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Influence of Olive Oil Phenolic Compounds on Headspace Aroma Release by Interaction with Whey Proteins

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Supporting Information

ABSTRACT: The release of volatile compounds in an oil-in-water model system obtained from olive oil−whey protein (WP) pairing was investigated by considering the effect of phenolic compounds. Human saliva was used to simulate mouth conditions by retronasal aroma simulator (RAS) analysis. Twelve aroma compounds were quantified in the dynamic headspace by SPME-GC/MS. The results showed significant influences of saliva on the aroma release of virgin olive oil (VOO) volatiles also in the presence of WP. The interaction between WP and saliva leads to lower headspace release of ethyl esters and hexanal. Salivary components caused lower decrease of the release of acetates and alcohols. A lower release of volatile compounds was found in the RAS essay in comparison to that in orthonasal simulation of only refined olive oil (without addition of saliva or WP), with the exception of hexanal and 1-penten-3-one, where a significantly higher release was found. Our results suggest that the extent of retronasal odor (green, pungent) of these two volatile compounds is higher than orthonasal odor. An extra VOO was used to verify the release in model systems, indicating that WP affected aroma release more than model systems, while saliva seems to exert an opposite trend. A significant increase in aroma release was found when phenolic compounds were added to the system, probably due to the contrasting effects of binding of volatile compounds caused by WP, for the polyphenol−protein interaction phenomenon. Our study could be applied to the formulation of new functional foods to enhance flavor release and modulate the presence and concentrations of phenolics and whey proteins in food emulsions/dispersions.

KEYWORDS: *phenolic−aroma interaction, volatile compounds, SPME-GC/MS, aroma release, human saliva, RAS*

INTRODUCTION

Olive oil is a staple in the Mediterranean diet, considered as one of the most health-promoting nutritional habits worldwide. It is characterized by a high intake of monounsaturated fatty acids, which have showed to help in lowering cholesterol and heart disease.[1] In comparison to seed oils, virgin olive oil (VOO) has a peculiar flavor and biophenol-rich polar fraction which gives rise to other benefits to human health, due to the important nutritional role played by the high percentage of oleic acid.[2,3] Therefore, an increase of VOO biophenol consumption, without an increase in fat intake, should be recommended by health authorities in order to encourage the general population to use it. The delicate flavor of VOO is first perceived during inhalation, when the odorants are released into the headspace; they pass through the external nostrils and stimulate the olfactory receptors in the nasal cavity (nasal route). Then the aroma is perceived while tasting VOO, when the odorants interact with the receptors by migrating from the mouth to the nasal cavity via the nasopharynx (retronasal route). Simultaneously other sensations take place: e.g., bitterness, sweetness, astringency, and pungency. VOO phenolic compounds are responsible for the astringency and pungency perceptions; the former sensation acts by stimulating taste receptors, while the latter is perceived by the trigeminal nerve. On the other hand, volatile compounds are responsible for the odor and aroma by stimulating the olfactory receptors.[4,5]

VOO is mainly employed in Mediterranean countries in many food preparations or as an ingredient in salad dressings. Generally, there are two distinct ways to pair VOO with food: complementary and contrasting approaches. A complementary flavor is obtained when two similar ingredients are blended. This results in the enhancement of primary flavors, while a contrasting approach consists in tasting each ingredient separately. Pairings of VOO with salads, vegetables, pesto, tomato sauces, etc. are examples of the first case. The latter example could be the case of pairing VOO and fresh mozzarella cheese in the famous and delicious "*insalata Caprese*." In fact, the unique flavor of a "strong" VOO (bitter, pungent, and fruity) is in contrast with the delicate texture and taste of fresh mozzarella (sweet, acid, and milk note). Frequently, ricotta cheese is also used as an ingredient in many recipes with VOO to obtain a sweetening effect of bitter-pungent notes in strong VOOs. It is not surprising that renowned chefs pair VOO with many different foods, in order to obtain new sensations and create new culinary experiences. When VOO is combined with dairy products, an oil-in-water emulsion or dispersion is produced. The presence of different phases and the level of volatile compounds affect their partitioning. In fact, lipophilic aroma compounds tend to move in the oil phase and their concentration considerably decreases in the continuous phase. In contrast, hydrophilic compounds tend to move in the aqueous phase.[6] In addition, nonvolatile matrix components, such as whey proteins, affect aroma release by interacting with aroma compounds.[7−9] Furthermore, it must be considered that

