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Effect of Compound Sequence on Bitterness Enhancement

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Abstract

The nature and occurrence of carry-over effects, i.e. the response to a stimulus is influenced by previous samples, were examined for selected bitter compounds. A time–intensity procedure was used to rate the bitterness of six compounds (caffeine, denatonium benzoate, limonin, naringin, quinine and sucrose octa-acetate). For each subject concentrations of these compounds were determined that were approximately equal in intensity to 1.18×10^{-5} M limonin. To test carry-over effects of each compound the 36 paired sequences (pairs) were evaluated. Within a session three pairs were tested, between which two-stage rinses were used to remove any effects of the previous pairs. Within a pair only water rinses were used between stimuli. For all compounds carry-over or sensitization effects were observed in which values for maximum intensity, rate of onset and total area under the time–intensity curve were higher for a compound when tested in the second position than in the first. In addition, the degree of sensitization and susceptibility to sensitization were compound-specific. Caffeine increased the bitterness by the largest amount for all other compounds, while it was least affected. Regardless of the compound in the first position, the bitterness of quinine and denatonium were most enhanced.

Introduction

Frequently a response to a stimulus or experimental variable is influenced by previous samples or treatments. In clinical trials or animal experiments these phenomena are called carry-over effects. The sensory effects of stimulus sequence on the chemical senses have been investigated in sensitization, adaptation and cross-adaptation studies (Durier *et al.*, 1997), although little research has addressed the role of sensitization and desensitization in bitterness perception. Cross-adaptation to bitterness has been reported between some but not all bitter compounds. For example, adaptation to quinine HCl strongly reduced the bitterness intensity of quinine sulfate, caffeine and sucrose octa-acetate (SOA), while having little effect on urea, magnesium sulfate and phenylthiocarbamide (PTC) (McBurney *et al.*, 1972). Consistent with that psychophysical study, receptor potentials to quinine sulfate were greatly reduced after adaptation to quinine HCl (Sato and Sugimoto, 1995).

In contrast to reported adaptation responses to bitter compounds, the second presentation in a pair of cider procyanidins was rated more bitter than the first presentation, regardless of the identity of the second compound (Lea and Arnold, 1978). Similarly, in time–intensity studies in which repeated sips of the same stimuli were rated, perceived bitterness increased with successive sips of beers containing hop acids (Guinard *et al.*, 1986). In preliminary evaluations of a range of bitter stimuli Cubero-Castillo observed that the intensity of a bitter stimulus was more intense when sipped after any other bitter tastant (Cubero-Castillo, 1999).

Bitter taste is elicited by very diverse chemical substances, such as alcohol, alkaloids, amino acids, catechins, denatonium benzoate, flavanols, isohumulones, inorganic salts, including KCl, $MgCl_2$ and aluminium sulfate, caffeine, sesquiterpenes, phenols, PTC, propylthiouracil (PROP), acetylated sugars (such SOA) and urea. The lack of a common structure–activity relationship among all classes of bitter compounds suggests that there may be several different receptors and/or transduction processes. For example, bitterness of some bitter compounds molecules is transduced by more than one mechanism (Spielman *et al.*, 1992). SOA increases the Ca^{2+} concentration via a G protein cascade involving phospolipase C (PLC) and inosine 1,4,5-triphosphate (IP_3), whereas denatonium benzoate generates IP_3 and stimulates transducin, resulting in activation of phosphodiesterase (PDE) and a decrease in cAMP (Ruiz-Avila *et al.*, 1995; Kinnamon, 1996; Spielman *et al.*, 1996; Ogura *et al.*, 1997). Yan *et al.* concluded that denatonium benzoate is transduced through a dual mechanism, involving both an α-gustducin-dependent, PDE-mediated reduction in cyclic nucleotide levels and a Gγ13-dependent, PLC β2-mediated increase in IP_3 (Yan *et al.*, 2000). In

addition, SOA and denatonium block K^+ channels, as does quinine (Kinnamon and Cummings, 1992). Peri *et al.* reported that quinine permeates into taste cells and is a potent direct activator of transducin which leads to a decrease in cAMP (Peri *et al.*, 1999). Caffeine permeates taste cells and directly activates calcium channels (Spielman *et al.*, 1992). Naringin induces production of IP_3, but does not affect cAMP levels (Naim *et al.*, 1997), while limonin was reported to decrease cAMP in the only study of this compound (Naim *et al.*, 1998). The transduction mechanism for PROP has not been studied, however, the reported distribution of threshold frequencies suggests that there are genetically influenced differences in the nature or number of receptors or transduction mechanisms.

