Quantitation of Potent Odorants of Virgin Olive Oil by Stable-Isotope Dilution Assays[1]

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The potent odorants of four olive oil samples differing in flavor were quantitated, and their odor activity values (OAVs) were calculated by dividing the concentrations of the odorants in the oil samples by the flavor threshold values in the oil. The odorants with higher OAVs were contrasted with the different notes of the flavor profiles of the olive oils. It was concluded that the following compounds contributed mainly to the flavor notes given in parentheses: (*Z*)-3-hexenal (green), ethyl 2-methylbutyrate, ethyl isobutyrate, ethyl cyclohexanoate (fruity), (*Z*)-2-nonenal (fatty) and 4-methoxy-2-methyl-2-butanethiol (black-currant-like). The results showed that the calculation of OAVs is an approach to objectify the flavor differences of olive oil samples.

KEY WORDS: Capillary gas chromatography, isotope dilution assay, mass spectrometry, odor activity value, odorant, odor evaluation, quantitation.

Recently (1), the following compounds were evaluated by means of an aroma extract dilution analysis (AEDA) as potent odorants of four olive oil samples with different flavor profiles: acetic acid (**I**), 3-methylbutanol (**II**), 2-phenylethanol (**III**), (*Z*)-3-hexenol (**IV**), ethyl 2-methylbutyrate (V), ethyl isobutyrate (**VI**), ethyl cyclohexanoate (**VII**), (*Z*)-3-hexenyl acetate (**VIII**), 4-methoxy-2-methyl-2-butanethiol (**IX**), hexanal (**X**), (*E*)-2- (**XI**) and (*Z*)-3-hexenal (**XII**), (*Z*)-2-nonenal (**XIII**), (*E,E*)-2,4-decadienal (**XIV**) and *trans*-4,5-epoxy-(*E*)-2-decenal (**XV**). Because AEDA is only a screening method (2,3), the quantitation of the potent odorants and the calculation of their odor activity values (OAVs, ratio of concentration to odor threshold) were necessary to show the actual contribution of each odorant to the flavor of a food.

The determination of each of the 15 odorants was performed by a stable-isotope dilution assay (SIDA), which allows the exact determination of odorants (4–6) and of other trace components of a food (7). In the SIDA applied here, the odorant labeled with deuterium (d) was used as an internal standard. Apart from a small isotope effect, the physical and chemical properties of the analyte and its labeled analogue were identical. Therefore, this internal standard was ideal in correcting for any losses that might occur during the distillation and purification of the odorant to be estimated.

EXPERIMENTAL PROCEDURES

Materials. The four samples of virgin olive oil were from Italy (I-1 and I-2) and from Spain (S-1 and S-2). The samples I-2 and S-1 were gifts (*cf.* Acknowledgments) and were of high flavor quality; the samples I-1 and S-2 were purchased from local markets. The following compounds were obtained commercially from the source given in parentheses: Nos. **I** to **VI**, **X** and **XI**, (*E,E*)-2,4-decadienal, sodium borodeuteride, ethanol-$2d_3$, acetic acid-$2d_3$ (**I**-d) and acetic acid-d_4 (Aldrich, Steinheim, Germany); iodomethane-d_3 (Sigma, Muenchen, Germany), deuterium gas (99.9% isotopic purity, Merck-Schuchardt, Darmstadt, Germany); 2-hexyn-1-ol (Atlanta, Heidelberg, Germany). All compounds and their assigned numbers are listed in Table 1. Silica gel 60 (0.053–0.2 mm; Merck) was treated with concentrated HCl and deactivated with water (4.1%, w/w) according to Esterbauer (8).

Instrumental analysis. High-resolution gas chromatography (HRGC) was performed with a Carlo Erba gas chromatograph (Type HRGC 5160, Carlo Erba, Hofheim, Germany) by using the fused silica capillaries SE-30 (25 m × 0.32 mm, Machery & Nagel, Dueren, Germany), DB-free fatty acid phase (DB-FFAP—, 30 m × 0.32 mm, J&W Scientific, Folsom, CA) and OV-1701 (30 m × 0.32 mm, J&W Scientific). The samples were applied by the on-column injection technique at 35°C (5,9). After the start, the temperature of 35°C was held for 2 min, then raised by 40°C/min to 50°C and held isothermally for 5 min; subsequently the temperature was raised by 4°C/min to 230°C and finally held at 230°C for 10 min.

