Test of Isotopic Fractionation during Liquid–Liquid Extraction of Volatile Components from Fruits

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Fruits constitute a natural source of flavoring molecules. Nevertheless, an isotopic fractionation could be induced by the extraction of volatile molecules from fruits. After the addition of six volatile molecules to Italia grape (hexyl acetate, acetic acid, linalool, butanoic acid, 3-hydroxy-2-methyl-4-pyrone, and methyl cinnamate) and five volatile molecules to Primofiori lemon (hexanal, *trans*-2-hexenyl acetate, 1-hexanol, *trans*-2-hexenol, and 4-decanolide) before extraction, it has been established that, for a particular extraction procedure, there is no significant difference between the ^{13}C enrichments of these molecules before and after extraction. Nothing has been concluded for acetic acid, which is not recovered for grape when the tested extraction procedures are used.

Keywords: *Fruit aroma; isotopic fractionation; GC–IRMS*

INTRODUCTION

In recent years, isotopic $^{13}C/^{12}C$ ratio measurement was suggested to be used to determine the origins of organic compounds (Krueger and Krueger, 1983, 1985; Butzenlechner et al., 1989). Comparing enrichment values, it is possible to determine whether a molecule is natural or not (Byrne et al., 1986; Bernreuther et al., 1990). Fruits constitute an important natural source of flavoring molecules. Before isotopic measurements, flavoring components must be extracted from fruits. During the extraction step, the sample handling (physicochemical process) may induce an isotopic fractionation, that is, a variation of the isotopic enrichment. Thus, isotopic fractionation has been tested during the extraction of vanillin. Nevertheless, it only concerns a chromatographic purification (Fellous et al., 1992). The influence of chromatographic and technological procedures on the ^{13}C enrichment was tested on aldehydes and linalool from orange oils. A variation of the $^{13}C/^{12}C$ isotope ratio was detectable in the case of special products containing single compounds (Braunsdorf et al., 1993). The aim of this work was to determine whether an isotopic fractionation could occur during a liquid–liquid extraction. Two kinds of fruits whose extract chromatograms showed a small number of peaks were chosen to be spiked by as many odoriferous molecules as possible. The molecules were spiked in these fruits before extraction, and their enrichments before and after extraction were compared. In that way, a liquid–liquid extraction and a liquid–liquid extraction saturated with sodium chloride were tested. For both kinds of extractions, the yields were calculated to explain a potential isotopic discrimination.

MATERIALS AND METHODS

Materials. Frozen Italia grape and frozen Primofiori lemon, commercially available, were used.

Standardization for the Extraction Yield Calculation. It was chosen to work without any internal standard owing to the fact that all spiked molecules were quite structurally different. All of the standard solutions and extracts were injected within the same period of time and under the same apparatus tuning conditions in order to cast off variations.

Grape. Six standard solutions were prepared. Each solution contained the following molecules: hexyl acetate, acetic acid, linalool, butanoic acid, 3-hydroxy-2-methyl-4-pyrone, and methyl cinnamate. The solutions were prepared in methylene chloride, and the concentrations were as follows: 100, 75, 50, 20, 15, 10, and 5 μg/mL.

Lemon. Eight standard solutions were prepared. Each solution contained the following molecules: hexanal, *trans*-2-hexenyl acetate, 1-hexanol, *trans*-2-hexenol, and 4-decanolide. The solutions were prepared in methylene chloride, and the concentrations were as follows: 100, 75, 50, 25, 20, 15, 10, and 5 μg/mL.

Solution Used To Spike the Grape Matrix. The blend was spiked with 3 mL of a solution containing the molecules mentioned above. This solution was prepared in methylene chloride at the following concentration: 100 μg/mL (solution A). This amount of spiked molecules was chosen to obtain final concentrations usually observed in fruits.

Solution Used To Spike the Lemon Matrix. The blend was spiked with 3 mL of a solution containing the molecules mentioned above. This solution was prepared in methylene chloride at the following concentration: 100 μg/mL (solution A').

Standard Solutions for the Isotopic Measurements. *Grape.* This solution contained the molecules mentioned above. It was prepared in methylene chloride at the following concentration: 10 mg/mL.

Lemon. This solution contained the molecules mentioned above. It was prepared in methylene chloride at the following concentration: 10 mg/mL.