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volatile compounds analyzed in foods per se could undergo significant differences when the food is consumed and could lead to differences in the final aromatic perception. In fact, considerable physicochemical changes which affect the release of volatile compounds from emulsions occur in the mouth while eating. This is the reason the sensations of odor (the so-called nasal route) and aroma (retronasal route) could be perceived differently, even though the same olfactory sense is involved.[10,11] In fact, salivation, mouth size, shear forces from tongue compression, breathing, pH, and temperature are factors able to change the food matrix structure and sensory properties.[12] Among the physiological parameters, saliva has been described in the literature as the most important. Its first effect is to dilute the oil-in-water emulsion when this latter is tasted. Then, the activity of salivary proteins (mucins, albumin, and proteins rich in proline) and enzymes (amylase, lipase, and lysozymes) are responsible for the emulsion destabilization.[13,14] In addition, it was reported that phenolic compounds, in olive oil-in-water emulsions stabilized by β-lactoglobulin, affect the droplet size, improving the dispersion degree.[15] These changes in the structure of the emulsion could also have an effect on the final perception of the products.[16] Several studies have been aimed at understanding the aroma release of volatile compounds in vegetable oil model solutions and emulsions.[6,17−22] Only two published research papers reported the release of only one volatile compound after the interaction between whey proteins and saliva by *in vivo* analysis. The first one studied the release of ethyl butanoate in gel, while the latter reported on 2-nonanone in aqueous solution.[23,24] Another study reported the aroma release of emulsions obtained by soybean oil and β-lactoglobulin under simulated mouth conditions.[25] Moreover, the majority of *in vitro* studies employed artificial saliva.[20−23] This could be a limit because the effect of numerous enzymes and proteins present in human saliva were not considered.[4,12,26] So far, only one published study has investigated the interaction of phenolic compounds from olive oil with different food proteins, including β-lactoglobulin.[27] From a food technology point of view, the polyphenol−protein interaction is interesting as a possible means to reduce bitterness, whereas the effects of such an interaction on the release of flavor compounds has still to be investigated. Up to now, no study has reported on the aroma release of VOO after the interaction with whey proteins and human saliva. The effect of these interactions could be important in understanding oral mechanisms of the emulsion in relation to sensory perception. This piece of information will also be useful to deepen consumer attraction toward foods or dishes made by pairing olive oil with dairy products. Therefore, it could be the basis of further studies in sensory science about consumer preferences.

The present work aims to study the aroma release of volatile compounds in an oil-in-water model dispersion by adding refined olive oil spiked with VOO biophenols to a whey protein solution (1/5 v/v). In particular, this research aims to verify the changes in headspace release of target volatile compounds due to the interactions between virgin olive oil biophenol extract and whey proteins, in the presence of human saliva, simulating mouth conditions.

MATERIALS AND METHODS

Samples, Standards, and Reagents. Refined olive oil and extra virgin olive oil (EVOO) from the Coratina cultivar were supplied by the IOBM (Industria Olearia Biagio Mataluni, Montesarchio, Benevento, Italy). EVOO from the Ravece cultivar was provided by APOOAT Soc. Coop a.r.l. (Avellino, Italy) in 250 mL green glass bottles. EVOO samples were stored under suitable conditions, avoiding light exposure and high temperatures in order to prevent oxidation, and were used within 8 months from their production (November 2013). For the analysis of volatile compounds, ethyl isobutyrate (99%), ethyl butyrate (99%), ethyl 2-methylbutyrate (99%), hexyl acetate (99%), *cis*-3-hexenyl acetate (98%), *trans*-2-pentenal (95%), *trans*-2-hexenal (98%), 1-hexanol (99%), *cis*-3-hexen-1-ol (99%), linalool (97%), and 1-penten-3-one (97%) were supplied by Sigma-Aldrich (St. Louis, USA). For the analysis, the reagents hexane (95%), methanol (99.9%), glacial acetic acid, trifluoroacetic acid, acetonitrile, diethyl ether, and distilled water were supplied by Romil (Cambridge, England). Potassium iodide and sodium carbonate were provided by AppliChem (Darmstadt, Germany). Ammonium acetate, Folin−Ciocalteu solution, and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide, phenolphthalein, and starch were provided by Titolchimica spa (Rovigo, Italy). Sodium thiosulfate was supplied by Fluka (Buchs, Switzerland), and chloroform was supplied by LabScan (Dublin, Ireland).

Sample Preparation. To study the effect of interactions between whey protein isolate and olive oil, added or absent, with biophenols from extra virgin olive oil on the aroma release, the experimental plan reported in the Supporting Information was applied. Six model systems were set up in order to obtain known initial concentrations of aroma compounds in olive oil added with phenolic compound extract and whey protein isolate. In order to verify our results, obtained by using model systems, a blank virgin olive oil was also analyzed. Human saliva was added to the model systems, and samples were subsequently analyzed by using a dynamic headspace simulating mouth conditions by a retronasal aroma simulator (RAS) device. In the systems without saliva, distilled water was added to obtain the same headspace volume in all of the samples, with the same pH of saliva.

