Descriptive sensory analysis of Aceto Balsamico Tradizionale di Modena DOP and Aceto Balsamico Tradizionale di Reggio Emilia DOP

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Running title: Traditional Balsamic Vinegar of Modena and Reggio Emilia

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ABSTRACT

BACKGROUND: Aceto Balsamico Tradizionale (ABT) is a typical Italian vinegar available in two different forms: “Aceto Balsamico Tradizionale di Modena DOP” (ABTM) and “Aceto Balsamico Tradizionale di Reggio Emilia DOP” (ABTRE). ABT is obtained by alcoholic fermentation and acetic bio-oxidation of cooked grape must and aged for at least 12 years in wooden casks and is known and sold around the world. Despite this widespread recognition, data on sensory characteristics of these products are very scarce. Therefore, a descriptive analysis was conducted to define a lexicon for the ABT sensory profile and to create a simple, stable and reproducible synthetic ABT for training panellists.

RESULTS: A lexicon of twenty sensory parameters was defined and validated and a synthetic ABT was prepared as standard reference. Simple standards for panellists training were also defined and the sensory profiles of ABTM and ABTRE were obtained.

CONCLUSION: The obtained results confirm that descriptive analysis can be used for the sensory characterisation of ABT and sensory profiles of ABTM and ABTRE are very different. Furthermore, the results demonstrate that a lexicon and proper standard references are essential to describing the sensory qualities of ABT both for technical purpose and to protect the product from commercial fraud.

Keywords: Aceto Balsamico Tradizionale; sensory analysis; descriptive analysis; vinegar; cooked must

INTRODUCTION

Aceto Balsamico Tradizionale (ABT) is a typical vinegar of the Emilia Romagna region of northern Italy. ABT is highly representative of artisanal Italian gastronomy and, despite its limited production, is recognised and commercially distributed around the world. There are two types of ABT. The first is produced in the province of Modena and is known as “Aceto Balsamico Tradizionale di Modena DOP“ (ABTM), while the second is produced in the province of Reggio Emilia and is known as “Aceto Balsamico Tradizionale di Reggio Emilia DOP” (ABTRE). Both of these products received Protected Denomination of Origin (DOP) certification from the European Commission in 2000 because their unique production technologies are typical of a well-defined geographical area of production.[1] In ABT production, the must of local grapes is first cooked in an uncovered pan over an open fire.[Errore. L'origine riferimento non è stata trovata.] After a partial alcoholic fermentation, the must-wine is transferred into a set of barrels (“batteria”) composed of 5 to 10 wooden casks decreasing in size and fashioned from different woods. The product stays in the barrel for a minimum of 12 years, a time during which the ethanol is bio-oxidised and fresh cooked must is added according to the “Solera” method. ABT produced in the province of Modena is labelled “ABTM” if aged a minimum of 12 years and “Extra Aged” if aged a minimum of 25 years while the ABT produced in the province of Reggio Emilia is labelled “ABTRE Red Label” or “ABTRE Silver Label” if aged a minimum of 12 years and “ ABTRE Gold Label” if aged a minimum of 25 years. Before marketing, ABTs of each type are examined by an official group of five expert tasters, nominated by the two Control Committees of ABT, and rated according to a hedonic evaluation card. The product may be bottled and sold only if the mean score measured during these evaluations is higher than of a standard.

Factors that contribute to the characteristic flavour, taste and physicochemical characteristics of ABT include the differences in grape varieties (typically Trebbiano, Lambrusco, Ancellotta and Sauvignon, plus additional cultivars authorised for each province), the technology of alcoholic fermentation and acetic bio-oxidation and, most importantly, the barrels utilised for aging.

A large number of papers have been published reporting studies intended to define the characteristics of ABT and to determine the relationships between these characteristics and ABT technology[1-5] for use in characterising ABT and protecting the product from commercial fraud.[6-10]

Sensory evaluation conducted by expert trained by the Control Committee of ABT, is the most important assessment of ABT because only those products scoring higher than a predefined standard may be sold; however, this evaluation has been subjected to minimal analysis. Evaluation scores provided by expert tasters have been used occasionally to classify ABT[11]**,** but these are more frequently used to explain chemical data[12,13] or to evaluate whether chemical analysis may replace sensory analysis by expert tasters.[14]

The work of Giudici *et al*.[15] provides the only examples in which useful descriptors for ABT sensory analysis were reported and standards were produced from white and red wines, despite these standard being very different in nature from the test product.

