend negligible significance to the initial starting point and, *ipso facto,* the measured thresholds. The size of the change from trial to trial is determined by a computational algorithm program that uses all the previous judgements to estimate by a least-squares method the best current value of the threshold (which is chosen as the level to be presented on the next trial) and to modulate the level of the track to give a correct response level of ≈80%. The response level can be set at any value desired, but psychophysicists who use tracking procedures generally opt for correct response rates in the vicinity of 75-80%. Tracks in the present experiment consisted of 20 forced-choice trials.

Each subject gave four such tracks in a test session, one for each of the component odors separately and one for the mixture. The order of the four tracks was counter-balanced across subjects. Only twice was a short rest break given between tracks, owing to a subject's request; otherwise, measurement was continuous.

The question arises how best to decide, among various possible alternative rules, on a threshold value from a particular track. The rule we adopted was the computer's least-square's estimate of the threshold after the last trial (the method used also by Simpson). For similar data, Stevens and Dadarwala (1993) compared several different rules (i.e. the least-squares method, the average level of the entire track, the average level of the last third and of the last half of the track, and the average of the transition levels of the tracks from up-to-down and from down-to-up, as is commonly done in up-down tracking). Thresholds thereby determined were, for all practical purposes, the same.

To assess the possible role of adaptation (or habituation, as some term it), we averaged each odorant's track, trial by trial, for both young and elderly subjects. In all cases, the drift was considerably lower than one dilution step throughout the 20 trials and was not systematic.

Results

Analysis of variance

All 320 thresholds, expressed in dilution steps, were submitted to analysis of variance, testing the variables age, gender, odorant (three compounds and the mixture), and day (test, retest), and their various interactions. Of these, only age ($P < 0.0002$) and odorant ($P < 0.0008$) were significant. *Post-hoc* tests indicate that there were no statistically significant differences among the three compounds, and that each of the three compounds was significantly different from the mixture ($P < 0.02$ in each case).

Nature of additivity

The mean thresholds for the three compounds and their mixtures are shown in Figure 2, plotted in terms of dilution steps, for the young and the elderly subjects separately.

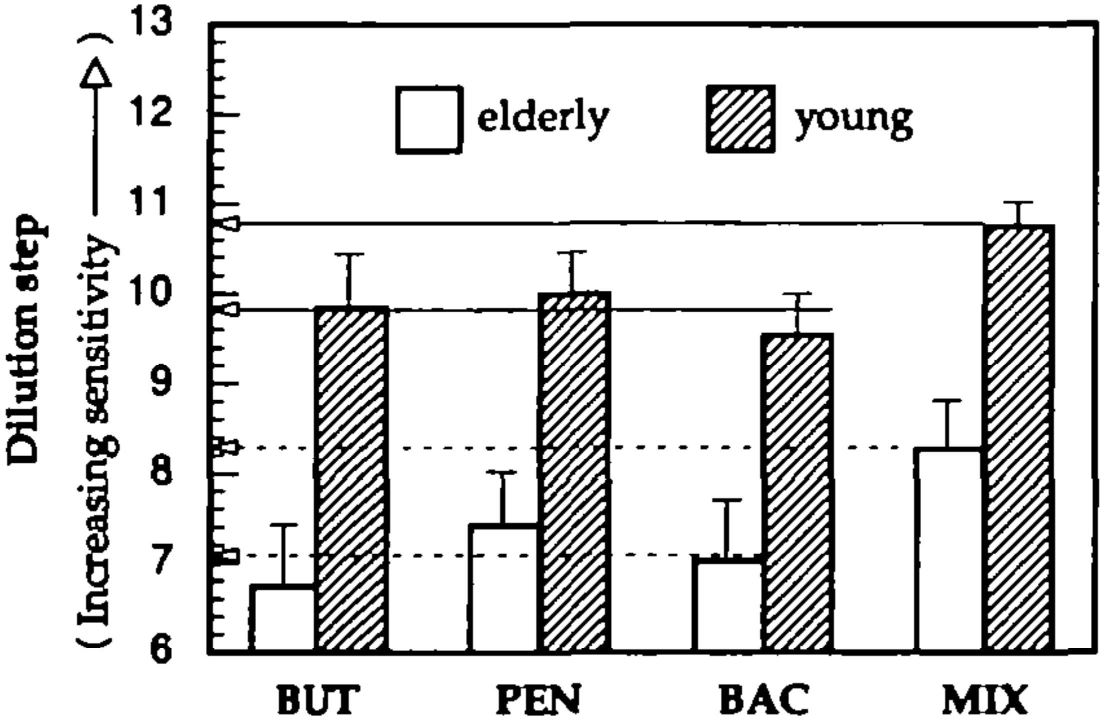

Figure 2. Mean thresholds (in dilution steps) for young and elderly subjects for 1-butanol (BUT), 2-pentanone (PEN), n-butyl acetate (BAC), and their mixture (MIX). The horizontal arrows are intended to facilitate inspection of the difference between the mixture's threshold and the average threshold of the three components measured individually. The vertical bars are standard errors, calculated using each subject's average of test and retest thresholds for each odorant.