Preparation of the Refined Olive Oil Sample with Added Virgin Olive Oil Biophenols (ROOP). The phenolic extract was obtained from extra virgin olive oil *cv.* Coratina, typically known for its high content of phenolic compounds. An aliquot of the oil sample (200 g) was dissolved in hexane (200 mL) and vigorously shaken for 10 s. A subsequent extraction was carried out using a water/methanol mixture (40/60 v/v) in a separatory funnel. This step was repeated three times by using a total of 420 mL of solvent. Subsequently, the obtained hydro-alcoholic extract was washed with hexane to remove any oil contamination and was centrifuged for 10 min at 3500 rpm (ALC International srl, PK-120, Milan, Italy). The organic phase was removed from the sample, and the hydro-alcoholic phase was collected in the flask and evaporated under vacuum in a rotary evaporator at 40 °C (Heidolph, VV 2000). The phenolic compounds were suspended using 40 mL of methanol. An aliquot of the extract was used for the HPLC and Folin−Ciocalteau analyses. A 2 mL amount of polyphenol extract was adjusted to volume using refined olive oil in a 100 mL volumetric flask, and the oil mixture was treated in an ultrasonic bath for 15 min. Then, methanol was evaporated in a vacuum evaporator (Heidolph VV 200) at 38 °C for 15 min.[28] The polyphenol extract was added to the refined oil all at once and stored in a refrigerated environment. Before the analyses were carried out, the solution was brought to room temperature. The amount of phenolic compounds (328 mg kg^{-1}) added to the refined olive oil was chosen on the basis of the average levels reported in the literature for extra virgin olive oil, equivalent to about 260 mg kg^{-1} of total phenolic compounds.[29] This level is between 220 and 340 mg kg^{-1}: i.e., it corresponds to the slightly bitter taste of VOO.[30]

Preparation of the Refined Olive Oil Sample (ROO). In the control sample the phenolic extract was not added; 2 mL of methanol was adjusted to volume using refined olive oil in a 100 mL volumetric flask. Then, the oil phase was submitted to the same protocol previously described for phenolic compound addition.

Preparation of the Oil Aroma Solution. The most abundant and significant volatile compounds of virgin olive oils were considered in our study for preparing the solutions of aroma compounds,

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Table 1. Chemical Standards, Concentration Added, Odor Descriptor, Physical−Chemical Properties, and MS Fragments Used for Quantitative Analysis[a]

compound	level added (mg kg^{-1})	odor descriptor[b]	MW[c]	C[d]	log P[e] o/w	log P[e] o/a	log P[e] a/w	VP (Pa at 25 °C)[e]	IF[f] (m/z)	r^2	calibration curve (mg kg^{-1})	n
Ethyl Esters												
ethyl isobutyrate	0.141	fruity[51]	116	6	1.77	3.546	−1.78	3226	**71**−116−88	0.9998	0.0360−1.4061	5
ethyl butyrate	0.129	cheesy,[51] fruity,[51] sweet[45]	116	6	1.85	3.637	−1.79	1946	**71**−88	0.9995	0.0329−1.2840	5
ethyl 2-methylbutyrate	0.120	fruity[51]	130	7	2.26	3.912	−1.65	1071	**57**−102	0.9998	0.0306−1.1970	5
hexyl acetate	0.219	fruity[52]	144	8	2.83	4.494	−1.66	193	**43**−56−84	0.9996	0.0224−2.1807	6
cis-3-hexenyl acetate	1.536	banana-like,[5,51] green,[5,52] fruity,[5,52] floral,[5] ester[5]	142	8	2.61	4.195	−1.59	152	**43**−67−82	0.9991	0.0627−15.2965	7
Aldehydes												
hexanal	1.605	green,[5,51] green apple,[5,45] grassy,[45] cut grass[5,52]	100	6	1.8	3.84	−2.06	1276	**56**−57−72	0.9996	0.0655−15.9858	7
trans-2-pentenal	0.122	green,[5,45,52] apple,[45] grassy,[52] pleasant[5]	84	5	1.09	3.607	−2.52	2466	**55**−84−83	0.9993	0.0123−1.2155	6
trans-2-hexenal	6.112	green,[5,45,51,52] apple-like,[5,51] bitter almond,[5,45] cut grass[5,52]	98	6	1.58	4.279	−2.70	629	**55**−69−83	0.9992	0.2493−60.8713	7
C_6-Alcohols												
1-hexanol	0.207	fruit,[5] grass,[5,52] floral,[52] aromatic[5]	102	6	1.82	5.185	−3.16	117	**56**−55−69	0.9988	0.0084−2.0589	7
cis-3-hexen-1-ol	0.209	leaf-like,[5,51] green,[5,44] herbal,[52] cut grass,[52] banana,[5] pungent[5]	100	6	1.61	4.808	−3.20	125	**67**−82−55	0.9998	0.0213−2.0811	6
Others												
linalool	0.086	lilac,[52] lavender[52]	154	10	3.38	6.026	−3.06	11	**71**−93−121	0.9998	0.0220−0.8615	5
1-penten-3-one	0.114	green,[5,45,51] pungent,[5,51] sweet,[5] strawberry,[5] sharp,[5] metallic[5]	84	5	0.9	3.748	−2.85	5092	**55**−84	0.9983	0.0726−1.1340	4