Wide variations in perception of intensity of selected bitter compounds have been demonstrated to occur among individuals (Cubero-Castillo, 1999), therefore compound-specific carry-over effects may occur. Sensitization or adaptation may be due to the nature of the transduction mechanisms by which bitterness of a compound is perceived. Evidence for this was cited in electrophysiological studies of *Manduca sexta*. Mixtures of aristocholic acid and caffeine (compounds which have different firing rates and different transduction pathways) produced higher responses than mixtures of caffeine and salicin, which have similar firing rates and transduction mechanisms (Glendinning and Hills, 1997).

The objective of this study was to quantify the carry-over effects of selected bitter stimuli and compare their sensitization and susceptibility to sensitization. It is hypothesized that two compounds which share the same transduction mechanism(s) will have different carry-over effects on each other than two compounds which do not share mechanisms of transduction. To test this hypothesis, compounds were selected which only elicit bitterness and which use the same (e.g. naringin, SOA, quinine and denatonium benzoate increase IP_3) or different mechanisms (e.g. only caffeine activates calcium channels directly).

Materials and methods

Subjects

Twelve subjects (seven male and five female, 23–51 years old) participated. As described elsewhere (Kalmus, 1971), one subject was classified as a non-taster, 11 as PROP tasters.

Stimuli

Six bitter compounds were assessed in deionized water: denatonium benzoate, limonin, naringin, SOA (Sigma, St Louis, MO), quinine sulfate (Fisher, Fair Lawn, NJ) and caffeine (Mallinckrodt, Paris, KY). In a preliminary experiment several rinse solutions were tested: 2 and 6% sucrose, 10 and 137 mM NaCl and 5 and 10% (v/v) ethanol. Ethanol solutions most effectively reduced carry-over effects, although the 10% level produced irritation or a burning sensation. Therefore, the 5% ethanol solution was chosen for rinsing between pairs to avoid carry-over effects.

In a previous study individuals were shown to differ widely in the perception of these six bitter tasting compounds (Cubero-Castillo, 1999). Hence, equi-bitter concentrations of the stimuli were determined separately for each subject. In two preliminary tests concentrations were selected for each individual that were approximately equal in bitterness to 1.18×10^{-5} M limonin. Bitterness of paired solutions was first compared using split-tongue tests to eliminate sequential effects. The solutions were applied on each side of the tongue with a medium sized cotton swab by the experimenter. Although the split-tongue protocol eliminated carry-over effects, subjects perceived only a very low intensity of bitterness. Therefore, additional paired comparison tests were performed using a sip and spit protocol. One compound was tested per day. In each session 1.18×10^{-5} M limonin was compared with four concentrations that bracketed the equi-bitter level found in the split-tongue tests. After each sample and between pairs individuals rinsed with 5% ethanol once and three times with water. Two training sessions were then conducted using the time–intensity protocol (T–I) described below. The maximum intensity values extracted from these T–I curves were used to determine the final equi-bitter concentrations for each subject.

Sensory protocol

To determine the carry-over effects of each compound on itself and on the other bitter tastants, each compound was presented before and after all other compounds, resulting in 36 sequence pairs (sequences). For each of two replicate evaluations the sequences were assigned in a random order to each subject in each of 12 sessions. At each session three sequences were served to each subject in an order balanced for carry-over and position. All stimuli were served in 15 ml aliquots in coded plastic cups.

Training and formal T–I evaluations were conducted in isolated booths under red light. T–I ratings were acquired each second using FIZZ software (Biosystemes, Dijon, France). Subjects initiated rating of bitterness at time 0, when the first sample was sipped. Bitterness intensity was continuously rated through expectoration at 15 s until extinction of bitterness (or 100 s). Within each paired sequence subjects rinsed for 20 s with deionized water before tasting the second compound. Between paired sequences subjects rinsed with 3% ethanol solution for 20 s, then rinsed with deionized water for 20 s, followed by an enforced break of 1 min before the next pair was presented.

Data analysis

T–I parameters were extracted from each curve: maximum intensity (I_{MAX}), time to I_{MAX} (T_{MAX}), total area under the curve (Area) and intensity at 60 s (I_{60}). The persistence was

expressed as percent decay in 60 s ($I_{MAX} - I_{60}/I_{MAX}$). Mixed model analyses of variance were conducted with subjects treated as random. To determine the significance of the carry-over effect within each paired combination, contrast tests were performed. All calculations were carried out using SAS for Windows v.6.1 (SAS, Cary, NC).

