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Accepted Version

# Perception of bitterness, sweetness and liking of different genotypes of lettuce.

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Keywords: lettuce, sesquiterpenoid lactone, bitterness, sweetness, sugar, bitter suppression

# Abstract

Lettuce is an important leafy vegetable, consumed across the world, containing bitter sesquiterpenoid lactone (SL) compounds that may negatively affect consumer acceptance and consumption. We assessed liking of samples with differing absolute abundance and different ratios of bitter:sweet compounds by analysing recombinant inbred lines (RILs) from an interspecific lettuce mapping population derived from a cross between a wild (*L. serriola* acc. UC96US23) and domesticated lettuce, (*L. sativa, cv.* Salinas). We found that the ratio of bitter:sweet compounds was a key determinant of bitterness perception and liking. We were able to demonstrate that SLs such as 8-deoxylactucin-15-sulphate contribute most strongly to bitterness perception, whilst 15-*p*-hydroxylphenylacetyllactucin-8-sulphate does not contribute to bitter taste. Glucose was the sugar most highly correlated with sweetness perception. There is a genetic basis to the biochemical composition of lettuce. This information will be useful in lettuce breeding programmes in order to produce leaves with more favourable taste profiles.

## Introduction

Sesquiterpene lactones are anti-feedants and phytoalexins produced by lettuce (*Lactuca sativa* L.). Selective breeding against the bitter taste imparted by them has reduced presence of these compounds in domesticated lettuce cultivars dramatically (Wink, 1988). Many modern varieties do still contain perceivable quantities of sesquiterpene lactones and this is particularly relevant with a move away from iceberg-type head-lettuce to bagged lettuce which contain fewer high yielding, sweet cultivars and more red-leaved varieties, which typically contain much higher concentrations of the bitter compounds (Price et al., 1990). The perceived bitterness is enough to reduce palatability and consumption in a westernised diet, where fruit and vegetables are already under-consumed (Casagrande et al., 2007; Rogers and Pryer, 2012). It is widely believed that this bitterness can be counteracted by sweetness (Bartoshuk, 1975; Keast and Breslin, 2003); an improvement in flavour is therefore likely to be a consequence of manipulating both factors. Although sensory perception of individual sugars (Pangborn, 1963) and SLs (Price et al., 1990; Seo et al., 2009; Sessa et al., 2000) has been previously assessed and sensory perception is well established in the case of sweet compounds, assessment of SL bitterness is sometimes contradictory and has not been considered with regards to tastant mixture suppression. Here we assess the interaction between sweet and bitter components within the natural food matrix of lettuce and additionally compare perception data to consumer liking.

Lettuce is a suitable crop in which to pursue flavour improvement as it is widely eaten across Europe and North America. Lettuce also contains a range of beneficial secondary plant metabolites including, phenolics, ascorbate, α-tocopherol, lignans, as well as SLs (García-Macías et al., 2007; Oh et al., 2009); consequently improving the flavour should increase consumer intake. Phytochemicals present in lettuce have been suggested as having a range of biological functions, from analgesic, anti-inflammatory, anti-tumour, and gastroprotective effect of the sesquiterpenoids (Giordano et al., 1990; Guzman et al., 2005; Sayyah et al., 2004), to cognitive effect of phenylpropanoid flavonoids (García-Macías et al., 2007; Spencer et al., 2009). Additionally lettuce, particularly the romaine type, is a source of iron and potassium and a good source of dietary fibre, folate and manganese, vitamins A, B1, B6, C, K, and omega-3 fatty acids (Belitz et al., 2009). Bitterness in lettuce is not thought to be linked to the beneficial biological effects of the same molecules, owing to distinct functional groups in the compounds (Chadwick et al., 2013; Behrens et al., 2009; Brockhoff et al., 2007) and so it is feasible to balance the reduction of those most bitter SLs while maintaining or increasing those with greatest biological function.

Sweet and bitter tastes are sensed through the binding of the tastants to G-protein coupled receptors located within papillae on the tongue. Sugars bind to type 1 receptors (T1R) (Meyers & Brewer, 2008) and bitter molecules to type 2 receptors (T2R) (Meyerhof, Batram, Kuhn, Brockhoff, Chudoba, Bufe, et al., 2010). Whereas there are just two T1R receptors involved in sweet perception (T1R2/T1R3) there are 25 T2Rs responsible for binding a broad range of bitter molecules. Whereas some T2Rs are generalists and bind to a wide range of structurally diverse molecules, others are specialists binding to a narrow range of compounds (Meyerhoff et al., 2010). SLs have been found to activate the T2R46, a generalist receptor (Brockhoff et al., 2007). Within the population it is common to categorise individual as “bitter sensitive” or “bitter blind”, where 25% of the population are “bitter blind” , however this categorisation is due to polymorphisms of the Tas2R38 gene (Menella et al., 2010). The receptor T2R38 is a specialist receptor binding to thiouracil groups (as found in Brassica vegetables) and not to SLs. We therefore propose that “bitter blindness” resulting from Tas2R38 will not effect consumers perception of bitterness in lettuce.

We hypothesise that consumers are able to accurately detect sweetness and bitterness in lettuce as imparted by the compounds of interest. We also propose that taste interaction between sweetness and bitterness as well as the absolute concentrations of the compounds will have a significant effect on taste perception and liking. Additionally, it is broadly believed that consumers prefer foods which they perceive as sweet. To most consumers a major factor in purchasing habits is liking for taste (Enneking et al., 2007) and so ultimately this will be the chief factor in delivering a positive change in consumer habits.

## Materials and Methods

### Plant Material and Growth Conditions.

$F_9$ recombinant inbred lines (RILs) were supplied by the Michelmore lab (Genome Center, UC Davis, USA) and 102 RILs plus their parents, *L. sativa* cv. Salinas and the wild *L. serriola* UC96US23, were propagated by A.L. Tozer. For these studies, plants were grown under glasshouse conditions at The University of Reading and watered once or twice daily in accordance with the weather. The glasshouse temperature ranged from 17 to 30°C. Seedlings were transferred from seed trays to 3 ½” pots with Osmocote after 3 weeks, and were given Sangral 1:1:1 liquid fertiliser weekly. Plants were harvested after 49 days, at a mature, commercially viable, stage and prior to floral transition.

The 102 RILs were analysed by HPLC-MS (see section below) to assess SL abundance and sugar assays to assess the concentrations of sucrose, fructose and glucose (see section below) in order to determine which lines would be most informative. Eight RILs were selected based on whether that line had high or low concentrations of sugar and SLs. The sample size was kept small to avoid fatigue in the consumer panel.

## Consumer Analysis and Sample Preparation

Lettuce samples were harvested daily on the morning of the tests and were used within an hour of preparation, being kept refrigerated and moist until they were needed in order to reduce respiration and sample wilting. Leaf samples were cut into strips 5cm by 1cm, avoiding the midrib as this can contain more variable levels of SLs (Sessa et al., 2000). Samples were labelled with arbitrary three digit codes in petri-dishes and three strips were provided per consumer. All consumer work took place in sensory booths at the University of Reading, with neutral odour, artificial daylight and controlled temperature. Forty three consumers took part in the study, consisting of eight men and 35 women. Ages ranged from 17 to 68 with 6 over the age of 40 (mean = 29.8 years, median = 25 years). This skew in participant age was due to primary recruitment taking place on the university campus. Participants were recruited after ethical approval of the study (University of Reading Research Ethics Committee, study number 08/13) via email notification and poster advertisement and volunteers were screened by questionnaire for any dietary restrictions, allergies or health conditions that may have affected their ability to participate the consumer study.

