A Psychophysical Investigation of Binary Bitter-compound Interactions

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Abstract

The aim of this study was to determine if taste interactions occur when bitter stimuli are mixed. Eight bitter stimuli were employed: denatonium benzoate (DB), quinine-HCl (QHCl), sucrose octaacetate (SOA), urea, L-tryptophan (L-trp), L-phenylalanine (L-phe), ranitidine-HCl, and Tetralone. The first experiment constructed individual psychophysical curves for each subject ($n = 19$) for each compound to account for individual differences in sensitivities when presenting bitter compounds in experiment 2. Correlation analysis revealed two groupings of bitter compounds at low intensity (1, L-trp, L-phe, and ranitidine; 2, SOA and QHCl), but the correlations within each group decreased as the perceived intensity increased. In experiment 2, intensity ratings and two-alternative forced-choice discrimination tasks showed that bitter compounds generally combine additively in mixture and do not show interactions with a few specific exceptions. The methods employed detected synergy among sweeteners, but could not detect synergy among these eight bitter compounds. In general, the perceived bitterness of these binary bitter-compound mixtures was an additive function of the total bitter-inducing stimuli in the mouth.

Key words: additive, bitter taste, suppression, synergy, taste interactions, taste psychophysics

Introduction

Psychophysical investigations of same-quality taste-mixture interactions have revealed non-linear enhancements that implicate taste-integration mechanisms. For both the sweet and savory (umami) qualities, certain same-quality binary mixtures stimulate a perceived intensity in excess of predicted additivity (synergy). The binary mixture of the sweeteners aspartame and acesulfame-K results in a synergy of sweet taste (McBride, 1988; Ayya and Lawless, 1992; Schiffman *et al.*, 1995; Schifferstein, 1996). Similarly, a binary mixture of monosodium glutamate and the sodium salt of 5′-inosine or guanosine monophosphate results in the synergy of savory taste (Yamaguchi, 1967; Rifkin and Bartoshuk, 1980). Very little is known, however, about same quality interactions within bitterness (Keast and Breslin, 2003), arguably the most physiologically complex taste. The aim of this study was to determine if taste interactions occur when bitter stimuli are mixed.

One reason for the dearth of data may be this complexity. Any investigation of human bitterness perception must contend with three complicating factors:

1. There are many chemically distinct compound classes that elicit bitter taste: alkaloids, amino acids, isohumulones, phenols, amines, thioureas, carbamates, ionic salts, etc. (Belitz and Wieser, 1985; Spielman *et al.*, 1992).
2. Bitter taste transduction involves many proteins. A large family (30–40) of putative 'bitter-compound' receptors (T2Rs) have been discovered (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000). There is also more than one post-receptor transduction sequence (Spielman *et al.*, 1992). With regard to coding, many different T2Rs were identified within individual bitter-sensitive cells (Adler *et al.*, 2000), indicating that each cell may respond to many bitter compounds (broad cellular tuning) (Chandrashekar *et al.*, 2000). An alternate hypothesis was suggested by Caicedo and Roper (Caicedo and Roper, 2001), who reported that bitter-sensitive taste cells generally responded to only one of five bitter stimuli, indicating that these stimuli activate different subpopulations of cells (more selective cellular tuning).
3. Individual observers vary in the quantity and presumably functionality of taste cells and receptors (Kim *et al.*, 2003), which causes large individual variation in bitter taste perception (Yokomukai *et al.*, 1993; Bartoshuk *et al.*, 1998; Delwiche *et al.*, 2001; Keast and Breslin, 2002a,b).

To address factor 3 above and determine if taste interactions occur, concentration–intensity psychophysical curves were constructed for each individual and each bitter compound in experiment 1, thereby allowing compounds to be mixed at the same perceived intensity for subjects with different sensitivities. Experiment 2 investigated whether

binary bitter-compound mixtures combined additively, or interacted synergistically or suppressively. This is a comprehensive investigation of binary interactions among eight compounds that stimulate bitter taste.

Materials and methods

Subjects

Twenty-two non-smoking volunteers (13 females, 9 males) between 21 and 52 years old (mean 30.1 years) were paid to participate in the study. Subjects were mostly employees of the Monell Chemical Senses Center (primarily Caucasian and African–American). They provided informed consent on an Institutional Review Board approved form. The subjects were asked to refrain from eating, drinking or chewing gum for at least 1 h before testing.

Subject training

Subjects were initially trained in the use of the Labeled Magnitude Scale (LMS) (Green *et al.*, 1993, 1996) except the top of the scale was described as the 'strongest imaginable' sensation of any kind (referred to as the general LMS, or gLMS) (Bartoshuk, 2000). The gLMS is a computerized psychophysical tool that requires subjects to rate the perceived intensity along a vertical axis lined with adjectives: barely detectable = 1, weak = 5, moderate = 16, strong = 33, very strong = 51, strongest imaginable = 96; the adjectives are spaced semi-logarithmically, based upon experimentally determined intervals to yield ratio quality data (Green *et al.*, 1993, 1996). The gLMS only shows adjectives, not numbers, to the subjects, but the experimenter receives numerical data from the computer program.

Subjects were trained to identify each of the five taste qualities by presenting them with 10 ml of prototypical stimuli: 150 mM sodium chloride (NaCl) salty, 0.05 mM quinine-HCl (QHCl) bitter, 300 mM sucrose sweet, 3 mM citric acid sour, and 100 mM monosodium glutamate (MSG) savory. In all cases, subjects were instructed to identify the labeled quality as the dominant one, but others may also be perceived to a lesser degree. To help subjects understand how a stimulus could elicit multiple taste qualities, 300 mM urea (usually bitter and slightly sour) and 50 mM NH_4Cl (usually salty, bitter, and slightly sour) were also employed as training stimuli.

A computerized data-collection program simultaneously presented subjects with 5 gLMSs corresponding to SWEET, SALTY, SOUR, SAVORY and BITTER. The order of the five scales on the monitor was randomized from session to session but remained constant within each test session.

Stimuli

Acesulfame-K, ammonium chloride, aspartame, citric acid, denatonium benzoate (DB), MSG, L-phenylalanine (L-phe), sucrose, sucrose octaacetate (SOA), NaCl, L-tryptophan (L-trp), and urea were all purchased from Sigma (St Louis,

MO) and were Sigma-ultra grade. QHCl was purchased from Fluka (Buchs, Switzerland), ranitidine from Medisca (New York) and Tetralone from Kalsec (Kalamazoo, MI). All solutions were prepared with deionized Millipore™ (Bedford, MA) filtered water and stored in amber glass bottles at 4–8°C and brought up to room temperature prior to testing with the aid of a water bath. Solutions were made fresh every 5 days. Millipore™ filtered deionized water was used as the blank stimulus and the rinsing agent in all experiments.

Stimulus delivery

An aliquot of 10 ml of each solution was presented in 30 ml polyethylene medicine cups (Dynarex, Orangeburg, NY) on a numbered tray. All samples were presented in random order with an interstimulus interval of 90 s unless otherwise stated. The tasting protocol asked subjects to sip, rate and expectorate each solution. On each trial, subjects held 10 ml of solution in their mouth for 5 s and rated the intensity of the taste qualities of the solution (sweet, bitter, sour, salty, savory) before expectorating. Subjects wore nose-clips (GaleMed, Taipei, Taiwan) to eliminate olfactory input while rating.

