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Chemical Characterization of Commercial Sherry Vinegar Aroma by Headspace Solid-Phase Microextraction and Gas Chromatography−Olfactometry

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ABSTRACT: The sensorial representativeness of the headspace solid-phase microextraction (HS-SPME) aroma extract from commercial Sherry vinegars has been determined by direct gas chromatography−olfactometry (D-GCO). Extracts obtained under optimal conditions were used to characterize the aroma of these vinegars by means of GCO and aroma extract dilution analysis (AEDA). Among the 37 different odorants determined, 13 of them were identified for the first time in Sherry vinegars: 2 pyrazines (3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine), 2 sulfur compounds (methanethiol, dimethyl trisulfide), 1 unsaturated ketone (1-octen-3-one), 1 norisoprenoid (β-damascenone), 1 ester (ethyl *trans*-cinnamate) and 6 aldehydes (2- and 3-methylbutanal, octanal, nonanal, (*E*)-2-nonenal and (*E*,*E*)-2,4-decadienal). The determination of the odor thresholds in a hydroacetic solution together with the quantitative analysis—which was also performed using the simple and fast SPME technique—allowed obtaining the odor activity values (OAV) of the aromatic compounds found. Thus, a first pattern of their sensory importance on commercial Sherry vinegar aroma was provided.

KEYWORDS: Sherry vinegar, key odorants, odor activity values, headspace solid-phase microextraction, gas chromatography−olfactometry

INTRODUCTION

Vinegars have been produced by humankind since the early days of agriculture until today, throughout the world and different cultures, and they have been employed as food ingredients and preservatives, as flavor enhancers, and also as ordinary remedies against illness.[1] Vinegars are the result of a two-step fermentation process over almost any fermentable carbohydrate source (fruits, honey, cereals, etc.). First, during alcoholic fermentation, yeasts transform sugars into ethanol, which is then converted into acetic acid during the second fermentation by acetic acid bacteria.

One of the most commonly used vinegars in the Mediterranean basin and central Europe is wine vinegar, a grape-derived product which can be produced by two different methods: a quick process involving a submerged culture of acetic acid bacteria and a slow, surface culture process.[2] The first method, where the rapid fermentation is forced thanks to a continuous oxygenation, provides most commercial vinegars, while the second process, which requires several months to reach the optimum acetic acid degree, produces traditional and high-quality vinegars.

Vinagre de Jerez or Sherry vinegar with Protected Denomination of Origin (PDO)[3] is one of those traditional and selected vinegars, highly appreciated in gastronomy due to its unique chemical and organoleptic properties. Its fermentation process takes place in wood barrels, and, as the acetic acid bacteria grow on the air−liquid interface, the oxygen availability is limited, which slows down the whole process. Moreover, the "criaderas y soleras" production system followed,[4] which consists of a dynamic aging, allows acetification and aging to occur simultaneously, so the product obtained presents a very special chemical composition and a great sensory complexity.

Wine vinegars are mainly employed due to their organoleptic properties, and, among them, flavor plays a relevant role as it is closely related to quality. Indeed, it is one of the first attributes perceived by consumers, and it will have a great influence on the acceptance or rejection of the product. Wine vinegar aroma, as the characteristic aroma of any food commodity, is due to numerous volatile and heterogeneous chemicals at very different concentrations, ranging from several mg L^{-1} to a few ng L^{-1}.[5] These volatile compounds have been studied and reported by different authors,[6,7] including a first attempt to analyze volatile components in Andalusian vinegars.[8] But not since the last ten years have Sherry vinegar volatiles been thoroughly analyzed.[9−13]

However, although all the odorants must be volatile to reach the nostrils and interact with the appropriate receptors located on the olfactory epithelium,[14] not all the volatiles are odor-active. To determine these compounds among the whole volatile fraction of complex mixtures such as foods, gas chromatography−olfactometry (GCO) is the most appropriate analytical tool, as it provides instrumental and sensory analysis simultaneously.[15,16] Thanks to GCO, the eluted analytes are perceived at the same time by the human nose and a conventional detector, like the flame ionic detector (FID) or the mass spectra detector (MSD), which turns this technique into a powerful one in food aroma characterization.

This technique is the one used by the few researchers that have focused their attention on the compounds that really contribute

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to wine vinegar aroma. Thus, Charles et al.[17] carried out a first determination of the odor-active compounds in two red wine vinegars by GCO based on detection frequency. Some years later, Callejón et al. used the GCO technique to screen targeting compounds with an impact on the perceived quality of Sherry vinegars[18] and to identify substances responsible for aromatic notes associated with some selected descriptors of the Sherry vinegar aroma.[19] All these studies reported an important number of aromatic components that, in all cases, had been isolated by liquid−liquid (L−L) extraction with dichloromethane. However, in the studies related to Sherry vinegar, the authors did not employ that aromatic extract obtained with L−L extraction to perform the quantification of the aromatic compounds. To achieve this purpose, they used three different techniques to extract and concentrate the aromatic compounds, which makes the proposed procedure very laborious, time-consuming, and quite tedious.