The most obvious feature of Figure 2 is the striking absolute difference between the young and the elderly—nearly three dilution steps. Less striking, but the central issue here, is the absolute difference between the threshold of the mixture and of each component separately—close to one dilution step in each case. We see from this inspection that the results approximate simple stimulus additivity, and this was true at the higher stimulus levels of the elderly subjects and at the lower stimulus levels of the young subjects. The results thus differ from the usual hypo-additivity exhibited by suprathreshold magnitudes and the hyper-additivity reported by Laska and Hudson (1991) for some of their mixture thresholds.

The simple additivity of our mixture can also be demonstrated by another kind of plot, as depicted in Figure 3. Here is plotted, averaging all subjects, the threshold concentrations (in p.p.m.) of all three compounds, as measured by the gas chromatograph both alone (separately) and in mixture. Note that the pattern of concentrations in the mixture is very nearly the same as the pattern of concentrations measured separately. What is more important, the threshold concentration of each component measured alone is approximately three times that of the same component measured in the mixture, again supporting a picture of complete stimulus additivity for our mixture. This result holds approximately the same for young and elderly subjects, as implied in Figure 2 where their results are plotted separately.

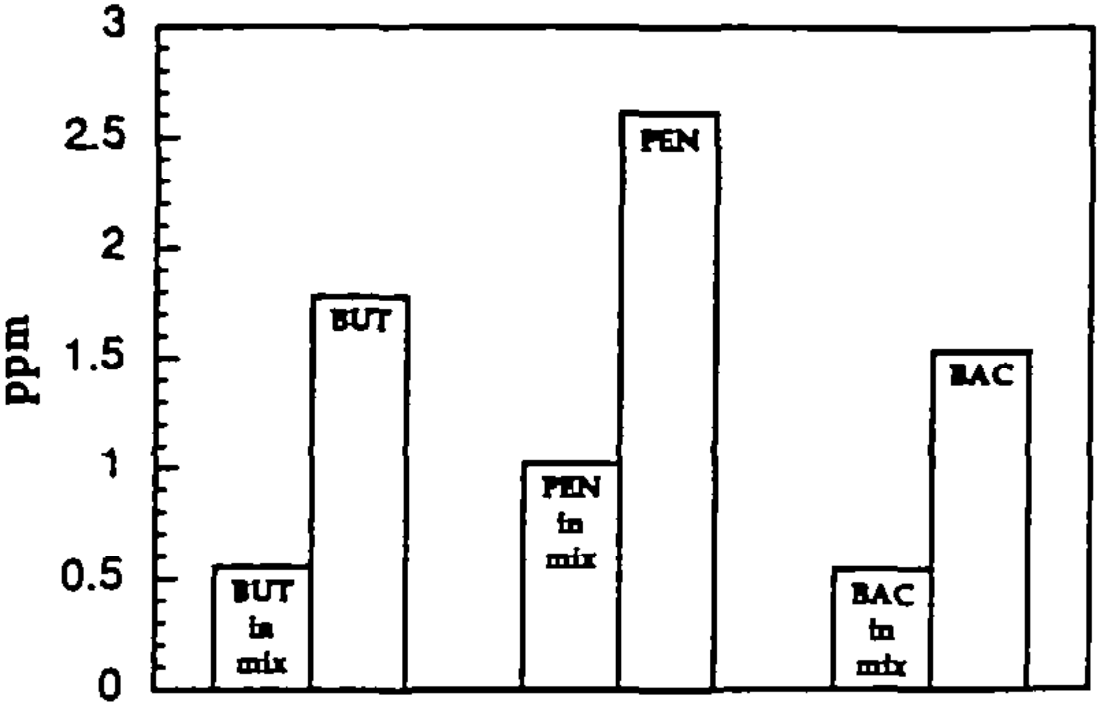

Figure 3. For all 40 subjects, mean threshold concentration (in p.p.m.) for the three components individually and in the mixture.

Complete additivity in mixture also characterized the behavior of several saturated aldehydes studied by Guadagni *et al.* (1963). An interesting feature was to show that additivity accurately predicted the results of a mixture of two odorants in a variety of proportions.

Recall that Laska and Hudson (1991) reported that smaller variability characterized mixtures, compared with their components; also, the larger the number of components, the smaller the variability of the mixture threshold. Our data agree with their overall conclusion. The error bars (standard errors) are in all six comparisons smaller for the mixture than for the individual compounds, by about 40% for the young subjects and about 12% for the elderly in each instance.

