Effect of Thermal Treatment during Processing of Orange Juice on the Enantiomeric Distribution of Chiral Terpenes

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ABSTRACT

Chiral terpenes in orange juices were examined by solid-phase microextraction–gas chromatography–mass spectrometry to study the dependence of their enantiomeric composition on the thermal treatment applied during the industrial manufacture. The experimental conditions used in the isolation and concentration of the compounds of interest produced relative standard deviations ranging from 2.9 to 15.1% when absolute areas were used and from 1.7 to 18.3% when normalized areas were used. Recovery varied between 8.8 and 56.1%, and detection limits ranged from 0.11 to 0.32 $\mu\text{g/ml}$. The enantiomeric compositions of the majority of the chiral terpenes varied within a reasonably narrow range. Nevertheless, the enantiomeric ratio of two monoterpene alcohols, α -terpineol and linalool, exhibited considerable variation according to the thermal treatment used in the manufacture of the juices. Therefore, the knowledge of the enantiomeric composition of α -terpineol and linalool might be useful for thermal treatment control purposes.

Orange juice is one of the most popular fruit beverages because it contains useful nutrients, vitamin C, fiber and antioxidants, which may reduce the risk of death from coronary heart disease (15, 34) and cancer (9). In recent reports, nutritionists have recommended the regular consumption of citrus fruits to promote health (5). The desirable and delicate flavor of juice is an important attribute of both fresh and processed citrus juices. Orange juice aroma is believed to result from the combination of various highly aromatic compounds (27, 28). Orange juice flavor components include aldehydes, esters, ketones, and hydrocarbons (21), some of which are terpenic compounds regarded as the main contributors to flavor (1). Some researchers have reported that processing techniques can affect orange juice compounds (31); specifically, aroma compounds of citrus juice can be strongly modified by the various treatments applied during industrial processing (18). Therefore, the efficient control of thermal treatments is important.

The evaluation of the enantiomeric composition of chiral compounds in foodstuffs has become an area of great interest over the last few years (2). The enantiomeric ratio can be altered by such things as storage, fermentation processes, and thermal treatments (11, 20, 29); therefore, this ratio may be indicative of the effects of processing, contamination, adulteration, and ageing. Thus, the enantiomeric ratio may be used as a reliable indicator of food quality. The evaluation of the enantiomeric distribution of chiral compounds has been used to assess processed foods that have undergone different heat treatments (22). Nonetheless, despite the recognized usefulness of chirality, it is not usually an aspect considered in food analysis, mostly because

of the lack of analytical methods suitable for reliable determination of the enantiomeric composition.

Some researchers (4, 6–8, 17) have been working on the development of new methods of analysis that overcome the limitations of conventional methods and will result in reliable chiral analysis of foodstuffs. Analysis is complicated because it requires the effective isolation of the compounds of interest from the matrix, but experimental conditions that may cause racemization and, thus, alter the original enantiomeric ratio must be avoided. We recently used solid-phase microextraction (SPME) methodology (3) for enantiomeric composition studies (24, 26) because it can be used to isolate the chiral compounds of interest from other components in the matrix under nonracemization conditions. SPME is a relatively new and simple technique for the isolation of headspace flavor compounds that does not involve solvent extraction. Over the past few years, researchers have applied this methodology to the isolation and concentration of a number of analytes from various matrices, although food aroma analysis is one of the most important applications of this technique (33). SPME has been used to extract volatile components from orange juices (13, 14), and to determine the enantiomeric composition of chiral components in foods and beverages (10, 19, 24). Although no adverse effects of the different enantiomers of the chiral terpenes in orange juices have been so far described, the determination of their enantiomeric distribution may be useful for quality assessment and specifically for the improvement of processing techniques. To our knowledge, no studies combining evaluation of the type of thermal treatment used in the manufacture of processed orange juices and the enantiomeric composition of chiral compounds have been reported.

The aim of this research was to evaluate the effect of

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the thermal treatment regimen utilized in the processing of orange juices on the enantiomeric composition of chiral terpenes in the juice. Another objective was to establish the usefulness of the enantiomeric distribution of chiral terpenes as an indicator to monitor the industrial processing of orange juice and as a contributor to the development of food-labeling regulations.