Among all the sampling techniques, the headspace solid-phase microextraction (HS-SPME) has been shown to be fast and simple because it allows extracting and concentrating the volatiles in a single step with very little sample handling.[20] Moreover, it has also been demonstrated to be reliable in the analysis of food aroma compounds[21] as well as in the characterization of the aroma of different commodities when applying an approach to AEDA developed in our laboratory.[22,23]

Therefore, the aim of this study was to characterize the commercial Sherry vinegar, labeled as "PDO Vinagre de Jerez", aroma by GCO and HS-SPME, with a previous optimization of the HS-SPME conditions to get a representative extract. The approach to the AEDA applied allowed us to obtain a first hierarchical classification of the most potent odorants present in the samples considered. Finally, to confirm the contribution of the most important odorants identified, also their odor activity values (OAV) were calculated.

MATERIALS AND METHODS

Samples. Eight commercial Sherry vinegars labeled as "PDO Vinagre de Jerez" were purchased in a specialized local shop. According to the current legislation,[3] all of them presented a six-month-aging in wood barrels, and their acetic degree and pH value were 7% (w/v) and approximately 2.90, respectively. Their sensory evaluation showed a similar aromatic profile, but some of them presented a higher complexity, so we decide to choose the three of them with the greatest aromatic richness. This greater richness was corroborated with the highest GCO response (both in number of odorants and intensity of the odors perceived).

Reagents and Chemicals. The chemical standards of the aroma compounds were supplied by Sigma-Aldrich (Madrid, Spain) and Lancaster (Bischheim, France). Their CAS numbers are specified in Table 1,

Table 1. Main Odorants Found in Sherry Vinegar with FD ≥ 64 in at Least One of the Three Samples

		RI on			FD[b] factor					identification					
										MSD					
odor-active regions	RT[a] (min)	CP-WAX	HP-5	odor description	A	B	C	odorant	CAS no.	A	B	C	RI[c]	odor	previously reported in Sherry vinegar
1	4.9	*d*	*e*	cabbage, sewer	1024	1024	64	methanethiol	74-93-1				*d*	X	
2	6.5	890	*d*	malty, bitter almonds	4096	16	16	2/3-methylbutanal	96-17-3	X	X	X	X	X	
									590-86-3	X	X	X	X	X	
3	7.1	950	*d*	strawberry	4096	4096	4096	ethyl isobutyrate	97-62-1	X	X	X	X	X	7, 12, 18, 19
4	7.4	970	*e*	butter	4096	1024	4096	diacetyl	431-03-8	X	X	X	X	X	18, 19
5	7.6	990		plastic, rubbery	1024	4096	256	unknown							
6	8.4	1032	801	fruity, strawberry	64	256	64	ethyl butyrate	105-54-4	X	X	X	X	X	7, 12, 18, 19
7	8.7	1050	843	fruity, pineapple	1024	8192	1024	ethyl 2-methylbutyrate	7452-79-1	X	X	X	X	X	12, 18, 19
8	9.1	1062	852	fruity, solvent	1024	8192	4096	ethyl isovalerate	108-64-5	X	X	X	X	X	7, 18, 19
9	9.9	1105	nd[f]	sulfury, disgusting	256	nd	4096	isobutanol	78-83-1	X		X	X	X	7, 8, 10, 12, 19
10	10.2	1119	881	banana, glue-like	4096	1024	4096	isoamyl acetate	123-92-2	X	X	X	X	X	7, 8, 10, 12, 18, 19
11	11.7	1168		fruity solvent, rancid	64	64	1024	unknown							
12	12.8	1214	756	solvent, bitter almonds	64	256	16	2-methyl-1-butanol	137-32-6	X	X	X	X	X	7, 8, 10, 12, 19
13	12.8	1219	710	solvent, bitter almonds	64	256	16	isoamyl alcohol	123-51-3	X	X	X	X	X	7, 8, 10, 12, 13, 18, 19
14	14.6	1290	1001	citrus, fresh	4096	1024	1024	octanal	124-13-0	X	X	X	X	X	
15	14.9	1306	975	mushroom	4096	4096	1024	1-octen-3-one	4312-99-6				X	X	
16	16.9	1368	965	sulfur-like	4096	64	64	dimethyl trisulfide	3658-80-8	X	X	X	X	X	
17	17.5	1397	nd	citrus, flowery	16	256	1024	nonanal	124-19-6	X	X	X	X	X	
18	17.6	1410		mushroom-like, earthy	256	4096	1024	unknown							
19	18.3	1427	nd	green, earth	4096	8192	4096	3-isopropyl-2-methoxypyrazine	25773-40-4	*g*	*g*	*g*	X	X	
20	18.6	1445		earthy, fatty	4096	4096	4096	unknown							
21	19.2	1459	907	cooked potato	1024	256	1024	methional	3268-49-3	*g*	*g*	*g*	X	X	18
22	19.8	1512		fresh, green	4096	1024	4096	unknown							
23	20.3	1529	nd	leaf-like	64	64	256	unknown							
24	20.6	1532	1191	green pepper	1024	1024	4096	3-isobutyl-2-methoxypyrazine	24683-00-9				X	X	
25	20.8	1538		anise-like, fresh	256	nd	256	unknown							