Extraction Procedure 1 for Volatile Components (Douillard and Guichard, 1990). Fruits (250 g) were ground in 250 mL of MilliQ water, in a domestic blender (whole grape and peeled lemon). The suspension was poured in a 1000 mL beaker, kept in ice. Three milliliters of solution A (grape) or solution A' (lemon) was added, and the mixture was stirred to homogenize it; 100 mL of redistilled methylene chloride was added. The blend was stirred for 30 min. Juice, pulp, and organic extract were separated by centrifugation (15 min, 2000*g*). Three more pulp extractions were achieved using 30 (grape) or 50 mL (lemon) of methylene chloride (15 min,

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Table 1. Extraction Yields of Molecules Added to Grape and Lemon[a]

fruit	molecule	water			water + NaCl		
		A ($\times 10^{-7}$)	C (μg/mL)	Y (%)	A ($\times 10^{-7}$)	C (μg/mL)	Y (%)
Italia grape	hexyl acetate	24.10	72.48	72.48	19.76	59.31	59.31
	acetic acid		too low			too low	
	linalool	17.53	58.64	58.64	15.89	53.07	53.07
	butanoic acid	1.64	9.58	9.58	4.12	20.86	20.86
	3-hydroxy-2-methyl-4-pyrone	4.22	33.79	33.79	5.20	39.71	39.71
	methyl cinnamate	8.22	55.71	55.71	7.93	53.79	53.79
Primofiori lemon	hexanal	5.42	66.00	66.00	4.82	58.6	58.36
	trans-2-hexenyl acetate	8.05	69.41	69.41	8.03	69.23	69.23
	1-hexanol	9.09	81.03	81.03	8.29	73.63	73.63
	trans-2-hexenol	6.95	75.21	75.21	6.71	72.51	72.51
	4-decanolide	1.96	32.08	32.08	3.64	60.03	60.03

[a] A, peak area; C, concentration; Y, yield.

Table 2. Isotopic Enrichments of Molecules Added to Grape and Lemon

fruit	molecule	standard			water			water + NaCl		
		δ	σ	n	δ	σ	n	δ	σ	n
Italia grape	hexyl acetate	−29.81	0.18	5	−29.83	0.15	5	−30.10	0.13	4
	linalool	−26.61	0.03	4	−26.62	0.25	6	−26.53	0.16	5
	butanoic acid	−27.93	0.05	4		too low		−28.14	0.21	4
	3-hydroxy-2-methyl-4-pyrone	−16.86	0.26	4	−16.91	0.24	4	−16.89	0.14	4
	methyl cinnamate	−29.18	0.20	4	−29.16	0.24	5	−29.34	0.16	5
Primofiori lemon	hexanal	−24.08	0.12	7	−23.94	0.11	5	−23.84	0.30	5
	trans-2-hexenyl acetate	−31.71	0.15	7	−31.68	0.19	4	−31.58	0.07	5
	1-hexanol	−25.96	0.22	5	−25.96	0.13	4	−25.93	0.09	6
	trans-2-hexenol	−23.96	0.07	8	−24.04	0.17	4	−25.00	0.22	6
	4-decanolide	−28.35	0.11	6	−28.41	0.11	5	−28.51	0.14	4

2000*g*). Then, the organic phase was added to the first extract. In the same way, two more juice extractions were carried out with 50 mL of solvent. All of the organic phases were combined, filtered, and concentrated to 3 mL (distillation column, Kuderna-Danish column; atmospheric pressure; 20 min; boiler temperature, 60 °C). To regulate the boiling, Carborundum (SiC) was used.

Extraction Procedure 2 for Volatile Components. This second procedure was the same as the first one except that MilliQ water was replaced by MilliQ water saturated with sodium chloride.

Gas Chromatography–Mass Spectrometry. Before standardization, GC–MS was used to clearly identify each component to correctly calculate the extraction yields.

GC–MS analysis was carried out using a HP 5890 gas chromatograph and a HP 5970 mass spectrometer. The mass spectra were obtained under electron impact. The chromatograms were recorded by monitoring the total ion current in the 30–320 amu range. The interface was maintained at 250 °C.

Chromatographic separations were performed using a DB FFAP column (J&W Scientific, 30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was held at 40 °C for 1 min and then programmed from 40 to 220 °C at 4 °C/min; after 10 min at 220 °C, the temperature was programmed from 220 to 250 °C at a rate of 8 °C/min. Then, the oven was maintained at 250 °C for 10 min. The injector was held at 250 °C.

The extracts and the standard solutions (micrograms per milliliter) were injected in the splitless mode. The injected volume was 1 μL.

