# Use of the Enantiomeric Composition for the Assessment of the Authenticity of Fruit Beverages

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Enantiomeric compositions of chiral terpenes in commercial fruit beverages were examined by solid phase microextraction–gas chromatography (SPME-GC). Optimization of the method was accomplished on the basis of some parameters involved in the extraction, such as heating temperature and extraction time, that provided the highest peak areas, 60 °C and 2 min being the optimal values. With the proposed method relative standard deviation (RSD) values from three replicates ranging from 2 to 12% were obtained. The enantiomeric distribution of some terpenes remained constant, whereas other terpenes (linalool, terpinen-4-ol, and α-terpineol) exhibited a considerable variation among samples. This can be indicative of the eventual addition of aromas to some fruit beverages.

**KEYWORDS: Chiral; SPME; fruit beverages; terpenes; enantiomeric distribution**

## INTRODUCTION

In recent years, fruit juices and concentrates have become valuable products in the food industry. Although commercial beverage production currently uses a variety of fruits, orange is by far the most popular because of its pleasant and complex citrus flavor (*1*). It has long been believed that the delicate and desirable flavor of freshly squeezed orange juice is due to a complex mixture of many volatile constituents blended in the proper proportions (*2*). These constituents have been described to include mainly alcohols, aldehydes, esters, ketones, and terpenic hydrocarbons (*3*), of which, limonene, linalool, and α-pinene are considered to be the main components of orange juice flavor (*4*).

Terpenes have been largely proven to play an important role as aroma-active components, and their importance in the flavor industry and, in particular, their contribution to fruit aroma have been widely reported (*5*–*7*). In addition, recently there has been growing interest in terpenic compounds due essentially to the biological activity shown by some of them. In this regard, some sesquiterpene hydrocarbons have exhibited antioxidant capacity (*8*), whereas (+)-limonene has demonstrated to be effective in the prevention of chemically induced tumors (*9*).

On the other hand, the adulteration of juices, specifically orange juice, has become a significant problem in the beverage industry (*10*), resulting, consequently, in the need for the development of new methodologies. In this regard, several studies have demonstrated the usefulness of stereochemistry in the assessment of foods and beverages over the past few years (*11*). In fact, the enantiomeric purity has already been used successfully to evaluate the handling and adulteration of food products or to differentiate natural compounds from those of

synthetic origin, enabling, in short, the authenticity of foodstuffs to be guaranteed (*12*). In this way, the use of chirality as a tool for the control of the composition of fruit beverages can offer an interesting alternative to conventional methods.

Nevertheless, despite the usefulness of the enantiomeric composition, its determination is not usual in food analysis due basically to the lack of analytical methods able to accomplish enantiomeric separations of chiral compounds in such complex matrices. In fact, at present the development of new analytical methodologies suitable for isolating efficiently the compounds of interest from the matrix, avoiding their overlapping with other components and minimizing, in turn, the use of racemization conditions, needs to be studied.

In this regard, solid phase microextraction (SPME) is an analytical methodology capable of overcoming these limitations. SPME headspace sampling presents also additional advantages: it requires neither solvent extraction and purification steps nor a complicated purge-and-trap apparatus; it is applicable to a variety of analytes and samples and is well suited to the analysis of volatile compounds by gas chromatography (GC). SPME has been previously applied to the extraction of volatile components from juices (*10*, *13*, *14*), and the enantiomeric composition of chiral components in beverages by using SPME has been occasionally considered (*15*–*17*). However, either previous work by other authors did not focus on the enantiomeric distribution of terpenes or only a limited number of them were considered. In this respect, the use of the enantiomeric distribution of a higher number of terpenes was believed to be of special importance to increase the reliability of the results obtained.

The purpose of this work was to determine the origin of aromas as well as their addition to commercial fruit beverages on the basis of the enantiomeric composition of certain chiral terpenes.

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**Table 1.** Commercial Beverages Considered in the Study

| beverage | commercial label designation |
|---|---|
| A | dairy product containing orange juice plus natural and nature-identical aromas |
| B | dairy product containing orange juice plus unspecified aromas |
| C | dairy product containing various fruit juices, including orange juices, plus unspecified aromas |
| D | orange soft drink containing natural aromas |
| E | dairy product containing peach juice plus unspecified aromas |