Table 1. Continued

odor-active regions	RT[a] (min)	RI on CP-WAX	RI on HP-5	odor description	FD[b] factor A	FD[b] factor B	FD[b] factor C	odorant	CAS no.	identification MSD A	MSD B	MSD C	MSD RI[c]	MSD odor	previously reported in Sherry vinegar
26	20.9	1546	1160	paper-like	1024	1024	1024	(*E*)-2-nonenal	18829-56-6		X		X	X	
27	21.8	1590	1232	lactic, cheese	64	4	256	isobutyric acid	79-31-2	X	X	X	X	X	7, 12, 13, 18
28	23.2	1644	nd	cheese, vomit	1024	4096	4096	butyric acid	107-92-6	X	X	X	X	X	7, 12, 13, 18
29	23.7	1675	879	blue cheese, sweat	4096	4096	4096	2-methylbutyric acid	116-53-0	X	X	X	X	X	10
								isovaleric acid	503-74-2	X	X	X	X	X	7, 12, 13, 18, 19
30	24.9	1728	1324	deep-fried	1024	4096	1024	(*E,E*)-2,4-decadienal	25152-84-5				X	X	
31	25.2	1739	nd	fresh, mint-like	1024	4096	1024	benzyl acetate	140-11-4	X	X	X	X	X	7, 10, 12, 13, 19
32	26	1770		sweet, flowery	256	64	4096	unknown							
33	26.6	1817	1243	flowery, rose, sweet	256	16	256	ethyl phenylacetate	101-97-3	X	X	X	X	X	10, 12, 13, 18, 19
34	27	1839		geranium	4096	8192	4096	unknown							
35	27.2	1845	1257	roses	4096	4096	1024	2-phenylethyl acetate	103-45-7	X	X	X	X	X	7, 10, 12, 13, 18, 19
36	27.4	1851	1383	sweet, peach jam	1024	4096	1024	β-damascenone	23726-93-4	X	X	X	X	X	
37	27.8	1870		flowery, sweet	nd	4096	256	unknown							
38	28.5	1899	1093	smoky, sweet	4096	4	1024	guaiacol	90-05-1	X		X	X	X	13, 18, 19
39	28.7	1915	1039	roses, sweety	4096	16	nd	phenylmethanol	100-51-6	X	X	X	X	X	7, 10, 12, 13, 18, 19
40	29.6	1957	1119	roses	256	256	4096	2-phenylethanol	60-12-8	X	X	X	X	X	7, 10, 12, 13, 19
41	30.2	1986	nd	disgusting, sour	4	nd	256	heptanoic acid	111-14-8				X	X	18
42	30.7	2022		roasted, burnt	1024	4096	1024	unknown							
43	31.7	2089	1281	smoky, clove	4096	16	4096	4-ethylguaiacol	2785-89-9	X	X	X	X	X	10, 12, 13, 18
44	33.4	2178	1466	sweet, honey	4096	4096	4096	ethyl *trans*-cinnamate	103-36-6				X	X	
45	34.3	2217	1182	animal, stall	1024	4096	4096	4-ethylphenol	123-07-9	X	X	X	X	X	10, 12, 13, 18, 19
46	34.6	2245		animal	nd	4096	1024	unknown							
47	35.1	2320	1377	dusty, spicy	4096	4096	4	decanoic acid	334-48-5	X	X	X	X	X	7, 10, 12, 13, 18, 19

[a] RT: Retention time on a CP-WAX 57CB. [b] FD: Flavor Dilution. [c] RI: Retention Index on different stationary phases. [d] : RI not calculated due to solvent interference. [e] : RI < RI of the first alkane (C6). [f] Not detected. [g] Identification not possible due to acetic acid interference.

and their purity was above 90% in all cases. 4-Methyl-2-pentanol (Fluka, Madrid, Spain) was employed as internal standard, and pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Acetic acid (HPLC grade), NaOH and NaCl (both reagent grade) were purchased from Scharlab (Barcelona, Spain).

To prepare the stock standard solutions (employed both for the odor threshold determination and for the calibration graph elaboration) and to dilute the samples on the AEDA study, we used an acetic acid water solution at 7% (w/v) with pH adjusted to 2.9 in order to get a similar matrix to real samples. To avoid odor interferences, this hydroacetic solution was filtered employing an Empore extraction disk with activated carbon (3M Bioanalytical Technologies, St. Paul, MN, USA). The cleaned hydroacetic solution was analyzed by HS-SPME and GCO, and the absence of odors in this analysis corroborated the effectiveness of this cleaning procedure.

The different stock standard solutions were stored at 4 °C.

SPME. The SPME holder for manual sampling and the polydimethylsiloxane (PDMS) 100 μm, carboxen−polydimethylsiloxane (Carboxen/PDMS) 75 μm and StableFlex divinylbenzene−carboxen−polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm fibers used in this study were purchased from Supelco (Bellefonte, PA, USA). All the fibers were conditioned before use and thermally cleaned between analyses by inserting them into the GC injection port at the temperature recommended by the producer.

Headspace Solid-Phase Microextraction (HS-SPME). The optimum conditions that allowed extracting the highest number and intensity of odorants were achieved by placing 10 mL of sample into a 20 mL glass vial with 3.5 g of NaCl and a little magnetic stir bar (as the extraction was carried out under constant magnetic stirring), the sample/headspace ratio being 1:1. After the vial was tightly capped with a silicon septum under nitrogen atmosphere, it was pre-equilibrated for 15 min at 50 °C in a thermostatic bath. Then, the SPME device was manually pushed through the vial septum and the fiber was exposed to the headspace vial for 2 h at 50 °C. Afterward, the fiber was pulled into the needle assembly and the SPME device was removed from the vial. Finally, it was inserted into the injection port of the GC-FID for thermal desorption of the analytes at 270 °C for 1 min in the splitless mode.