Thus, the aim of this work was to conduct a sensory descriptive analysis (DA)[18-19] to define a lexicon for characterising the sensory profile of ABT and to apply this lexicon in comparing samples of ABTM to samples of ABTRE. Sensory DA has been successfully used to obtain detailed descriptions of the aroma, flavour and oral texture of food and beverages[20-28], and thus could be applied to describing ABT.

Given the importance of using trained panellists for the sensory DA, a new synthetic ABT was also created in this research for use as a standard reference for ABT sensory analysis.

MATERIALS AND METHODS

Samples

Samples of ABTM (19 products; 17 with at least 12 years of ageing and 2 with at least 25 years of ageing) and ABTRE (13 products; 11 with at least 12 years of ageing and 2 with at least 25 years of ageing) were furnished directly by the producers. Only those samples that had been evaluated as "suitable" (i.e., marketable) by the official group of expert tasters were used for this work. All products were stored in opaque, closed vessels at 20±2 °C and labelled with a 3-digit code for sensory analysis.

Synthetic ABT

According to ISO 11035[29] it is necessary to define a standard reference for each descriptor that must be simple, stable and reproducible over time. A pure chemical is not appropriate as a standard for the descriptor because the assessor must be able to recognise the descriptor among the complex sensations of the stimulus.

Giudici *et al*.[15] used white or red wines as standard references, as these products are stable and commercially available. ABT is a brown vinegar characterised by a very sweet flavour, a high viscosity and a caramel/burnt sugar odour. However, it is not stable nor reproducible, so a new synthetic ABT was defined in this study to serve as a more authentic standard. According to previous scientific reports[30-31], the most important components of ABT are glucose and fructose which are present at similar concentrations (approximately 700-800 g L^{-1}), glycerol (approximately 18 g L^{-1}), acetic acid (approximately 20 g L^{-1}), gluconic acid (approximately 11 g L^{-1}), succinic acid (approximately 11 g L^{-1}), malic acid (approximately 7 g L^{-1}), tartaric acid (approximately 6 g L^{-1}),

citric acid (approximately 5 g L^{-1}) and lactic acid (approximately 2 g L^{-1}). Polyphenolic compounds such as tannins and anthocyanins (approximately 2-3 g L^{-1}) are also very important due to the taste and colour of balsamic vinegar. Working in collaboration with experts on ABTM and ABTRE, a synthetic ABT with composition and sensory characteristics similar to those of natural ABT was produced. The final composition of the synthetic product included lactic acid (48 g L^{-1}), acetic acid (15 g L^{-1}), glycerol (18 g L^{-1}), liquid caramel E150d (10 g L^{-1}), liquid caramel from sucrose E150a (80 g L^{-1}), Arabic gum E414 Oliver Gum 30® (100 g L^{-1}), fructose (400 g L^{-1}) and glucose (400 g L^{-1}).

Glucose, fructose, lactic acid, acetic acid and glycerol were purchased from Sigma-Aldrich (Milan, Italy). All chemicals were of high purity. Liquid caramel E150d and E150a were purchased from Sicna SpA (Cassina Nuova di Bollate, Milan, Italy) and Arabic gum E414 Oliver Gum 30® was purchased from Oliver Ogar Italia SpA (Montebello Vicentino, Vicenza, Italy). Ultra-pure water was produced with a Milli-Q System (Millipore, Milan, Italy).

A quantity of lactic acid was added to the synthetic ABT to achieve the same acidity present in natural ABT. Moreover, lactic acid was chosen because it is liquid, thereby simplifying preparation of the standard reference. The quantity of acetic acid added to the reference was lower than that reported for ABT to produce a lesser sensorial impact. Viscosity in the synthetic ABT was achieved using glycerol, whereas the typical brown colour and odour/aroma of caramel/burnt sugar, generated in the natural product during the concentration phase of must cooking, were obtained with the two liquid caramels. The sweetness and density of natural ABT were recreated in the synthetic ABT by the addition of glucose and fructose. A very important characteristic of ABT is its adhesivity, which result from the presence of significant polymeric compounds produced by acetic bacteria[32] and polyphenolic compounds provided by grapes. To obtain the same adhesivity in the synthetic ABT, Arabic gum was used since polyphenolic compounds give a strong bitter taste.