Experiment 1: covariation of bitterness among compounds at three concentrations

Bitterness perception among individuals is highly variable, but the bitterness elicited by two compounds may correlate. For example, at a fixed concentration of QHCl and a fixed concentration of DB one individual may be sensitive to the bitterness of both (rate them as 'strong' on the gLMS), while a second individual may be insensitive to the bitterness of both (rate them as 'weak' on the gLMS). While there are large differences in the perceived bitterness of DB and QHCl between the two individuals, each individual responds similarly to the two.

Psychophysical curves were constructed for each bitter compound for each individual subject to enable us to deliver bitter additives that were in the same intensity range for all subjects (experiment 2). These functions provided the opportunity to investigate bitterness correlations as a function of individual sensitivities among bitter compounds at three different concentration levels. First, we adjusted intensity ratings for bias in scale use.

PROP (n-propylthiouracil) bitterness ratings and standardization of gLMS ratings with tone and weight ratings

The PROP assessment and gLMS standardization followed previously published methods used in our laboratory (Delwiche *et al.*, 2001). Briefly, subjects rated the bitterness and total intensity of 10 ml samples of five concentrations of PROP (5.5×10^{-5}, 1.7×10^{-4}, 5.5×10^{-4}, 1.7×10^{-3} and 5.5×10^{-3} M). Between each sample, subjects rinsed four times with deionized water. Subjects also rated the loudness of six tones [generated by a Maico Hearing Instruments

tone generator (Minneapolis, MN), presented via head-phones at 4000 Hz for 2 s at levels 0, 20, 35, 50, 65 and 80 dB] and the heaviness of six visually identical weights (opaque, sand-filled jars at levels 225, 380, 558, 713, 870 and 999 g). All three types of ratings were made on a computerized gLMS. Subjects were asked to rate the intensity of taste, or loudness, or heaviness, and all judgments were made within the context of the full range of sensations experienced in life on the gLMS. All stimuli were presented twice in blocks of ascending order. Subjects first rated the intensity of weights, then tones, and finally PROP solutions.

There were significant correlations between PROP bitterness ratings, heaviness ratings and loudness ratings. Since these three sensory modalities were assumed to be unrelated, the significant correlations indicated that the gLMS ratings were subject to individual scale-use bias and required standardization across subjects.

To determine a standardization factor, each subject's average intensity for heaviness was divided by the grand mean for heaviness across weight levels and subjects. This procedure for determining a correction factor was repeated with loudness ratings (averaging across decibel levels). The two correction factors (one for weights and one for tones) were averaged, and each individual's bitter intensity ratings for all eight bitter compounds, in subsequent tests, and all five levels of PROP were multiplied by his or her personal standardization factor for scale-use bias.

Psychophysical curves for bitter compounds

The concentration ranges for constructing a psychophysical curve for the bitter stimuli were: DB (7.5×10^{-8}–1×10^{-4} M), L-phe (0.016–0.16 M), L-trp (0.01–0.06 M), SOA (1×10^{-5}–1×10^{-3} M), urea (0.15–2.5 M), QHCl (1×10^{-5}–1×10^{-2} M), ranitidine (1×10^{-4}–2×10^{-2} M), Tetralone (1.37×10^{-5}–1×10^{-2} M). Subjects were presented with numbered trays that contained 10 randomized solutions (10 ml) of one bitter stimulus (nine concentrations from the psychophysical curve and one deionized water control). The nine concentrations for each bitter stimulus ranged from below 'weak' on the gLMS to maximum solubility (L-trp, L-phe, SOA) or maximum practical tasting limit (near 'very strong'). Each point on an individual psychophysical curve was tested at least four times. Subjects were excluded from the study (3 of 22 subjects screened), if bitterness concentration–intensity curves were not ordinal (defined here as a change of direction of slope >30% of the y-axis values) over the range of concentrations tested.

Statistical analysis

Data used for correlation and cluster analysis were the individual bitterness intensity ratings of concentration levels (associated with average ratings of gLMS 4, 8 and 12). Note that individual ratings of the compounds were free to vary at each level; the concentrations were selected so that the average ratings would be perceived at particular intensities. Correlation analysis (Pearson's product moment coefficients) and cluster analysis (single linkage joining, Euclidean distances) were performed using Statistica version 6.0. To reduce Type I errors, a Bonferroni correction for multiple comparisons was made by dividing the P value ($P < 0.05$) by 36, the total number of correlations. Statistical significance of correlation therefore was $P < 0.0014$.

Experiment 2: bitter–bitter interactions

Subjects

All subjects had participated in experiment 1. Due to the large number of sessions to complete experiment 2 (eight sub-experiments each comprising at least 16 sessions) and some subject's insensitivity to the bitterness of certain compounds, only five subjects completed all of the sub-experiments (128 sessions). Other subjects completed partial sets of separate sub-experiments. For each bitter stimulus used as a target compound to which other compounds were added, the number of subjects who completed each test matrix was: DB $n = 14$ (eight females), L-phe $n = 15$ (seven females), L-trp $n = 14$ (seven females), SOA $n = 15$ (nine females), urea $n = 10$ (seven females), QHCl $n = 15$ (nine females), ranitidine $n = 15$ (nine females), Tetralone $n = 14$ (eight females).

Design and rationale

All bitter compounds were both a 'target' (four concentrations from the dynamic portion of the psychophysical curve) and an 'additive' (a weak intensity added to the four concentrations of the target compound). During each session, subjects were presented with the target concentrations of a bitter compound, and binary combinations of the target concentrations with the weakly bitter additives [including self-addition of a weak intensity (the additive control)]. There were some binary combinations that were not included due to physical limitations: QHCl–Tetralone mixtures at all concentrations precipitated when mixed, and the amino acids (L-phe and L-trp) when combined with the additive urea at their highest concentration also precipitated.
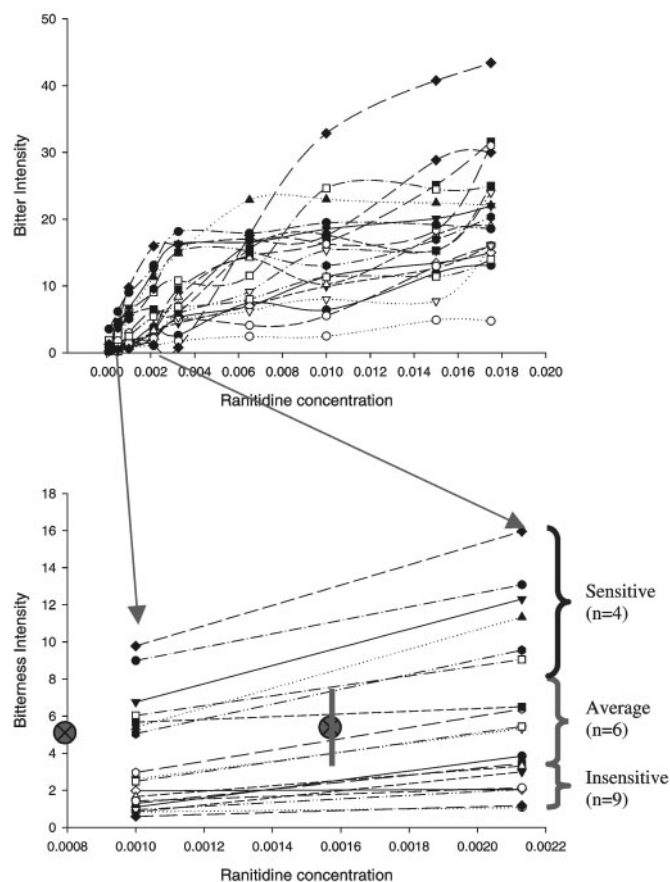
The group psychophysical curves for all eight bitter compounds were examined and four concentrations corresponding to varying bitter intensities were chosen for the bitter–bitter interaction experiment. The four concentrations were from the dynamic phase of the group psychophysical curve, determined in experiment 1, and corresponded to increasing bitter intensity (Figure 1C1–C4). These are referred to as the 'target' compound concentrations.