## MATERIALS AND METHODS

**Materials.** $\beta$-*trans*-Caryophyllene, linalool, $\alpha$-phellandrene, $\alpha$-pinene, $\beta$-pinene, terpinen-4-ol, and limonene were supplied by Sigma-Aldrich (Dorset, U.K.), whereas dihydrocarveol, isopulegol, menthol, and $\alpha$-terpineol were purchased from Sugelabor (Madrid, Spain) and citronellal and $\beta$-citronellol from Fluka (Steinheim, Switzerland). Linalool, $\alpha$-phellandrene, dihydrocarveol, isopulegol, citronellal, menthol, and $\beta$-citronellol were achieved as racemic mixtures, whereas terpinen-4-ol, limonene, and $\alpha$-terpineol standards were obtained as the (+)-enantiomers, with enantiomeric purities of 94, 97, and 96%, respectively, and $\alpha$- and $\beta$-pinene and $\beta$-*trans*-caryophyllene were acquired as the (−)-enantiomer (96% enantiomeric purity in all cases). A 0.5-mL volume of a standard solution (1 $\mu$L of each terpene/10 mL of MeOH, HPLC grade) containing the investigated compounds was used to perform the optimization. The elution order for racemic mixtures was obtained from previous studies (*18*) and from the literature. Identifications of terpenes in samples were based on comparisons of retention times to standards run under identical experimental conditions. All of these identities were also confirmed by adding the reference compounds to each sample.

Various commercially available products including an orange soft drink and different dairy products containing fruit juices, mostly orange juice, were obtained from the supermarket. For their subsequent identification under Results and Discussion, these samples were labeled as indicated in **Table 1**.

**Procedures.** *Extraction.* All samples were analyzed immediately after they were acquired. An SPME holder (Supelco, Madrid, Spain) was used to perform the experiments. A fused silica fiber coated with a 100-$\mu$m layer of poly(dimethylsiloxane) (PDMS) was utilized to trap volatile terpenes released from the fruit beverages. Prior to use, the fiber was conditioned in the injection port of the gas chromatograph at 250 °C for 1 h. To perform the extraction procedure, 1.0 mL of the fruit beverage was placed in a 5.0-mL vial, which was sealed with plastic film. The extraction was accomplished by exposing the SPME fiber to the headspace of the sample for 2 min at 60 °C. These conditions were selected as a result of an optimization of the method. This optimization was as follows.

As the starting point, the procedure to follow, including the application of certain parameters involved in the extraction such as fiber type [100-$\mu$m layer of poly(dimethylsiloxane)] and vial size (5.0 mL), was chosen on the basis of data reported in the literature (*14*). Subsequently, heating temperature and extraction time were carefully optimized as they are regarded as critical variables in the SPME procedure. For this purpose, several temperatures (30, 45, and 60 °C) and times (2, 5, and 15 min) were tested. In all cases, the variables were selected on the basis of the highest peak areas obtained by SPME-GC. Because 60 °C for 15 min resulted in the loss of terpenes and both 30 and 45 °C for 2, 5, or even 15 min provided unacceptable peak areas, 60 °C and 2 min were the experimental conditions finally selected as the most advantageous. Constant sample stirring was necessary to promote the release of the compounds of interest. Once the extraction was finished, the analytes were thermally desorbed in the GC injector at 250 °C for 5 min and subsequently analyzed by GC.

*GC-Analysis.* A Hewlett-Packard model 5890 gas chromatograph fitted with a split/splitless injector and a flame ionization detector (FID) was used. GC separations were performed on a chiral column (a 25-m × 0.25-mm i.d. fused silica column coated with a 0.25-$\mu$m layer of Chirasil-$\beta$-Dex, Restek, Bellefonte, PA). After the initial temperature had been held at 40 °C for 10 min, the column was programmed at 2 °C/min to 170 °C. In all analyses the FID temperature was set at 250 °C and helium was used as the carrier gas at an initial flow rate of 1 mL/min. Splitless mode was used in all cases. Data acquisition from the FID was performed using adequate software (TotalChrom, Perkin-Elmer, Madrid, Spain).

## RESULTS AND DISCUSSION

Certain terpenes ($\alpha$-pinene, $\beta$-pinene, $\alpha$-phellandrene, limonene, linalool, citronellal, terpinen-4-ol, $\alpha$-terpineol, and $\beta$-citronellol), the presence of which in orange juice or beverages had been previously described by different authors (*10*, *13*, *14*, *19*), were the compounds initially selected for analysis. Other chiral terpenes ($\beta$-*trans*-caryophyllene, menthol, dihydrocarveol, and isopulegol) were also considered in our study, although only $\beta$-*trans*-caryophyllene was subsequently found in real-life samples.

Taking into account that during the sample preparation step the enantiomeric composition may be easily altered, we established as a priority the isolation of the compounds of interest from the matrix under nonracemization conditions. In this respect, heat treatment was particularly minimized. Although higher temperatures might provide higher peak areas, temperatures >60 °C were not tested in order to prevent possible alterations in the enantiomeric composition.