Gas Chromatography Analysis. *GC-FID and GCO.* Samples were analyzed on a Hewlett-Packard (HP, Palo Alto, CA, USA) 6890 gas chromatograph equipped with a flame ionization detector (FID) and an olfactory detector. To carry out the chromatographic separations, a Chrompack (Varian, Middelburg, The Netherlands) CP-WAX 57CB (50 m × 0.25 mm i.d., 0.2 μm film thickness) fused silica capillary column was employed and the oven temperature was programmed as follows: 40 °C (2 min), 5 °C min^{-1} to 220 °C (22 min). To verify the identity of the compounds, an HP-5 (Agilent Technologies, Santa Clara, USA) (30 m × 0.32 mm i.d., 0.25 μm film thickness) fused silica capillary column was used with the following oven temperatures: 40 °C (5 min), 3.5 °C min^{-1} to 120 °C, 10 °C min^{-1} to 210 °C (10 min). In both cases, helium was the carrier gas at a constant flow rate of 1 mL min^{-1}, the split−splitless injection port operated in the splitless mode at 270 °C for 1 min and the temperature of the FID was set at 250 °C.

GCO analyses were carried out using an olfactory detector commercialized by SGE International (Ringwood, Australia) that presented

an outlet splitter system (ODO-I) which provided a continuously variable range of split ratios thanks to a micro control valve (OSS-2). The chosen split ratio for the olfactometric analysis was 1:10 (FID: sniffing port). Two deactivated and uncoated fused silica capillaries of the same length and width were used as transfer lines between the valve and the detectors. In addition, the olfactory detector control module incorporated a heated transfer section from the GC oven to the glass detection cone that kept the unit at a suitable temperature to transfer the volatiles to the detection cone without losses due to condensation. Furthermore, the glass cone is purged with humidified air to prevent nasal mucous membranes from drying out in order to maintain olfactory sensitivity.

Timing and odor descriptions were recorded by two trained sniffers (replaced at 15 min intervals to avoid fatigue) after each sample injection. Each trained researcher analyzed each extract in triplicate, and three different fibers were employed to take into account the efficiency variability of the SPME StableFlex fibers.

GC−MS. A Hewlett-Packard (HP, Palo Alto, CA, USA) 6890 gas chromatograph coupled to an HP-5973 mass selective detector (MSD) was used to perform the GC−MS analyses. The mass spectrometer operated in the EI mode (70 eV), and the mass range was from 35 to 300 amu, while interface, source and quadrupole temperatures were 200 °C, 230 °C and 150 °C, respectively. Separation was achieved under the same operating conditions mentioned before and using the same columns as in the GC-FID and GCO analyses. The split−splitless injection port operated in the splitless mode at 270 °C for 1 min.

Aroma Extract Dilution Analysis (AEDA). The aroma extract dilution analysis (AEDA) involves stepwise dilution of the aromatic extract with a solvent followed by an evaluation of each dilution by GCO, which leads to a hierarchical classification of the most odor-active compounds based on the flavor dilution (FD) factors. However, the usual AEDA cannot be used when dealing with the SPME technique because no physical extract is obtained. As the analytes are retained on the fiber and it is not possible to dilute them, an approach to the AEDA developed in our laboratory[22] was applied. It consists of successive dilutions (1:4) of the Sherry vinegar samples with a hydroacetic solution before performing the SPME. Dilutions were carried out until no odorant was detected by sniffing the highest dilution. Two experienced sniffers performed the AEDA experiments in triplicate, and their response to the individual compounds did not differ by more than 2 FD-factors.

Compound Identification. The odorants perceived in the olfactometric study were identified by comparison with the reference compounds (analyzed under identical conditions) on the basis of the following criteria: odor quality detected at the sniffing port, mass spectra obtained and retention indices (RI) determined on the two stationary phases of different polarity employed (CP-WAX 57CB and HP-5). Retention indices were calculated from the retention times of a series of *n*-alkanes (from 6 to 26 carbon atoms) injected under the same chromatographic conditions.

Sensory Analysis. Twelve trained nonsmoker individuals (8 women and 4 men, between 25 and 42 years old) constituted the test panel that performed the sensory experiments, all of them belonging to the Department of Analytical Chemistry of the Rovira i Virgili University and with previous sensory analysis experience. To get used to testing acetic acid matrices, they were subjected to a specific training: different single standard solutions, and also mixtures of them, were prepared in a 7% (w/v) hydroacetic solution at various concentration levels. The panelists were asked to describe the odor detected and to rate the intensity perceived in a scale from 0 to 5 for each standard (i.e., each odor quality) and concentration, which was also used to evaluate the panel response. The training was carried out twice a week during five consecutive weeks, each session lasting 60 min. The coefficients of variance of each single panelist for every sample replicate were less than 10%. To evaluate the panel performance as a whole, one-way analysis of variance (ANOVA) was carried out.

Threshold Determination. Threshold values were determined by the three-alternative forced choice (3-AFC) test,[24,25] that consists of three samples, two controls and one spiked sample, among which assessors are instructed to guess (forced choice) when they cannot perceive a difference.

First, the threshold values of the different odorants were looked up in the literature,[26] although most of them had not been calculated in a vinegar matrix. Even so, these thresholds were chosen as the odorant concentrations (x values) to delimit a first concentration working range. Then, five 2-fold and 8-fold dilutions ($x/8$; $x/2$; x; $2x$ and $8x$) were prepared in a 7% (w/v) acetic acid water solution for each substance whose threshold was to be determined, and the five 3-AFCs were given to the panelists in ascending order (most diluted first). For each level, all the solutions were labeled with a randomized three-digit number, and the appearance order for the spiked sample was also random.

