Quantitative analysis of flavour volatiles detects differences among closely related traditional cultivars of tomato

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Abstract: Volatile compounds with a major contribution to aroma have been quantitatively determined in four traditional tomato cultivars and one commercial F1 hybrid. One of the traditional cultivars was the most appreciated for flavour and overall acceptability in tests performed using a panel of 30 untrained tasters. The same cultivar showed significantly higher contents of hexanal and *cis*-3-hexenal volatile compounds, which have been previously reported to be two of the most important contributors to tomato flavour. On the basis of a small number of fruits per cultivar, significant differences among very closely related tomato cultivars can be detected for volatile aromas, thus allowing the use of the determination of volatiles as a possible tool in tomato breeding programs, making even the selection of single plants possible.
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INTRODUCTION

Poor flavour in tomato fruit is a serious consumer concern. It could be said that tomato flavour has declined as variety selection and tomato production has emphasized yield, fruit size, firmness, lack of defects, disease resistance and processing performance (°Brix, consistency), and not the sensory aspects of fruit quality.[1,2] With the availability of tomato all the year round and with the spread of long shelf-life varieties, consumers have begun to complain about tomato flavour. Indeed, consumers frequently associate recent varieties with a lack of flavour, although such an association has not been proved.[3] Sugars and organic acids are major components of tomato fruit and account for *ca* 60% of dry matter. They contribute to soluble solids (°Brix) and are essential factors in overall flavour intensity.[4,5] Flavour is also a function of aroma components, and it is clear that the aroma of tomatoes plays an important role in consumer acceptability.[6] However, the importance of taste and aroma in tomato flavour has never been firmly established.[7] Volatile compounds contribute to the tomato overall aroma intensity and numerous studies have been devoted to identifying the major constituents responsible for tomato aroma. Some fruits or vegetables have one or two odour-impact compounds that dominate the flavour of that particular commodity. This is not the case for tomato, however, since no single compound has been found in this fruit that is reminiscent of a ripe tomato.[8] Over 400 compounds have been identified in tomato,[9] and some of them, such as *cis*-3-hexenal, *trans*-2-hexenal, 1-hexanal, *cis*-3-hexan-1-ol, hexanol, 2-isobutylthiazole and 6-methyl-5-hepten-2-one, are considered important flavour-contributing agents.[10,11] However, the effect of genetic variation and growing conditions of tomato on aroma compounds is not well understood. Reasons for this lack of information are the complexity of analysis of volatiles,[10] the difficulty in developing a consistent methodology for sensory evaluation, and the challenge to link these analytical tools to well-defined raw materials.[12] In addition, little quantitative data is available on tomato flavour volatiles.

The tomato was probably domesticated in Mexico, although the first transfer of varieties to Europe

was made by Spanish explorers.[13] Spain and Italy were the first European countries in which the tomato gained agricultural importance. Starting with its introduction into Spain, tomato began a process of diversification and adaptation to the different agroclimatic conditions of different localities. In this process of selection–differentiation, special attention was paid to organoleptic quality, and a great array of traditional tomato cultivars have originated in many Spanish regions.[14] Several traditional cultivars still survive in the orchards of southern Spain. They are frequently ignored outside their production area, but all of them are highly esteemed by local people due to their excellent quality. In local markets, traditional cultivars sell for three to six times the price of the hybrid varieties, as is the case for two types of local cultivars, the 'Muchamiel' and the 'De la pera'. Although cultivated tomato has a very narrow genetic base,[15] there is a considerable diversity of cultivars which differ in characteristics such as shape, firmness, soluble solid contents, etc. For example, we have found considerable levels of diversity for micronutrient content between different forms of the 'Muchamiel' and the 'De la pera' cultivars.[16] It would be interesting to find out whether there is also variability of the volatile aroma compounds. In the present study we have used a system specifically designed for the analysis of volatile organic compounds in water, called the purge-and-trap sample concentrator, which has been set up to quantify the volatile aroma constituents of fruits.[17] Among the high number of volatiles identified in tomato, a few make a major contribution to aroma, such as hexanal, cis-3-hexenal trans-2-hexenal, cis-3-hexenol and hexanol.[6,10,18,19] These are the compounds that show highest differences in level between varieties,[3,7] and the compounds most influenced by factors such as storage[20] or water supply.[21] The objective of this study is to try to find the differences among closely related traditional tomato varieties for some important volatile compounds. The development of a simple procedure to determine volatile aroma concentrations could be very useful as a possible tool in tomato breeding programmes for selecting genotypes with better quality characteristics.