Results

Effectiveness of rinse between pairs

Across the six compounds evaluated within a session a significant difference in maximum intensity occurred due to order of presentation [$F(5,55) = 2.48$, $P < 0.0001$]. Stimuli presented fourth in order of presentation (second in the second paired sequence) and sixth (second in the third sequence) were significantly more bitter than the samples presented first, third and fifth (which were in the first position in each sequence) within the session. The compounds presented second in order of presentation were only significantly higher than the first stimuli. Ratings for stimuli presented first, third and fifth within a session were not significantly different from each other. Thus the rinse between pairs reduced, but did not completely eliminate, the effect of sensitization of the first sequence on subsequent pairs.

Effect of position within paired sequences

Across all compounds I_{MAX} varied significantly [$F(1,11) = 26.93$, $P < 0.0003$] as a function of position within a pair. Compounds presented in the second position in a paired combination were more bitter than when presented first. To illustrate these results, average T–I curves for each compound in the first and second positions are provided in Figure 1.

The analyses of variance of I_{MAX} for the 12 paired sequences in which each compound appeared (i.e. before six compounds and after six compounds) are shown in Table 1. Within each paired combination the maximum intensity for the compound when evaluated in the second position was higher than when tasted in the first position, although the magnitude of the increase in intensity was clearly compound-specific. This is shown in Figure 2, in which the bottom of each bar is the I_{MAX} when the component was tasted in first place in the paired combination and the total height of the bar is the I_{MAX} when the compound was presented in the second position.

Not all enhancements were significant, as determined by contrast tests. In Figure 2 asterisks denote those increases in I_{MAX} due to position which were significant. All compounds significantly enhanced the bitterness of denatonium and quinine, while all compounds except denatonium significantly increased limonin bitterness. In contrast, significant increases in caffeine bitterness occurred only after presentation of caffeine. Both caffeine and SOA had a significant effect on SOA and naringin. Caffeine exerted the largest carry-over effect on all other compounds.

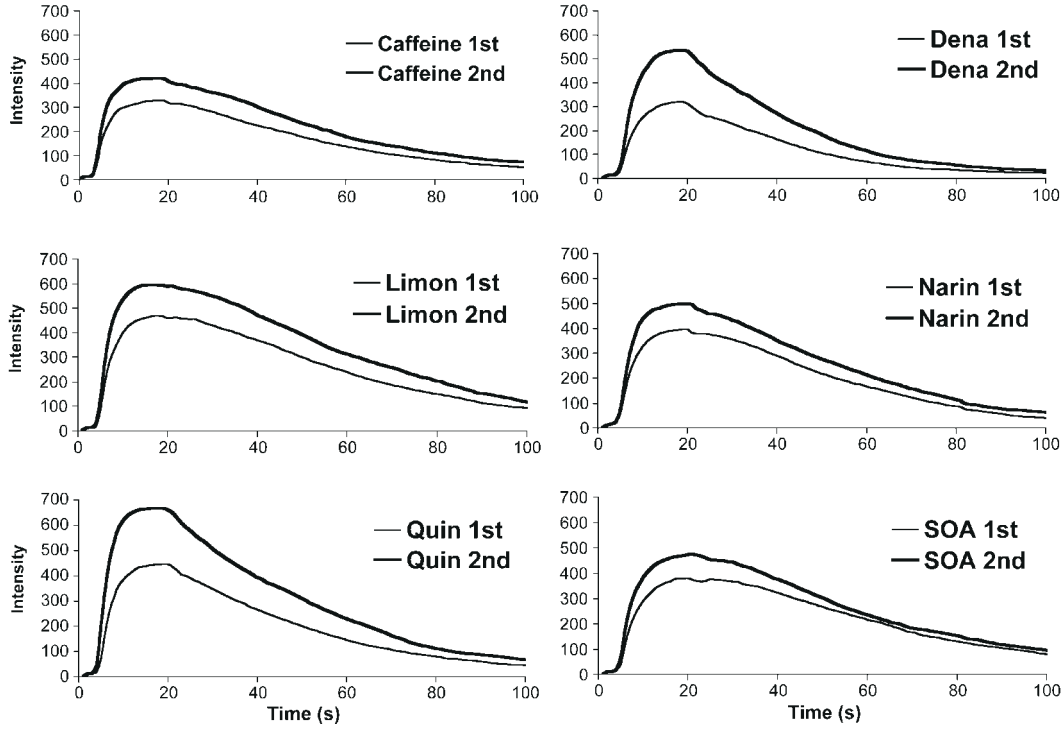

Figure 1 Average T–I curves for each compound when presented in the first position and in the second position before (1st) and after (2nd) all other compounds (n = 12 subjects × 2 repetitions × 6 compounds).