Aging

As shown in Figure 2, the threshold for the elderly for all three compounds and their mixture exceeded that for the young by ≈20 times (2.7 dilution steps). This study adds to the considerable list of studies (see Cain and Stevens, 1989; Cain and Gent, 1991; Stevens and Dadarwala, 1993) reporting that the elderly's olfactory threshold averages one or two orders of magnitude higher than the young's. The exact difference may depend on the particular compound tested (Stevens and Cain, 1987). The present study shows that aging can also dull the detectability of mixtures. Also, no evidence emerged that the features of the mixture (simple additivity, lower variance) vary with age, and *ipso facto,* the exact concentration levels of the constituents.

Test-retest reliability

The experimental design permits computation of correlations across compounds (and their mixture), including thresholds measured in succession on the same day and thresholds measured on separate days, on all subjects regardless of age, and on young and elderly subjects separately. The main question is, to what extent are subjects consistently relatively sensitive or insensitive from test to test? In other words, does a relatively high (low) score on one compound (or mixture) predict a relatively high (low) score on another compound (or mixture)? How does this relationship hold for repetitive measurements on the same day? On different days?

Table I. For all 40 subjects, and for 20 young and 20 elderly subjects separately, the median correlation coefficient, Pearson *r,* (and range) relating all six pairs of four odorants (three compounds and mixture) on the same day (Day 1 and Day 2 separately) and all 16 pairs on different days (each odorant on Day 1 paired with each odorant on Day 2).

	Day 1	Day 2	Day 1/Day 2
Young	0.33 (0.04–0.62)	0.48 (0.33–0.85)	0.10 (–0.27–0.44)
Elderly	0.45 (0.21–0.60)	0.71 (0.58–0.90)	0.50 (0.35–0.71)
All	0.53 (0.38–0.61)	0.71 (0.58–0.81)	0.50 (0.33–0.60)

Table I is a condensation of 84 Pearson correlation coefficients relating thresholds obtained on the same day (36 *r*'s) and on different days (48 *r's),* for young and elderly subjects separately, and for all subjects combined. Each cell in Table I is the median of six r's (same-day correlations) or 16 r's (different-day correlations). The table reveals five features of inter-test reliability.

(i) All nine median correlations are positive in sign and seven are significant at $P<0.05$. (Of the matrix of 84 r's on which these are based, 79 were positive and 57 were individually significant). The overall picture is thus persistent low-to moderate positive correlation.

(ii) The correlations depend on the ages of the subjects; they are plainly higher for the elderly subjects than for the young subjects. This reflects the small age range of the young group and perhaps also a differential rate of physiological aging in the elderly. Removing the (chronological) age variable by partial correlation (not shown in the table) reduced, but far from eliminated, the disparity between the young and the elderly's correlations. Apparently, within the elderly group some subjects have become more impaired than others, regardless of exact chronological age. Combining the young and the elderly subjects had only small effect on the correlations. The main lesson to be learned is that the evaluation of test-retest reliability must take into account the age composition of the subjects. Including subjects of greatly non-homogeneous age (especially elderly) will tend to enhance the appearance of reliability as gauged by the size of *r* (see also Stevens and Dadarwala, 1993).

(iii) Same-day correlations are higher than different-day correlations. In fact, same-day correlations across compounds were higher than different-day correlations for the same compound. Likewise, different-day correlations appear to be little or no higher for the same compound than for different ones. This implies that thresholds for different compounds tend to fluctuate in step with each other from day to day. This fact supports the idea that a subject's overall olfactory sensitivity varies day by day.

(iv) Same-day correlations are higher for the second day of testing than the first. This probably means that a day's practice causes subjects to become more consistent with themselves. This is essentially the same finding and interpretation made by Cain and Gent (1991).

(v) These correlations are reasonably similar to ones previously reported. For example, Punter (1983) reported average test-retest correlations of 0.40 and 0.27 for the same and different compounds respectively (young subjects). Stevens and Dadarwala (1993) also reported an average of 0.40 relating intra odorant pairs of tests for young and elderly subjects separately, but 0.70 for the combined results of young and elderly. Cain and Gent (1991) reported inter-odorant correlations of about 0.60 for a group of diverse ages.

We need to clarify further the sources of the large threshold variability that characterize olfaction. The present study adds some fresh information, while corroborating earlier information. Eventually, these facts may translate into a unified theory of olfactory sensitivity, suggest more efficient and reliable ways to assess it in the individual, and help the olfactory scientist explain the mechanisms whereby chemical stimulations transmute into odor experience.