HRGC-mass spectrometry (MS) was carried out either with an MS 8230 or with an ion trap detector, ITD-800, (both Finnigan, Bremen, Germany) in tandem with the above-mentioned capillaries. In the MS 8230, mass spectra in the electron impact mode [MS(EI)] were generated at 70 eV, and in the chemical ionization mode [MS(CI)] at 115 eV with isobutane as the reagent gas. The ITD was used only in the CI mode with methanol as the reagent gas (10). Mass chromatograms were recorded at the ions selected in Table 1. Comparison of the integrated abundance of the selected ion of the odorant to that of the abundance of the selected ion of the deuterated internal standard (Table 1) provided the data needed to carry out the quantitative calibration of the method (5,10). A calibration factor was determined for each of the 15 odorants as exemplified for (*Z*)-2-nonenal in (5). The factors calculated are listed in Table 1.

Synthesis. Compounds **VII** to **IX** (1), **XII** (11), **XIII** (12), **XIV** (6) and the following labeled compounds were synthesized as reported earlier: **II**-d and **III**-d (13), **IV**-d, **X**-d, **XII**-d to **XV**-d (5). (*E*)-2-Hexenal-d (**XI**-d) was synthesized by the route reported for (*E*)-2-nonenal-d (5), but starting with 2-hexynol instead of 2-nonynol. The esters **V**-d, **VI**-d and **VII**-d were obtained by a proton-catalyzed reaction of the corresponding acid (isobutyric acid, 2-methylbutyric acid, cyclohexanoic acid) with deuterated ethanol as follows: mixture consisting of the acid (50 mmol), ethanol-$2d_3$ (5 mmol) and concentrated sulfuric acid (50 mg) was refluxed for 4 h. After addition of water (20 mL), the ester was extracted with pentane (30 mL). The organic layer was washed twice with aqueous sodium bicarbonate (0.5 mol/L; 30 mL) and then dried over sodium sulfate. The esters were further purified by chromatography on silica gel (1). The esters **V**-d, **VI**-d and **VII**-d were

[1]Presented at the 83rd Annual Meeting of the American Oil Chemists' Society, Toronto, Canada, May 10–14, 1992.

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TABLE 1

Selected Ions in the Mass Spectra of the Flavor Compounds I to XV and Their Deuterated Standards I-d to XV-d—Calibration Factors for Quantitative Analysis [a]

Flavor compound ion (*m/z*)		Deuterated compound ion (*m/z*)		Calibration factor
Acetic acid (**I**)[b]	60	I-d	63	0.72
3-Methylbutanol (**II**)[b]	70	II-d	72-75[c]	1.08
2-Phenylethanol (**III**)[b]	122	III-d	123	1.15
(*Z*)-3-Hexenol (**IV**)[d]	83	IV-d	85	0.73
Ethyl 2-methylbutyrate (**V**)[d]	131	V-d	134	1.12
Ethyl isobutyrate (**VI**)[d]	117	VI-d	120	0.92
Ethyl cyclohexanoate (**VII**)[d]	157	VII-d	160	1.03
(*Z*)-3-Hexenyl acetate (**VIII**)[e]	143	VIII-d	146	0.94
4-Methoxy-2-methyl-2-butanethiol (**IX**)[d]	101	IX-d	104	0.99
Hexanal (**X**)[c]	101	X-d	103-105[c]	0.73
(*E*)-2-Hexenal (**XI**)[d]	99	XI-d	101	0.62
(*Z*)-3-Hexenal (**XII**)[e]	99	XII-d	101	0.93
(*Z*)-2-Nonenal (**XIII**)[e]	141	XIII-d	143	0.90
(*E,E*)-2,4-Decadienal (**XIV**)[d]	153	XIV-d	155-157[c]	1.01
trans-4,5-Epoxy-(*E*)-2-decenal (**XV**)[e]	169	XV-d	171	0.67