Consumer response was recorded using Compusense 5 software (Compusense Ontario, Canada). The study was divided into three sections. First, participants were asked to familiarise themselves with a labelled magnitude scale, rating their most bitter, sweet, salty and sour experiences on the scale. This was used to normalise their scores against other participants, to allow for high and low scale users. The main study involved rating lettuce samples presented to them one at a time in a balanced design for liking on a 9 point hedonic category scale (anchored from dislike extremely to like extremely), then for perception of sweetness and bitterness using labelled magnitude scales (where semantic descriptors from weak to strongest imaginable are positioned on a logarithmic scale, and scored 0 to 1.97). Participants were asked to taste each sample three times, once for liking, then sweetness and again for bitterness. Finally perception of aftertaste intensity was rated on a 5 point category scale (anchored from no after taste to very strong) after a 10 second wait period. Participants were also asked to give any additional comments on the samples. Once the assessment of one lettuce line was completed, participants were given the next sample after a 30 second rest period. Participants were given water and plain water crackers (Carr's, United Biscuits, UK) to cleanse their palate during this rest period. See supplementary data for a transcript of the questions exactly as posed. After the test participants were given an exit questionnaire asking for further information on age, gender, frequency with which they consume lettuce, and also the regularity of their consumption of bitter foods in their diet, based on a list of 12 common bitter foods (white cabbage, green cabbage, red cabbage, cauliflower, kale, brussels sprouts, watercress, rocket, radish, coffee, tonic water, and broccoli). Finally they were phenotypically tested for bitter blindness using PTC (Phenylthiocarbamide) strips. Bitter blindness occurs in around 25% of people as the result of an inactive hTAS2R38 receptor and, while it is not directly responsible for detection of SLs, it is a widely accepted indicator of bitter taste acuity.

## Chemical analysis

Sesquiterpene lactones and some polyphenols in the main population of 102 RILs were analysed by HPLC and identities confirmed by HPLC-MS based on details published in Sessa et al. (2000), mass data for each compound was as follows; Lactucin m/z 277; Lactucopicrin m/z 411; 8-deoxylactucin m/z 332; 15-p-hydroxyphenylacetylactucin-8-oxalate m/z 490; Lactucin oxalate m/z 348; Lactucopicrin oxalate m/z 482. Full spectra are presented in Supplementary Figure 1. Plant samples from each individual genotype were replicated in quadruplicate and analysed individually for determination of SLs. These were extracted as follows: 0.5g of frozen homogenised leafy plant material was added to 2ml of 70% MeOH, shaken for 10 minutes, centrifuged (13000 x g, 4°C, 5 min) and filtered through a 0.45μm filter attached to a syringe; the supernatant was run in an Agilent 1100 HPLC system (Agilent Technologies, Wokingham, UK) coupled to a Bruker Microtof high resolution quadrupole-time of flight mass spectrometer (QToFMS) (Bruker Daltonics Ltd, Coventry, UK). Samples were separated on an ACE $C_{18}$ 15 cm x 2.1 mm, 5 µm, 100 Å HPLC column (Advanced Chromatography Technologies, UK). Running conditions were as according to Table S1 with a flow rate of 0.5ml/min; 50µl injection and UV response measured at 264nm, 280nm, 320nm, and 365nm, 520nm.

Sugars were assessed by high throughput plate assays using a modified version of Wingler *et al*. (2006). Lettuce samples were first weighed and solutes extracted by heating to 80°C in 80% ethanol; the supernatant was dried under vacuum (Savant Speed Vac, Thermo Scientific, MA, USA). Sugars were then resuspended in 100µl of sterile deionised water. Sugars were assessed by hexokinase (Roche; 1500units/ml diluted 1:30 in HEPES buffer) directed phosphorylation of glucose, leading to reduction of NAD+ to NADH whereupon a change in absorbance at 340nm proportional to sugar content can be measured. Sucrose was converted to glucose by hydrolysis of sucrose by invertase (Sigma; 355 units/ml diluted 1:150 in HEPES buffer) and fructose-6-phosphate converted to glucose-6-phosphate by phosphoglucose isomerase (Roche; 2mg/ml diluted 1:10 in HEPES buffer).

### Statistical Analysis

In order to determine whether there were significant differences in consumer perception and liking between the RILs, response data were normalised and assessed for variance by Kruskal-Wallis with Dunn's procedure. Correlation statistics assessed by Spearman's rank were completed using Prism 6 (GraphPad Software, Inc., La Jolla, USA). Significant differences were determined at 95% confidence intervals ($P<0.05$). An internal preference map was attained by carrying out a principle component analysis of the individual liking data and fitting the mean ratings for bitter and sweet perception, as well as the mean liking ratings, onto the plot as supplementary variables using XLStat (AddinSoft, version 2012.1.01, Paris, France).

## Results

### Sample Selection

Lines within the mapping population were selected for extreme values in concentrations of sugars, total SLs, and for specific SLs according to previously reported bitterness ratios. This was done to maximise qualitative data from a small number of samples, hence while others were selected for overall profile, RILs 41 and 122 were selected on account of having particularly high concentrations of lactucin-15-oxalate, which was reasoned to be the most bitter individual SL based on correlation data in previous research by Price et al. (1990). Absolute concentration of each assessed compound is given in Table S2, along with the rationale for the RIL's selection.

### Demographic factors

Regularity of lettuce consumption was ascertained by individual recall. There was no significant link to perception of bitterness, nor to liking of certain samples. Of the participants only a single participant reported never eating lettuce, while 19 responded with 'more than once per week' which was the highest category on our scale. There was no trend for participants who regularly consumed lettuce to prefer bitter or sweeter genotypes, nor did this show any influence on bitter perception; however, the study size was not large enough to conclude whether preference for bitter or sweet genotypes influences frequency of lettuce purchase or consumption.

Volunteers reported the regularity with which they consumed other bitter foods. Frequency of consumption of foods with known bitter components, such as a range of Brassicaceous plants, coffee, and tonic water, were assessed and related to liking and perception scores, with the conclusion that this does not affect preference for lettuce genotypes, nor does it alter perception of bitterness or sweetness, within the population assessed. These findings were anticipated, as there is little relationship between the SL structure and those of brassica glucosinolates, or alkaloids such as caffeine present in coffee, or quinine used to flavour tonic water; such compounds typically have a range of different structures and bind to structurally different receptors. Age and gender data were also recorded, with no significance found across age groups or gender. Finally, participants were tested for bitter blindness using PTC strips. Eleven volunteers were found to be bitter blind, while the remaining 32 were tasters, as predicted for a Mendelian segregation of a phenotype controlled by a single gene. There was no significant difference between liking or perception scores of either bitter tasting or non-tasting consumer groups, indicating that, unlike hTAS2R46, the hTAS2R38 receptor has no role in detecting the bitter taste derived from SL compounds.

## Taste perception

We found that there was significant variation in reported bitterness, sweetness, aftertaste and in consumer liking between different lines (Figure 1). This showed that consumers were able to detect the differences between the samples in terms of the major sensory parameters related to sesquiterpene lactones and to sugars; bitterness, aftertaste, sweetness, and these attributes influenced preference. The perceived bitterness and sweetness correlated to absolute phytochemical levels with high statistical significance in most cases.

In terms of sweetness perception (Figure 2) consumers ranked RILs 41 and 123 as the least sweet, and these RILs indeed had relatively low sugar contents, however RIL 61 also had a very low total sugar content and was rated relatively high for sweetness. RIL 61 has the lowest levels of fructose, the sugar with the highest relative sweetness of the sugars present in this lettuce population. This was expected therefore the be perceived as least sweet, however it also contained the least total SL content of all the tested samples, showing that interaction of the bitter SLs suppressed the sweetness of the other lines (Figure 3A). The sweetness of RIL 61 can largely be attributed to its glucose content, which was considerably higher than either fructose or sucrose. Glucose levels had the greatest correlation with perceived sweetness ($r=0.2266$ $P<0.0001$) across all lines, possibly because it was the most abundant sugar in the lines perceived as being the most sweet (RILs 61 and 19). RILs 41 and 123 were significantly the least liked and perceived as the least sweet (at $p<0.05$); these samples had significantly less sugar than the other selected lines in terms of total sugar and for each of the individual sugars tested for. Correction for the relative sweetness of each sugar present (glucose; 0.74, sucrose; 1, fructose; 1.73; Koehler and Kays, 1991) was used to determine an expected total sweetness level (Figure 2E). This shows a positive relationship between perceived sweetness and relative sucrose equivalent concentration ($r = 0.961$, $P = -0.002$) despite other confounding effects, such as influence of bitterness. RILs 61 and 122 maintain higher perceived sweetness compared to predicted sweetness scores, due to their relatively low concentration of total SLs, at a factor of 4-24 fold less than RIL 123 which had the highest SL content. RIL 94, which was selected for high concentration of SLs in combination with high sugar content, was marginally less sweet than might be anticipated from sugar content alone, owing to sweetness suppression by the bitter compounds.