A weak intensity 'additive' control concentration of each compound was also required for the study so that a compound could be added to itself. Due to the large individual differences in bitterness perception at a single concentration of stimulus (as detailed in experiment 1), it was necessary to divide the subject population into three sub-groups, a sensitive group, an insensitive group, and the middle group (Figure 2). Psychophysical curves were plotted for the sub-
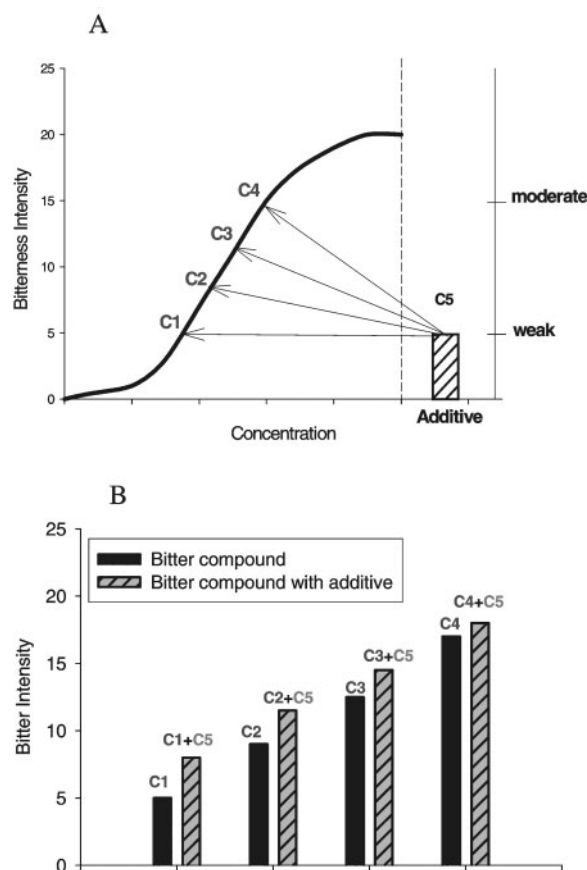
Figure 1   Schematic design of bitter–bitter interaction methodology. **(A)** A hypothetical psychophysical curve for a bitter compound. Four points corresponding to increased concentration and intensity from the dynamic phase of the curve are chosen (C1–C4). To those four points, a 'weak' (C5) intensity of a second bitter compound is added. **(B)** The effect the weak intensity additive has on the intensity of C1–C4. Note that the influence of C5 on bitterness is greater at C1 than C4. The effect can be graphically observed in **A**, where above C4 we see an asymptote of bitterness for the hypothetical compound. (Reprinted from *Food Quality and Preference*, 14, R.S.J. Keast and P.A.S. Breslin, An overview of binary taste–taste interactions, 111–124, 2003, with permission from Elsevier.)

Figure 2   Schematic representation of how to select a weak additive for subjects of different bitter sensitivities. The upper graph shows actual psychophysical curves for all subjects for the bitter compound ranitidine. From the group mean, calculations show that gLMS 'weak' intensity corresponds to 0.00159 M ranitidine. The lower graph shows an enlarged portion of the upper graph that corresponds to the group mean for 'weak' intensity. Subjects with ratings within 2 gLMS points of 'weak' of the group mean concentration were termed average ($n = 6$), those with ratings greater than 2 gLMS points above 'weak' were termed sensitive ($n = 4$), and those with ratings less than 2 gLMS points below 'weak' were termed insensitive ($n = 9$). The three additive concentrations were selected to generate a 'weak' intensity for each of the three groups. The insensitive group would require a higher concentration of ranitidine to elicit a weak intensity, while the sensitive group would require a lower concentration to elicit a weak intensity.

groups for each compound and the three concentrations that corresponded to a 'weak' intensity were determined, one for each sub-group for each compound. Thus, the insensitive group had a concentration for their additive that was higher than the average, and the sensitive group an additive concentration that was lower. Across these sub-groups the average bitterness experienced for each additive was the same intensity, 'weak'. This approach was necessary since the intensity of the additive could influence the type of perceived interaction that would occur between bitter compounds. Although it would be theoretically ideal, the preparation of individual concentrations of additives for every subject would have greatly increased the stimulus preparation time. The 'additive' control concentration was mixed with the four 'target' concentrations and subjects

rated the taste intensities of sweet, sour, salty, bitter, and savory.

The additive control bitter mixture was made by adding a compound to itself at the four target concentrations.

A set concentration of sucrose corresponding to 'weak' sweetness (gLMS = 5.76) was included as a taste quality control and a confirmation of the methods. It was expected that the cognitive influence of sweetness would inhibit bitterness in general (Kroeze and Bartoshuk, 1985; Calvino *et al.*, 1990, 1993; Frijters and Schifferstein, 1994; Breslin and Beauchamp, 1997).

Table 1   Molarity (mM) of bitter compounds determined from group psychophysical curves at intensity ratings gLMS 4, 8 and 12. The range of individual subjects gLMS ratings at the mean concentration is included

Bitter compound	Concentration (mM) and (LMS range)		
	gLMS = 4	gLMS = 8	gLMS = 12
n-6-Propylthiouracil	0.16 (0–13)	0.57 (0–26)	1.8 (1–43)
Denatonium benzoate	0.00015 (0–13)	0.00044 (0–16)	0.0011 (3–23)
L-Phenylalanine	41 (0–13)	93 (2–24)	148 (2–34)
L-Tryptophan	20 (1–11)	39 (1–17)	59 (4–19)
SOA	0.023 (1–13)	0.054 (1–16)	0.19 (3–20)
Urea	850 (0–8)	1750 (1–15)	2500 (2–25)
Quinine–HCl	0.06 (1–14)	0.21 (4–20)	0.4 (4–24)
Ranitidine	1.14 (1–10)	3.08 (2–17)	6.5 (3–23)
Tetralone®	0.1 (0–10)	0.281 (1–16)	0.68 (2–27)

Methodology

Subjects were given numbered trays of randomized bitter tasting solutions. For each session, the solutions included deionized water as a control for spurious ratings ($n = 1$), self-addition concentrations of the target bitter stimuli ($n = 4$), and one 'target' concentration with the 'additive' concentrations of the other seven bitter compounds ($n = 7$). The testing protocol was as follows. Randomized solutions (12 solutions containing 10 ml) were presented in 30 ml plastic medicine cups on numerically labeled trays. Subjects rinsed with deionized water at least four times over a 2 min period prior to testing. Each subject tasted and then rated each solution for sweetness, sourness, saltiness, bitterness and savoriness, on the gLMS before expectorating, while wearing nose-clips (GaleMed) to minimize any olfactory input. All subjects rinsed with deionized water four times during the interstimulus interval of 85 s. All binary bitter combinations were tasted on at least four separate occasions.