More diluted or concentrated solutions were prepared when necessary, for each panelist so, finally, it was possible to relate each assessor to two concentration values for each odorant: the lowest was the one where the odor was not still perceived and the highest, where the odor was detected for the first time. Individual thresholds were calculated by the best-estimate criterion: the threshold for each individual was the geometric mean between the last concentration missed and the first concentration detected for all the sensory sessions. The final threshold was the geometric mean of each panelist's best estimates for each compound.[24,25]

RESULTS AND DISCUSSION

Optimization of HS-SPME Parameters. The chromatographic areas of the extracted compounds (FID response) do not always present a correlation with the sensory perception, so it is not enough to consider this fact when optimizing the parameters that affect the aroma extraction efficiency. Therefore, we decided to evaluate the number and intensity of the odorants perceived (GCO response) as well. All the experiments were performed in triplicate, and we used more than one SPME fiber to take into account their variability response, although the reproducibility of the fibers has considerably been improved during the last years.

The first parameter considered was the SPME fiber coating chosen. The coatings checked were those that, according to their specifications, were the most appropriate to extract aroma compounds: CAR/PDMS (low molecular weight compounds), PDMS (volatiles) and DVB/CAR/PDMS (volatiles and semivolatiles). The results obtained, when extracting the same vinegar with different fibers, showed that the DVB/CAR/PDMS fibers presented higher extraction efficiency. Thus, whereas with PDMS the number of odor-active regions was between 35 and 40 and for CAR-PDMS was between 55 and 60, when using DVB/CAR/PDMS, up to 79 odorant regions were detected, so this last fiber coating was selected.

The extraction efficiency is greatly affected by the headspace volume,[27] so the next parameter studied was the sample/headspace ratio. Experiments with 20 and 50 mL vials and different sample volumes (5, 10, and 25 mL) were carried out. The results showed that the extraction efficiency improved as the headspace decreased, 1/1 being the optimum sample/headspace ratio, whatever the vial considered. However, in order to use the minimum amount of sample in each analysis, it was decided to work with 10 mL of sample in a 20 mL vial. Related to ionic

strength, the higher it was the better response obtained, so samples were saturated by adding 3.5 g of NaCl.

In SPME, extraction time and temperature are deeply related,[22,27] so both parameters were studied simultaneously. Thus, different experiments in the range from 2 to 4 h (times under 2 h did not ensure suitable aromatic perception of all the odorants) and from 30 to 50 °C were carried out. The experiments showed that the shortest sampling time and the lowest temperature (2 h, 30 °C) provided the poorest chromatographic and olfactometric profiles (nearly 10% smaller than the ones obtained when working with other sampling times and temperatures). Meanwhile, the largest time and the highest temperature (4 h, 50 °C) did not improve the overall extraction efficiency because of the exothermic character of the SPME technique. Therefore, as the chromatographic and ofactometric responses obtained at 2 h and 50 °C did not significantly differ (less than 5%) from the ones obtained at 3 h and 40 °C, we decided to reduce the sample preparation step by employing the shortest extraction time (2 h, 50 °C).

As a result, the optimum HS-SPME conditions were achieved by placing 10 mL of sample into a 20 mL glass vial with 3.5 g of NaCl and a little magnetic stir bar. Extraction was performed during 2 h at 50 °C under constant magnetic stirring.

Regarding the precision of the method, it was assessed in terms of within-day repeatability and between-days repeatability (intermediate precision). Although both precision parameters were sensory evaluated in the sniffing port, it was difficult to quantify them. Therefore, to obtain an objective evaluation we used the chromatographic response of the aromatic compounds and we expressed both parameters by means of the percentage of relative standard deviation (% RSD) of that response. Thus, while the repeatability was calculated by the consecutive injection of 5 different extracts obtained the same day, the intermediate precision was calculated after the injection of 6 different extracts obtained over a month. In both cases the results were very good since we obtained RSD < 4.7% for repeatability and RSD < 7.3% for intermediate precision.

Extract Representativeness. Although the best conditions above specified allowed obtaining the highest number and intensity of odorants, it should not be forgotten that the aroma representativeness of the sample extract is the most important parameter to ensure the reliability of the results when characterizing that sample aroma. Therefore, 12 trained assessors evaluated the similarity between the aroma of the three Sherry vinegars considered and the aroma extracted by the HS-SPME technique. To evaluate the global perception of the compounds retained on the SPME fiber, the direct gas chromatography−olfactometry technique (D-GCO)[23,28] was employed. Since a short deactivated capillary column is used, this technique avoids chromatographic separation of the extract constituents, so the analyst perceives the extract as a whole at the sniffing port. When asking the panelists about the degree of similitude between the extracts and the real sample, they found an 85% (5% RSD) of likeness whatever the Sherry vinegar evaluated. Therefore, this great similitude indicated that the SPME is a good and fast technique to obtain representative aroma extracts of samples so complex such as vinegars.

HS-SPME-GCO. The aromas of the three Sherry vinegars considered in this study were extracted and concentrated by HS-SPME and analyzed by GCO. The results showed slight differences in the number of odor-active regions detected: while 70 aromatic areas were recorded for the A sample, 65 and 79 were perceived for the B and C samples, respectively. Furthermore, most of the odor-active regions coincided in the three samples both in their retention times and in the descriptors used to describe the odors detected. In all cases, as happens when a polar column is employed to carry out similar flavor studies,[18,22,23] chemical and fruity odors appeared at lower retention indices. Then earthy and green notes were detected, followed by lactic and fatty ones. Afterward, sweety and flowery odors appeared, and finally, at the end of the GCO analysis, smoky and disgusting notes were perceived.