EXPERIMENTAL
Plant material and growing conditions
Four traditional varieties, two of the 'Muchamiel' type (MUCH4 and MUCH18), two of the 'De la pera' type (PER1 and PER5), and the most frequent commercial variety grown in the area (BOND, an F1 hybrid cultivar distributed by the commercial company Seminis Iberica SA), were grown in hydroponics in greenhouses under homogeneous conditions, over an autumn–winter growing cycle. Fruits of the 'Muchamiel' cultivars are large in size (>200 g), flattened and strongly ribbed, while those of the 'De la pera' type weigh between 100 and 200 g, varying from rectangular to an elongated-oval shape, without ribs.

Samples
Fruit in the same stage of ripening, with >90% of the surface showing red colour, were harvested for the five cultivars. Fruits were randomly separated into two groups for chemical and sensory analysis.

Organoleptic tests
For sensory analysis fruits were washed and cut into wedges. The organoleptic quality of four cultivars (MUCH4, MUCH18, PER5 and BOND) was evaluated through the panel difference method. The tests were conducted according to the ranking method.[22] An incomplete-block design was used,[23] with 40 blocks of three samples per block. Using an index of 1 (bad) to 3 (good), 30 untrained tasters ranked the three fruit samples from the four cultivars, according to flavour, texture and overall acceptability. Significant differences between cultivars were determined by comparing the Durbin statistic to the X^2 value at t-1 degrees of freedom, with $t = 4$.

Analytical determinations
Based on previous experiments, four fruits per variety were individually analysed. Three repeated analytical measurements were made for each fruit. Immediately after the fruits had been juiced, using a domestic juice extractor, 10 ml samples were frozen and stored at $-81\,^{\circ}$C until they were analyzed for volatile composition. The soluble solids content (SSC) was determined in the remaining juice with an Atago PR-100 digital refractometer (Atago Co Ltd, Tokyo, Japan), the results being expressed in °Brix. Total acidity (TA) was measured by titration with 0.1 M NaOH, and presented as g kg^{-1} of citric acid. Fruit juice colour was measured with a Minolta CR-200 colorimeter (Minolta Camera Co Ltd, Osaka, Japan) adapted for juice, in order to obtain the CIELAB L, a and b parameters. Parameter a is a green-to-red scale, and b a blue-to-yellow scale.

Volatile composition
A system specifically designed for the analysis of volatile organic compounds in water, called the purge-and-trap sample concentrator, has been set up to quantify the volatile aroma constituents of tomato fruits. The purge-and-trap system shows greater sensitivity than other techniques in the analysis of the volatile aroma components, so it needs lower amounts of sample per run (less than one fruit). This allows us to study the reproducibility of the analysis as well as the variability from one fruit to another, and even to analyze different parts taken from the same fruit.[17]

Equipment
An OI Analytical (College Station, Texas) 4560 purge-and-trap and a Hewlett-Packard 5890 (Wilmington, Delaware) gas chromatograph were used in the analysis of volatile aroma constituents. Some of

the instrumental parameters for the purge-and-trap and gas chromatography (GC) are as follows: purge-and-trap sample, 1 ml; trap, OI Analytical #10 (Tenax/silica gel/carbon molecular sieve); purge, He at 10 psi for 11 min at 25 °C; desorption, 4 min at 180 °C; transfer line, 180 °C; valve oven, 100 °C; gas chromatography column, DB-624 (J&W Scientific, Folsom, CA) 0.25 mm × 30 m × 1.4 mm; oven, 60 °C (5 min) then 3 °C min^{-1} to 220 °C (10 min); carrier gas, He at 1 ml min^{-1}; transfer line, 225 °C; FID, 250 °C. A flame ionization detector (FID) (Wilmington, Delaware, USA) is used to measure the separated compounds.