Method verification

To ensure the method could detect non-additive interactions in taste intensity, we conducted a parallel experiment with aspartame and acesulfame-K (both sweeteners), which, when mixed, exhibit synergy of sweet taste (McBride, 1988; Ayya and Lawless, 1992; Schiffman *et al.*, 1995; Schifferstein, 1996). Sucrose was used as a control sweetener, since it does not synergize with either sweetener (Schifferstein, 1995). All subjects ($n = 16$) matched the intensity of sweeteners to gLMS 5 and 10 prior to the experiment. The group mean concentration required for each of the sweeteners to elicit gLMS 5 or 10 intensity was determined. The method for intensity matching followed previously published methods used in our laboratory (Keast and Breslin, 2002a). During each session, subjects were presented with a single concentration of a sweetener, a double concentration of the same sweetener (self-addition control) and binary combinations of sweeteners. The tasting procedure was the same as above. Each sample was tasted only once per session and every binary sweet combination was tasted on at least three separate occasions. There were a total of six sessions, three for gLMS 5 and three for gLMS 10 solutions.

Alternative forced-choice methodology

Subjects ($n = 10$) were asked to determine whether a bitter-tasting additive was more bitter than a self-addition control with a two-alternative forced-choice (2-AFC) method. The 2-AFC method is more sensitive than the rating method and could identify deviations from bitter-taste additivity that were not statistically significant using the rating data. The 2-AFC procedure was used to determine if either urea (as a bitterness inhibitor) or DB (as a bitterness enhancer) could be distinguished from the self-addition target. The choice of urea and DB provided the best chance to confirm a suppression or enhancement of taste because urea tended to suppress and DB tended to enhance bitterness. Each session consisted of six discrimination tasks with an interstimulus interval of 85 s. Each sample pair was repeated three times for the 10 subjects yielding 30 trials per pair. For a result to be statistically significant ($P < 0.05$) using a chi-square test, one of the two samples must be chosen as more bitter on 20 or more of the 30 trials. All sample pairs were presented in random order.

Normalization of gLMS ratings

The standardized bitterness rating for bitter compounds tended to follow a log-normal distribution. A normal distribution was approximated by taking the log value of the ratings. Therefore, the log was taken of all standardized gLMS ratings before any statistical analyses were conducted. Before taking the log, all zero values were converted to 0.24, the lowest possible value above zero that can be measured on the computerized gLMS.

Statistical analysis

Numerical results are expressed as geometric means + geometric standard error [see Breslin and Tharp (Breslin and Tharp, 2001) for calculation of geometric standard error]. Statistical variation was determined by one- or two- or three-way analysis of variance (ANOVA) using Statistica 6.0 software package. P values < 0.05 were considered statistically significant. Individual's mean bitterness intensity data from the binary bitter-compound experiment were analyzed by an $8 \times 8 \times 4$ (target × additive × concentration) repeated-measures ANOVA.

Results

Experiment 1

Table 1, Figure 2 (top) and Figure 3 illustrate the wide range in the perceived bitterness intensity of compounds used in this study. Table 1 shows concentrations of the bitter compounds that correspond to three intensities, gLMS 4, 8 and 12, as well as the range of individual ratings of bitterness at those concentrations. Figure 3 shows psychophysical curves plotted for the group, and representative curves from typical insensitive and sensitive subjects (sensitivities for an individual varied from compound to compound). These results complement other studies that illustrate the high variability of bitterness perception within a population (Yokomukai *et al*., 1993; Delwiche *et al*., 2001; Keast and Breslin, 2002b). PROP's psychophysical curve was included in this phase of the research, although PROP was not one of the compounds used in the binary bitter interactions phase due to the high proportion of the population that is insensitive. Urea, L-phe, and L-trp were perceived as being the least bitter. The limitations of solubility for L-trp, L-phe, and SOA in aqueous solutions determined the maximum bitterness of those compounds. Thus, for these three compounds, the highest concentration tested was the maximum practical solubility.

Table 2 shows the results of the correlation analyses at gLMS 4, 8 and 12. In general, the correlations between bitter compounds were more frequent at gLMS 4 and diminished as the intensity increased. For example, at gLMS 4 the bitterness of L-phe was correlated with five other compounds. At gLMS 8 (Table 2), the bitterness of L-phe was only correlated with one other compound, and at gLMS 12 (Table 2) L-phe did not correlate with any compounds. This illustrates that the concentrations of bitter compounds is an important variable to account for when assessing bitter taste interactions. The bitterness of PROP did not correlate with the other bitter compounds at any intensity.

Figure 4 shows the results of the descriptive cluster analyses (single linkage, Euclidean Distance) at the three concentration levels. The placement of compounds at the three intensities is similar to results from the correlation matrices. As the perceived intensity increased, the linkage distance among compounds also increased. There were two tight groupings at gLMS 4, the first being ranitidine, L-trp and L-phe, while the second was SOA and QHCl. As the intensity of bitterness increased, the separation of these tight groupings was evident. PROP was always the outlier in these analyses.

The analysis indicates that at higher concentrations the compounds become more distantly connected and linkages appear more uniform. Data from Figure 2 (bottom graph) support these observations where three groupings of subjects are evident according to perceived intensity at low concentrations of ranitidine, while at higher concentrations of ranitidine (upper graph), the perceived bitterness intensity for the majority of subjects is more evenly distributed over a wide range of intensities. Thus, at low concentrations, some low sensitivity subjects become moderately sensitive at high concentrations, and some high sensitivity subjects become moderately sensitive at high concentrations. This results in both weaker correlations and weaker linkages among compounds at higher concentrations.

Experiment 2

Figure 5 shows the pooled (across four target concentrations and across all the target compounds) effects of the bitter compounds as additives. This figure illustrates the overall influence of these additives on bitterness in mixture. There were no significant differences between bitter compounds as additives. Figure 6A–H shows the effects of additives on specific target compounds pooled across all four concentrations of the targets, which indicates how each target compound was generally influenced by each additive. The bitter additives did not significantly alter the bitterness of the target compound.

Verification of the method with sweetness

The results reveal that there are significant differences in sweetness of binary mixtures of sweeteners: gLMS5 [$F(5,55) = 9.75$, $P < 0.05$]; gLMS10 [$F(5,55) = 12.4$, $P < 0.05$] (Figure 7). The mixture of aspartame and acesulfame-K significantly ($P < 0.05$) increase sweetness (synergy) relative to the self-addition controls, which verifies that the methodology is sensitive enough to confirm non-linear taste interactions that are known to exist.

Binary bitter interactions

Results from an $8 \times 8 \times 4$ (target × additive × concentration) repeated-measures ANOVA follow. There was a significant main effect of the 'target' compounds [$F(7,35) = 3.2$, $P < 0.05$]. This indicates that the bitterness of the 'target' compounds differed overall.

There was a significant main effect of concentration [$F(3,15) = 19.4$, $P < 0.001$], indicating that the bitterness significantly increased as the concentration of the target compound increased.