[a]The relative abundances of the ions of the labeled and unlabeled compound were recorded (details in the Experimental Procedures Section).
[b]In the mass spectra in the electron impact mode obtained by the MS 8230.
[c]The sum of the relative abundances of the ions was calculated.
[d]In the mass spectra in the chemical ionization mode (MS(CI))obtained by the ion trap detector ITD-800.
[e]In the MS(CI) obtained by the MS 8230.

TABLE 2

Mass Spectral Data[a]

Deuterated compound	MS(EI)	MS(CI)
Ethyl 2-methylbutyrate (**V**-d)	57 (100), 105 (96), 85 (42) 41 (35), 75 (24), 118 (14) 56 (14), 89 (17), 42 (17)	134 (M + 1, 100)
Ethyl isobutyrate (**VI**-d)	43 (100), 71 (64), 119 (M, 36), 41 (25)	120 (M + 1, 100)
Ethyl cyclohexanoate (**VII**-d)	83 (100), 55 (92), 104 (82) 159 (M, 60), 111 (38), 41 (32) 110 (28), 91 (26), 82 (22), 74 (20)	160 (M + 1, 100)
(*Z*)-3-Hexenyl acetate (**VIII**-d)	67 (100), 46 (98), 82 (70), 41 (14)	146 (M + 1, 100)
4-Methoxy-2-methyl-2-butanethiol (**IX**-d)	48 (100), 88 (64), 69 (58) 41 (38), 103 (32), 137 (M, 18) 104 (17)	104 (M + 1 − H_2S, 100)

[a]M, molecular mass; MS(EI), mass spectra in the electron impact mode; MS(CI), mass spectra in the chemical ionization mode.

characterized by HRGC-MS; the most intense signals are presented in Table 2. Ester **VIII**-d: (*Z*)-3-hexenol (5 mmol) and concentrated sulfuric acid (50 mg) were dissolved in acetic acid-d_4 (50 mmol). After refluxing the mixture for 4 h, the ester formed was isolated and purified as described above. Mass spectral data are given in Table 2. Compound **IX**-d was synthesized by following the route reported for unlabeled 4-methoxy-2-methyl-2-butanethiol (1) but with iodomethane-d_3 instead of the unlabeled reagent. Mass spectral data are given in Table 2.

Concentrations of deuterated compounds. The concentrations of compounds **I**-d and **IV**-d to **VIII**-d were determined by HRGC with methyl octanoate as the internal standard. HRGC was performed with the apparatus, the SE-54 and OV-1701 thin film capillaries and the conditions reported above. The correction factors were estimated by HRGC analysis of mixtures consisting of known amounts of methyl octanoate and of unlabeled compounds **I** and **IV** to **VIII**. The concentration of compound **IX**-d was determined without a correction factor with 1-methoxy-3-methyl-3-butene as the internal standard. The concentrations of the other deuterated compounds were determined by HRGC as reported earlier (5,13).

Analysis of olive oils. The oil sample (500 g) was spiked with known amounts of the 15 deuterated standards listed in Table 1. The procedures of spiking, distillation in high vacuum (T: 34°C, pressure: 6 mPa) and concentration of the volatile fraction, dissolved in diethyl ether to a volume of 300 μL, were the same as reported for the analysis of flavor compounds from a soybean oil (5). Aliquots (0.5 μL) of the volatile fraction were separated by HRGC on capillary OV-1701 for the determination of **II** to **VIII**, **X**, **XI** and **XIV** and on capillary FFAP for the determination of **I** and **XV**.