To evaluate the sensory contribution of each odorant to the whole sample aroma and to get a first hierarchical classification of the odorants in commercial Sherry vinegar, the volatiles extracted with the HS-SPME technique were analyzed by using the approach to the AEDA previously developed in our laboratory.[22] This approach has been demonstrated to be a valuable screening tool for ranking odor-active compounds in a sample according to their relative odor potency.[22,23] It involves stepwise dilution of the sample (therefore, of its aroma) followed by SPME and the subsequent evaluation of each dilution by GCO until no odors are perceived in the GCO effluent. As defined when using the classical AEDA,[29,30] the last dilution step where an odorant is perceived is its flavor dilution (FD) factor, which can be regarded as a good indicator of the odor potency of that compound.

The odor-active regions with higher FD factors (ranging from 64 to 8192 for at least one of the three samples) are reported in Table 1, where they have been arranged according to their retention indices in the polar column. As can be seen, among the different odor-active regions only 2 of them were not detected for the A sample (region numbers 37 and 46, which were not identified), 3 of them were not perceived for the B sample (area numbers 9, identified as isobutanol; 25, unknown; and 41, identified as heptanoic acid) and only the flavor-active region number 39 (benzyl alcohol) was not detected in the C sample. From these results, as usual when dealing with different samples of the same product, we could conclude that the odorants responsible for the Sherry vinegar aroma are almost the same whatever the sample considered and that the differences among samples are given by the perception intensity of some of these odorants (i.e., different flavor dilution (FD) factors). In fact, only 5 odor-active regions were detected with the same FD in all the samples: 4 regions with a FD of 4096, described as "strawberry", "earthy, fatty", "blue cheese, sweat", and "sweet, honey", which corresponded to region numbers 3, 20, 29 and 44, and 1 with a FD of 1024 that was described as "paper-like" (region 26).

As shown in Table 1, some of the identified odorants had been previously reported in Sherry vinegars either as volatile[7,8,10,12,13] or as aromatic[18,19] compounds, but 13 of them were detected in Sherry vinegar aroma for the first time. These are the ones summarized in Table 2, whose odor thresholds in an acetic acid water solution were also determined: 2 pyrazines (3-isopropyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine), 2 sulfur compounds (methanethiol and dimethyl trisulfide), 1 unsaturated ketone (1-octen-3-one), 1 norisoprenoid (β-damascenone), 1 ester (ethyl *trans*-cinnamate) and 6 aldehydes (2- and 3-methylbutanal, octanal, nonanal, (*E*)-2-nonenal and (*E*,*E*)-2,4-decadienal). The presence of 3-alkyl-2-methoxypyrazines could be due to their natural origin in grapes,[31] and, although they are characteristic of some varieties such as Cabernet sauvignon, Merlot noir and Sauvignon blanc,[32] they have also been identified in some aged red wines.[33] Regarding sulfur

Table 2. Sensory Thresholds of the Odorants Found in Sherry Vinegar for the First Time

odorant	sensory threshold (ng L^{-1})	FD factor A	FD factor B	FD factor C
3-isopropyl-2-methoxypyrazine	10	4096	8192	4096
3-isobutyl-2-methoxypyrazine	22	1024	1024	4096
dimethyl trisulfide	35	4096	64	64
methanethiol	160	1024	1024	64
β-damascenone	190	1024	4096	1024
(*E*,*E*)-2,4-decadienal	435.6	1024	4096	1024
ethyl *trans*-cinnamate	480	4096	4096	4096
(*E*)-2-nonenal	1500	1024	1024	1024
1-octen-3-one	6800	4096	4096	1024
nonanal	18200	16	256	1024
octanal	22500	4096	1024	1024
3-methylbutanal	31600	4096	16	16
2-methylbutanal	84300	4096	16	16

compounds, they are mostly released during alcoholic fermentation by the degradation of odorless precursors, such as *S*-cysteine conjugates. However, the origin of these compounds in wines is still being investigated and alternative biogenetic pathways involving conjugated carbonyl compounds, or the *S*-glutathione conjugate, have either been hypothesized or evidenced.[34,35] Related to 1-octen-3-one, it is a common constituent of the gas chromatographic odor (GCO) profile of normal dry wines and it is a naturally occurring compound in grapes.[36] On the other hand, β-damascenone has its source in grape carotenoid degradation,[37] ethyl *trans*-cinnamate comes from alcoholic fermentation in wines,[38] 3-methylbutanal can be considered an amino acid derivative[39] and (*E*)-2-nonenal is a product of the oxidative degradation of unsaturated fatty acid.[40] Moreover, the formation of aldehydes in vinegars, as occurs in wine, could be related to changes in aroma properties linked to oxidation,[41] and it is also known that the aldehydes with 8−10 carbon atoms can be considered strong odorants because of their sensory properties.[42]

Table 2 shows also the FD factors of these odorants for each sample and their sensory thresholds, which in some cases are at ng L^{-1} levels. Because of their high FD values, it could be thought that *a priori* they could contribute significantly to the overall aroma of the sample, despite their low concentrations. Indeed, these low concentrations imply the use of a concentration technique such as SPME to be detected. Even so, although we could perceive all of them by GCO, only some of them were detected when applying a conventional detector.