Sensory analysis

Tasting panel. Three different tasting panels were use. The first, the University panel, was made up of eight tasters (2 males and 6 females between 25 and 42 years old) who were recruited according to ISO regulations[33-36] and who had previous experience in sensory analysis. This panel was used for all phases of work. The second, the ABTM panel, consisted of 26 tasters (21 males and 5 females between 35 and 58 years old), all of whom were member of the official ABTM certification group. The third, the ABTRE panel, was made up of 25 tasters (24 males and 1 females between 32 and 60 years old), all of whom were members of the official ABTRE certification group. Due to their minimal prior experience in sensory analysis, members of these two panels were used only in generating and selecting descriptors.

Tasting procedure. The University panel analysis sessions were conducted in the morning (11:00-13:00) in white light. The sensory laboratory was designed according to ISO 8589 with separate booths.[37] The room temperature was 22±1 °C. Mineral water (Sant'Anna, Fonti di Vinadio, Torino, Italy) was provided for palate cleansing during sessions. Analysis sessions with the ABTM and ABTRE groups were carried out in the evening (20:00-22:00) in white light in a room maintained at 20±1 °C. As described above, mineral water (Sant'Anna, Fonti di Vinadio, Torino, Italy) was provided for palate cleansing during sessions. Samples (50 mL) were furnished in an ISO wine tasting glass[38] covered with a Petri dish. A plastic teaspoon was provided for taste analysis. For each sample, panellists were directed to first note the sample odour and then to sample a small quantity of product with the teaspoon for the taste and aroma analysis.

Statistical analysis

A two-way analysis of variance (ANOVA) was employed to study the ABT types, using judges and their interactions to highlight the differences among samples for each sensory parameter. A one-way ANOVA was used to highlight significant differences among ABT samples for each term of the sensory lexicon. A Principal Component Analysis (PCA) with Varimax rotation of factors was also performed to show the relationships among ABT samples and the variables investigated. All calculations were performed with the STATISTICA program for Windows (Release 7.0; StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Generating descriptors

First, the three tasting panels constructed a list of attributes for odour, aroma and taste. The University panel was subjected to three 45-minutes sessions. For each session, 4 samples of ABTM (3 aged a minimum of 12 years and 1 aged a minimum of 25 years) and 4 samples of ABTRE (3 aged a minimum of 12 years and 1 aged a minimum of 25 years) were used.

The same samples were also examined by ABTM and ABTRE panels in two 90-minutes sessions per group.

Assessors were asked to generate the maximum number of descriptors possible for these products excluding colour and structure descriptors, as these parameters could be evaluated with simple physical analysis.

A total of 173 descriptive terms were generated from these initial tasting sessions.

Each group then participated in a single 1-h round-table discussion to establish the initial lexicon. In accordance with ISO 11035[29], the terms that were judged to be inappropriate descriptions of the sensory attributes of ABT were eliminated, including, for example, “excellent” and “good”, or

hedonic or “typical” terms. The panellists grouped also any synonyms and eliminated duplications. A revised list of 86 descriptive terms was produced (Table 1) from the discussion. With the exception of “sour”, “bitter” and “sweet”, all the descriptors were characterised as both orthonasal and retronasal and were categorised as both odours and aroma.

Selection of descriptors

In the second phase of the work, the data set of 86 attributes of ABT was then refined. The procedure for selecting and identifying the descriptors was adapted from ISO 11035[29] and Meilgaard[19] and their adjusted frequencies (AFs) were applied. Two 30-minute sessions were conducted for each of the three panels, and ten samples were examined (5 ABTM and 5 ABTRE samples, using 4 products aged a minimum of 12 years of ageing and 1 aged a minimum of 25 years per type). Each panellist was asked to judge the perceived intensity of each of the descriptors from the initial list. The perceived intensity was scored on a 5-point scale from 0 (none) to 5 (strong), in accordance with ISO 11035.[29] For each descriptor, the **AF** was calculated as $\mathbf{AF} = (\mathbf{F} \times \mathbf{I})^{½}$ in which **F** is the number of times a descriptor was mentioned divided by the total number of times that descriptor could have been mentioned, expressed as a percentage. **I** is the sum of the intensities reported by all panellists for a given descriptor divided by the maximum possible intensity reported for that descriptor, expressed as a percentage. This calculation method also accounted for descriptors that were rarely mentioned but that were important in terms of perceived intensity, as well as descriptors with low perceived intensity that were mentioned frequently. Classifying descriptors according to the size of their means allowed the elimination of a number of descriptors with relatively low geometrical means.