[a] n = number of calibration points. r^2 = linear regression coefficient. [b] The odor descriptors were indicated as reported in the literature. [c] Molecular weight. [d] Number of carbon atoms. [e] The logarithms of octanol/water, octanol/air, and air/water partition coefficients (log P) and vapor pressures of the volatile compounds were calculated using EPI Suite v.4.1 software, U.S. Environmental Protection Agency and Syracuse Research Corp. [f] Bold numbers indicate quantifier ions.

according to the literature.[31] They included five esters, three aldehydes, two alcohols, one ketone and one terpene compound (Table 1). Volatile compounds were dissolved in 10 mL of refined olive oil and homogeneously mixed. The aroma solution was obtained by diluting 4 mL of each volatile compound in oil to 100 mL of refined oil. The aroma solution was added to the oil sample 1 h before the analysis, in order to allow its stabilization. The final concentration for each volatile compound in an oil sample is reported in Table 1. The concentrations were chosen to stay within the range typically found in extra virgin olive oil.[5,31]

Preparation of the Whey Protein Solution (WP). We considered the amount of whey proteins and the pH of the relative solution (11% and 5.8−6.6, respectively) to simulate a real food product commonly used in many food preparations: i.e., ricotta cheese (obtained from whey by adding sodium chloride and thermally treated to separate the upper phase). Whey protein isolate (95%) was supplied by ACEF s.p.a. (Fiorenzuola d'Arda, PC, Italy). Therefore, a water solution at 11% (WP) was prepared in an ammonium acetate buffer solution. The buffer solution was added to 27.5 g of whey protein isolate. The 0.4% ammonium acetate buffer solution (Sigma-Aldrich, Germany) was acidified to pH 6 by using acetic acid solution (Romil, U.K.) by using a few drops in 250 mL of the buffer solution. Whey protein isolate was characterized by 96.7% proteins, 2% ashes, 0.3% fat (dry weight), and 4.7% humidity.

Human Saliva Sampling. Mixed whole saliva (about 150 mL) was separately collected from 14 nonsmoking subjects (8 males and 6 females, 23−48 years of age) from the Department of Agriculture, University of Naples Federico II.[32] Saliva was separated into aliquots of 2 mL and frozen at −20 °C. Before being used, the samples were held in a thermal bath at 37 °C and shaken in order to dissolve any suspension.

Free Acidity, Peroxide Value, and Specific Ultraviolet Absorbances. Refined olive oil (ROO) and EVOO (from Ravece cultivar) were analyzed to determine their acidity levels, peroxide value, and K_{232}, K_{270} and ΔK values according to UE Reg. 2568/91. Acidity was expressed as oleic acid percentage (%), while the peroxide value (PV) was expressed as mequiv of O_2 per kg of oil. For the analysis of spectrophotometric indices a UV−visible Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) was used. All of the analyses were performed in triplicate.

Extraction and Analysis of Phenolic Compounds. The phenolic compounds were analyzed in both ROO and EVOO Ravece samples. The extraction of phenolic compounds was performed according to Vásquez-Roncero,[33] but slightly modified, in order to minimize the volume of solvent used. A 10 g portion of he sample was dissolved in 10 mL of hexane and vigorously shaken for 10 s. Then, the sample was extracted via a separating funnel by using three 7 mL portions of a water/methanol mixture (40/60 v/v). The obtained hydro-alcoholic extract was washed with hexane to remove any oil contamination and centrifuged (ALC International Ltd., mod. PK 120, Milan, Italy) for 10 min at 3500 rpm. The organic phase was removed from the extract. Thereafter, the lower phase was collected in a flask and evaporated under vacuum in a rotary evaporator (Heidolph, VV 2000) at 40 °C. The obtained residue was dissolved in 2 mL of methanol to obtain the final phenolic extract, and an aliquot of this solution was used for the analysis of phenolic compounds. All of the extractions were performed in duplicate. The quantification of total phenolic compounds was carried out by using the Folin−Ciocalteau colorimetric method according to Gutfinger.[34] The samples were diluted 1/50, and the solvent was completely removed by using a constant nitrogen flow. Distilled water (200 μL) was added to the polyphenol extract, and 800 μL of 7.5% sodium carbonate (Na_2CO_3)

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Table 2. Quality Indices for Olive Oil and Total Phenolic Compounds by the Folin−Ciocalteau Method for Refined Olive Oil (ROO) and Extra Virgin Olive Oil (EVOO) from Ravece Cultivar[a]

	free acidity	peroxide value	K_{232}	K_{270}	ΔK	total phenolic compounds (mg kg^{-1})
ROO	0.09 ± 0.01	0.8 ± 0.0	1.82 ± 0.05	0.69 ± 0.02	0.08 ± 0.00	44.0 ± 7.1
Ravece EVOO	0.21 ± 0.03	11.8 ± 0.4	2.18 ± 0.01	0.16 ± 0.00	−0.07 ± 0.01	431.2 ± 93.2
ROO spiked with phenolic extract						328.0 ± 59.1
EVOO accepted values	≤0.80	20.0	≤2.50	≤0.22	≤0.01	
ROO accepted values	≤0.30	≤5.0		≤1.10	≤0.16	