The repeatability of the headspace sampling technique was estimated by measuring the relative standard deviation (RSD) from three replicates of the standard solution when the extraction was accomplished under the optimum experimental conditions. The values ranged from 2 to 12% for all of the investigated terpenes.

To assess the authenticity of foods and beverages, it is necessary first to take into account the fact that the determination of the genuineness of natural and processed foodstuffs by means of chirality requires knowledge of the enantiomeric ratio found in nature and of its consistency, so that any modification observed can be attributed to handling or a technological process. Considering this, the determination of the origin of aromas in the present work was based on the enantiomeric composition of the target compounds detected in natural orange samples (*20*, *21*).

**Figure 1** illustrates the chromatograms resulting from SPME-GC analysis of (a) a dairy product containing orange juice and unspecified aromas, (b) an orange soft drink with natural aromas, and (c) a dairy product containing peach juice and unspecified aromas. These samples had been previously labeled as beverages B, D, and E, respectively, in **Table 1**.

From **Figure 1** it seems that other juice volatile components were also extracted and analyzed along with the compounds of interest, although they were not further considered in the present work.

As can be seen, highly similar terpene profiles were obtained from beverages B and D. As expected (*14*), (+)-limonene represented the major component followed by linalool, terpinen-4-ol, and $\alpha$-terpineol, which were present at far lower levels. Similarly, $\alpha$-pinene, $\alpha$-phellandrene, citronellal, $\beta$-citronellol, and $\beta$-*trans*-caryophyllene were detected as minor constituents. With regard to $\beta$-pinene, it was found at a reasonable level in beverage B. This is relatively surprising as this terpene, when present in orange juice, occurs just as a minor component. The considerable concentration found in this sample suggests the addition of other fruit juice(s) apart from orange juice.
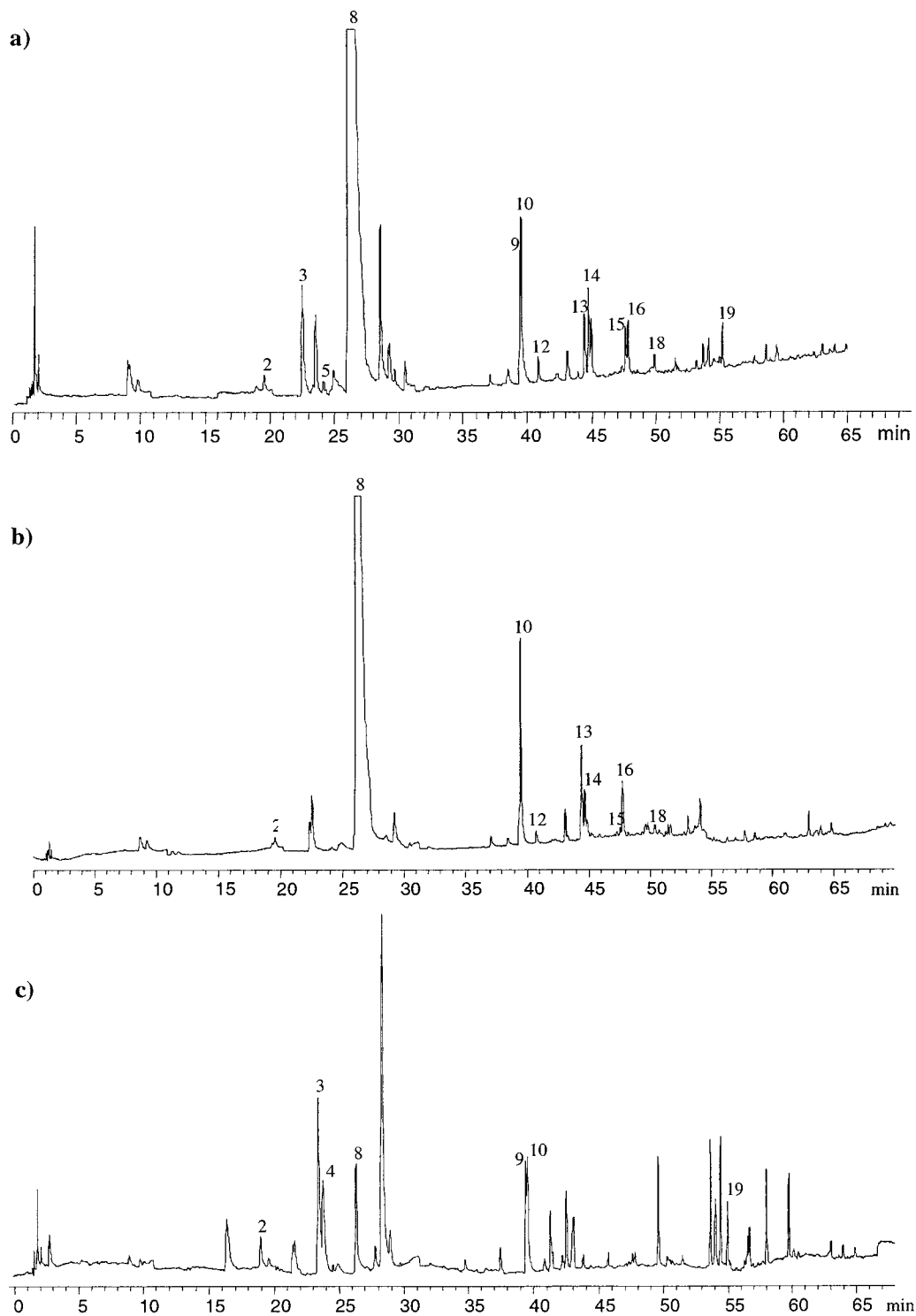
**Figure 1.** SPME-GC analysis of commercial fruit beverages containing (a) orange juice plus unspecified aromas, (b) orange soft drink plus natural aromas, and (c) peach juice plus unspecified aromas. Peaks: 1, (−)-α-pinene; 2, (+)-α-pinene; 3, (−)-β-pinene; 4, (+)-β-pinene; 5, (−)-α-phellandrene; 6, (+)-α-phellandrene; 7, (−)-limonene; 8, (+)-limonene; 9, (−)-linalool; 10, (+)-linalool; 11, (−)-citronellal; 12, (+)-citronellal; 13, (+)-terpinen-4-ol; 14, (−)-terpinen-4-ol; 15, (−)-α-terpineol; 16, (+)-α-terpineol; 17, (−)-β-citronellol; 18, (+)-β-citronellol; 19, (−)-β-*trans*-caryophyllene; 20, (+)-β-*trans*-caryophyllene.