Table 1 presents the mean values of the AFs calculated for all descriptors from responses across the three panels. Because there is no set value for defining a meaningful descriptor, the most

important descriptors were arbitrarily defined as those with AF > 18.[28] This AF value was calculated by assuming that half of the panellists identified a descriptor for half the samples with an intensity value of half the maximum potential. Only twenty descriptors experimentally met this value. For ABT Giudici *et al*.[15] listed twenty-four descriptors, including one descriptor for appearance, fifteen for aroma, four for taste, one for texture and three for trigeminal sensations. Eight of the descriptors of aroma (caramel, cooked must, wood, dried plum, vinegar, honey, liquorice, vanilla) reported by Giudici *et al.*[17] were also used in this work. Furthermore, Giudici *et al*.[15] cited sour, sweet, bitter and salty as taste descriptors, while in this work salty was never reported by panellists.

Sample evaluation to verify the lexicon

The third phase of the study, directed to verify the lexicon, was performed exclusively with the University panel, as its members had previous experience in sensory analysis.

The synthetic ABT was used as a base to prepare the reference standards reported in Table 2. Simple, standard, commercially available products were used as additives. Over the course of ten 1-h round-table discussions, the standards were explained in detail to the panellists. The attributes of these standards corresponded to the highest possible intensity score on the sensory description rating scale.

These scores were discussed until a consensus was reached among all panellists.

After completing the training, the panellists were asked to describe during two sessions the sensory profile of ten ABTM and four ABTRE (two “Red Label” and two “Silver Label”) samples, all aged at least 12 years. All samples were tested in duplicate. Each parameter was rated on a 10 cm unstructured straight line labelled “no sensation” and “extremely intense sensation” at the left and the right end points, respectively.[39]

F-ratios of the two-way ANOVA (Table 3) allowed verification of whether assessors agreed or disagreed with respect to the three type of ABT (ABTM, ABTRE “Red Label” and ABTRE “Silver Label”).

The results indicated that, in general, the intensities of the various descriptors were significantly different among ABT types (ABTM, ABTRE “Red Label” and ABTRE “Silver label”), implying that these descriptors are useful in differentiating products according to their origins. Only three descriptors - lemon, plum and cider – could not be used to differentiate the samples.

No significant assessors effect was noted, likely on account of the effective training sessions. An assessor effect was noted only for caramel and wood descriptors, indicating that a new training strategy may be necessary for these descriptors.

None of the judges $\times$ product (J $\times$ P) interactions showed significant interaction, and as a result, the disagreement among the judges in the evaluation of these descriptors may be considered negligible.

A one-way ANOVA was then performed using the three types of ABT as variables and the resulting mean intensity ratings of descriptive attributes are shown in Table 4.

Blackcurrant, tamarind, raisin, caramel, honey, cooked must, cooked apple and wood descriptors were strongly associated with ABTM and could be combined with sweetness and bitterness to generate a sensory lexicon for this product.

The two ABTRE samples showed greater similarity. The “Red Label” (a lower quality of ABT from the ABTRE group) had high intensity ratings for the descriptors liquorice, cooked apple and, most prominently, vinegar and sourness. The “Silver Label” product was most strongly associated with the descriptors orange, dried plums, cherry jam and cooked plum, but had a low intensity rating for sourness.

The intensity values for each sensory attribute were submitted to PCA, from which two principal components were obtained. Figure 1 is a bi-plot demonstrating the variable distribution of sensory attributes on the plane defined by the two factors.

The plot reveals strong correlations among blackcurrant, plum, honey, raisin, cooked must, caramel and bitterness. The descriptors sweet and bitter are directly correlated, whereas sour correlates with cider and lemon. Vinegar and liquorice also demonstrate a different trend.