Gas Chromatography–Isotopic Ratio Mass Spectrometry. The chromatographic separation was performed on a HP 5890 gas chromatograph. Isotopic measurements were achieved on a ISOCHROM SIRA 10 VG ISOTECH. The results of the stable isotope ratio analysis (SIRA) were expressed as δ ^{13}C:

$$\delta\ ^{13}C = [(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}]/(^{13}C/^{12}C)_{standard} \times 1000$$

The standard for carbon isotopes is PDB. The CO_2 gas is obtained by reacting the Cretaceous belemnite, *Belemnitella americana*, with 100% phosphoric acid. This CO_2 gas represents the zero for the δ ^{13}C scale. Since this primary standard is not routinely used, a calibrated working standard is used.

The same columns and oven temperature programs were used for both GC–MS and GC–IRMS analyses. The standard solutions for the isotopic measurements (10 mg/mL) were injected in the split (1/50; injected volume, 1 μL) and the extracts in the splitless mode (injected volume, 5 μL).

RESULTS AND DISCUSSION

The molecules added to the matrices to test the potential isotopic fractionation have been chosen not to present the same retention times as the native volatile compounds extracted from fruits.

Extraction Yields. The values of the extraction yields are gathered into Table 1 (grape and lemon). It can be observed that the extraction yields obtained by both procedures are not quite different. However, it can be noticed that for the lower yields the use of MilliQ water saturated with NaCl increases the values. Acetic acid is not recovered by the used extraction procedures. This can be explained by the fact that acetic acid has a lower solubility in methylene chloride than in water. Moreover, the chromatographic limit detection is inferior to 10 μg/mL. Consequently, it is possible to say that the extraction yield of acetic acid is inferior to 10%.

Isotopic Fractionation. The results of the isotopic analysis are presented in Table 2 (grape and lemon). Isotope effects on equilibria are thermodynamically well established. Their practical importance will mainly become manifest when the distribution coefficient in question is not extremly in favor of one phase or when the extraction is incomplete. Therefore, in the present example a measurable isotope fractionation is not to be expected with the exception for acetic acid but, unfortunately, this compound has not been recovered. It can be observed that the values of the isotopic enrichment of the extracted molecules from each fruit are close to those obtained from the standard solutions. There is no significant difference between all enrichments (Stu-

dent test, $P < 0.05$—standard values vs extracted values), except for the isotopic values of standard hexyl acetate and hexyl acetate extracted using procedure 2 (MilliQ water saturated with NaCl, grape). However, it is impossible to conclude that there is an isotopic fractionation. Indeed, the repeatability is really better than the reproducibility (0.3‰) and a Student test, $P < 0.05$, will always present a significant difference. Nevertheless, the nonsignificant difference level is $P < 0.03$, for hexyl acetate. In consequence, it cannot be concluded that there is an isotopic fractionation for this molecule handled using this procedure. Therefore, it seems that even if the extraction procedure of volatile components from fruits induces losses, it does not induce an isotopic fractionation.

Aspect of Chromatography. It appears that the chromatographic separation in the GC–IRMS system also occurred without a shift of the δ value. It indicates that the whole peak has been collected. Moreover, with complex chromatograms, peaks can appear to be superimposed upon a high sample background. If the sample peak is small and the background large, then significant errors can result. Extreme caution must be exercised in the interpretation of such peaks. To realize background subtraction, specific background ratio points along the chromatogram are selected and fitted to polynomial curves. In the studied case, background appears along the chromatogram and background subtraction was necessary. Consequently, the results show that the background subtraction system is very reliable.

Conclusion. This study showed that for both extraction procedures (aqueous phase, organic phase; aqueous phase saturated with sodium chloride, organic phase) isotopic fractionation does not occur for some molecules (hexyl acetate, acetic acid, linalool, butanoic acid, 3-hydroxy-2-methyl-4-pyrone, hexanal, *trans*-2-hexenyl acetate, 1-hexanol, *trans*-2-hexenol, and 4-decanolide). These conclusions are consistent with expectation. Whereas when volatile compounds are distilled, one might expect the higher molecular weight containing molecules (those containing 10 ^{13}C as in the linalool molecule) to be higher boiling, leading to enrichment on distillation of part of the total amount. However, extraction (liquid–liquid) is more a function of the chemistry of the molecule rather than the boiling point (a physical property). Therefore, all like molecules (i.e. linalool) would behave similarly. Therefore, the SIRA values measured after such extraction on these molecules can be used to determine their origin or to detect flavor adulterations. It must be noted that these results (no isotopic fractionation) are available only for the tested extraction and that isotopic fractionation can occur during other sample handling.

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