Discussion

Regardless of the exact degree of additivity—whether complete, partial, or synergistic in nature—its existence to any degree is remarkable. Essentially, it says that two or more individual compounds at levels each too weak to make conscious impact on their own are able to do so in concert, despite diversity of their chemical structure. From this point of view, a useful biological function of mixture additivity may be to enhance sensitivity to the typical olfactory stimuli of everyday life. More often than not these stimuli are complex blends of organic compounds. From the point of view of biological economy, the number of different chemical signals impinging on the odor sense may count as much as the strength of any one of them. The three-fold gain in sensitivity we report here may seem somewhat modest in view of the variability of thresholds, but it remains to be seen just how much could be gained by mixtures of many more components. In hearing, perfect stimulus (energy) additivity can take place for at least ten different frequencies spaced within a so-called 'critical band' and further (imperfect) additivity for at least an additional 40 or so frequencies beyond the critical band (Gassier, 1954). Thus, extensive additivity at the detection threshold characterizes a modality that we think of as impressive for quality (pitch) discrimination. It is possible that olfaction works similarly.

A second possible biological function of mixture interaction is, according to the limited evidence so far, that detection of mixtures may be more *stable* than detection of individual compounds. That is, intra- and inter-individual variation in detection thresholds of mixtures may be less variable than those of their components. If so, this principle, especially when coupled with enhanced sensitivity, might help to explain how detection, which has hitherto revealed a picture of bewildering fickleness, might nevertheless promote survival.

The generality of these two possible biological roles of mixture—enhancement of sensitivity and stability—is far from established given the very limited scope of mixture studies to date. [Another possible generality of mixtures is their resistance to adaptation, or 'durability' as noted by Schiet and Cain (1990)]. We need to know how mixtures grow in sensitivity and stability as the number of components increases. Laska and Hudson (1991) suggest increasing stability with increasing complexity. This question is also under further study in our own laboratory. The point has more than academic interest. For example, being easier to detect and more reliable, mixtures may (other things being equal) serve as better gas-warning agents than single compounds (Cain and Turk, 1985).

Although the present results exhibit complete (not hyper- or hypo-) additivity, its generality is, of course, very far from proven by a single case. Its demonstration is nonetheless significant in revealing that different chemical stimuli can, like different auditory frequencies (Gassier, 1954) perfectly sum their effects. Whether, as in hearing detection, there are limits on such additivity, remains to be learned. Perfect energy additivity in hearing takes place only within a critical band. Are there perhaps similar limits on additivity within the olfactory domain? Guadagni *et al.* (1963) suggested that the exact rules of detection additivity might depend on chemical similarity; according to this conjecture, mixtures within a class (saturated aldehydes in their study) might exhibit complete additivity (as theirs did), while those across classes might exhibit a less complete additivity. Just how to define 'chemical similarity' is, of course, an open question, but the compounds of the present study yielded virtually complete additivity despite considerable chemical dissimilarity. In defining similarity, it is possible that what really counts is not so much chemical structure as subjective similarity or 'thematic' commonality, as discussed in the context of choice of compounds in Materials and methods. Either way, our compounds exhibit large differences.

Much remains to be learned. Yet what we do know already poses interesting questions for those who inquire about the underlying mechanisms of olfactory perception. Does, for example, additivity take place because of imperfect stimulus selectivity at the level of the receptor? Or, perhaps, does it represent a kind of pooling or funneling of the outputs from multiple receptors? What is the relation between *stimulus additivity* (ability to sum the effects of stimulation of different compounds) and *stimulus selectivity* (ability to differentiate quality among different compounds) in the olfactory world? Why does additivity at threshold appear to be complete, or in some instances perhaps even synergistic, while above threshold it clearly tends toward hypo-additivity?

When it comes to the thermal senses, Stevens *et al.* (1974) demonstrated that the same difference between complete additivity and hypo-additivity at and above threshold characterizes spatial summation of thermal sensation—apparently for good biological reasons. When it comes to taste mixtures, analogous issues characterize this sense, too. Although the exact nature of taste mixtures is under intense scrutiny by various investigators (Frijters, 1987; Frijters and De Graaf, 1989; Kroeze, 1989; McBride, 1989; Schifferstein and Frijters, 1993), it is generally acknowledged that the degree of mixture additivity may depend on the level of the perceptual response (i.e. threshold versus suprathreshold measures) and whether a mixture is homogeneous (i.e. mixture of like-quality taste components) or heterogeneous (i.e. mixture of different-quality components).

Finally, that aging elevates the detection threshold (by some 20-fold in the present study) is immaterial to the nature of additivity and variability of mixtures. The rule of complete additivity seems to hold even at a concentration range that is suprathreshold for the young and, therefore, presumably hypo-additive for them. Greater stability of the mixture thresholds also tended to characterize elderly as well as young persons.

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