Figure 1 also shows the locations of the three types of ABTs on the factor plane. The first component (explained variance of approximately 54%) readily distinguishes the ABTM (characterised by strong intensities of plum, blackcurrant, raisin, caramel, honey, cooked must, sourness, sweetness and wood and low intensities of dried plum and vinegar) from the two ABTREs. The second component (explained variance of approximately 22%) serves to distinguish the two ABTREs. To illustrate, ABTRE “Red Label” (ABTRERED) is characterised by high intensity values for liquorice, sour, cooked apple and vinegar, whereas ABTRE “Silver Label” (ABTRESILV) demonstrated high correlation with the descriptors dried plum, cherry jam, orange and cooked plum and vinegar.

CONCLUSIONS

The sensory DA approach allowed the definition of the sensory attributes of different types of ABT for the first time. Twenty terms (seventeen for odour/aroma and three for taste) were selected and referenced during a sensory evaluation of ABTs ans subsequent discussion. A simple and reproducible synthetic ABT standard was produced for use as a standard reference in panellist training. The lexicon was validated by its use in defining the sensory profile of three types of ABT, one from Modena and two from Reggio Emilia. These results indicate that most of the selected terms are appropriate for differentiating sensory qualities among samples. Furthermore, they

demonstrate that the defined lexicon can be used to describe the sensory qualities of ABT during research studies, such as those on the effect of different woods or ageing technologies on ABT, and, most importantly, during product development to protect ABTs from commercial fraud.

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Table 1. Descriptive terms selected by panellists after the round table discussion and the adjusted frequencies (AFs) for sensory descriptors determined during the selection phase.

	AF value		AF value		AF value
Odour/Aroma		Geranium	0	Tar	1
Acetaldeyde	0	Grapefruit	6	Thyme	2
Almond	0	Grass	8	Tobacco	5
Anise	3	Hay	4	Truffle	0
Apple	6	Honey	19	Vanilla	3
Apricot	4	Juniper	10	Vinegar	77
Banana	0	Kerosene	9	Violet	4
Bilberry	9	Lactic acid	5	Walnut	4
Blackberry	7	Leather	8	Wet wool	2
Blackcurrant	20	Lemon	28	Wistaria	4
Burnt match	0	Liquorice	23	Wood	18
Butyric acid	0	Methanol	8	Yeast	0
Caoutchouc	1	Molasses	11	**Taste**	
Caramel	51	Mouldy	0	Sour	71
Cherry	12	Mushroom	4	Sweet	60

Cherry jam	21	Nutmeg	3	Bitter	20
Cherry stone	4	Oak wood	11		
Chocolate	6	Onion	4		
Cider	19	Orange	23		
Cinnamon	12	Orange blossoms	10		
Citron tree	0	Oregano	3		
Cloves	8	Peach	0		
Coffee	4	Peanut	0		
Cooked apple	23	Pear	2		
Cooked cream	7	Pepper	9		
Cooked must	36	Phenol	5		
Cooked plum	45	Pineapple	3		
Diacetyl	3	Plum	22		
Dried plum	19	Raisin	23		
Dried tomato	10	Raspberry	7		
Dust	5	Resin	5		
Elder flowers	6	Rose	7		
Ethanol	6	Smoked	7		
Ethyl acetate	6	Strawberry	5		
Fermented must	11	Sulphur dioxide	2		
Fig	8	Tamarind	20		

Table 2. Reference standards used for training of panellists on ABT descriptors

Odour/Aroma	
Liquorice	0.10 g liquorice Amarelli[a] in synthetic ABT[x]
Orange	0.25 μL orange aroma[b] in synthetic ABT
Lemon	5.00 μL lemon aroma[c] in synthetic ABT
Blackcurrant	Fresh blended blackcurrant
Plum	Fresh blended plum
Tamarind	Tamarind extract[d]
Dried plums	1.00 g dried plums[e] in synthetic ABT
Raisin	1.00 g raisin[f] in synthetic ABT
Cider	Cider[g]
Caramel	Synthetic ABT
Cherry jam	1.50 g cherry jam[h] in synthetic ABT
Honey	5.00 μL honey aroma[c] in synthetic ABT
Cooked must	Synthetic ABT
Cooked apple	1.00 g Pink Lady apple baked to 180°C/60' and blended in synthetic ABT
Cooked plum	1.50 g plum jam[h] in synthetic ABT
Wood	0.50 mg wood aroma[c] in synthetic ABT
Vinegar	Synthetic ABT
Taste	
Sour	Synthetic ABT without sugars
Sweet	Synthetic ABT without acids, caramel E150d and caramel E150a
Bitter	Synthetic ABT without sugars