There was no main effect of the 'additive' [$F(7,35) = 1.9$, $P = 0.09$] and no interaction between the 'target' compound and the 'additive' [$F(49,245) = 1.4$, $P = 0.051$], indicating
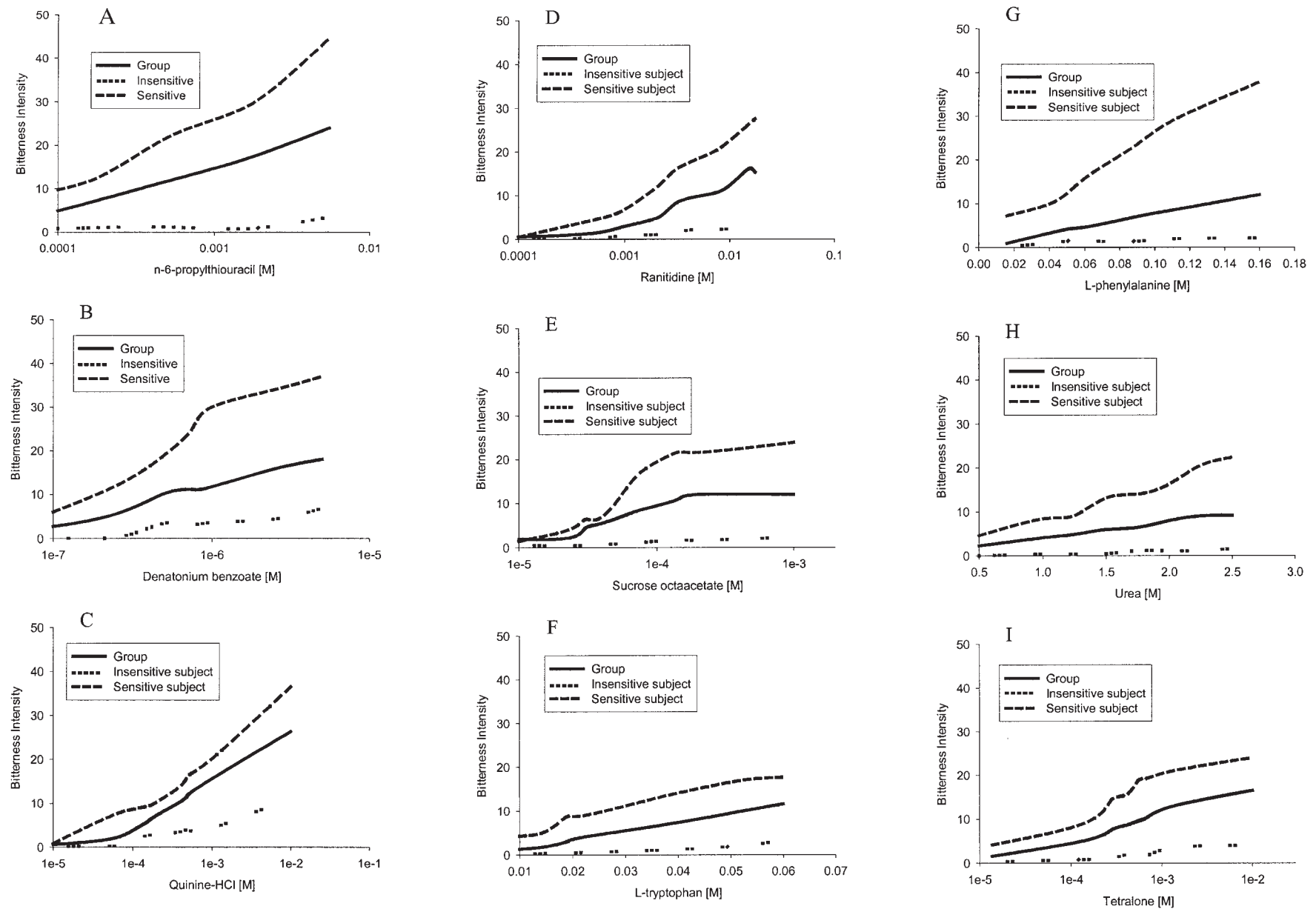
Figure 3 **(A–I)** Psychophysical curves of the sample population mean and the least and most sensitive subjects for PROP and for the eight bitter compounds used in the bitter–bitter mixture interaction phase. Included in each graph is a typical sensitive (highest curve) and insensitive subject (lowest curve) for that compound as well as the mean psychophysical curve (the typical curves for sensitive and insensitive subjects are not from the same subjects in each graph). The *y*-axis is a numerical measure of bitterness intensity ratings from the general Labeled Magnitude Scale (gLMS).

Table 2 Pearson's product moment correlation coefficients of bitterness intensity between compounds. Three intensities are represented: (A) gLMS 4 and (B) gLMS 8, and (C) gLMS 12

	PROP	DB	L-phe	L-trp	SOA	Urea	QHCl	RAN	TET
(A) Intensity data gLMS = 4									
PROP		$P = 0.93$	$P = 0.9$	$P = 0.79$	$P = 0.95$	$P = 0.92$	$P = 0.51$	$P = 0.54$	$P = 0.68$
DB	$r^2 = 0.02$		$P = 0.04$	$P = 0.01$	**$P = 0.000$**	$P = 0.31$	$P = 0.02$	$P = 0.1$	$P = 0.03$
L-phe	$r^2 = 0.03$	$r^2 = 0.47$		**$P = 0.000$**	$P = 0.001$	**$P = 0.000$**	**$P = 0.000$**	**$P = 0.001$**	$P = 0.07$
L-trp	$r^2 = 0.06$	$r^2 = 0.54$	**$r^2 = 0.77$**		$P = 0.008$	$P = 0.06$	$P = 0.006$	**$P = 0.000$**	$P = 0.2$
SOA	$r^2 = 0.01$	**$r^2 = 0.77$**	**$r^2 = 0.71$**	$r^2 = 0.59$		$P = 0.002$	**$P = 0.000$**	$P = 0.006$	**$P = 0.000$**
Urea	$r^2 = 0.02$	$r^2 = 0.25$	**$r^2 = 0.75$**	$r^2 = 0.45$	$r^2 = 0.66$		**$P = 0.000$**	$P = 0.01$	$P = 0.08$
QHCl	$r^2 = 0.16$	$r^2 = 0.53$	**$r^2 = 0.76$**	$r^2 = 0.61$	**$r^2 = 0.88$**	**$r^2 = 0.75$**		$P = 0.006$	**$P = 0.001$**
RAN	$r^2 = 0.15$	$r^2 = 0.39$	**$r^2 = 0.69$**	**$r^2 = 0.77$**	$r^2 = 0.61$	$r^2 = 0.56$	$r^2 = 0.6$		$P = 0.2$
TET	$r^2 = 0.1$	$r^2 = 0.5$	$r^2 = 0.42$	$r^2 = 0.29$	**$r^2 = 0.73$**	$r^2 = 0.41$	**$r^2 = 0.71$**	$r^2 = 0.32$	
(B) Intensity data gLMS = 8									
PROP		$P = 0.14$	$P = 0.94$	$P = 0.83$	$P = 0.34$	$P = 0.58$	$P = 0.23$	$P = 0.68$	$P = 0.54$
DB	$r^2 = 0.35$		$P = 0.32$	$P = 0.05$	$P = 0.002$	$P = 0.06$	$P = 0.005$	$P = 0.1$	$P = 0.06$
L-phe	$r^2 = 0.02$	$r^2 = 0.24$		$P = 0.003$	$P = 0.01$	$P = 0.22$	$P = 0.01$	**$P = 0.000$**	$P = 0.31$
L-trp	$r^2 = 0.05$	$r^2 = 0.45$	$r^2 = 0.64$		$P = 0.07$	$P = 0.86$	$P = 0.01$	$P = 0.01$	$P = 0.38$
SOA	$r^2 = 0.23$	$r^2 = 0.67$	$r^2 = 0.55$	$r^2 = 0.42$		$P = 0.009$	**$P = 0.000$**	**$P = 0.000$**	**$P = 0.000$**
Urea	$r^2 = 0.14$	$r^2 = 0.07$	$r^2 = 0.29$	$r^2 = 0.04$	$r^2 = 0.58$		$P = 0.09$	$P = 0.05$	$P = 0.01$
QHCl	$r^2 = 0.29$	$r^2 = 0.62$	$r^2 = 0.57$	$r^2 = 0.57$	**$r^2 = 0.85$**	$r^2 = 0.4$		**$P = 0.000$**	$P = 0.007$
RAN	$r^2 = 0.1$	$r^2 = 0.39$	**$r^2 = 0.72$**	$r^2 = 0.58$	**$r^2 = 0.72$**	$r^2 = 0.45$	**$r^2 = 0.73$**		$P = 0.02$
TET	$r^2 = 0.15$	$r^2 = 0.45$	$r^2 = 0.25$	$r^2 = 0.21$	**$r^2 = 0.72$**	$r^2 = 0.57$	$r^2 = 0.57$	$r^2 = 0.54$	
(C) Intensity data gLMS = 12									
PROP		$P = 0.35$	$P = 0.64$	$P = 0.37$	$P = 0.23$	$P = 0.76$	$P = 0.16$	$P = 0.78$	$P = 0.28$
DB	$r^2 = 0.22$		$P = 0.18$	$P = 0.1$	$P = 0.12$	$P = 0.44$	$P = 0.03$	$P = 0.004$	$P = 0.002$
L-phe	$r^2 = 0.11$	$r^2 = 0.32$		$P = 0.04$	$P = 0.04$	$P = 0.25$	$P = 0.18$	$P = 0.05$	$P = 0.21$
L-trp	$r^2 = 0.22$	$r^2 = 0.38$	$r^2 = 0.49$		$P = 0.09$	$P = 0.46$	$P = 0.006$	$P = 0.19$	$P = 0.28$
SOA	$r^2 = 0.29$	$r^2 = 0.37$	$r^2 = 0.46$	$r^2 = 0.4$		$P = 0.51$	$P = 0.003$	$P = 0.29$	$P = 0.05$
Urea	$r^2 = 0.08$	$r^2 = 0.19$	$r^2 = 0.27$	$r^2 = 0.18$	$r^2 = 0.16$		$P = 0.36$	$P = 0.005$	**$P = 0.001$**
QHCl	$r^2 = 0.34$	$r^2 = 0.51$	$r^2 = 0.32$	$r^2 = 0.6$	$r^2 = 0.64$	$r^2 = 0.22$		$P = 0.08$	$P = 0.01$
RAN	$r^2 = 0.07$	$r^2 = 0.62$	$r^2 = 0.45$	$r^2 = 0.32$	$r^2 = 0.26$	$r^2 = 0.62$	$r^2 = 0.41$		**$P = 0.000$**
TET	$r^2 = 0.26$	$r^2 = 0.66$	$r^2 = 0.30$	$r^2 = 0.26$	$r^2 = 0.45$	**$r^2 = 0.72$**	$r^2 = 0.57$	**$r^2 = 0.82$**	