Consumers perceived RIL 123 and 41 (selected for high total SL and high lactucin-15-oxalate respectively) as the most bitter, significantly different from all others ($P=<0.0001$; Figure 3). Of our detected SLs, only 15-*p*-hydroxylphenylacetyllactucin-8-sulphate (Figure 3G) showed no correlation with bitterness, while 8-deoxylactucin-15-sulphate (Figure 3B) showed the most divergence between lines and had the strongest positive relationship when content was correlated with bitter perception, suggesting that this is the compound which most strongly drives the bitter taste in our lettuce population. The sample perceived to be least bitter compared to the others was RIL 61 ($P<0.0001$), consistent with it having the lowest concentrations of most SLs, including 8-deoxylactucin-15-sulphate, and the least total SL content. The low SL content also means that there would be less suppression of sweet taste, hence the higher than anticipated sweetness perception for this line even though it had low sugars (which can mask bitterness) (Figure 2E).

## Consumer Liking

Spearman correlation was conducted to relate liking to perception of each of the 3 sensory attributes. Sweetness was seen as the main positive influence on liking ($r=0.40$, $P<0.0001$; Figure 4A), whereas perceived bitterness gave a strong negative correlation (r-0.56, P<0.0001; Figure 4B). Consumers' perceptions accurately matched the chemical analysis, once both bitter and sweet compounds were considered together, and have highlighted the differences between compounds in terms of their contribution to overall taste perception. For this reason, RIL 61 was the most liked sample, despite the fact that it does not have the highest sugar content or the lowest content of every SL. Aftertaste perception was negatively correlated to liking (r=-0.31, P<0.0001), and SL content (r=-0.27, P<0.0001; Figure 4C) and positively correlated with bitterness perception (r=0.61, P<0.0001). Aftertaste was correlated to all SLs with the exception of 15-*p*-hydroxylphenylacetyllactucin-8-sulphate (r= 0.07687 P= 0.1835), which was the compound which did not appear to have any association with bitter taste, but correlated best with 8-deoxylactucin-15-sulphate (r= 0.2687 P= < 0.0001) which was the most bitter compound. RIL 61 was perceived as imparting significantly less aftertaste than the other samples, while RILs 123 and 41 grouped as imparting the most aftertaste, implying that the most bitter compounds are the principle contributors to aftertaste. Consequently, we can assume that modifying concentrations of these compounds in novel cultivars will have a perceivable positive effect on consumer liking.

Our consumers reported that they most liked RILs 19, 61 and 89, and disliked the RILs 41 and 123. RILs 19 and 89 had the highest total sugar content, while 61 was selected on account of having low total SL content, explaining preference for these samples over others. RILs 41 and 123 were perceived as the most bitter as well as being the least liked; 41 was selected for high lactucin-15-oxalate, while 123 was selected for high total SL content. A preference map was derived using principle component analysis to relate the consumer perception of the taste attributes to the individual consumer liking ratings (Figure 5), where the positioning of the samples on the map is derived from the individual liking data. This showed an overall preference for sweeter lines and dislike for bitter lines with the first principal component accounting for 28.7 % of the variance in liking. The secondary principal component, accounting for 19.7 % of the variance, was not related to any of the assessed parameters, and may not relate to taste, but another sensory parameter such as colour or texture. Dimensions 3 and 4 accounted for 29.2 % of the variance (plot not shown), dimension 3 separated RILs 89 and 121 and dimension 4 separated RILs 19 and 61, where in both cases these RILs were positioned together on PC1 and 2. This shows that no all consumers gave them equal liking scores although, in both cases, their mean liking ratings were not significantly different (see Fig 1). Interestingly in Figure 4, RIL 94, which contained both high SLs and high sugar, fell in the centre of the PCA and in the middle grouping for preference, supporting the concept that high sugar concentrations do help to counteract high SL content.

### Tastant Mixture Suppression

Mixing suppression is thought to influence taste perception in food samples. Liking, bitterness perception, and sweetness perception were plotted against sugar to sesquiterpene ratios (Figure 6). There was a negative correlation between sugar:SL ratio and perceived bitterness ($r=-0.280$, $P<0.0001$) and a weaker correlation between sugar:SL ratio and perceived sweetness ($r=0.171$, $P=0.0015$), although liking was not significant with sugar:SL ratio ($r=0.042$, $P=0.2338$). Taking mixing suppression into account moved outliers such as RIL 61 back toward their anticipated ranking of sweetness perception relative to the other RILs, confirming that mixing interaction between the taste factors was driving overall perception of taste.

## Discussion

Sesquiterpene lactones in a natural food matrix can impart a bitter taste to consumers, with our consumer panel reliably scoring samples correctly in terms of bitterness with regards to the quantities of their determining compounds as derived by biochemical measurement using HPLC. While it is known that there is great variety in the detection threshold between individual SLs, there is some disagreement as to which SL is the most influential on taste (Sessa et al., 2000; Van Beek et al., 1990). We found that our consumers' bitterness detection positively correlated to 5 of the 6 SLs present in our samples as determined previously by HPLC and confirmed by HPLC-MS/MS. The only SL from our population not found to be correlated to bitterness was 15-p-hydroxylphenylacetyllactucin-8-sulphate, which makes it a strong candidate for counterbalancing any reduction in other SLs as it is unlikely to impart a perceptible change in bitterness. Maintaining concentration of less bitter SLs will potentially keep the analgesic and anti-inflammatory function of lettuce for consumers (Bork et al., 1996). This strategy may also retain the ability of the plant to survive field stress through the anti-feedant (Bennett, 1994; Cowan, 1999) and antimicrobial activities of the SL (Koul, 2008; Wedge et al., 2000), depending on whether herbivores and microbes are using the same mechanism as humans to structurally detect and respond to individual SL compounds. Consumers were able to accurately rate bitter taste based on the content of SLs and primarily 8-deoxylactucin-15-sulphate, which has been previously rated as one of the most bitter of the SLs present in lettuce (Peters and van Amerongen, 1998; Price et al., 1990). The perceived sweetness scores correlated with total sugar content, but the primary factor appears to be glucose content, which accounts for the majority of lettuce sugar content, despite fructose being the sweetest of the sugars present. It has been reported that fructose is detected as 173% as sweet as sucrose on a *pro rata* basis and glucose is considered the least strong tasting with a relative sweetness 74% that of sucrose (Pangborn, 1963). The correlation between sugar concentrations and perceived sweetness was less strong than that of SL concentrations and perceived bitterness. It is important to consider the availability of compounds to taste receptors as a result of the natural food matrix, which is not currently known for lettuce, and may vary with physiological composition of the samples. Other interactions such as the effect of acidity on complementing sweetness may also play a part as it does in tomato fruit (Baldwin et al., 2008); however, acidity was not directly assessed for the present study. Additionally, cross-modal interactions of small volatile molecules such as geranial and apocarotenoids are thought to impact on the perceived sweetness of fruit (Green et al., 2010; McMath et al., 1991; Tieman et al., 2012) and are also likely to affect the taste of lettuce.