Procedure

The sample is placed in a purge-and-trap sample concentrator where an inert gas is purged through the sample. The volatiles travel out with the gas flow and are trapped onto a sorbent trap. After sample purging is completed, the trap is heated, and the volatiles are desorbed to the injector whose temperature is −100 °C (cooled with liquid nitrogen). When the desorption is finished, the injector is quickly heated to 210 °C, and the volatiles are injected 'on-column' into a gas chromatograph containing a capillary column. The separated compounds are detected by using an FID. During the GC separation the trap is baked at 180 °C with an inert gas (He) flowing in opposite direction to the purge flow. This prepares the trap for the next sample. Two repeated measurements were made for each fruit.

It is important to point out the differences between the head-space technique and the purge-and-trap system, also called 'dynamic head-space technique'. The static head-space method extracts a small amount of volatiles from a sample, since this technique only analyses the volatiles contained in the vapour phase which is in equilibrium with the liquid sample. Therefore, each peak of the chromatogram only represents a part of the total content of the sample. In contrast, the purge-and-trap technique is based on an efficient transference of all the volatile organic compounds from the aqueous to the gaseous phase by bubbling at room temperature an inert gas through a liquid sample contained in a specifically designed purging chamber. In preliminary studies, standards of each volatile compound were added to tomato samples in order to select convenient bubbling times for achieving complete transference of all the volatiles. The chromatogram peak then, represents the total content of the sample. This was true even with the compound *cis*-3-hexenal; although it is very unstable, the short time of bubbling (11 min at 25 °C) allowed total recovery of the compound. In order to check that a total transference has been achieved, no volatiles should be detected by a second bubbling using an already processed sample. This aspect of the purge-and-trap system, the total recovery of the compounds, allows the use of external standards to obtain calibration curves.[17]

Concentrations of the volatile compounds hexanal, *cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexenol and hexanol were calculated using regression equations, determined by placing four different concentrations of each standard in the purge-and-trap sample concentrator to obtain calibration curves as described previously.[17] The logarithm of odour unit values (log odour) was calculated from the ratio of the concentration of a component to its odour threshold, using the values of threshold determined by Buttery *et al*.[24] Volatile compounds with positive odour units are assumed to contribute to the flavour of a food, while those with negative units are not.[8]

A variance components analysis (ANOVA with a nested or hierarchical design) was undertaken to estimate the amount of variability provided by each of the three factors in the experiment: variability among cultivars, among fruits within each cultivar and among repeated measurements of the same fruit. Duncan's multiple-range test was used to establish possible significant differences among the volatile content of the different cultivars.

RESULTS AND DISCUSSION
Organoleptic tests

Tomato fruits from the 'Muchamiel' type are clearly distinguishable from those of the 'De la pera' type and from those of the hybrid variety on the basis of morphological characteristics, but fruits from cultivars of the same type are very similar and it is impossible to visually differentiate between them. However, in previous experiments all the cultivars analyzed in the present work have differed in yield characteristics (data not shown) and in some analytical parameters, such as micronutrient content.[16] The results of the organoleptic test presented in Table 1 suggest that these cultivars also differ in sensory aspects of fruit quality. Although the test panel was made up of untrained tasters, significant differences for flavour and overall acceptability, but not for texture, were detected. The cultivar PER1 was not included in the test in order to reduce the number

Table 1. Flavour, texture, and overall acceptability values for the four cultivars evaluated

Cultivar[a]	Flavour		Texture		Overall acceptability	
	Score[b]	Mean	Score	Mean	Score	Mean
MUCH4	71	2.4	64	2.1	74	2.5
MUCH18	58	1.9	66	2.2	57	1.9
PER5	66	2.2	60	2.0	63	2.1
BOND	47	1.6	55	1.8	53	1.8
T value[c]	12.375**		2.89^{NS}		9.86**	

[a] Only one of the two cultivars of the De la pera type was evaluated.
[b] Each value is the sum of scores of 30 tasters, on a 1 (bad) to 3 (good) scale. Maximum = 90.
[c] T = Durbin statistic. ** Significant at $p \leq 0.01$. NS = not significant at $p \leq 0.01$.