MATERIALS AND METHODS

Materials. Linalool, β -*trans*-caryophyllene, α -phellandrene, α -pinene, β -pinene, terpinen-4-ol, and limonene were obtained from Sigma-Aldrich (Dorset, UK). Dihydrocarveol, isopulegol, menthol, and α -terpineol were supplied by Sugelabor (Madrid, Spain), and citronellal and β -citronellol were supplied by Fluka (Steinheim, Switzerland). Linalool, α -phellandrene, dihydrocarveol, isopulegol, citronellal, menthol, and β -citronellol were purchased as racemic mixtures, but terpinen-4-ol, limonene, and α -terpineol standards were obtained as the (+)-enantiomers, with enantiomeric purities of 94, 97, and 96%, respectively, and α - and β -pinene and β -*trans*-caryophyllene were acquired as the (–)-enantiomer (96% enantiomeric purity in all cases). A 0.5-ml volume of a standard solution (1 μ l of each terpene per 10 ml of MeOH, high-performance liquid chromatography grade) containing the compounds of interest was used to estimate recoveries, detection limits, and linear range. The elution order for racemic mixtures was obtained from previous studies (24, 25) and from other literature. Identification of terpenes in samples was carried out by comparison of retention times with standards run under identical experimental conditions and by mass spectrometry (MS).

The orange juice samples considered in this study consisted of five commercially available processed orange juices purchased from the supermarket and declared to have been subjected to pasteurization (samples A, B, D, and E) or to ultrahigh temperature (UHT) treatment (sample C) and one nonprocessed fresh-squeezed orange juice (sample F).

SPME. An SPME holder (Supelco, Bellefonte, Pa.) was used to carry out the experimentation. A fused silica fiber coated with a 100- μ m layer of polydimethylsiloxane was used to trap volatile terpenes released from the orange juices. Prior to use, the fiber was conditioned at 250°C for 30 min.

To perform the extraction, 1.0 ml of the juice was placed in a 5.0-ml vial, which was sealed with plastic film. Subsequently, the SPME fiber was exposed to the headspace of the sample for 2 min at 60°C. Constant sample stirring was essential at all times to facilitate the release of the investigated compounds from the matrix. The analytes then were thermally desorbed into the gas chromatography (GC) injector at 250°C for 5 min and analyzed by GC.

GC-MS analysis of extracts obtained by SPME. A Hewlett-Packard model 6890 gas chromatograph coupled to an Agilent 5989A quadrupole instrument (Palo Alto, Calif.) was used to perform all analyses. The source and the quadrupole temperatures were 230 and 100°C, respectively. Peak identification was accomplished by comparison of the mass spectra with those contained in the Wiley library and with those provided by standard terpenes. The GC separation was performed on a fused silica column (inside diameter, 25 m by 0.25 mm) coated with a 0.25- μ m layer of Chirasil- β -Dex (Chrompack, Middelburg, The Netherlands). Helium was used as the carrier gas at an initial flow rate of 1 ml/min, and the splitless mode was used in all cases. The injector was kept at 250°C throughout the experiment, and the GC column was programmed at 2°C/min from 70°C (10 min) to 170°C. Data

TABLE 1. Average recoveries and detection limits for the standard mixture

Compound	Recovery (%)	Detection limit (μ g/ml) ^a
(–)- α -Pinene	17.7	0.22
(+)- α -Pinene	17.7	0.21
(–)- α -Phellandrene	11.6	0.25
(+)- α -Phellandrene	11.6	0.25
(–)-Limonene	14.9	0.31
(+)-Limonene	14.9	0.26
(+)- β -Pinene	15.2	0.26
(–)- β -Pinene	15.2	0.25
(–)-Citronellal	8.8	0.31
(+)-Citronellal	8.8	0.31
(–)-Linalool	20.2	0.11
(+)-Linalool	20.3	0.12
(+)-Terpinen-4-ol	22.1	0.17
(–)-Terpinen-4-ol	22.1	0.17
(–)- α -Terpineol	22.2	0.15
(+)- α -Terpineol	22.2	0.15
(–)-Citronellol	13.7	0.32
(+)-Citronellol	13.7	0.32
(–)- β - <i>trans</i> -Caryophyllene	56.1	0.18
(+)- β - <i>trans</i> -Caryophyllene	56.1	0.18

^a Estimated from three replicates.

acquisition from the MS was performed using the Hewlett-Packard G1701BA ChemStation (revision B.01.00), which allows the control of both the GC and the MS systems.

RESULTS AND DISCUSSION

Recovery, detection limit, linear range, and repeatability of terpenes. The experimental conditions used in the extraction of the compounds of interest were selected on the basis of our previous experience (24). Because temperature and time were the most critical parameters for the SPME procedure, different experimental values (30, 45, and 60°C; 2, 5, and 15 min) were used to establish the best set of parameters for the highest recoveries of the terpenes. As a result, 60°C and 2 min were finally selected as the best conditions; temperatures lower than 60°C yielded unacceptable peak areas when the material absorbed on the fiber was analyzed by GC. Moreover, the use of high extraction times at 60°C also resulted in loss of terpenes.