It is important to consider the relative quantities, as well as simple detection thresholds, in determining the net flavour profile of a food. RIL 61, which contained the second lowest total sugars (only 40.6% that of RIL 19, the highest total sugars) and the lowest total SLs (6.1% that of RIL 94) was consistently rated as one of the sweetest varieties and was rated the most sweet overall. The most likely explanation is that there is a lower suppression effect of bitter SLs on the sweetness of the sugars, leading to an increased perception of the sugars present. In contrast, RIL 122, which had high concentrations of many SLs, was perceived as less bitter than may be predicted on account of low content of 8-deoxylactucin-15-sulphate which the SL most strongly correlated to bitterness. Price (1990) and van Beek (1990) did not assess the conjugated form of this compounds but this is in dissent with their findings, which implicate lactucopirin as the most bitter SL backbone, but is in keeping with their conclusion that conjugated forms of SLs are more bitter than those which are not.

Taste perception is known to deteriorate with age, especially with regards to bitterness perception due to its natural association with harmful toxins which are a presumed as a greater hazard to children (Mennella et al., 2010), but we lacked the sample size and range of ages to look into this further. We were also unable to determine gender differences though there is some indication that women are more likely to be 'supertasters' and therefore have increased taste and flavour perception on a population level (Bartoshuk et al., 1994; Doty et al., 1985). We also looked at how regular consumption of bitter foods affects bitterness perception, with regards to sensitisation due to frequent exposure to bitter flavours, or a tolerance factor for the same reasons. We found that there was no significant change in either direction; however, there was a trend toward people who infrequently ate lettuce to prefer sweeter lettuce, possibly accounting for their lack of consumption. This subgroup remains an important target group for marketing novel, sweeter varieties. Some breeding to this end has already taken place resulting in the commercially available Little Gem and O' So Sweet varieties which are small and sweet romaine type lettuces. Bitter blindness to PTC had no effect on perception of bitter SLs. It is known that the receptor involved in detection of sesquiterpene lactones is separate to that which detects glucosinolates and which can cause 'bitter blindness' in 25% of people in response to glucosinolate-derived compounds. The receptor known as hTAS2R46 has been reported to be responsible for detection of SL compounds and other bitter substances, such as clerodane and labdane diterpenoids, strychnine, and denatonium (Brockhoff et al., 2007).

Kim et al. (2005) found that there are inactive polymorphisms of the HTAS2R46 receptor, which would result in bitterness insensitivity in around 24% of the general population; however, inability to detect sesquiterpene lactones has not been reported.

This study supports our hypothesis that consumers are capable of detecting the sweet and the bitter compounds in lettuce, as well as our hypothesis that most consumers have a preference for sweeter and less bitter genotypes.  Our data suggest that the bitter and sweet components act to counterbalance each other and that ratios of key compounds are more important drivers of taste perception than concentrations of individual metabolites. It is not entirely understood what the functional groups involved in SL bitterness are, but the dienone system has been implicated (Ivie et al., 1975), in addition to steric interference from other large modifications to the primary SL backbones.  However, it is commonly accepted that the while biological function is primarily attributed to the αMγL group, bitterness is not (Brockhoff et al., 2010).  The fact that the SLs show varying degrees of influence on bitter perception, with one SL showing no significant correlation, is therefore a promising result.  8-deoxylactucin-15-sulphate showed the strongest correlation to perceived bitterness in this study where the oxalates of lactucin and lactucopicrin were also strongly correlated with bitterness, consistent with previous reports (Peters and van Amerongen, 1998; Van Beek et al., 1990).

Lettuce breeding programmes should therefore target an increase in sugar compounds against a reduction in specific SLs, such as 8-deoxylactucin-15-sulphate. Our work therefore enables a refinement of breeding for metabolic composition in lettuce and directly relates biochemical composition to consumer preference. Reducing the content of all sesquiterpene lactones would potentially decrease ability of the plant to defend itself from attack, thereby decreasing yield and shelf life, but our approach enables a balanced breeding strategy by maximising the most sweet sugars and minimising only the most bitter of the sesquiterpene lactones.

## Acknowledgements

MJC would like to thank both Tozer Seeds Ltd and the BBSRC for their financial backing and support of this work via a CASE studentship BB/G017670/1

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## Tables (supplementary)

***Table S1. Buffer conditions and gradient for HPLC used for SL Analysis***

| Time (mins) | % A | % B |
|---|---|---|
| 0 | 5 | 95 |
| 5 | 5 | 95 |
| 40 | 50 | 50 |
| 55 | 100 | 0 |
| 59.9 | 100 | 0 |
| 60 | 5 | 95 |

A= 50% Acetonitrile 50% H2O, 0.1% HCl
B= 95% H2O, 5% Methanol, 0.1% HCl

***Table S2. Concentration of sugars and SLs present in selected lines.*** Values given in µg/g dry weight. SLs were analysed by HPLC and confirmed by MS. RILs were selected for extremes in concentrations of the compounds listed from within the whole population of 96 lines. Table A gives the mean values, Table B gives the raw values, mean values, and standard error of mean (n=4 biologically distinct samples). Quantity of sesquiterpene lactones in lettuce RILs was relative to the wild parent *L. serriola* UC96US23, which was given a value of 100 in each case. Values were determined from total peak area. Sugars were analysed by high throughput plate assay as described in the methods.

Table A.

| RIL | 19 | 41 | 61 | 89 | 94 | 121 | 122 | 123 |
|---|---|---|---|---|---|---|---|---|
| **Total Sugar** | 1433.0 | 635.6 | 583.0 | 1178.0 | 1363.0 | 1036.0 | 766.1 | 568.0 |
| **Total SL** | 70.8 | 262.1 | 32.3 | 93.3 | 529.5 | 56.1 | 222.5 | 803.8 |
| **Fructose** | 279.7 | 189.2 | 137.0 | 443.3 | 475.1 | 388.0 | 206.6 | 214.8 |
| **Sucrose** | 436.3 | 252.4 | 160.0 | 264.0 | 313.7 | 222.4 | 301.9 | 127.2 |
| **Glucose** | 605.3 | 149.2 | 256.0 | 397.3 | 481.1 | 377.1 | 184.9 | 182.4 |
| **Lactucin** | 7.6 | 14.8 | 1.4 | 5.1 | 7.3 | 9.0 | 16.2 | 11.8 |
| **Lactucopicrin** | 18.2 | 27.7 | 4.9 | 6.9 | 14.5 | 19.9 | 35.9 | 20.2 |
| **8-deoxylactucin-15-sulphate** | 0.6 | 23.9 | 4.0 | 14.4 | 432.6 | 17.8 | 8.3 | 682.5 |
| **Lactucin-15-oxalate** | 8.8 | 71.2 | 3.8 | 21.9 | 28.6 | 1.9 | 78.0 | 43.6 |
| **Lactucopicrin-15-oxalate** | 28.7 | 85.7 | 18.1 | 34.2 | 46.3 | 7.3 | 83.9 | 45.4 |
| **15-*p*-hydroxylphenylacetyllactucin-8-sulphate** | 6.9 | 38.7 | 0.1 | 10.9 | 0.2 | 0.1 | 0.3 | 0.4 |
| **Summary of Attributes and rationale to select for consumer study.** | High Sugar | High lactucin-15-oxalate | Low SL | High Sugar | High SL High Sugar | Low SL | High lactucin-15-oxalate | High SL Low Sugar |

Table B.