of samples presented to the panellists, since a large number of samples makes the detection of differences more difficult In addition, previous tests had shown only slight sensory differences between 'De la pera' cultivars. The cultivar MUCH4 received the highest scores for flavour and overall acceptability, being significantly better even than the other cultivar of the same type, MUCH18. The second most appreciated cultivar was PER5, both for flavour and for overall acceptability. The hybrid BOND, in spite of being more adapted to greenhouse-growing conditions than the traditional varieties, received the lowest scores for the three aspects evaluated, although the differences for texture were not significant.

Analytical determinations

Colour parameters, SSC and TA

We are trying to develop a simple analytical procedure to compare quantitatively desirable flavour volatiles in tomato lines, since it would be a useful tool in selecting for a better tasting tomato. For practical reasons, fruits were visually selected on the basis of external colour, trying to obtain the most uniform stage of maturity for the fruits of each of the five genotypes. A variance components analysis was then performed on simple maturity analytical parameters to estimate the amount of variability provided by the factors 'cultivars', 'fruits within each cultivar' and 'error' (repeated measurements of the same fruit) (Table 2). For the juice colour parameters, as expected, cultivar was the factor that gave rise to most variance. There was low variability among fruit samples and very low variance due to the error. In spite of the fruits being selected according to a homogeneous degree of maturity, for SSC and TA the amount of variability among fruits was important and higher than the variation associated with differences among cultivars. This result should be taken into account when considering volatile aroma analysis, since volatile aroma content also varies with maturity.[8,25] Part of the variability for the volatile compounds concentrations will be due to inevitable differences among the internal maturity stages of the fruits analyzed.

Volatile compounds

Figure 1 shows a typical purge-and-trap/GC pattern of tomato fruit. Differences among varieties represented between 23 and 52% of the total variation for volatile contents. Between 5 and 47% of the variability was due to differences among fruits of the same cultivar, and the error associated to the analytical determination ranged from 21 to 72% (Table 2). This analytical error was particularly high for the compound $trans$-2-hexenal. Due to the complexity of volatile compounds analysis, there is always a high intrinsic variability associated with quantitative data, as in this case. However, the ANOVA detected significant differences among cultivars for all the analyzed compounds ($p < 0.05$). The differences for $trans$-2-hexenal, cis-3-hexenol and hexanol were very low, and those for hexanal and cis-3-hexenal were of greater importance.

Data available in the scientific literature about quantitative analysis of tomato volatiles, in addition to being scarce, shows values over a wide range. This may be partially due to differences in the plant material analysed, but the main factor is probably the analytical method used by the different authors. For example, the values we have obtained for hexanal content (ranging from 2.33 to 5.16 mg kg^{-1}) are similar to the 3.1 mg kg^{-1} cited by Buttery et al[26] in fresh tomato, although others authors give values ranging from 0.05 to 12.3 mg kg^{-1}.[27,28] For cis-3-hexenal content, our data varies between 1.51 and 2.47 mg kg^{-1}, while data reported by other authors ranged from 0.004 to 12 mg kg^{-1}.[26,29] The same is true for the other compounds analyzed.

All the cultivars were grown under greenhouse conditions over an autumn–winter cycle and, as mentioned before, the modern variety BOND should be more suited to these growing conditions. However, BOND showed the lowest concentrations of all the compounds analyzed (although BOND yield under greenhouse is two to three times that of traditional cultivars). For some compounds we have found significant differences even between cultivars of the same type. For example, the cultivar MUCH4 showed higher content for the hexanal and cis-3-hexenal

Table 2. Variance components (%) obtained from a nested ANOVA for the analytical parameters

Source of variation	df	Colour parameters, SSC and TA				
		a	b	L	SSC	TA
Among cultivars	4	73.4	48.8	81.6	40.6	29.2
Among fruits	15	25.8	44.2	15.0	55.6	66.5
Error	40	0.8	7.0	3.4	3.8	4.3
Total		100	100	100	100	100