Bonferroni correction was made to all P values by dividing it by 36. The level of significance was $P < 0.05/36 = 0.00139$. Bold indicates a significant correlation ($P < 0.00139$). Abbreviations of bitter compounds are: PROP (n-6-propylthiouracil), DB (denatonium benzoate), L-phe (L-phenylalanine), L-trp (L-tryptophan), SOA (sucrose octaacetate), QHCl (quinine hydrochloride), RAN (ranitidine), TET (Tetralone).

that additives affected the bitterness of all compounds equally (Figures 5 and 6A–H).

There was a significant interaction between the 'target' compound and the concentration [$F(21,105) = 5.9$, $P < 0.001$], indicating the bitterness intensity of target compounds increased differentially as the concentration increased. There was a significant interaction between the 'additive' compound and concentration [$F(21,105) = 1.93$, $P < 0.05$], indicating the some additives interact with target concentrations differently than other additives.

There was a significant three-way interaction between the 'target' compound, the 'additive' compound, and the concentration [$F(147,735) = 1.3$, $P < 0.05$], indicating that specific 'target', 'additive' and 'concentration' combinations were different in bitterness from each other. Overall there were very few significant differences among the bitter compounds (see below for specific interactions). Note that these effects do not appear in Figure 6, since responses have been averaged across concentration levels in the figure.

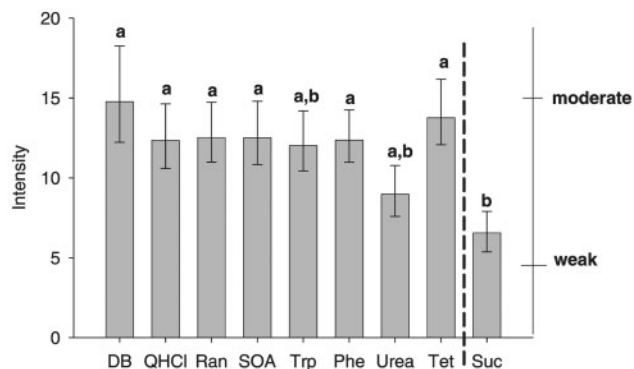### Bitter compounds as 'additives'

Figure 5 shows the average bitterness intensity ratings when the bitter stimuli and sucrose were added to the target bitter compounds. There were no significant differences between bitter compounds ($8 \times 8 \times 4$ ANOVA). Results from an $8 \times 9 \times 4$ (target × additive × concentration) repeated-measures ANOVA revealed that sucrose (sweet), as an additive, was significantly ($P < 0.05$) more effective at suppressing bitterness than most bitter compounds, except urea and L-trp.