**Raw HPLC-MS relative quantities of sesquiterpenes**

| Line | Lactucin | Lactucopicrin | p-15-hydroxyphenyllacetyllactucin-8-sulphate | 8-deoxylactucin-15-oxalate | lactucopicrin-15-oxalate | lactucin-15-oxalate | Total SL |
|---|---|---|---|---|---|---|---|
| **19** | 1.92 | 6.01 | 3.59 | 0.42 | 20.01 | 6.03 | 37.98 |
| **19** | 4.97 | 12.95 | 2.08 | 0.24 | 3.10 | 1.42 | 24.76 |
| **19** | 7.34 | 15.21 | 3.30 | 0.18 | 6.70 | 2.67 | 35.40 |
| **19** | 0.92 | 2.16 | 4.83 | 0.45 | 27.62 | 7.54 | 43.52 |
| **41** | 17.96 | 32.50 | 41.13 | 28.72 | 87.62 | 80.80 | 288.74 |
| **41** | 2.52 | 3.96 | 11.13 | 4.99 | 26.27 | 19.41 | 68.28 |
| **41** | 1.79 | 5.09 | 5.76 | 2.12 | 14.71 | 6.65 | 36.12 |
| **61** | 0.68 | 2.83 | 0.03 | 1.47 | 9.97 | 1.73 | 16.70 |
| **61** | 1.62 | 4.34 | 0.06 | 1.48 | 11.58 | 3.48 | 22.57 |
| **61** | 0.34 | 1.88 | 0.05 | 4.09 | 8.76 | 1.66 | 16.79 |
| **61** | 0.17 | 0.85 | 0.07 | 0.91 | 5.87 | 0.75 | 8.63 |
| **89** | 3.69 | 6.30 | 5.20 | 3.92 | 9.71 | 5.40 | 34.22 |
| **89** | 2.88 | 2.75 | 4.85 | 7.45 | 17.18 | 12.79 | 47.90 |
| **89** | 2.87 | 3.08 | 7.07 | 13.52 | 25.61 | 18.23 | 70.38 |
| **89** | 0.73 | 1.67 | 4.60 | 3.95 | 15.88 | 7.30 | 34.13 |
| **94** | 1.75 | 6.74 | 0.08 | 579.99 | 11.34 | 22.27 | 622.18 |
| **94** | 8.53 | 13.40 | 0.20 | 84.84 | 37.31 | 19.27 | 163.55 |
| **94** | 2.70 | 6.21 | 0.01 | 82.42 | 17.80 | 6.71 | 115.85 |
| **94** | 1.56 | 2.70 | 0.14 | 117.92 | 26.10 | 9.02 | 157.44 |
| **121** | 1.42 | 5.50 | 0.03 | 8.90 | 3.43 | 0.76 | 20.04 |
| **121** | 11.40 | 24.03 | 0.13 | 0.87 | 1.52 | 0.33 | 38.28 |
| **121** | 0.97 | 2.51 | 0.02 | 20.18 | 7.98 | 2.02 | 33.68 |
| **121** | 4.31 | 7.75 | 0.05 | 5.73 | 1.72 | 0.66 | 20.22 |
| **122** | 4.04 | 18.54 | 0.08 | 8.83 | 25.63 | 59.51 | 116.62 |
| **122** | 6.35 | 11.40 | 0.10 | 1.01 | 17.91 | 8.83 | 45.59 |
| **122** | 18.76 | 35.94 | 0.35 | 5.27 | 101.36 | 76.29 | 237.96 |
| **122** | 3.22 | 5.86 | 0.08 | 1.48 | 22.90 | 11.29 | 44.83 |
| **123** | 2.66 | 8.81 | 0.26 | 624.57 | 15.17 | 32.01 | 683.48 |
| **123** | 11.39 | 16.20 | 0.11 | 71.41 | 18.08 | 12.58 | 129.78 |
| **123** | 5.50 | 8.44 | 0.31 | 249.96 | 33.31 | 26.53 | 324.05 |
| **123** | 3.99 | 6.96 | 0.18 | 149.16 | 24.16 | 16.09 | 200.52 |

**Average HPLC-MS relative quantities of sesquiterpenes**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **19** | 3.79 | 9.08 | 3.45 | 0.32 | 14.36 | 4.41 | 4.41 |
| **41** | 7.42 | 13.85 | 19.34 | 11.94 | 42.87 | 35.62 | 35.62 |
| **61** | 0.70 | 2.47 | 0.05 | 1.99 | 9.05 | 1.91 | 1.91 |
| **89** | 2.54 | 3.45 | 5.43 | 7.21 | 17.09 | 10.93 | 10.93 |
| **94** | 3.63 | 7.26 | 0.11 | 216.29 | 23.14 | 14.32 | 14.32 |
| **121** | 4.52 | 9.95 | 0.06 | 8.92 | 3.66 | 0.94 | 0.94 |
| **122** | 8.09 | 17.93 | 0.15 | 4.15 | 41.95 | 38.98 | 38.98 |
| **123** | 5.88 | 10.10 | 0.22 | 273.78 | 22.68 | 21.80 | 21.80 |

**SEM of HPLC-MS relative quantities of sesquiterpenes**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **19** | 1.46 | 3.03 | 0.56 | 0.07 | 5.72 | 1.43 | 3.94 |
| **41** | 5.27 | 9.33 | 11.01 | 8.43 | 22.63 | 22.89 | 79.39 |
| **61** | 0.32 | 0.74 | 0.01 | 0.71 | 1.20 | 0.57 | 2.86 |
| **89** | 0.63 | 1.00 | 0.56 | 2.26 | 3.27 | 2.89 | 8.54 |
| **94** | 1.65 | 2.23 | 0.04 | 121.50 | 5.61 | 3.80 | 119.61 |
| **121** | 2.41 | 4.82 | 0.02 | 4.10 | 1.50 | 0.37 | 4.67 |
| **122** | 3.62 | 6.54 | 0.07 | 1.83 | 19.87 | 17.05 | 45.47 |
| **123** | 1.93 | 2.07 | 0.05 | 122.51 | 4.01 | 4.51 | 123.07 |

**Sugar content (mg/g DW)**

| | Total sugar | | Glucose | | Fructose | | Sucrose | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| **19** | 1237.47 | 149.60 | 574.00 | 89.90 | 285.41 | 51.28 | 378.06 | 59.28 |
| **41** | 407.72 | 102.54 | 116.28 | 36.09 | 141.21 | 29.62 | 150.23 | 57.30 |
| **61** | 667.22 | 203.16 | 242.54 | 52.85 | 163.07 | 41.14 | 261.61 | 126.98 |
| **89** | 1263.25 | 109.09 | 491.59 | 55.54 | 478.99 | 75.79 | 292.66 | 18.74 |
| **94** | 1265.04 | 429.50 | 488.65 | 178.69 | 444.09 | 133.72 | 332.30 | 117.65 |
| **121** | 1258.54 | 306.06 | 377.09 | 84.55 | 387.99 | 68.49 | 222.41 | 68.31 |
| **122** | 720.14 | 157.20 | 206.56 | 40.93 | 220.49 | 53.73 | 293.09 | 87.43 |
| **123** | 643.33 | 147.53 | 236.22 | 63.46 | 262.55 | 49.49 | 144.56 | 35.60 |

## List of Figure Legends

***Figure 1. Mean scores for bitterness, sweetness, aftertaste, and liking.*** Consumer scores for sweetness and bitterness (A) and for aftertaste perception and consumer liking by RIL. (B) Bitterness and sweetness scores as log mean values from the LMS scale as described in Green (1993). Aftertaste was assessed on a 5 point hedonic scale, while liking was measured on a 9 point hedonic scale. Error bars show standard error, n=43. Categories a-d denote significantly different groupings as determined by Kruskal-Wallis, Dunn's procedure.

***Figure 2. Quantified Sugar Concentration vs Perceived Sweetness in Lettuce Lines***

Total sugar (A)(r=0.1747 P<0.0001) correlates to perceived sweetness less well than does glucose (D)( r=0.2266 P<0.0001). Fructose (B) is not significant, owing to RIL 61, which has the lowest levels of fructose, yet the highest perceived sweetness. Sucrose (C)( r=0.1543 p-0.0041) has less correlation to perceived sweetness that does glucose despite a higher relative sweetness. Taking into account relative sweetness (E) highlights the lack of sweetness suppression in RILs 61 and 122.

***Figure 3. Quantified sesquiterpene concentration vs perceived bitterness in lettuce lines.***

Total SL (A)(r=0.56 P<0.0001) correlates best. The most significant individual SL is 8-deoxylactucin-15-sulphate (B) (r=0.3403 P<0.0001) possibly due to the very high levels observed in some samples. Lactucin and lactucopicrin (C and D) had equal effect as scored by consumers (r=0.1817 P=0.0007) and were each less bitter than their oxalates (E and F). Lactucin-15-oxalate (r=0.1986 P=0.0002) was less bitter than lactucopicrin-15-oxalate(r=0.226 P<0.0001) as was expected. 15-p-hydroxylphenylacetyllactucin-8-sulphate was not significantly correlated to bitterness in our samples.