Source of variation	df	Aroma volatiles				
		Hexanal	cis-3-Hexenal	$trans$-2-Hexenal	cis-3-Hexenol	Hexanol
Among cultivars	4	52.0	25.3	23.3	28.0	30.6
Among fruits	15	26.7	24.1	5.0	24.4	46.8
Error	20	21.3	50.6	71.7	47.6	22.6
Total		100	100	100	100	100

1 hexanal
2 *cis*-3-hexenal
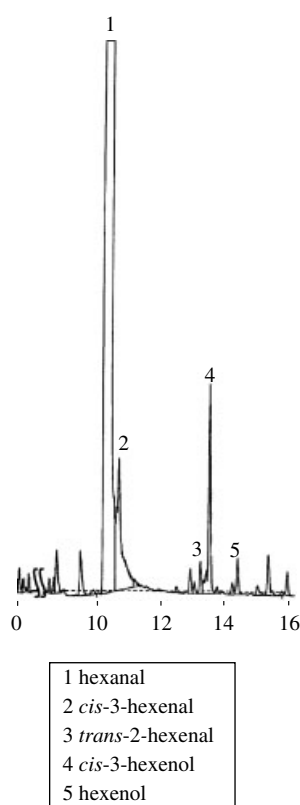3 *trans*-2-hexenal
4 *cis*-3-hexenol
5 hexenol

Figure 1. Purge-and-trap/GC pattern of tomato fruit, cultivar MUCH4.

compounds than the two 'De la pera' cultivars and MUCH18. Cultivars PER1 and PER5 showed the highest content of *trans*-2-hexenal, *cis*-3-hexenol and hexanol, although the differences were not important and in only a few cases were statistically significant (Table 3). All the compounds analyzed showed positive odour units (were present at a concentration above their threshold) and, according to Baldwin *et al*,[8] are assumed to contribute to the flavour of the fruits. None the less, because of possible interactions with other compounds, odour unit values might not give a clear indication of an individual aroma compound's contribution when in a complex mixture.[2]

Using principal component analysis,[21] relationships between aroma volatiles and sensory attributes determined by quantitative description analysis were

found in tomatoes. *cis*-3-Hexenal, the most odour-active compound, was associated with the flavour attributes 'fruity' and 'sweet'. This compound is probably the most important contributor to tomato aroma and flavour.[6,30] The hexanal odour has been described as 'green, herbaceous'. In three seasonal studies, sweetness intensity was related to hexanal, with contributions from *cis*-3-hexenal, *trans*-2-hexenal or *cis*-3-hexenol.[8]

The fact that MUCH4, the most appreciated cultivar in the organoleptic test, was also the cultivar with the higher content of hexanal and *cis*-3-hexenal, two of the most important contributors to tomato aroma, is a suggestive result. However, we should not forget that both the absolute concentrations of sugars and organic acids and the balanced ratio between them are also important factors in consumer acceptance. Increasing total sugar and organic acid levels of fresh tomato improved flavour acceptability,[31,32] and a balanced sugar/organic acid ratio was preferred by a panel examining the flavour characteristics of cherry tomato.[33] However, since we have found no important differences between cultivars for SSC and TA, it is therefore more likely that the contribution from the aroma of volatiles was more important in the panellist appreciation. In this sense, Baldwin *et al*[7] found that tomato-like flavour intensity ratings for seven cultivars were almost identical to the rating for overall acceptability, indicating the close relationship between flavour and tomato quality.

The situation, however, is not so straightforward; even after extensive research on tomato flavour compounds there is little definitive information on the relationship between flavour/aroma compounds and sensory flavour perception.[2] Baldwin *et al*[7] conducted extensive research to find correlations between sensory data and measurements of volatile compounds, but their conclusions were not definitive. Thus far, flavour quality for tomato has been an elusive trait and we still lack quantifiable definition for tomato flavour.[8] There is no complete agreement about which flavour compounds are important and what the appropriate levels and balance for good flavour are. For example, the recommendations of Tandon *et al*[30] directly contradict those of Gray *et al*,[34] who recommended increasing levels of C6 aldehydes. Its is likely that the desirable levels of