For quantitation of **IX**, **XII** and **XIII**, the major part of the volatile fraction was separated by high-performance liquid chromatography (HPLC) with the column and the apparatus previously described (14). Elution (flow rate: 2 mL/min) was performed with pentane (30 mL) and with pentane/diethyl ether (98:2, vol/vol; 30 mL). The last 10

TABLE 3

Odor Profiles of Four Virgin Olive Oil Samples

Odor characteristic	Olive oil sample			
	I-1	I-2	S-1	S-2
	Mean panel score of odor intensity[a]			
Fruity	1.0	1.1	1.6	1.8
Green	0.7	1.5	0	0
Blackcurrant-like	0	0	2.5	2.7
Fatty	1.5	1.7	0.6	0.4
Floral	0.2	0.3	0.3	0.3
Spicy	1.0	0.5	0.5	0.3

[a]Rating scale of the odor intensity: 0, odor quality is lacking; 1, bland; 2, moderate; 3, strong.

mL of the effluent (total volume: 60 mL) were collected and concentrated by distillation and microdistillation (5) to a volume of 50 μL. Aliquots (0.5 μL) of the HPLC fraction were separated by HRGC on capillary SE-30 for the determination of **IX** and on capillary OV-1701 for the determination of **XII** and **XIII**.

Odor evaluation of olive oils. The odor profiles of the four olive oil samples were evaluated in an isolated sensory panel room at 21 ± 1°C. The panel consisted of seven experienced assessors, who were trained with solutions of reference odorants in sunflower oil, *e.g.*, (*Z*)-3-hexenal for the green odor. In each session, the four oil samples (10 mL each) were presented in covered glass beakers (diameter, 40 mm; capacity, 45 mL) at 21 ± 1°C. The beaker was swirled and, after removing the cover, the sample was sniffed by the panelist. The odor characteristics of the samples were evaluated in the first session, and the intensity of the odor characteristics was determined in the second session as a point on a continuum between 0 and 3 (Table 3). The results obtained by the seven panelists were averaged.

Threshold values. A defined amount of each compound, dissolved in 0.1 mL ethanol, was added to a sample of freshly refined sunflower oil (100 g). After stirring for 30 min, this stock solution was diluted stepwise with sunflower oil (50:50, w/w) and stirred for 10 min after each dilution step. Immediately after preparation, the diluted samples (10 mL) were presented in covered glass beakers (diameter, 40 mm; capacity, 45 mL) at 21 ± 1°C to individual panel members (at least three trained judges). The cap was removed, the sample was rinsed into the mouth and the odor was then retronasally perceived. Sensitivity odor threshold values were determined by the triangle test by using odorless refined sunflower oil as a blank. The samples were presented in order of decreasing concentration, and the threshold values evaluated in two sessions were averaged.

RESULTS AND DISCUSSION

Quantitative method. A model experiment was performed to check the accuracy of the method. The model mixture consisted of odorants **I** to **IX** and **XI** (Table 4) as well as of the labeled internal standards **I**-d (627 μg), **II**-d (71 μg), **III**-d (116 μg), **IV**-d (94 μg), **V**-d (16 μg), **VI**-d (23 μg), **VII**-d (9.5 μg), **VIII**-d (212 μg), **IX**-d (5.0 μg) and **XI**-d (210 μg). The model was added to a sunflower oil (500 g) which was stirred for 45 min and then analyzed as reported in the Experimental Procedures Section. Compounds **X**, **XII** to **XV** were excluded from the model, as their recoveries had been determined earlier (5) in a similar experiment. As summarized in Table 4, the recovery of each compound was determined by using the SIDA developed here. The results listed in Table 4 indicate that the differences between the theoretical values and the values measured amounted to not more than 10%, as found for 2-phenylethanol. This analytical error was considered adequate for quantitation of the flavor compounds.

To show the application of the method to the quantitation of odorants in olive oil, the SIDA of the thiol **IX** is elucidated. The volatiles isolated by distillation from the sample S-1 (spiked with 3.5 μg of **IX**-d) were fractionated by HPLC. An aliquot of the fraction collected was then separated by HRGC on capillary SE 30. The numbered smaller peak in the gas chromatogram (Fig. 1) was identified by MS(EI) as a mixture of compounds **IX** and **IX**-d. To differentiate between the unlabeled odorant (from the olive oil) and the deuterated internal standard, mass chromatograms were recorded for the ions *m/z* 101 (**IX**) and *m/z* 104 (**IX**-d), which were selected for quantitation as shown in Table 1. The mass chromatograms obtained are displayed in Figure 2. The concentration of 4-methoxy-2-methyl-2-butanethiol (**IX**) in sample S-1 (1.8 μg/kg) was calculated from the areas of the two peaks (**IX** and **IX**-d in Fig. 2) by using the calibration factor of 0.99 (Table 1).