Some terpenes (α -pinene, β -pinene, α -phellandrene, limonene, linalool, citronellal, terpinen-4-ol, α -terpineol, β -citronellol, and β -*trans*-caryophyllene), whose presence in orange juice and other beverages has been described previously (13, 14, 24, 30), were initially selected for analysis. Other chiral terpenes (dihydrocarveol, menthol, and isopulegol) were included later, although none of these terpenes were found subsequently in orange juice samples.

Table 1 includes the average recoveries and detection limits obtained for the standard mixture from at least three replicates. Recoveries were estimated by comparison of the areas of the compounds extracted using SPME from the pure standard mixture of terpenes and the areas obtained by direct injection (splitless mode) of 0.4 μ l of the same

TABLE 2. Relative standard deviation (RSD) obtained from SPME-GC analysis of an orange juice sample

Compound	RSD (%) ^a	
	Absolute areas	Normalized areas ^b
(+)-α-Pinene	8.1	2.1
(-)-α-Phellandrene	2.9	18.3
(-)-Limonene	14.7	1.8
(+)-Limonene	6.9	1.7
(-)-Linalool	14.6	8.3
(+)-Linalool	12.7	5.0
(+)-Terpinen-4-ol	11.8	8.4
(-)-Terpinen-4-ol	14.8	9.4
(-)-α-Terpineol	15.1	6.8
(+)-α-Terpineol	6.3	6.5
(-)-β-trans-Caryophyllene	14.6	6.6

^a Estimated from the average value ($\bar{x}$) of three replicates and the standard deviation (σ_{n-1}): $RSD = (\sigma_{n-1}/\bar{x}) 100$.
^b Calculated by dividing each individual peak area by the total peak area (sum of the areas of all recorded terpene peaks).

pure standard mixture into GC. Likewise, detection limits were calculated from the peaks giving a signal equal to five times the detector baseline noise (established from the width of the baseline over a certain period of time). Recovery values ranged from 8.8% for (-/+)-citronellal to 56.1% for (-/+)-β-trans-caryophyllene (Table 1). Similar results have previously been reported by other authors for typical volatile components of fruits (12) and for volatile hydrocarbons in water (32), reaching recovery values ranging between 10.7 and 45.3% and between 4.0 and 25.0%, respectively. These relatively low recoveries can be explained by the fact that during the SPME procedure equilibrium is established among the concentrations of the analyte in the headspace above the sample and in the polymer coating on the fused silica fiber. As a result, the mass of analyte absorbed by the coating depends on the partition of this analyte between the fiber coating and the sample (i.e., the distribution constant for the analyte). This approach may make it difficult to establish equilibrium in such a way that high percentages of the analyte are eventually retained

on the fiber. Moreover, the small diameter of the fiber and consequently the small volume of the fiber coating also contribute to making the complete extraction of analyte difficult. Regarding detection limits of the target compounds, values ranged from 0.11 and 0.12 μg/ml for (-)- and (+)-linalool, respectively, to 0.32 μg/ml for (-/+)-citronellol. Recoveries in the present study reflect the semiquantitative character of SPME (24), whose main advantages are speed, simplicity, and low cost.

The linear range, obtained from SPME extraction of the standard solution under the same experimental conditions as used for the orange juice samples, ranged from 0.5 to 50 μg for all compounds. The terpene concentrations were within this range in all samples.

The repeatability of the headspace sampling technique was estimated by measuring the relative standard deviation from a minimum of three replicates of all orange juices under the experimental conditions.

Table 2 displays the relative standard deviations for orange juices in terms of absolute peak areas and peak areas normalized to the sum of the areas of all recorded terpene peaks obtained from the GC analysis. The relative standard deviations ranged from 2.9 for (-)-α-phellandrene to 15.1% for (-)-α-terpineol when absolute areas were used and from 1.7% for (+)-limonene to 18.3% for (-)-α-phellandrene when normalized areas were employed. Although this latter range appeared to be slightly wider than that for absolute areas, generally speaking, the relative standard deviations obtained from normalized areas were lower than those from absolute areas. Actual average for absolute areas was around 10%; that for normalized areas was around 6%.