A

LMS 4

B

LMS 8

C

LMS 12
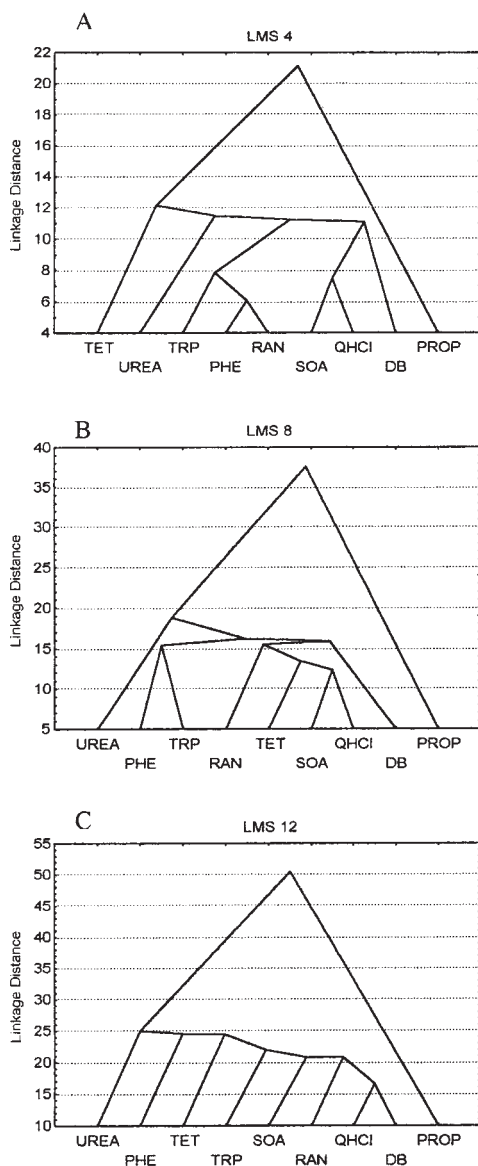
Figure 4 Cluster analysis (single linkage joining, Euclidean distances) of individual bitter intensity ratings at three concentrations. The top panel **(A)** is the weakest concentrations and the bottom panel **(C)** is the highest concentrations. Abbreviations are: PROP = *n*-6-propylthiouracil, DB = denatonium benzoate, PHE = L-phenylalanine, TRP = L-tryptophan, SOA = sucrose octaacetate, QHCl = quinine, RAN = ranitidine, TET = tetralone. Note that the *y*-axis scale (linkage distance) increases with increasing intensity.
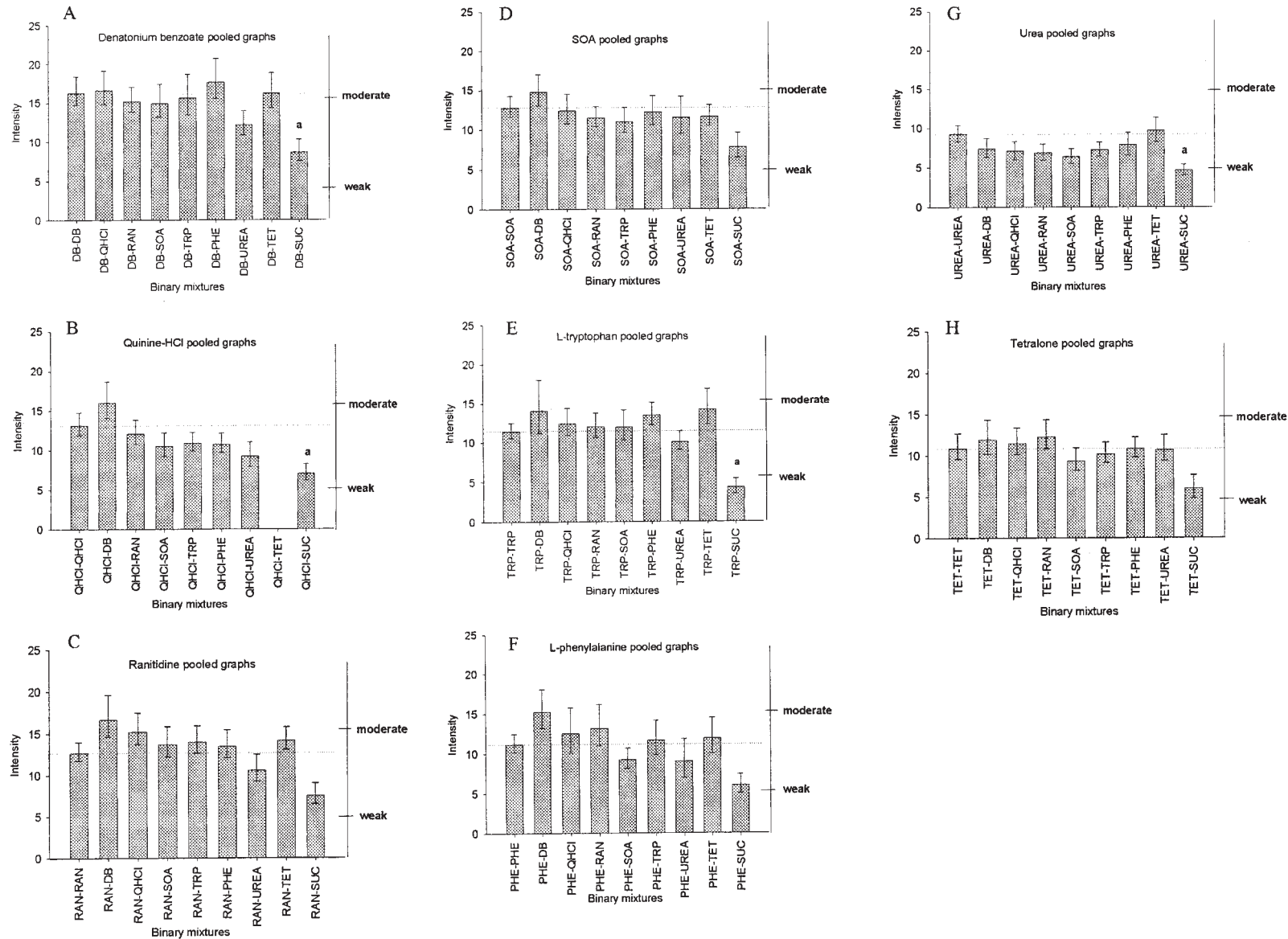

Figure 5 The average influence of bitter compounds as weak intensity 'additives' on bitterness of target compounds, pooled across the four target concentration levels and across the target compounds. The *x*-axis lists the bitter additives and abbreviations are the same as in Figure 4. The *y*-axis represents the mean bitterness rating for every binary mixture in which the compound given on the *x*-axis was 'added'. There was no statistical difference between the bitter compounds as additives [8 × 8 × 4 (target × additive × concentration) repeated-measures ANOVA]. Results from 8 × 9 × 4 (target × additive × concentration) repeated-measures ANOVA show sucrose suppressed bitterness more than bitter compounds. Bars that do not share a letter in common (top of bars) were statistically ($P < 0.05$) different in bitterness. Error bars represent geometric standard errors. The right-side *y*-axis provides verbal descriptors on the gLMS.

In general, the vast majority of the 218 unique binary interactions between bitter compounds were not statistically significant, meaning that the bitterness among these compound mixtures at a variety of concentrations and intensities combined additively.

Two-alternate forced-choice method assessing urea and denatonium benzoate as 'additives'

Figure 5 shows that bitter mixtures with DB as an additive were rated on average LMS15 and bitter mixtures with urea as a component were on average LMS10. While an ANOVA failed to find a significant difference in bitterness between these additives, the difference was large enough to warrant further investigation. A two-alternative forced-choice procedure was used to directly assess whether the bitter compounds DB or urea, as additives, significantly affected bitterness in relation to self-addition controls. Results from this highly sensitive method showed that subjects were unable to distinguish between the intensities of DB as an additive or the self-addition control, thereby illustrating that the bitterness of DB was perceptually additive. Urea suppressed the bitterness of QHCl and L-phe at all four concentrations, SOA and ranitidine at all concentrations except the lowest, and DB and L-trp all concentrations except the highest ($P < 0.05$). Addition of urea to Tetralone had no effect on bitterness. This demonstrated that urea inhibits bitterness, although the effect is both compound and concentration dependent.

There were concentration specific non-additive binary interactions (results not shown). Tukey HSD analysis of target–additive concentration interactions revealed that urea inhibited the bitterness of L-phe, QHCl and ranitidine at low intensities ($P < 0.05$) (see below for urea's forced choice results). SOA suppressed the bitterness of urea and QHCl at low intensities ($P < 0.05$). In addition, the amino acids L-trp and L-phe suppressed QHCl bitterness at low intensity ($P < 0.05$).

Figure 6 The influence of additives on target compounds pooled across their four concentration levels. The *x*-axis shows binary pairs of bitter stimuli. The first compound is the target and the second compound is the additive. Comparisons were made with the first bar on the graph (the self-addition control note horizontal dotted line) and abbreviations are the same as in Figure 4. Each graph represents a target compound: **(A)** Denatonium benzoate, **(B)** quinine-HCl, **(C)** ranitidine, **(D)** sucrose octaacetate, **(E)** L-tryptophan, **(F)** L-phenylalanine, **(G)** urea, **(H)** Tetralone. The *y*-axis represents the bitterness for each binary pair pooled across all four concentrations of the target. There was no statistical difference between the self-addition target and the target with weakly bitter

Discussion

Experiment 1

Increasing the concentration of bitter compounds decreases the differences among individuals in bitterness sensitivities

The correlation and cluster analysis from the lowest intensity level (LMS 4) supports the hypothesis that bitterness in humans appears to be transduced via several heterogeneous mechanisms. The individual differences in bitter intensity ratings of the nine compounds indicate three clusterings: one for PROP; one for L-trp, L-phe and ranitidine; and one for SOA and QHCl.