However, although the FD factors provided a first hierarchical classification of the odorants detected, it was necessary to use other parameters to get more precise information about the real contribution of each odorant to the overall sample aroma.

Odor Activity Value (OAV). Odor activity value (OAV) was obtained by dividing the concentration of the compound in a matrix by its odor threshold in that matrix.[5] Although this parameter provides a rough pattern of the sensory importance of the odorants, it allows turning the quantitative data into sensorial information. So it is generally assumed that the odorants with higher OAVs contribute in a stronger manner to the overall aroma.

Table 3. Mean Concentrations of the Potent Odorants Found in Sherry Vinegars

compound	mean concn ± SD (μg L^{-1}) A	B	C
ethyl isobutyrate	462.9 ± 8.5	303.1 ± 12.7	391.9 ± 17.6
diacetyl	117.5 ± 4.9	60.8 ± 13.8	177.7 ± 16.7
ethyl butyrate	16.3 ± 0.7	23.1 ± 3.7	17.2 ± 2.1
ethyl 2-methylbutyrate	10.0 ± 0.7	26.5 ± 0.4	7.2 ± 0.2
ethyl isovalerate	277.5 ± 7.2	301.6 ± 1.7	299.5 ± 2.5
isobutanol	1752.4 ± 55.4	nd[a]	2042.9 ± 27.6
isoamyl acetate	1071.7 ± 7.9	840.2 ± 62.67	1315.6 ± 24.6
2-methyl-1-butanol	2949.1 ± 33.0	3086.6 ± 69.1	2774.9 ± 14.2
isoamyl alcohol	3674.9 ± 35.5	3812.1 ± 17.5	3306.0 ± 12.2
octanal	14.1 ± 0.9	10.8 ± 1.8	11.7 ± 1.1
isobutyric acid	2460.5 ± 19.1	1801.9 ± 69.6	3622.0 ± 111.7
2-methylbutyric acid	2301.0 ± 107.6	1989.3 ± 81.9	2523.0 ± 181.4
isovaleric acid	6643.0 ± 226.9	6110.1 ± 325.0	5830.8 ± 120.7
benzyl acetate	13.2 ± 0.2	20.5 ± 0.6	10.4 ± 0.4
ethyl phenylacetate	988.3 ± 2.6	117.2 ± 0.4	385.5 ± 1.7
2-phenylethyl acetate	462.3 ± 16.0	388.4 ± 21.0	240.9 ± 21.0
β-damascenone	0.21 ± 0.03	0.29 ± 0.02	0.14 ± 0.03
phenylmethanol	120.2 ± 12.7	63.4 ± 6.6	nd
2-phenylethanol	6514.2 ± 132.4	7993.1 ± 133.0	9511.3 ± 161.0
4-ethylguaiacol	2971.4 ± 87.5	664.4 ± 1.8	1603.9 ± 29.6
4-ethylphenol	71.7 ± 0.5	81.3 ± 4.7	79.8 ± 6.0
decanoic acid	126.7 ± 3.326	122.7 ± 1.5	31.5 ± 5.5

[a] Not detected.

Therefore, once the most potent odorants were determined, it was necessary to quantify them with the aim to calculate their OAVs. To make as simple as possible the methodology used to carry out the quantification, the extract employed for this purpose was the same as the one used to perform the GCO analysis. Taking into account the matrix interference that hindered the use of calibration curves constructed with model solutions, we elaborated the calibration graphs by using the addition standard method and extracting the spiked samples under the same HS-SPME extraction conditions above specified (Headspace Solid-Phase Microextraction (HS-SPME)). Thus, the most potent odor-active compounds according to the AEDA results were quantified, except those with so extremely low concentrations that it was not possible to obtain any instrumental signal when using a FID or a MSD. This is what happened with 3-alkyl-2-methoxypyrazines, 1-octen-3-one or methanethiol, among others. Table 3 summarizes the mean concentrations of the odorants quantified, and the values obtained are very similar to those determined by other authors when analyzing Sherry vinegar,[10,12,19] except for 2- and 3-methylbutyric acid. For these compounds we found lower concentrations than those determined by other researchers. It could be due to the fact that we have quantified both odorants separately but, in other studies, they have been quantified together. Other small differences could be attributed to the different length of aging in wood because in some of the previous studies[10,12] the authors did not specify such length.

In addition, we determined the sensory thresholds of the most potent odorants identified by means of GCO and AEDA. In order to determine these values in a matrix as similar as possible

Table 4. Odor Thresholds and Odor Activity Value (OAV) Calculated for the Quantified Odorants

		odor activity values (OAV)		
odorant	sensory threshold (μg L^{-1})	A	B	C
ethyl isovalerate	2.2	126	137	136
ethyl isobutyrate	5.2	89	58	75
isoamyl acetate	22.2	48	38	59
isovaleric acid	144.4	46	42	40
4-ethylguaiacol	69.5	43	9.6	23
2-methylbutyric acid	200.5	11	9.9	12
2-phenylethanol	1400	4.6	5.7	6.8
2-phenylethyl acetate	97.8	4.7	4.0	2.5
isobutyric acid	1500	1.6	1.2	2.4
4-ethylphenol	51	1.4	1.6	1.6
ethyl phenylacetate	148.2	6.7	0.79	2.6
diacetyl	95.3	1.2	0.64	1.9
β-damascenone	190[a]	1.1	1.5	0.74
isoamyl alcohol	3500	1.0	1.1	0.94
ethyl 2-methylbutyrate	16.5	0.61	1.6	0.44
octanal	22.5	0.63	0.48	0.52
ethyl butyrate	73	0.22	0.32	0.24
2-methyl-1-butanol	12200	0.24	0.25	0.23
isobutanol	7500	0.23	nd[b]	0.27
decanoic acid	1100	0.11	0.11	29×10^{-3}
phenylmethanol	5100	23×10^{-3}	12×10^{-3}	nd
benzyl acetate	2300	5.7×10^{-3}	8.9×10^{-3}	4.5×10^{-3}