Enantiomeric compositions and concentrations of chiral terpenes in orange juice samples subjected to different thermal treatments. Table 3 shows the concentrations (μg/ml) and enantiomeric excesses (%) of the investigated compounds in orange juice samples subjected to different thermal treatments. Concentrations were estimated from the orange juice samples spiked with the standard mixture of pure terpenes. In all instances, the enantiomeric excess was calculated from peak areas, and excess of the dominant enantiomer was expressed as a percentage:

TABLE 3. Dominant enantiomer, average (n = 3) enantiomeric excess (ee), and concentrations of the investigated terpenes in orange juice samples determined by SPME-GC-MS

Dominant enantiomer	Orange juice samples ^a											
	A		B		C		D		E		F	
	ee (%)	μg/ml	ee (%)	μg/ml	ee (%)	μg/ml	ee (%)	μg/ml	ee (%)	μg/ml	ee (%)	μg/ml
(+)-α-Pinene	100	5.9	100	35.2	100	12.5	100	9.9	100	2.7	100	4.3
(-)-α-Phellandrene	100	3.7	100	12.2	100	4.1	100	4.3	100	1.8	100	3.6
(+)-Limonene	98.9	1472.7	99.1	40.6	99.7	2866.9	98.9	1788.8	99.1	728.1	98.8	928.8
(+)-Linalool	85.5	319.8	82.2	29.7	79.4	29.9	64.1	205.9	36.6	63.2	77.8	115.7
(+)-Terpinen-4-ol	20.9	24.8	51.0	34.2	42.7	43.1	30.5	64.2	29.2	52.2	28.3	10.9
(+)-α-Terpineol	64.5	20.9	79.8	27.4	54.4	55.3	81.3	108.7	75.9	59.9	100	13.1
(+)-β-trans-Caryophyllene	100	43.5	100	20.3	100	83.2	100	21.5	100	8.4	100	4.6

^a A, B, D, and E, pasteurized orange juice; C, UHT-treated orange juice; F, nonprocessed fresh-squeezed orange juice.

$$\frac{[(\text{dominant enantiomer} - \text{minor enantiomer})/(\text{dominant enantiomer} + \text{minor enantiomer})] \times 100.}$$

The terpene (+)-limonene was clearly the major component, followed in most cases by linalool, α -terpineol, and terpinen-4-ol. Likewise, (+)- α -pinene and (–)- α -phellandrene were minor constituents and the concentration of β -*trans*-caryophyllene differed across samples. Samples A, C, and D were in general richer in volatile terpenes than were samples E, F, and B. In particular, sample B concentrations of the main terpene, (+)-limonene, were substantially lower than those of the rest of the orange juice samples studied.

With regard to the enantiomeric composition, (+)- α -pinene, (–)- α -phellandrene, and (–)- β -*trans*-caryophyllene were present as a pure enantiomer in all samples, including the fresh-squeezed orange juice (sample F). In the same way, the enantiomeric distribution of (+)-limonene appeared to be very close to 100% in all cases. These results are in agreement with those reported by other authors, who also established the presence of the pure (+)-enantiomers of α -pinene and limonene in orange essential oils (16) and with data obtained in our laboratory using the same approach in orange beverages (24) and concentrates (23).

Although to our knowledge the presence of β -*trans*-caryophyllene in orange beverages had not been previously reported, the enantiomeric excess value found in the present study supports statements by others who have considered the natural occurrence of both enantiomers for sesquiterpenes unusual (16). These results indicate that heat treatment does not affect the enantiomeric purity of (+)- α -pinene, (–)- α -phellandrene, and (–)- β -*trans*-caryophyllene in orange juices. In contrast, linalool, terpinen-4-ol, and α -terpineol varied considerably among samples, with the (+)-enantiomer dominant in all instances.

The compound terpinen-4-ol has already been proven to be variable in nature, exhibiting not only different enantiomeric composition values but also a different dominant enantiomer according to the natural or artificial character of the aroma (24) or to geographic origin (23). In a study of various blackcurrant varieties (25), we established that although the (+)-enantiomer always prevailed, the enantiomeric purity of terpinen-4-ol was significantly variable depending on the cultivar. In the present work (Table 3), the value obtained for fresh-squeezed juice was very similar to those obtained for samples D and E. However, the variation observed in samples A, B, and C appears to support the uselessness of this terpenic compound for thermal treatment evaluation.