***Figure 4. Perceived taste parameters vs liking for lettuce lines***

Sweetness (A) positively correlates with liking, (r=0.4026 P<0.0001), while bitterness (B) and aftertaste (C) negatively correlate (r=-0.56 P<0.0001 and r=0.3075 P<0.0001 respectively). As all results are so significant, it is clear to us that consumers have a strong and reliable aversion to bitterness and preference for sweetness in lettuce.

***Figure 5. Consumer Preference Map***

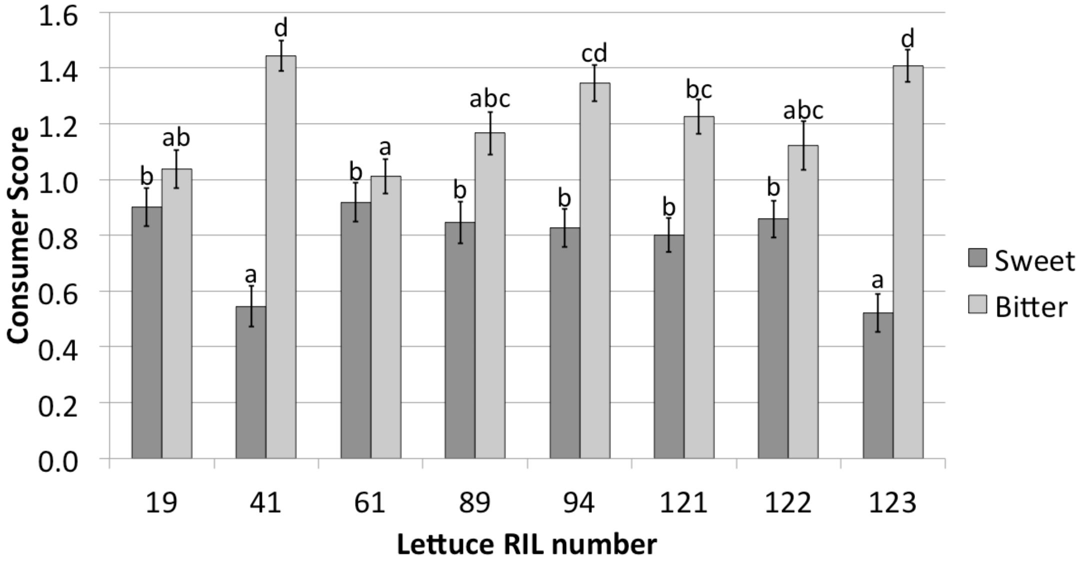
28.74% of variance in reported liking is a consequence of sweet-bitter balance. The secondary and subsequent components relate to traits which were not assessed in this study, but participants were able to distinguish lines based upon this, with RILs 122 and 94 driving this trait positively and negatively respectively.

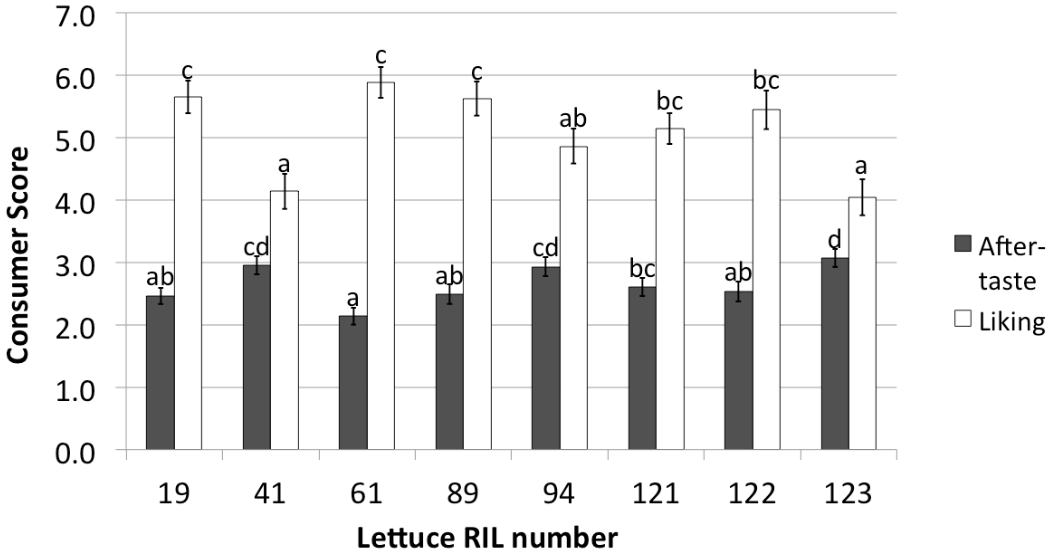
***Figure 6. Consumer Perception vs Predicted Perception***

Taking into account the ratio of sugars to sesquiterpene lactones take into account the mixing suppression to an extent and corrects outliers affected by this. Using this method the correlation to preference (A) was no longer significant, and the correlation to sweetness dropped (B), (r=0.171 P=0.0015) though the correlation to bitterness (C) remained strong (r=-0.2803 P<0.0001).

***Supplementary Figure 1. MS/MS fragmentation of assessed sesquiterpene lactones.***

MS/MS fragmentation spectra of each sesquiterpene lactone, determined by Agilent 1100 HPLC with QToFMS.

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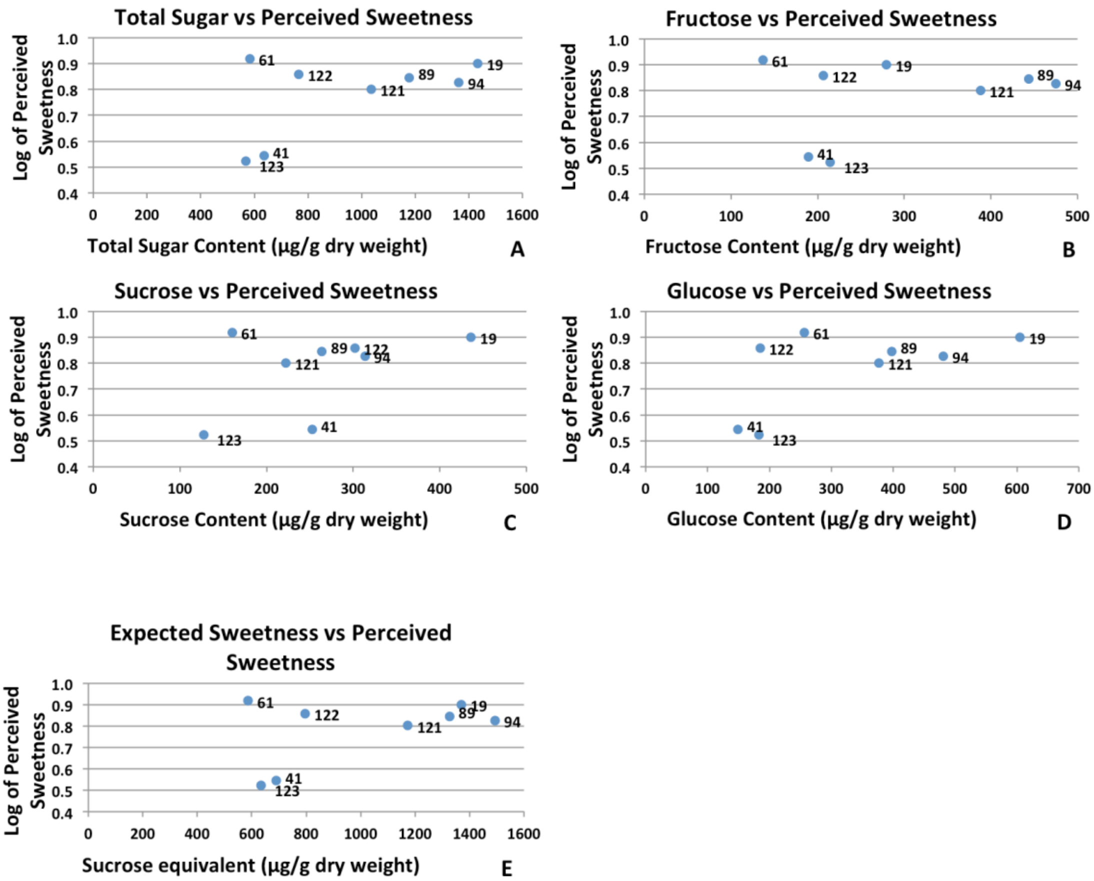