TABLE 4

Isotope Dilution Assay of a Model Mixture of the Odorants I to IX and XI Dissolved in a Freshly Refined Sunflower Oil

Compound	Amount (μg)		Recovery (%)
	Added	Measured	
Acetic acid (**I**)	695	753	108
3-Methylbutanol (**II**)	78	85	108
2-Phenylethanol (**III**)	120	108	90
(*Z*)-3-Hexenol (**IV**)	104	108	104
Ethyl 2-methylbutyrate (**V**)	16	17	106
Ethyl isobutyrate (**VI**)	23	24	104
Ethyl cyclohexanoate (**VII**)	10	10	100
(*Z*)-3-Hexenyl acetate (**VIII**)	235	232	99
4-Methoxy-2-methyl-2-butanethiol (**IX**)	1.0	0.9	92
(*E*)-2-Hexenal (**XI**)	213	231	108

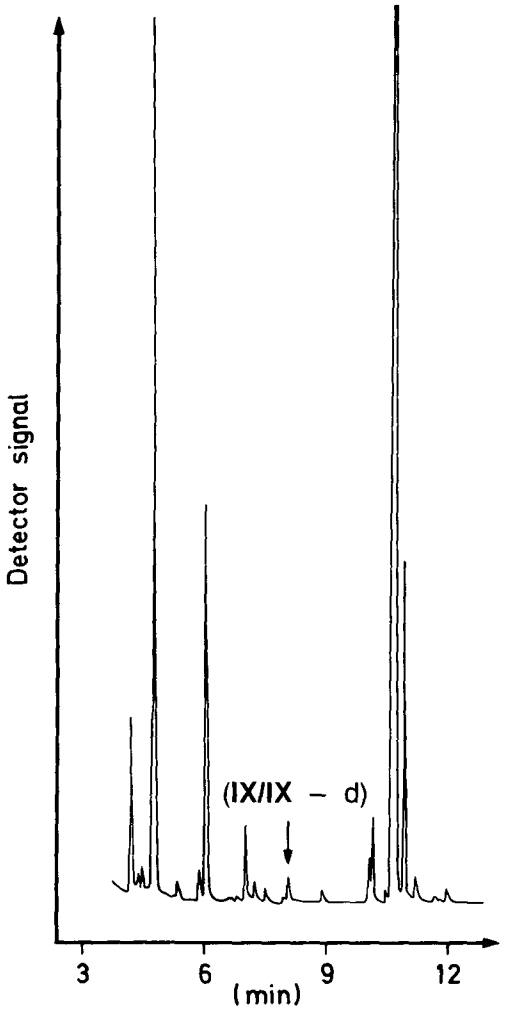

FIG. 1. High-resolution gas chromatogram (cutting) of the high-performance liquid chromatography fraction of olive oil volatiles containing the thiol IX and its internal standard IX-d.

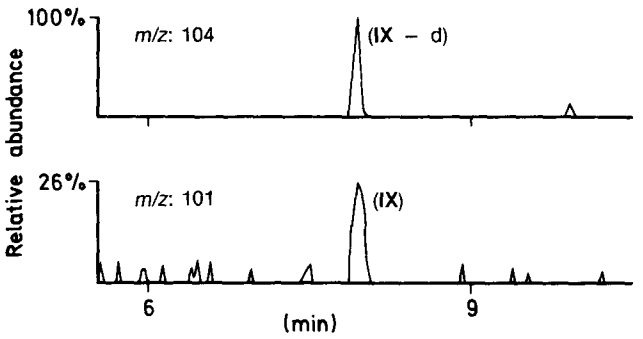

FIG. 2. Mass chromatograms of the high-performance liquid chromatography fraction of Figure 1 recorded at the ions *m/z* 101 (*IX*) and *m/z* 104 (IX-d).