Table 1 Summary of mixed model analysis of variance testing the main effect of position within each sequence in which the compound was presented [*F* values, significance levels and least significant differences for each compound are given (<0.05)]

Compound	I_{MAX}		LSD
	F(11,121)	P	
Caffeine	2.56	0.006	0.896
Denatonium	8.46	0.0001	1.140
Limonin	4.23	0.0001	1.067
Naringin	2.15	0.02	1.088
Quinine	9.98	0.0001	1.127
SOA	3.04	0.001	0.932

Temporal properties

To compare the temporal characteristics of these bitter compounds, the mean values for time to maximum (T_{MAX}), I_{MAX}, area under the T–I curve (Area) and percent decay in intensity at 60 s are presented for each compound rated in the first and second position in Figure 3. I_{MAX}, Area and rate of onset (not shown) increased from the first to second position, while T_{MAX} generally decreased. The time to maximum for caffeine was significantly shorter than for the other compounds in the first position, although no difference across compounds was found in the second. The persistence of bitterness, estimated as percent decay, did not change from the first to the second position, with the exception of SOA.

Despite efforts to select equi-bitter levels, caffeine and denatonium had significantly lower I_{MAX} and Area than the other compounds in the first position. Inspection of the average curves shows a difference in persistence of the compounds. Typically, in T–I data the I_{MAX} is highly significantly correlated with duration, as observed here with a highly significant correlation between percent decay and I_{MAX}. Although denatonium and caffeine were equi-bitter at I_{MAX}, caffeine has a longer persistence (percent decay) and slower rate of decay (not shown). Denatonium and quinine had the shortest duration, with intensity reduced to <20% of I_{MAX} at 60 s. Consistent with these data, caffeine was previously reported to have a shorter T_{MAX} and longer duration than quinine (Leach and Noble, 1986).

Discussion

All compounds sensitized the response to the stimulus which followed them, although to different degrees. Despite the efforts to establish equi-bitter levels of all compounds for each subject, overall caffeine and denatonium were slightly, but significantly, lower in I_{MAX} than the other compounds (see Figure 3). Despite this, caffeine was the least affected by other compounds and exerted the largest sensitization effect on all other compounds. Similarly, denatonium was one of the two compounds most enhanced,

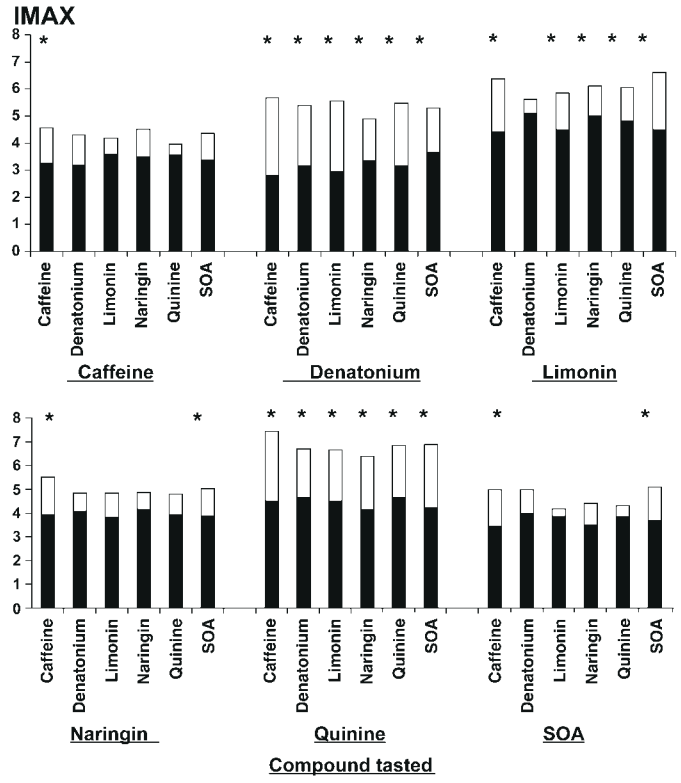

Figure 2 Mean maximum intensity (I_{MAX}) across subjects for each compound in each paired sequence. The shaded lower portion of each bar is I_{MAX} for the component tasted in the first position; total height of the bar is I_{MAX} for the same compound when tasted in the second position in the same paired combination. Increases which are significant ($P < 0.05$) are marked with asterisks ($n = 12$ subjects $\times$ 2 repetitions).

although it was lower in I_{MAX} than the others. These results suggest that the effects are compound-specific and not a function of intensity *per se*.