Likewise, beverages A and C, the main constituent of which was also orange juice, exhibited qualitative and quantitative profiles identical to those of beverages B and D. Once more, (+)-limonene appeared to be the main terpenic component, whereas linalool, α-terpineol, and terpinen-4-ol proved to occur as the most important secondary compounds.

On the contrary, substantial differences in the chromatographic profile were obtained from beverage E. First, although

the presence of (+)-limonene could also be established, its concentration was considerably lower due to the lack of orange in its composition. Second, β-pinene, which was not detected in the rest of the fruit beverages, except, as already mentioned, in beverage B, also occurred at reasonably higher proportion in beverage E, and, finally, citronellal, terpinen-4-ol, and α-terpineol, present in every orange sample, could not be detected on this occasion. Moreover, a great number of

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**Table 2.** Enantiomeric Excess (ee), Predominant Enantiomer, and Level of Chiral Terpenes in Commercial Fruit Beverages by SPME-GC

| | fruit beverage | | | | | | | | | |
| | A | | B | | C | | D | | E | |
| terpene | ee, % | level, $\mu$g/mL | ee, % | level, $\mu$g/mL | ee, % | level, $\mu$g/mL | ee, % | level, $\mu$g/mL | ee, % | level, $\mu$g/mL |
|---|---|---|---|---|---|---|---|---|---|---|
| (+)-$\alpha$-pinene | nd[a] | 0.22 | 100 | 0.15 | 100 | 0.32 | 100 | 0.17 | 100 | 2.32 |
| (−)-$\beta$-pinene | nd | nd | 100 | 1.82 | nd | nd | nd | nd | 20.7 | 4.81 |
| (−)-$\alpha$-phellandrene | nd | nd | 100 | 0.22 | 100 | 0.15 | nd | nd | nd | nd |
| (+)-limonene | 100 | 29.0 | 100 | 37.0 | 100 | 25.0 | 100 | 29.5 | 100 | 2.56 |
| (+)-linalool | 52.2 | 3.97 | 32.9 | 4.26 | 74.0 | 3.15 | 100 | 3.72 | 7.2 | 4.59 |
| (+)-citronellal | 100 | 0.12 | 100 | 0.16 | 100 | 0.21 | 100 | 0.14 | nd | nd |
| terpinen-4-ol | 16.3(−)[b] | 0.71 | 2.6(+) | 2.12 | 45.7(+) | 1.18 | 37.0(+) | 1.95 | nd | nd |
| (+)-$\alpha$-terpineol | 51.4 | 0.19 | 13.3 | 1.78 | 54.6 | 1.52 | 76.6 | 2.11 | nd | nd |
| (−)-$\beta$-citronellol | nd | nd | 100 | 0.17 | 100 | 0.12 | nd | nd | nd | nd |
| (−)-$\beta$-*trans*-caryophyllene | 100 | 0.22 | 100 | 0.38 | 100 | 0.32 | nd | nd | 100 | 2.81 |

[a] Not detected. [b] Predominant enantiomer.

unidentified extra peaks present at significant levels were found in beverage E, reflecting its different qualitative composition with respect to the other samples.