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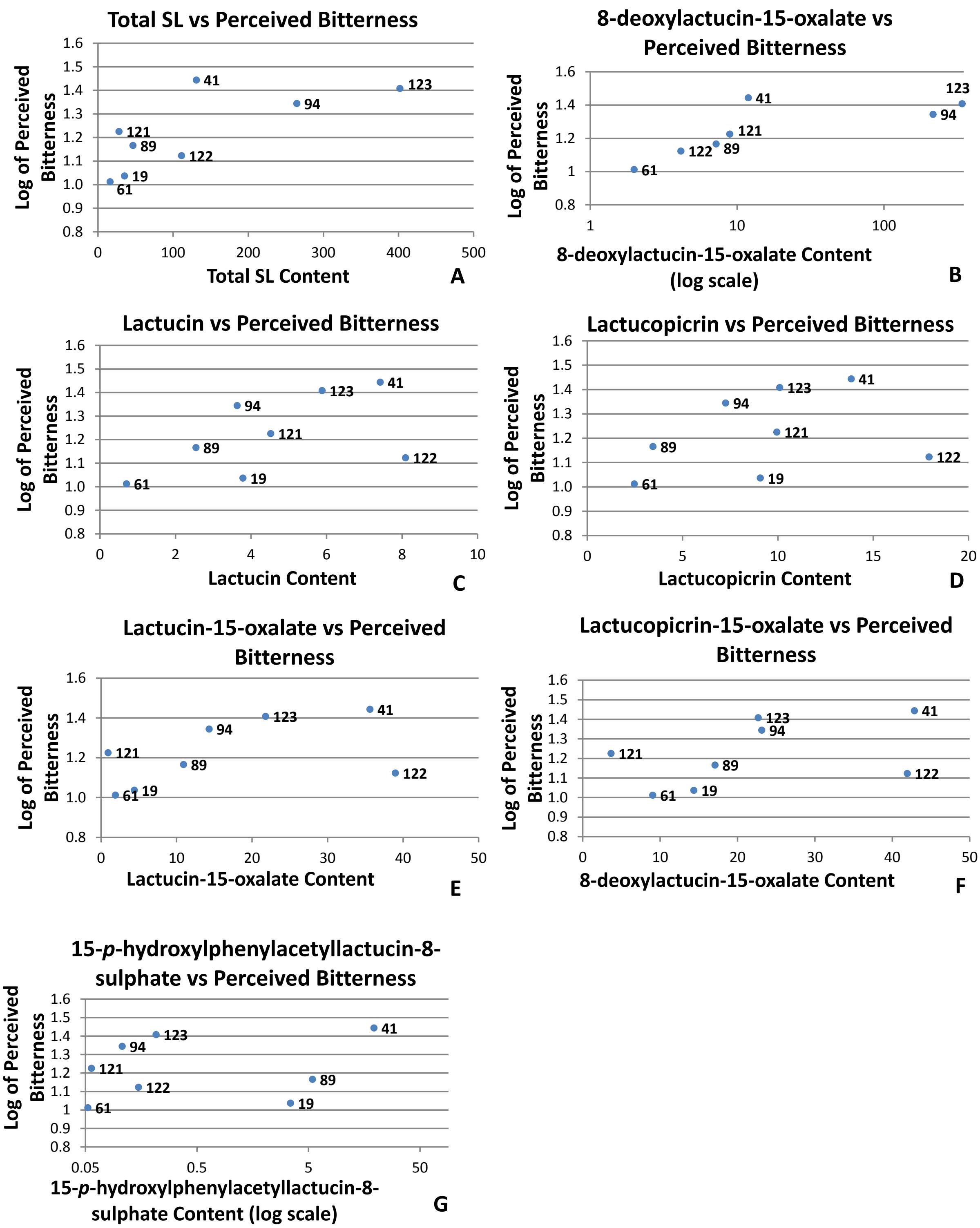


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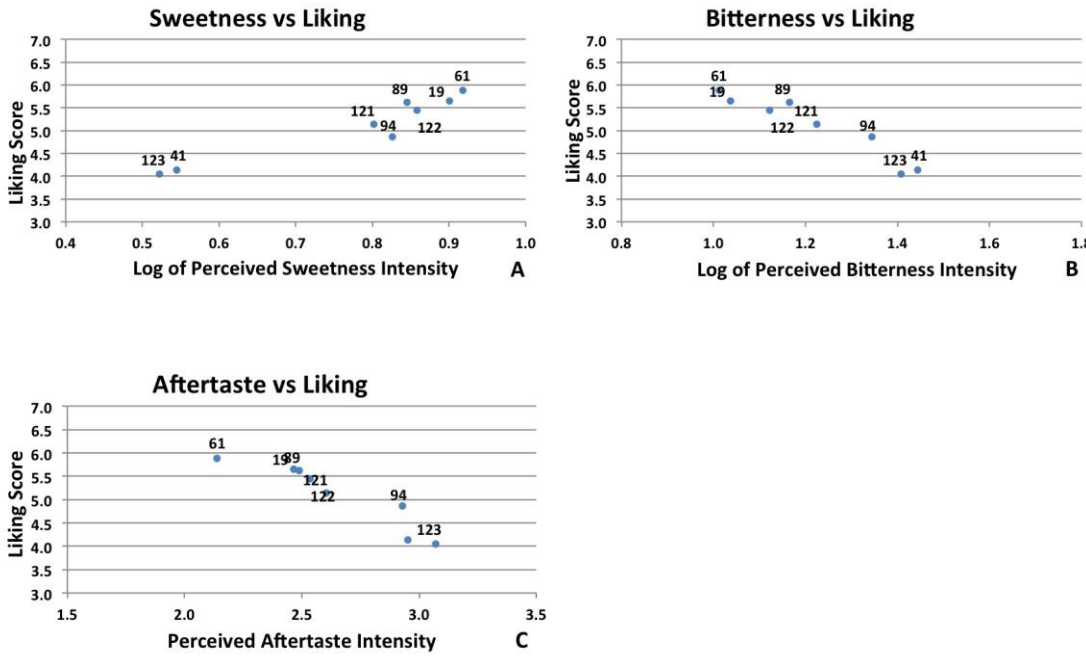