Overall, caffeine had the largest carry-over effect. Caffeine penetrates cell membranes to activate the calcium channels directly. Thus it can be speculated that caffeine may take longer to leave the taste cell than the other compounds which act on apical receptor sites, membrane ionic channels or penetrate the membrane to act directly on G proteins. This physical constraint for caffeine may result in a slower recovery of the cell, which may enhance the response to the following compound. This may also explain why the only significant carry-over effect on caffeine was produced by itself.

Quinine and denatonium were most sensitized, followed by limonin (Figure 2). With the exception of sensitization of limonin by denatonium, all compounds produced significant increases in bitterness intensity of these three compounds when tasted in the second position. Both denatonium and quinine increase IP_3, block potassium channels and reduce levels of cAMP. Generalizing from the broad spectrum of transduction mechanisms for denatonium and quinine, it could be speculated that sensitization results from residual

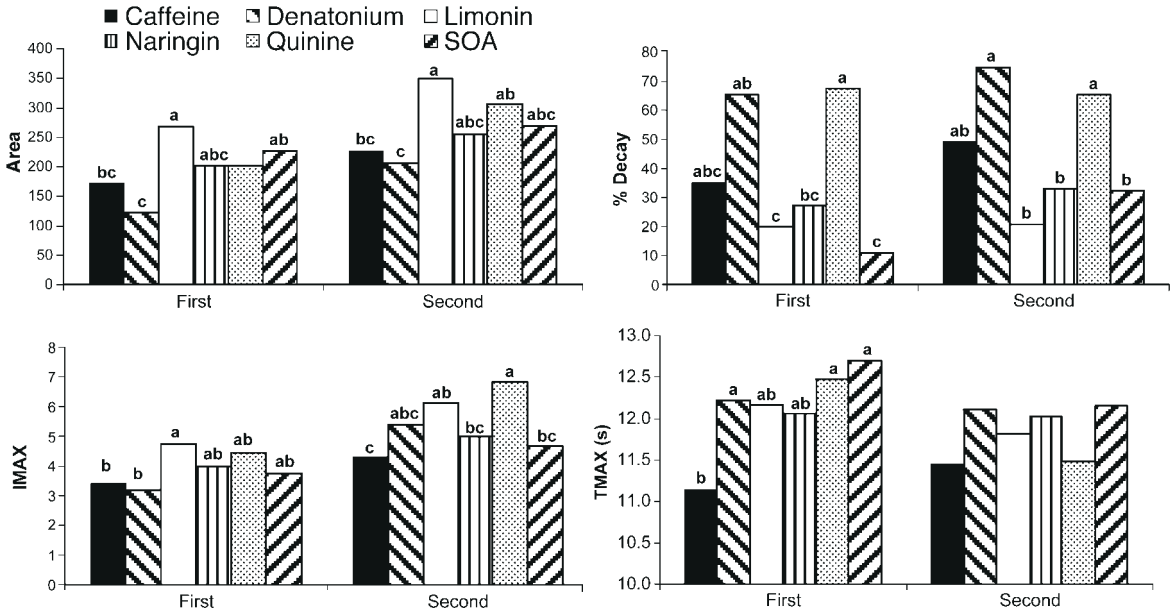

Figure 3 Mean values for time to maximum intensity (T_{MAX}), maximum intensity (I_{MAX}), total area (Area) and percent decay at 60 s (% Decay) for each compound when presented in the first and second positions ($n = 12$ subjects $\times$ 2 repetitions $\times$ 6 compounds). For each parameter and position bitterness values of compounds with different letters varied significantly ($P < 0.05$).

effects of the response to a preceding compound which shares one or more transduction mechanisms with denatonium or quinine. However, this conflicts with previous results in which a mixture of two compounds using different transduction mechanisms produced a more intense response in *Manduca sexta* than a solution of two compounds which used the same transduction mechanism (Glendinning and Hills, 1997).

From this investigation and with the present knowledge of transduction mechanisms it is not possible to explain fully why caffeine sensitizes the responses to all other compounds yet is least affected by the others. Nor is it understood why bitterness of compounds such as quinine and denatonium are so susceptible to sensitization. However, measuring carry-over effects might be very useful in further studies of the mechanisms by which bitterness is perceived.

Conclusion

Significant carry-over effects were observed which are compound-specific. The compounds that most sensitize responses to subsequent stimuli are the least susceptible to sensitization. Conversely, quinine and denatonium, which are most affected by carry-over effects, have little or no effect on potent sensitizers such as caffeine. However, both quinine and denatonium can self-sensitize and sensitize each other. Unfortunately, no clear interpretation of these responses is provided by the present knowledge of transduction mechanisms for these compounds. These results clearly demonstrate that carry-over effects may invalidate the results of simple pair tests of bitter compounds unless the protocol is modified to minimize sequential sensitization.

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