Analysis of olive oils. Four olive oil samples differing in odor, as shown in Table 3, were analyzed. The concentrations of the odorants that were found in these samples are listed in Table 5. To get an insight into the potent odorants causing different notes in the odor profiles of the oil samples (Table 3), the OAVs of the 15 odorants were calculated by dividing the concentrations given in Table 5 by the odor threshold values of the compounds in a sunflower oil. For compounds **X**, **XII** to **XV**, the threshold data published earlier (5) were used, and for the remaining compounds the values were specifically evaluated in this study and are shown in Table 6. The OAVs calculated for the odorants of the four olive oil samples are listed in Table 7.

The most striking difference in the odor profiles of samples I-1 and I-2 was the more intense green note in the latter (Table 3). A comparison of the OAVs of the green-smelling aldehydes [hexanal, (*E*)-2- and (*Z*)-3- hexenal (5,15,16)] suggests (Table 7), on the basis of the much higher OAV in sample I-2, that (*Z*)-3-hexenal was responsible for the stronger green odor note of I-2. In particular, sample I-1 but also sample I-2 contained much more (*E*)-

TABLE 5

Concentrations (μg/kg) of the Odorants in Four Virgin Olive Oils[a]

Compound	Olive oil sample			
	I-1	I-2	S-1	S-2
Acetic acid (I)	10494	2449	6683	970
3-Methylbutanol (II)	1592	116	904	273
2-Phenylethanol (III)	1134	363	345	201
(*Z*)-3-Hexenol (IV)	777	662	796	765
Ethyl 2-methylbutyrate (V)	3.9	2.0	14	41
Ethyl isobutyrate (VI)	2.7	1.8	7.9	17
Ethyl cyclohexanoate (VII)	1.6	1.2	4.3	3.6
(*Z*)-3-Hexenyl acetate (VIII)	113	3212	3383	1672
4-Methoxy-2-methyl-2-butanethiol (IX)	<0.1	<0.1	1.8	1.8
Hexanal (X)	964	1274	388	644
(*E*)-2--Hexenal (XI)	10574	4296	365	497
(*Z*)-3-Hexenal (XII)	33	325	53	29
(*Z*)-2-Nonenal (XIII)	9	14	10	8.2
(*E,E*)-2,4-Decadienal (XIV)	112	224	111	145
trans-4,5-Epoxy-(*E*)-2-decenal (XV)	20	n.a.	13	n.a.

[a]The data are means of two assays; maximum SD: ± 10%; n.a.; not analyzed.

TABLE 6

Sensitivity Odor Threshold Values of the Odorants I to IX and XI Dissolved in a Freshly Refined Sunflower Oil

Compound[a]	Mean odor threshold value[a] (μg/kg)
Acetic acid (I)	1050
3-Methylbutanol (II)	225
2-Phenylethanol (III)	113
(Z)-3-Hexenol (IV)	6000
Ethyl 2-methylbutyrate (V)	0.75
Ethyl isobutyrate (VI)	0.75
Ethyl cyclohexanoate (VII)	0.38
(Z)-3-Hexenyl acetate (VIII)	750
4-Methoxy-2-methyl-2-butanethiol (IX)	0.045
(E)-2-Hexenal (XI)	1125

[a]The odor threshold value was retronasally determined.