[a]Acidity is expressed as oleic acid equivalent. Peroxide value is expressed as mequiv of O_2 per kg of oil. Values are the average of three replicates (n = 3).

and 1 mL of Folin−Ciocalteau (2 N) previously diluted by using distilled water (1/10) were finally added to the solution. The samples were left for 30 min in the dark at room temperature.[35] Absorbance was measured at 765 nm wavelength by using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank samples consisted of 200 μL of distilled water and all of the other reagents previously described. The total phenolic content was determined by using a calibration curve constructed with caffeic acid and expressed as mg of caffeic acid per kg of oil. The analyses were performed in triplicate for each extraction.

Release of Aroma Compounds in Model Mouth System. The measurement of aroma compound headspace release was performed under dynamic conditions using an experimental RAS, as previously reported in the literature.[6,17,36,37] A 10 μL portion of aroma solution was added to 1.66 mL of the oil sample, and the solution was left to react for 50 min. Subsequently, 8.33 mL of buffer or whey protein solution was added to the system and gently mixed by using a magnetic stirrer (200 rpm) for 10 min. After 1 h, 10 mL of oil-in-water emulsion to which 10 μL of internal standard was previously added (500 mg L^{-1} isobutyl acetate, 99.8% purity, Fluka, Buchs, Switzerland) was transferred into a cylindrical glass jar (100 mL) of the model mouth system. Then, human saliva or distilled water (2 mL), at the same pH of saliva, was added to the system, which was kept in a water bath (Analitica De Mori, Milano, Italy) at 37 °C. The SPME fiber was inserted through the Teflon septum in the RAS and exposed to the sample headspace. Nitrogen flow (20 mL s^{-1}) was passed through the sample for 4 min. During this time, the volatile compounds were trapped on the fiber. The exposure time was chosen according to data previously reported by other authors.[6,38] In addition, this exposure time could represent a realistic application to study the retronasal aroma perception of a food during the simulation of its consumption, as longer times would represent an unrealistic situation.

Dynamic Headspace−Solid Phase Microextraction (SPME) and GC/MS Analysis. The SPME device (Supelco Co., Bellefonte, PA, USA) was equipped with a 50/30 μm thickness divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber coated with a 2 cm length stationary phase. Volatile compounds were analyzed by GC coupled with a mass spectrometer using a GC/MS Shimadzu Model QP5050A instrument (Kyoto, Japan) equipped with a Supelcowax-10 capillary column (60 m × 0.32 mm i.d., with 0.5 μm thickness) (Supelco Co., Bellefonte, PA, USA). The temperature was set at 40 °C for 4 min followed by an increase of 3.5 °C min^{-1} up to 240 °C and was held at that temperature for 3 min. The injector was kept at 230 °C. Helium was used as the carrier gas (1.4 mL min^{-1}). Volatile compound thermal desorption was carried out by exposing the SPME fiber in the injector for 10 min. The compound identification was performed by comparing retention times and mass spectra obtained by analyzing pure reference compounds under the same conditions. Moreover, the identification was confirmed by comparing mass spectra with those of the NIST database. Mass spectra were recorded at 70 eV. The source temperature was 200 °C, and the interface temperature was 250 °C. Before it was used, the fiber was conditioned at 270 °C for 1 h for the analysis. A blank test was performed before each analysis to prevent the release of undesirable compounds. All of the analyses were performed in triplicate.

Quantitative Analysis. The quantitative analysis of olive oil volatile compounds was carried out by using selected ion monitoring (SIM). The selected ions for each volatile compound are given in Table 1. The peak areas of each compound were normalized with respect to the area of the internal standard peak and interpolated on the calibration curve. A calibration curve for each molecule was constructed by preparing a solution containing known amounts of analyte in the oil and the internal standard. The oil was diluted in order to obtain one to seven solutions with decreasing values of the concentration of analytes, and each of these solutions was analyzed by SPME-GC/MS in selected ion monitoring, by applying the same conditions previously reported. The concentration range considered for the calibration curve of each molecule (Table 1) was within the values typically found in virgin olive oil.[5,31] The peak areas were calculated by using a Lab solutions acquisition system (GCMS solutions version 1.20; Shimadzu, Kyoto, Japan). The linear regression coefficient (r^2) for the studied volatile compounds was satisfactory, resulting in a value higher than 0.9983 (Table 1).