[a] Concentration in ng L^{-1} [b] Not detected.

to the real one, an acetic acid water solution was employed. The values obtained can be seen in Table 4 together with the OAVs calculated for each quantified compound in every Sherry vinegar sample.

When comparing these values with those obtained by other authors that previously identified some of these compounds in Sherry vinegar aroma, we could check that the relative OAVs for ethyl isovalerate, ethyl isobutyrate, isoamyl acetate, isovaleric acid or 2-phenylethyl acetate agreed with the ones reported by Callejón et al.[19] However, there was no coincidence when comparing 4-ethylphenol and diacetyl, as these authors obtained higher OAVs. In fact, diacetyl presented the highest OAV in their study. But these differences could be attributed to the different extraction procedures employed and also to the lower odor threshold reported by Callejón et al. for both compounds: 4 μg L^{-1} vs 51 μg L^{-1} regarding 4-ethylphenol, and 40 μg L^{-1} vs 95.3 μg L^{-1} regarding diacetyl.

Finally, focusing again on the results shown in Table 4, there are 10 compounds with OAV > 1 in the three vinegars analyzed. These compounds, together with their FD values, are displayed in a spider-web (Figure 1). As it can be seen from this figure, the profile obtained is quite similar whatever the sample considered, except for isobutyric acid, 4-ethylguaiacol and, to a lesser extent, 2-phenylethanol. To check the real contribution of these 10 compounds to the Sherry vinegar aroma, a very simple similarity test was performed: we added all these compounds to a 7% (w/v) hydroacetic solution at the same concentration as found in each vinegar. Then, panelists were asked to evaluate the similarity between the spiked solutions and the real vinegars. They compared the odor perceived for each spiked solution with that

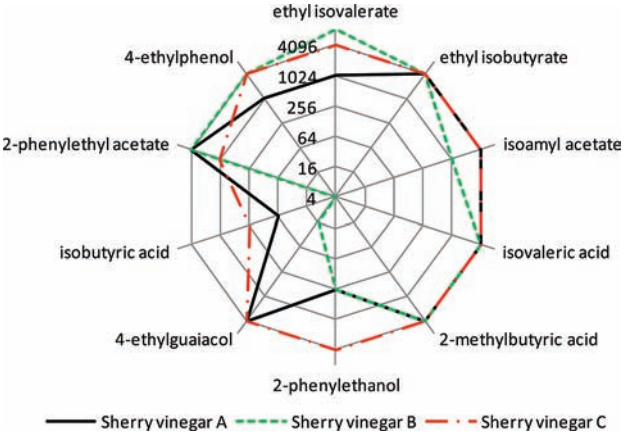

Figure 1. Spider-web for the odorants with OAV ≥ 1 for the three Sherry vinegar samples.

of its real vinegar, as a pair, on a discontinuous scale from 0 (no similarity) to 5 (maximum similarity). The results showed a degree of similarity of 49, 46 and 51% (RSD 5%) for vinegars A, B and C, respectively. According to the panelists' description, this relatively low percentage was related to a much higher perception of the pungent sensation in the spiked sample mainly due to the acetic acid. Therefore, we performed a second similarity test, but this time adding all the compounds quantified. In this case, panelists agreed with the fact that the pungent sensation was balanced thanks to the higher aromatic richness of the spiked solution, and, when rating the degree of similarity, they gave a

grading of 67, 63 and 70% (RSD 6%) for vinegars A, B and C, respectively. So, these results agree with the ones obtained in the GC−olfactometric study because, as it can be seen, the most important aromatic compounds, those that play the most relevant role in Sherry vinegar aroma, are almost the same whatever the sample considered. Only slight differences are perceived among samples regarding the perception intensity of the odorants, which makes possible the characteristic organoleptic properties of every Sherry vinegar sample.

From the results, we can conclude that the HS-SPME technique when using a DVB/CAR/PDMS fiber is suitable for extracting and analyzing the odor compounds in vinegar matrices. Moreover, it requires shorter sampling times and minimum sample handling compared with the conventional extraction methods (i.e., liquid−liquid extraction).[17−19,23] As explained, some of the key odorants determined in this study have been reported as aromatic compounds in Sherry vinegar aroma for the first time and their odor thresholds have been determined in a hydroacetic solution. Then, the approach to the AEDA applied has allowed us to establish a hierarchy on the contribution of each compound to Sherry vinegar aroma, which has been confirmed by the OAVs calculated. Nevertheless, to finally give more accurate results about the real contribution of the different constituents to the overall Sherry vinegar aroma it will be necessary to overcome the limitations of the instrumental detector (e.g., by using a MS detector in SIM mode) to make possible the quantification of all the odorants detected by GCO and to perform complete reconstitution studies, which will be the future aim of our studies.

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