TABLE 7

Odor Activity Values of the Odorants in the Four Olive Oil Samples[a]

Compound	Olive oil sample			
	I-1	I-2	S-1	S-2
Acetic acid (I)	10	2.3	6.4	<1
3-Methylbutanol (II)	7.1	<1	4	1.2
2-Phenylethanol (III)	10	3.2	3.1	1.8
(Z)-3-Hexenol (IV)	<1	<1	<1	<1
Ethyl 2-methylbutyrate (V)	5.2	2.7	19	55
Ethyl isobutyrate (VI)	3.6	2.4	11	23
Ethyl cyclohexanoate (VII)	4.2	3.2	11	9.5
(Z)-3-Hexenyl acetate (VIII)	<1	4.3	4.5	2.2
4-Methoxy-2-methyl-2-butanethiol (IX)	<1	<1	40	40
Hexanal (X)	13	17	5.3	8.8
(E)-2--Hexenal (XI)	9.4	3.8	<1	<1
(Z)-3-Hexenal (XII)	12	116	19	10
(Z)-2-Nonenal (XIII)	15	23	17	14
(E,E)-2,4-Decadienal (XIV)	2.7	5.5	2.7	3.5
trans-4,5-Epoxy-(E)-2-decenal (XV)	6.7	n.a.	4.3	n.a.

[a]n.a.; Not analyzed.

2- than (Z)-3-hexenal (Table 5). The odor thresholds of (E)-2- and (Z)-3-hexenal amounted to 1125 μg/kg oil (Table 6) and 2.8 μg/kg oil (5), respectively. On the basis of this great difference, the OAVs of (E)-2-hexenal were lower in samples I-1 and I-2 than the OAVs of the (Z)-3-isomer (Table 7). This result suggests that (Z)-3-hexenal contributed more than (E)-2-hexenal to the green odor in I-1 and, in particular, in I-2.

The fruity odor note in samples I-1 and I-2 (Table 3) was caused by the esters **V** to **VII**, which have relatively low flavor thresholds (Table 6). Compared to these esters, (Z)-3-hexenyl acetate (**VIII**) was not a potent odorant in terms of its high flavor threshold (Table 6). The OAV of the acetate **VIII** was less than unity in sample I-1 (Table 7) indicating no effect on the odor. Sample I-2 was much higher in **VIII** than sample I-1 (Table 5), and consequently the OAV of **VIII** amounted to 4.3 (Table 7), which was in the range of the OAVs of the esters **V**, **VI** and **VII**. This suggests that the four esters together contributed to the fruity odor note of I-2. The fruity odor note was more intense in samples S-1 and S-2 (Table 3) than in I-1 and I-2. This agrees with the higher OAVs of the esters **V**, **VI** and **VII** in samples S-1 and S-2 (Table 3).

The high OAV of (Z)-2-nonenal in samples I-1 and I-2 (Table 7) suggests that this aldehyde was strongly involved in the fatty notes of these oils (Table 3). The relatively high values of this aldehyde in samples S-1 and S-2 may have been masked by other compounds as discussed in the next paragraph. Acetic acid showed such a high OAV in sample I-1 that it belonged to the major odorants (Table 7). Possibly, it contributed to the spicy odor note, which was relatively intense in I-1 (Table 3).

A blackcurrant-like note predominated in the odor profiles of samples S-1 and S-2 (Table 3). Thiol **IX**, smelling blackcurrant-like (1,17), likely caused this note in both oil samples. Even though the concentration of **IX** amounted only to 1.8 μg/kg (Table 5), it had a great impact on the odors of S-1 and S-2 due to its low odor threshold (Table 6). Thiol **IX** was not detectable in samples I-1 and I-2, from which the blackcurrant-like odor note was lacking.

The concentration of (Z)-3-hexenal in samples S-1 and S-2 was comparable to that in sample I-1 (Table 5), but there was no perceptible green note (Table 3). It was, therefore, assumed that the odor of (Z)-3-hexenal in samples S-1 and S-2 was masked by the intense odors of thiol **IX** and of esters **V** to **VII**. Presumably, this mask-

ing effect also reduced the intensity of the fatty note caused by (Z)-2- nonenal in the odors of S-1 and S-2 as well as the contribution of acetic acid to the odor of S-1.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Cert (Instituto de la Grasa Y sus Derivados, Sevilla, Spain) and Prof. Dr. E. Fedeli (Centro Nazionale per La Lipochimica del C.N.R., Milano, Italy) for supplying the olive oil samples, and I. Hubensteiner for skillful technical assistance.

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[Received August 23, 1992; accepted March 9, 1993]