A different situation was observed for linalool and α -terpineol. Both compounds have already proven to have stable enantiomeric composition in orange concentrates (23) and blackcurrant varieties (25) and therefore their usefulness in determining both the geographic origin and any variation in the manufacturing process of this kind of foodstuffs. On this occasion, a reasonably high enantiomeric excess for (+)-linalool was found in fresh-squeezed juice (77.8%). This observation agrees well with data obtained previously (23, 25). These values were stable across samples, except for sample E where a value as low as 36.6% was obtained. Considering the thermal treatment that this

juice had undergone, as indicated by the label designation, pasteurization and hence extensive heat treatments apparently greatly affect the enantiomeric composition of linalool. However, other samples subjected to pasteurization (A, B, and D) did not exhibit such a remarkable drop in the enantiomeric distribution of this terpene and therefore do not support this statement. As a consequence, the thermal processing used in the manufacture of sample E might be different from that declared in the label of the product.

As indicated in Table 3, enantiomeric excesses of around 80% were found for α -terpineol in most processed samples. These results are in agreement with those obtained previously for orange concentrates from different geographic areas (23). In that study, no alteration of the enantiomeric distribution of (+)- α -terpineol was found, even when racemization conditions were used during the extraction procedure. A clear exception was found for sample C, which had undergone UHT treatment during its industrial manufacture. The enantiomeric purity of the (+)- α -terpineol obtained for fresh-squeezed juice (sample F) and the previously mentioned stability of this compound in orange concentrates processed under racemization conditions indicate that any heat treatment used during the manufacturing process might alter slightly the original enantiomeric excess of this terpene. However, the lower enantiomeric excess obtained for sample C (54.4%) suggests that UHT treatment and, thus, higher temperatures even for short periods of time might result in a more remarkable decrease of the enantiomeric composition of (+)- α -terpineol by promoting the presence of the (–)-enantiomer, which is naturally absent in orange juice.

Figure 1 displays the chromatograms resulting from SPME-GC-MS analysis of orange juices subjected to pasteurization (Fig. 1a, sample E in Table 3), subjected to UHT treatment (Fig. 1b, sample C in Table 3), and fresh squeezed (Fig. 1c, sample F in Table 3). From a quantitative standpoint, (+)-limonene was by far the major component in all samples. This finding was not surprising because this compound has been regarded by others as one of the main aroma constituents in orange juice (13).

Although this study was mainly focused on chiral terpenes, some additional components of orange juice (Figure 1) were also detected by MS. In this way, although terpenic compounds were the most representative group in orange juice aroma, aldehydes, esters, and alcohols also were found. Among them, octanal and decanal were present in all samples. These compounds have been previously described as more relevant aroma-active constituents of orange juice than are terpenes (13); thus, their presence in the samples included in this study was expected. A reasonably similar profile was obtained for the chromatograms in Figures 1a and 1b (samples E and C). The fresh-squeezed orange juice (Fig. 1c, sample F) contained some constituents not detected in the rest of the samples. These compounds were some minor phenols, valencene, Δ -cadinene, and *E/Z*-citral.

The enantiomeric composition of some of these terpenes (α -pinene, α -phellandrene, limonene, and β -*trans*-caryophyllene) remained stable regardless the thermal treat-

FIGURE 1. Chromatograms resulting from SPME-GC-MS analysis (fiber coating, polydimethylsiloxane; vial size, 5 ml; extraction temperature, 60°C; extraction time, 2 min) of chiral terpenes from (a) orange juice subjected to pasteurization, (b) orange juice subjected to UHT treatment, and (c) fresh-squeezed orange juice. Lanes are numbered 1 through 23: 1, (+)- α -pinene; 2, (-)- α -phellandrene; 3, (-)-limonene; 4, (+)-limonene; 5, undecanoic acid, ethyl ester; 6, octanal; 7, (-)-linalool; 8, (+)-linalool; 9, acetic acid, 2-ethyl hexyl ester; 10, decanal; 11, Z-citral; 12, (+)-terpinen-4-ol; 13, (-)-terpinen-4-ol; 14, E-citral; 15, (-)- α -terpineol; 16, (+)- α -terpineol; 17, β -elemene; 18, geranyl acetate; 19, dodecanal; 20, valencene; 21, farnesene; 22, (-)-trans- β -caryophyllene; 23, Δ -cadinene. All chromatograms were recorded at the same full range.

ment employed during industrial processing. However, linalool, terpinen-4-ol, and α -terpineol showed variations with the heat treatment used. Although terpinen-4-ol has not been useful for thermal treatment monitoring purposes, the determination of the enantiomeric composition of linalool and α -terpineol might contribute to the efficient control of the processing of orange juice in industry.

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