When comparing experiment 1 to the parallel study of Delwiche *et al.* (Delwiche *et al.*, 2001) there were 29 binary combinations of bitter compounds in common, and on only five occasions were there differences in binary-pair bitterness correlations between the two experiments. Cluster analysis also revealed strong similarities between the two studies. Delwiche *et al.* reported tight clusters among L-trp, L-phe and urea and among QHCl, SOA and DB. In the present experiment, Figure 4A shows that L-phe and L-trp cluster tightly with urea less related, and SOA and QHCl cluster tightly with DB somewhat less related.

Interestingly, as the concentration of the bitter compounds was increased, the correlations between bitter compounds decreased (Table 2). For example, no intercompound correlations persisted at all three intensity levels; and only three pairs of compounds correlated at two intensities (ranitidine and L-phe, QHCl and SOA, and Tetralone and SOA). Cluster analyses in Figure 4A–C, show a similar pattern; the tight clusters loosen as the bitterness intensity increases. At the highest intensity, the clusters of bitter compounds are more evenly distributed (except for PROP), essentially forming one large cluster. These data indicate that individual differences to bitter tasting compounds that were evident at low intensity levels become less prominent the more intense the bitter compounds are. That is, the population becomes more evenly distributed about the *y*-axis at higher concentrations (see Figure 2 for example).

PROP

Many studies report that sensitivity to the compound PROP correlates with sensitivities to several other bitter compounds (Hall *et al.*, 1975; Bartoshuk, 1979; Lawless, 1979; Gent and Bartoshuk, 1983; Leach and Noble, 1986; Bartoshuk *et al.*, 1988) and an equal number of studies show no correlations with PROP (Mela, 1989; Schifferstein and Frijters, 1991; Yokomukai *et al.*, 1993; Schiffman *et al.*, 1994; Delwiche *et al.*, 2001). In the present study, the perceived bitterness of PROP did not correlate or cluster with the bitterness of any other compounds at any intensity. We conclude that one's sensitivity to PROP does not predict sensitivity to the bitterness of these other compounds (Delwiche *et al.*, 2001).
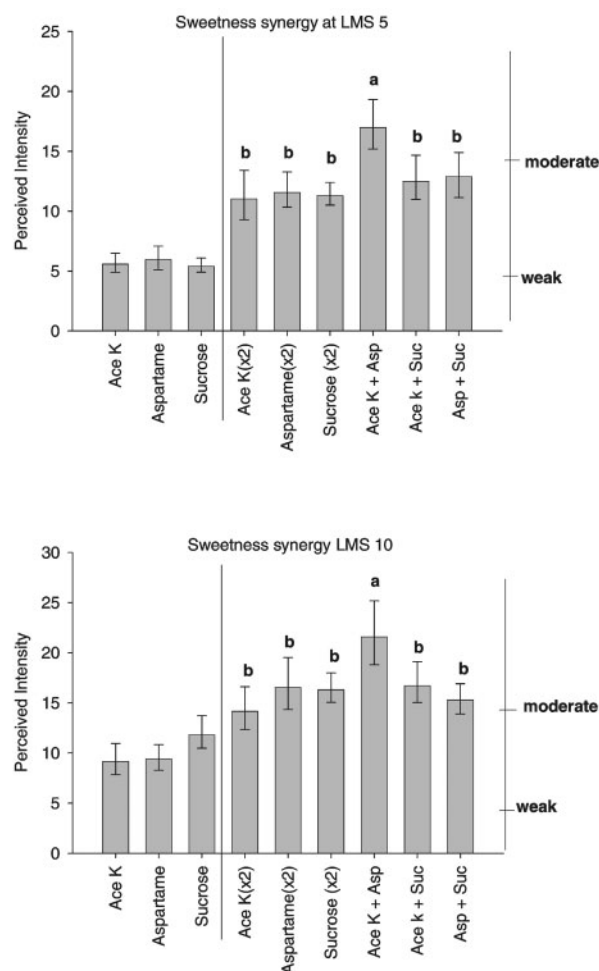
Figure 7 A test of the methods ability to detect synergy with sweeteners. Two sweetness intensities were used, corresponding to gLMS 5 (top) and 10 (bottom). The left *y*-axis represents the sweetness intensity ratings and the *y*-axis on the right displays the corresponding intensity adjectives. The *x*-axis shows the sweeteners and the binary combination of sweeteners. To the left of the vertical line are the sweetness ratings of the individual compounds. To the right of the vertical line are the mixtures (X2) designates self-mixture and the others are the binary sweetener mixtures. The mixture of acesulfame-K and aspartame was significantly sweeter than the self-addition controls or the mixes with sucrose. This figure provides verification that the method used in this study can detect mixture synergy. Abbreviations are Ace K = acesulfame-K, Asp = aspartame, Suc = sucrose. Error bars represent the geometric standard error. Bars with different letters on top are significantly different (*P* < 0.05).

Experiment 2: bitter–bitter interactions

While there were exceptions, most binary bitter mixtures combined additively with respect to taste and did not show interactions. The few interactions that occurred were suppressive and only occurred at weak intensities, with the added compound decreasing the bitterness in comparison to the target compound's self-addition control.

Urea as a component in a binary mixture of bitter compounds

Urea was effective at suppressing the bitterness of most compounds with the exception of Tetralone using 2-AFC.

Therefore, we suggest that the bitter tasting compound urea is a bitter taste suppressor (Keast and Breslin, 2002a). Urea's influence over bitterness may be due to an oral peripheral effect, rather than a cognitive effect. The primary reason for suggesting an oral peripheral effect is that urea did not suppress the bitterness of Tetralone. Such compound-specific differences indicate that the site of urea's bitterness suppression is likely in the oral periphery and is independent of mechanisms involved with Tetralone, rather than a cognitive influence affecting perceived bitterness generally. This latter type of cognitive interaction was found with the additive sucrose, which was effective at inhibiting the bitterness of all compounds tested, including Tetralone. At present, the mode of bitterness inhibition by urea is unknown.

Rejection of false negatives

The primary finding of this study is that bitter-tasting compounds do not interact when in binary mixtures. There were a couple notable exceptions to this rule, mentioned above, but they were suppressions rather than synergies. Therefore, the question arises as to whether the methods employed in the present study could detect taste synergy. The sweet taste control study demonstrated that compounds that are expected to show synergy (aspartame and acesulfame-K) in fact do, and those that are not expected to show synergy (sucrose and aspartame or sucrose and acesulfame-K) do not (Figure 7). Thus, it appears that if bitter mixtures were synergizing perceptually, the present methods would have detected this.

Bitter taste as a linear, additive combinatorial system

The majority of 'bitter' compound binary mixtures did not interact significantly (bitterness was additive). Therefore, taste receptor cells and higher taste relays generally act as simple, additive, bitter-taste integrators and convey a signal to higher cognitive centers that reflects the total amount of bitterness-inducing compounds present in the mouth. Since it may be important to accurately relay information regarding amounts of toxins being ingested in foods (including foods with multiple classes of toxins), this strategy may be the most informative and maximize survival. Although we recognize that not all bitter-tasting compounds are toxic and not all toxins taste bitter, we believe that the bitter taste system evolved to detect toxins in foods. Virtually all foods contain relatively low levels of bitter-tasting toxins (Leiner, 1969); yet we must eat them. The strategy of the taste system appears to be to keep an additive tally of what bitter toxins are in the mouth and track total levels of different potential toxins ingested.

Acknowledgments

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