Statistical Analysis of Data. Significant differences among the different model systems were determined for each compound by one-way ANOVA statistical analysis. Tukey's test was used to discriminate among the means of the variables. Differences with $p < 0.05$ were considered significant. In order to better understand the influence of saliva, biophenols, and WP presence in the food model system, as well as their interactions, on the volatile compound concentration, a multifactor analysis of variance (ANOVA) with second-order interactions was carried out. The data elaboration was carried out using XLStat (version 2009.3.02), an add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France).

RESULTS AND DISCUSSION

Table 2 reports the free acidity, peroxide value, ultraviolet indices (K_{232}, K_{270}, ΔK), and total phenolic compounds in refined and extra virgin olive oils. Free acidity, peroxide value, K_{232}, K_{270}, and ΔK of all olive oil samples were within the legal limits of the category they belong to: i.e., extra virgin olive oil (EC 2568/91). VOO total phenolic compounds, quantified by the Folin−Ciocalteau colorimetric method, were 431 ± 93 mg kg^{-1}. With regard to the refined olive oil, the phenolic compounds were about 44 ± 7 mg kg^{-1}, while in the refined olive oil with an extract of VOO biophenols added their level was 328 ± 59 mg kg^{-1}.

Effect of WP Addition on VOO Aroma Release in the Presence of Biophenols. To verify the effect of the interaction between WP and VOO on aroma release, several model systems were added to VOO phenolic compounds, to reach a final concentration of 328 mg kg^{-1}. The aroma compounds were set up and were kept to interact with WP. In these systems, VOO aroma compounds are partitioned between the two liquid phases (water and oil) and liquid and gas phase (air). In addition, by its emulsion destabilization effect,[13,14] saliva could further affect the volatile compound partition. This complex balance could lead to a different aroma release in comparison to the initial level of volatile compounds in olive oil. In order to better detect these differences in headspace aroma release, a comparison was carried out by

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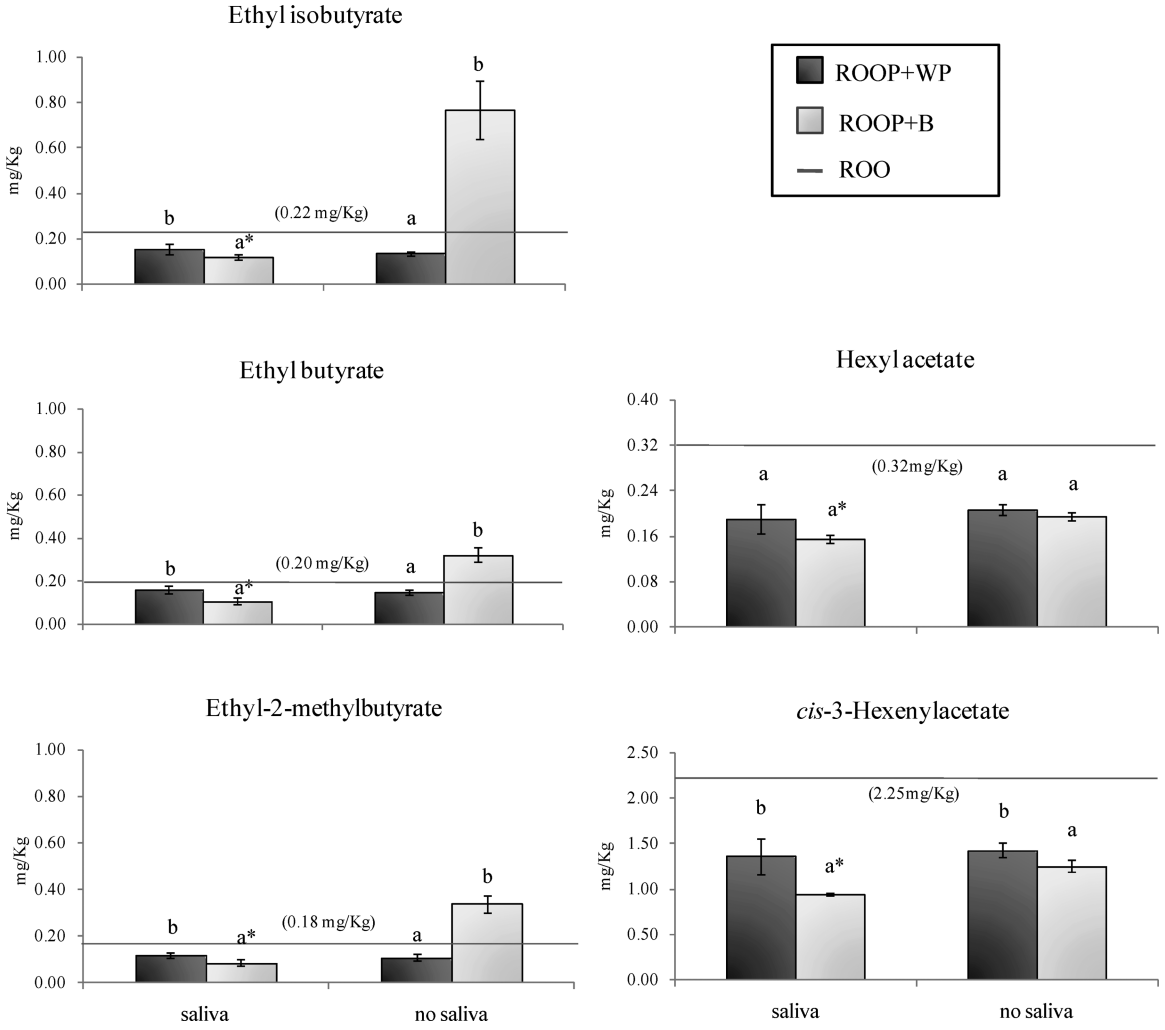