Table 3. Volatile compounds concentration (mg kg^{-1}) and their log odour units (log *U*)

Cultivar	Hexanal		*cis*-3-Hexenal		*trans*-2-Hexenal		*cis*-3-Hexenol		Hexanol	
	mg kg^{-1}	log *U*	mg kg^{-1}	log *U*	mg kg^{-1}	log *U*	mg kg^{-1}	log *U*	mg kg^{-1}	log *U*
MUCH4	5.16[a]	3.06	2.47[a]	3.99	0.93[b]	1.74	0.96[b]	1.14	0.73[b]	0.16
MUCH18	3.35[b]	2.87	1.93[ab]	3.89	0.96[b]	1.75	0.98[b]	1.15	0.77[a]	0.19
PER1	3.26[b]	2.86	1.01[c]	3.61	1.03[ab]	1.78	1.03[ab]	1.17	0.79[a]	0.20
PER5	3.61[b]	2.90	0.89[c]	3.55	1.13[a]	1.82	1.12[a]	1.20	0.78[a]	0.19
BOND	2.33[c]	2.71	1.51[bc]	3.78	0.91[b]	1.73	0.93[b]	1.12	0.71[b]	0.15
SD	0.58		1.04		0.12		0.11		0.05	

Values followed by different letters within a column are significantly different at $p < 0.05$ (Duncan's test). SD: standard deviation; sample size: $n = 40$.

the different volatiles depend on the target market. In the southeast of Spain consumer preferences for traditional cultivars varies even between neighbouring regions. For future breeding purposes, consumer testing might be required to determine which are the levels of compounds that would enhance the acceptability of tomatoes.

Poor flavour quality in tomato appears to be, in part, a result of breeding practices that do not select for flavour, because of lack of information.[8] Sensory parameters that could assist the breeders in an efficient selection for flavour have not been characterized. The definition and use of markers that correlate with tomato flavour could improve this situation and provide the breeder and processor with analytical tools for flavour enhancement.[12] Breeders could also use sensory analysis, but this is often difficult to perform and requires access to a panel and considerable expertise.[8] Quantitative comparison of desirable flavour volatiles, in addition to reducing sugars and free acids, in tomato breeding lines would be a useful tool in selecting for a better tasting tomato. However, several authors have reported that differences among tomato cultivars for volatile compounds are not important. For example, Buttery et al[11] found no marked differences for the concentrations of volatiles of more than 10 different tomato commercial lines. The main quantitative differences seemed to be caused by variations in degrees of ripeness or by the storage conditions. In contrast, Baldwin et al[8] have reported significant differences between cultivars in levels of important aroma compounds. The different analytical methods used could probably justify these discrepancies. Our results show that the analytical method we used is valid to detect quantitative differences among tomato varieties. It is particularly interesting that we were able to detect differences among very closely related cultivars of the same type. Modern genetic and genomic tools are currently being intensively applied to the tomato. Simple sequence repeat (SSR) or microsatellites are becoming the preferred molecular markers in crop breeding, and they are the most practical markers for variety identification. He et al[35] have recently found that the combination of only five selected SSR loci could differentiate all the 19 tomato cultivars they were studying. However, using these five SSRs we have found no differences between the cultivars MUCH4, MUCH 18, PER1 and PER5 (Ruiz JJ unpublished), indicating the great similarity among them.

CONCLUSIONS

Methods to analyze volatile compounds that need large amounts of tomato samples are not useful for selecting individual genotypes. As analysis of flavour compounds in the aromatic component requires expensive equipment and training; if determination of volatiles is to be used as a tool in selection programs,

a low number of samples should be needed. We have found significant differences among closely related cultivars for selected volatile compounds using a small number of fruits per cultivar whose maturity stage had been visually judged. Tomato aroma is complex, probably a combination of more than 16 compounds give tomato its unique odour characteristics. However, reducing the number of compounds to a few with major contributions to aroma could increase the usefulness of volatile determinations in tomato breeding programmes. In this sense, although further investigations are needed, it is interesting that the most appreciated cultivar in the organoleptic tests was the one with high content of the volatile compounds hexanal and cis-3-hexenal.

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