[a] Amarelli, Rossano, CS, Italy

[b] Pane Angeli, Desenzano sul Garda, BS, Italy

[c] FlavourArt, Oleggio, NO, Italy

[d] Cedral Tassoni S.p.a., Salò, BS, Italy

[e] Sunsweet Growers Inc, Yuba City, California, USA

[f] Abicci Frutta Secca S.r.l., Somma Vesuviana, NA, Italy)

[g] Il Frutto Permesso, Bibiana, TO, Italy

[h] Zuegg S.p.a., VR, Italy

[x] For all solution 10 g of synthetic ABT were used

Table 3. F-ratios and corresponding significance levels for the two-way ANOVA (judges, products) performed for each sensory descriptor

	Judges	Products	J × P
Liquorice	1.23 ns	63.87 ***	0.34 ns
Orange	0.68 ns	93.28 ***	0.99 ns
Lemon	0.45 ns	0.87 ns	1.28 ns
Blackcurrant	0.70 ns	9.24 **	0.65 ns
Plum	1.62 ns	1.03 ns	0.70 ns
Tamarind	2.36 ns	289.13 ***	0.30 ns
Dried plum	0.21 ns	185.85 ***	0.65 ns
Raisin	0.31 ns	14.34 **	0.73 ns
Cider	1.27 ns	0.62 ns	0.45 ns
Caramel	3.44 *	285.37 ***	1.72 ns
Cherry jam	0.92 ns	123.62 ***	0.21 ns
Honey	1.09 ns	361.89 ***	0.21 ns
Cooked must	0.81 ns	177.55 ***	0.46 ns
Cooked apple	1.58 ns	76.55 ***	0.47 ns
Cooked plum	0.76 ns	247.41 ***	0.16 ns
Wood	3.14 *	171.11 ***	0.80 ns
Vinegar	1.18 ns	65.59 ***	0.11 ns
Sour	0.71 ns	65.50 ***	0.49 ns
Sweet	0.82 ns	18.67 ***	0.69 ns
Bitter	1.13 ns	183.05 ***	0.40 ns

(F-ratios marked with asterisk indicate significance at: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns not significant difference; J × P = judges × products)

Table 4. Mean intensity rating of sensory descriptors of three type of ABT and results of one-way ANOVA with Duncan's test (ABTM: Aceto Balsamico Tradizionale di Modena; ABTRERED: Aceto Balsamico Tradizionale di Reggio Emilia "Red Label"; ABTRESILV: Aceto Balsamico Tradizionale di Reggio Emilia "Silver Label")

	ABTM	ABTRERED	ABTRESILV	Significance
Liquorice	3.4 a	5.2 b	3.1 a	***
Orange	2.9 b	1.2 a	4.2 c	**
Lemon	1.5	1.4	1.2	ns
Blackcurrant	2.1 b	1.4 a	1.4 a	***
Plum	0.8	0.5	0.5	ns
Tamarind	6.3 c	2.3 a	4.4 b	**
Dried plum	3.3 a	4.0 b	7.3 c	**
Raisin	3.2 b	2.1 a	2.3 a	***
Cider	0.4	0.4	0.3	ns
Caramel	4.6 c	1.3 a	2.3 b	**
Cherry jam	7.2 b	6.2 a	9.2 c	**
Honey	6.0 b	1.2 a	1.2 a	**
Cooked must	7.5 c	3.4 a	5.2 b	**
Cooked apple	3.2 c	2.2 b	1.2 a	***
Cooked plum	7.5 b	4.3 a	9.1 c	**
Wood	2.7 b	0.4 a	0.4 a	**
Vinegar	6.1 a	8.2 c	7.1 b	***
Sour	9.2 b	9.1 b	7.3 a	***
Sweet	2.2 b	1.2 a	1.5 a	***

Bitter	3.6 c	0.4 a	1.2 b	**

(Mean values within column with the same letter are not significantly different at $P \leq 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; “ns” indicates not significant)