Figure 1. Headspace concentration of ethyl esters and acetates in the refined olive oil model system with the addition of VOO phenolic compounds (ROOP) in the presence (WP) and absence of whey proteins (B) and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between WP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

analyzing the levels of added volatile compounds by using only refined olive oil (ROO): i.e., without interaction of WP and saliva (it is possible to refer to it as orthonasal odor). In the systems without whey proteins (WP) a buffer solution (B) was added to obtain the same headspace volume in all of the samples. The extent of simulated retronasal release of ethyl esters (Figure 1) was similar among the three molecules belonging to this chemical class (ethyl isobutyrate, ethyl butyrate, and ethyl 2-methylbutyrate). Saliva seems to cause a significant decrease in the release of the three esters analyzed without whey protein interaction (ROOP+B). This result could be explained by the binding effects of human saliva, as demonstrated by other researchers, who attributed it to both the chemical binding or hydrolysis and/or physical trapping of volatile compounds mainly caused by mucin.[17,32,36,39] In addition, in the whey protein system (ROOP+WP) a significant and considerable decrease in the release of the ethyl esters was obtained, and this result is in accordance with the literature,[9] but in the systems in which WP interacted with saliva, the decrease was lower. This behavior could be explained by a possible interaction between human salivary constituents and whey proteins, with the result that their action on these volatile compounds could be limited. In fact, it was shown that mucins form aggregates with β-lactoglobulin in aqueous and in oil-in-water emulsions.[40,41] These kinds of interactions, which occur *in vivo* during the consumption of dairy products, might be responsible for the sensory astringency.[42] In fact, in agreement with the well-accepted astringency model of wine polyphenols,[43] this sensation is due to the loss of lubrication of saliva when mucin and lactoglobulin interact to form an aggregate, which precipitates and leads to a dryness and roughness perception in the mouth. Therefore, as reported for red wine in the case of mucin and polyphenols,[36] in addition to sensory astringency, mucin and whey protein interaction affect the extent of ester release. A similar behavior was observed for acetates (hexyl acetate and *cis*-3-hexenyl acetate). In the case of this class of compounds, the level of aroma release was lower in the presence of saliva in the buffer system (ROOP+B) with respect to that of distilled water (no saliva), while in the systems with WP and saliva the lower decrease of aroma compounds was observed only for *cis*-3-hexenyl acetate (Figure 1). The result obtained for hexenyl acetate was in line with that for other acetates, whereas, in this case, statistical significance was not reached. In fact, it was demonstrated that, within one chemical class, the affinity for β-lactoglobulin increases with hydrophobic chain length or overall hydrophobicity (expressed