**Table 2** depicts the enantiomeric purity as well as the levels (micrograms per milliliter) of the investigated compounds in commercial beverages. In all cases, the enantiomeric excess was calculated from peak areas, and excess of predominant enantiomer was expressed as a percent, that is,

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

As can be seen, (+)-$\alpha$-pinene, (−)-$\alpha$-phellandrene, (+)-limonene, (+)-citronellal, (−)-$\beta$-citronellol, and (−)-$\beta$-*trans*-caryophyllene, when present, occurred as a pure enantiomer in all samples. These results are in good agreement with those reported by other authors, who also established the presence of the pure (+)-enantiomers of $\alpha$-pinene and limonene in orange essential oils (*20*) and with data obtained in our laboratory by using the same approach in juice concentrates (*22*). Although, to our knowledge, the presence of $\beta$-*trans*-caryophyllene in orange beverages had not been previously reported, it is worth pointing out that the enantiomeric excess value found in this study supports the statement by other authors who consider as unusual the occurrence of both enantiomers for sesquiterpenes in nature (*20*).

On the contrary, $\beta$-pinene, linalool, terpinen-4-ol, and $\alpha$-terpineol varied substantially depending on the studied beverage. In this regard, linalool and terpinen-4-ol have already demonstrated their variability in nature. In fact, linalool has been reported to be present from the almost pure (−)-enantiomer in lavender flowers (*23*) to the existence of high proportions of the (+)-isomer (94−96%) in sweet orange oil (*24*). This was also observed in the present work as enantiomeric distributions of (+)-linalool varying from 7.2% in beverage E to 100% in beverage D were obtained. This latter result agreed well with data reported in sweet orange oil (*24*), as well as with data obtained in our laboratory from nonprocessed orange concentrates (*22*), suggesting the use of natural aromas obtained from orange in the manufacture of beverage D. This result was also consistent with the label designation. Likewise, the wide range of enantiomeric excesses (from 32.9 to 74.0%) found for (+)-linalool in beverages A−C suggests either the employ of a technological process involving heat treatment or the addition of aromas to improve the sensorial perception of these beverages. This latter explanation is most likely, as samples A−C were declared to have been submitted to the same handling during processing as sample D, which showed no racemization

for linalool. As a conclusion, the addition of aromas not obtained from orange to beverages A−C appeared to lead to the presence of (−)-linalool, naturally absent in orange juice.

With regard to terpinen-4-ol, its enantiomeric composition ranged from 45.7% in beverage C to 2.6% in beverage B, exhibiting the (+)-enantiomer as predominant. A value of 16.3% was found in beverage A in which the (−)-isomer prevailed. This considerable variation had already been observed in a previous study on fruits in which the enantiomeric ratio of terpinen-4-ol depended significantly on the cultivar (*18*). This phenomenon makes it extremely difficult to know the enantiomeric distribution of this terpene in nature and, as a consequence, makes terpinen-4-ol useless in the determination of the genuineness of any food product.

Interestingly, $\alpha$-terpineol seemed to be particularly valuable in the confirmation of the natural origin obtained from orange of the aromas present in beverage D as it displayed an enantiomeric distribution (76.6%) very close to that obtained previously in orange concentrates (80%) (*22*). On the other hand, a considerably wide range of enantiomeric ratios was found for the rest of the studied samples. This variation supported the previously mentioned addition of aromas not obtained from orange to samples A−C.

$\beta$-Pinene was detected in sample B in the (−)-enantiomer pure form, whereas an enantiomeric composition of 20.7% was estimated in sample E. This can be easily explained by the presence of a blend of different fruit juices, which did not seem to include orange juice.

In conclusion, the determination of the enantiomeric distribution of chiral terpenes, mainly limonene, linalool, and $\alpha$-terpineol, can be used as a valuable tool in the detection of the addition of aromas to fruit beverages. These compounds occur in nature with enantiomeric distributions of 100, 100, and 80%, respectively, the (+)-enantiomer being the predominant isomer in all cases. Any alteration in these values may be attributable to the addition of aromas. Likewise, the consideration of further terpenes may contribute to the improved reliability of the determination of aroma origin.

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