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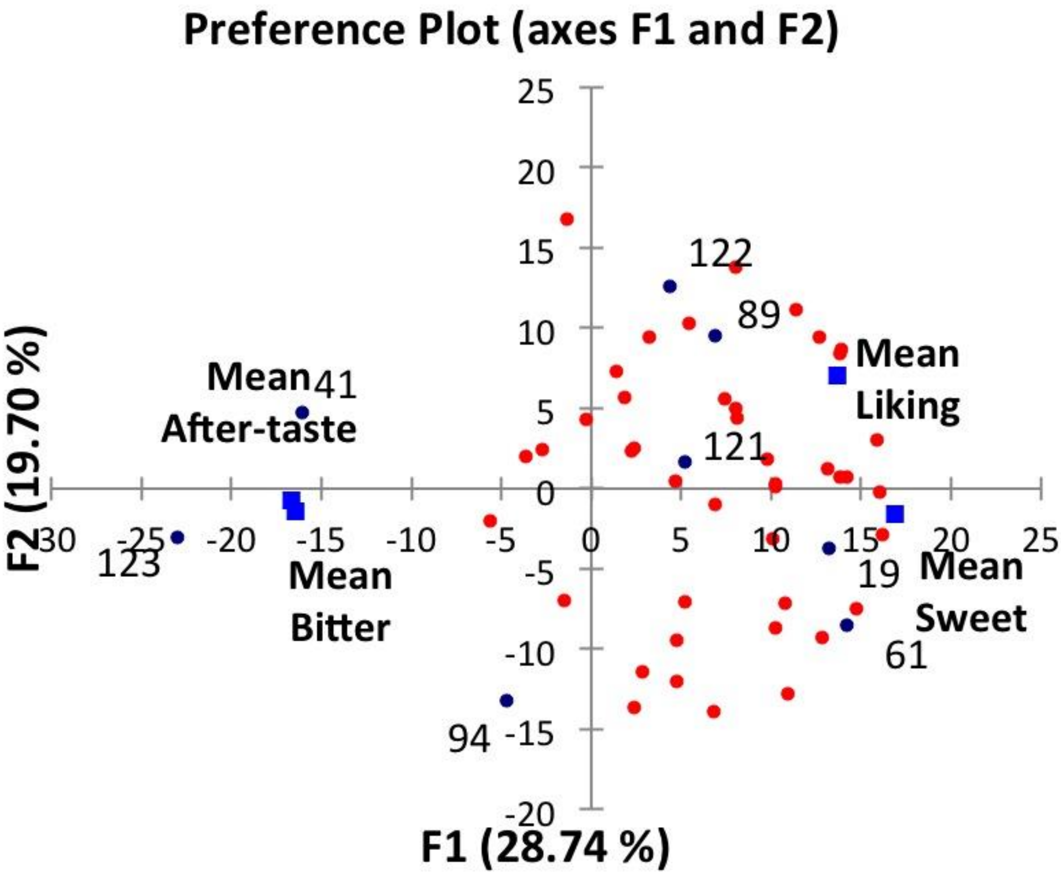


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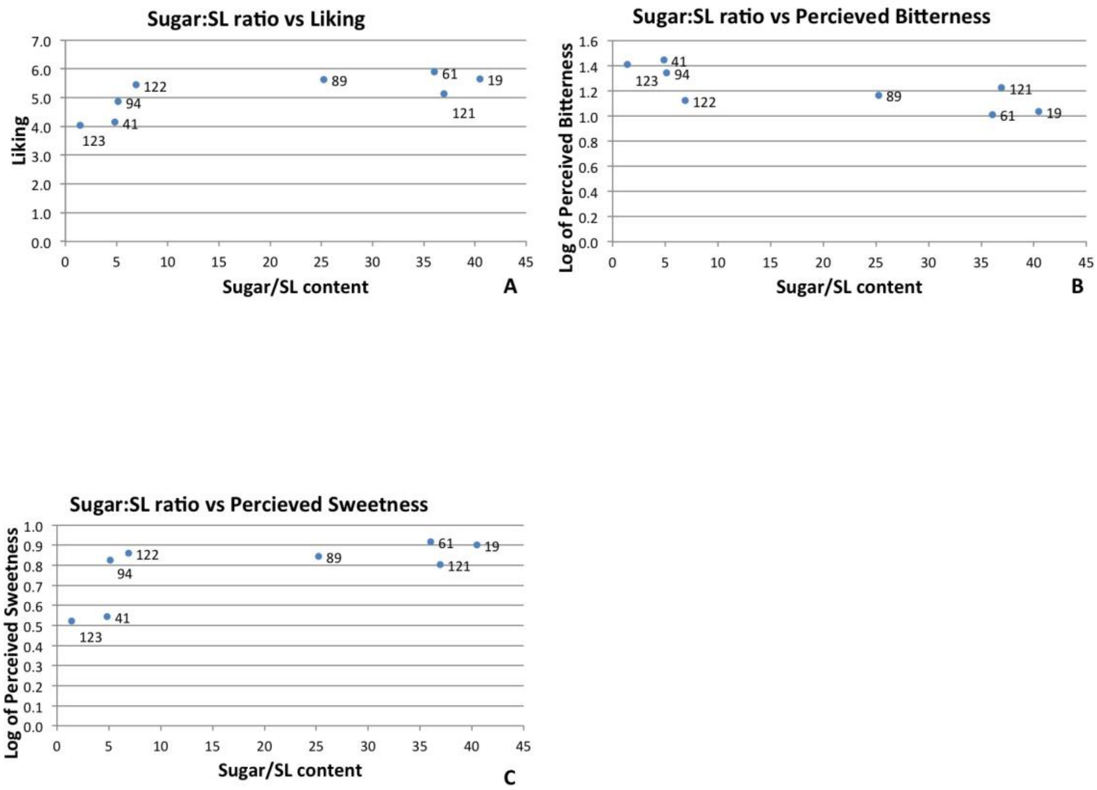


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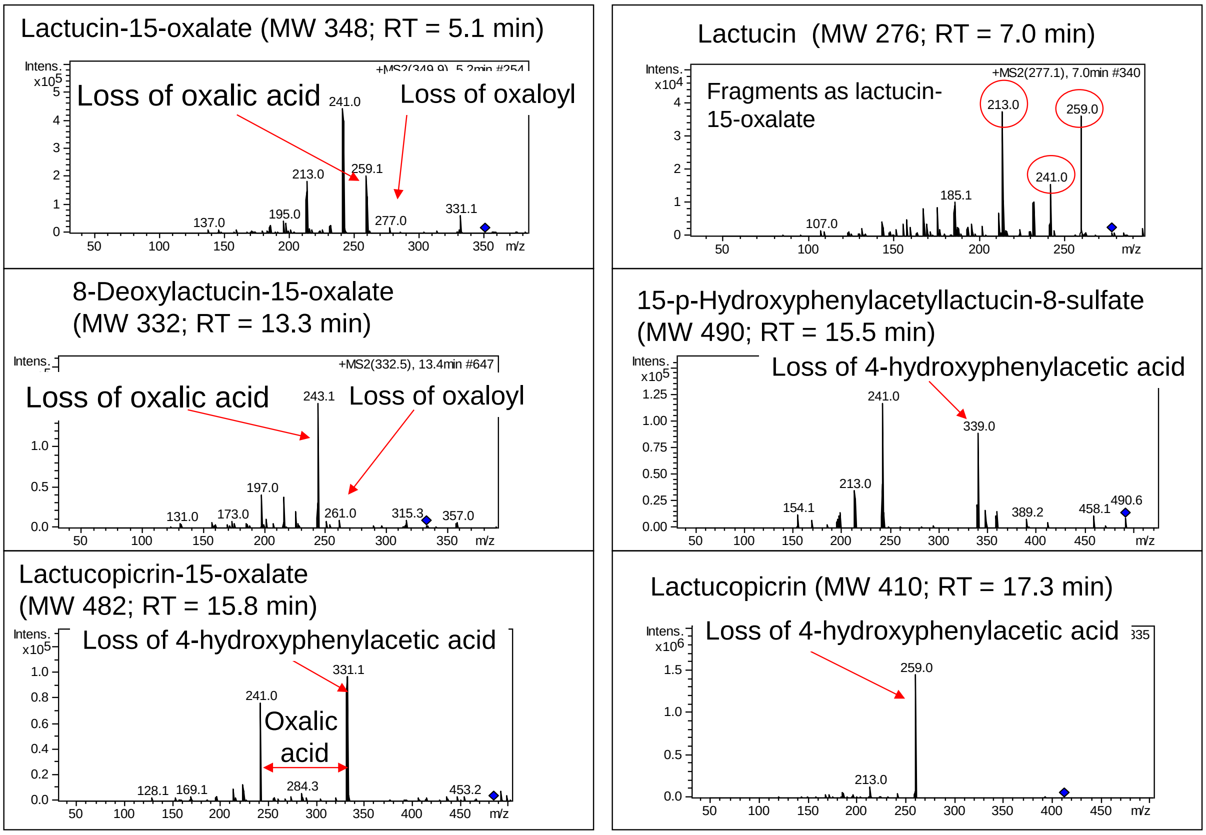


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