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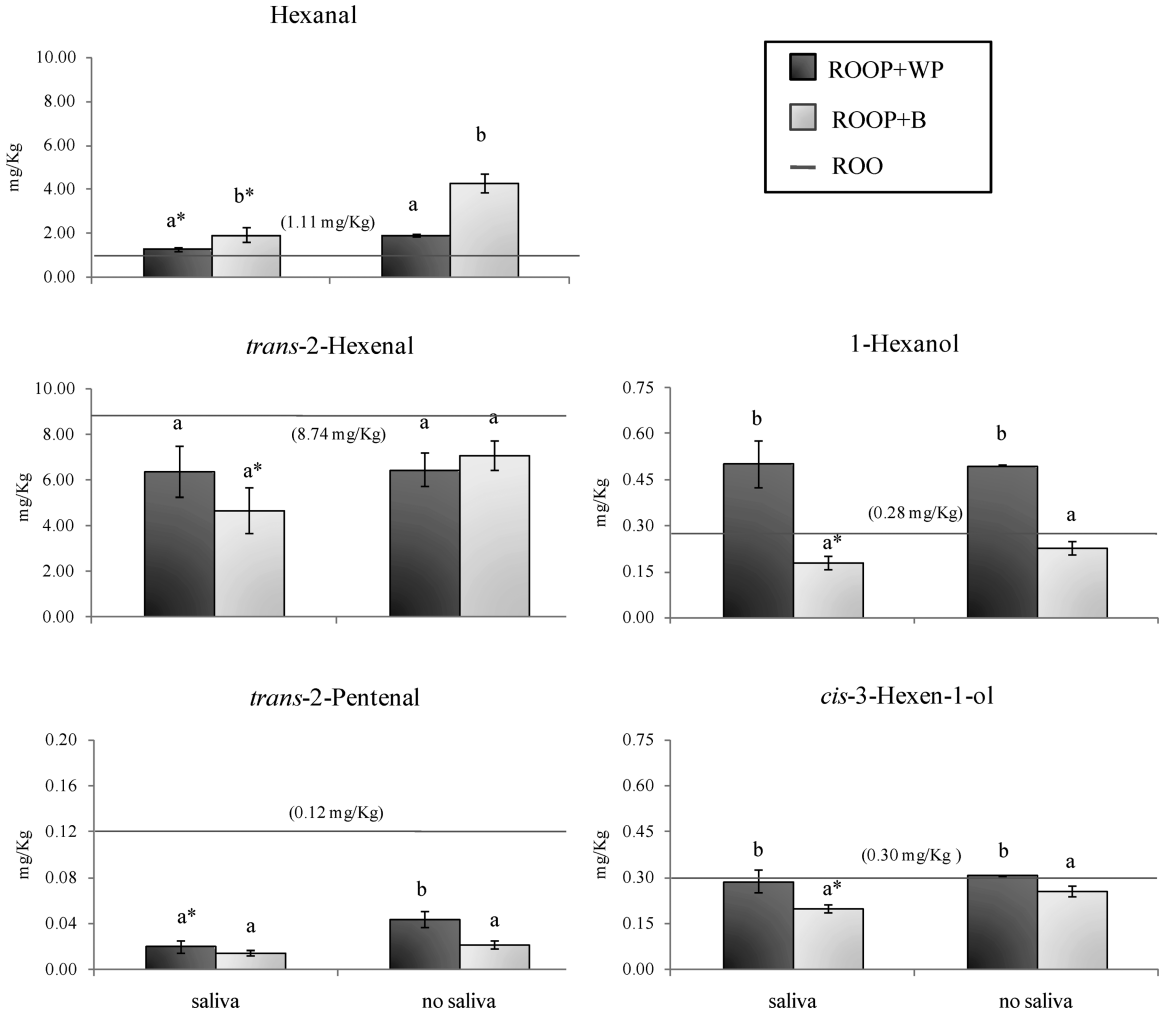

Figure 2. Headspace concentration of aldehydes and C_6-alcohols in the refined olive oil model system with the addition of VOO phenolic compounds (ROOP) in the presence (WP) and absence (B) of whey protein and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between WP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

as log $P_{o/w}$ values).[9] Hexenyl acetate, which has a log $P_{o/w}$ value of 2.83, showed a higher affinity to whey proteins than the other volatile compounds. Therefore, this compound gave a lower release than *cis*-3-hexenyl acetate (log $P_{o/w}$ 2.61), which had lower affinity and higher headspace release.

In all of the systems in which saliva or whey proteins were added, a lower release of ethyl esters was obtained under simulated retronasal conditions with respect to the orthonasal simulation assay, only for refined olive oil (ROO without addition of saliva and whey proteins). In contrast, in the system without saliva and whey proteins, a higher release of ethyl esters was measured. This could indicate not only that aroma release is influenced by the phase separation but also that the increased viscosity of the emulsion could play an important role in the aroma release.

The behavior obtained for aldehydes (hexanal, *trans*-2-pentenal, *trans*-2-hexenal) was quite different (Figure 2). The addition of saliva clearly resulted in a decrease of hexanal headspace concentration with respect to distilled water. When WP was added to the systems, a significantly lower headspace concentration was observed, which indicates a possible further binding of hexanal by whey proteins. However, the behavior was different in comparison to those discussed for esters: i.e., when WP interacted with saliva there was not a significant increase with respect to whey protein or saliva alone, where a further decrease in headspace concentration was observed. Probably, this occurs because most of the aliphatic aldehydes are also linked to proteins by covalent irreversible bonds, as reported for β-lactoglobulin[8,9] and mucin,[7,39] to form Schiff bases. In contrast, in the case of esters and acetates, the interactions between protein and aroma compounds are reversible, involving only hydrophobic binding sites. In the first case, when the bonds to proteins are covalent, i.e. irreversible, a cumulative effect of the individual reaction occurs. Otherwise, in the latter case reversible hydrophobic or hydrogen bonds occur, resulting in an antagonistic and not a cumulative effect. Hexanal headspace concentration decreased in the presence of human saliva and WP, in accordance with Weel et al.,[44] who reported an *in vivo* study. A dissimilar phenomenon was observed in the case of *trans*-2-hexenal and *trans*-2-pentenal. The first showed a lower headspace concentration only in the buffer system with saliva, while the latter had a lower concentration in the presence of whey proteins. As discussed for acetates, this difference could be due to different hydrophobicities (expressed as log $P_{o/w}$ values) of aldehydes.[9] In fact, hexanal, which has a log P value of 1.8,

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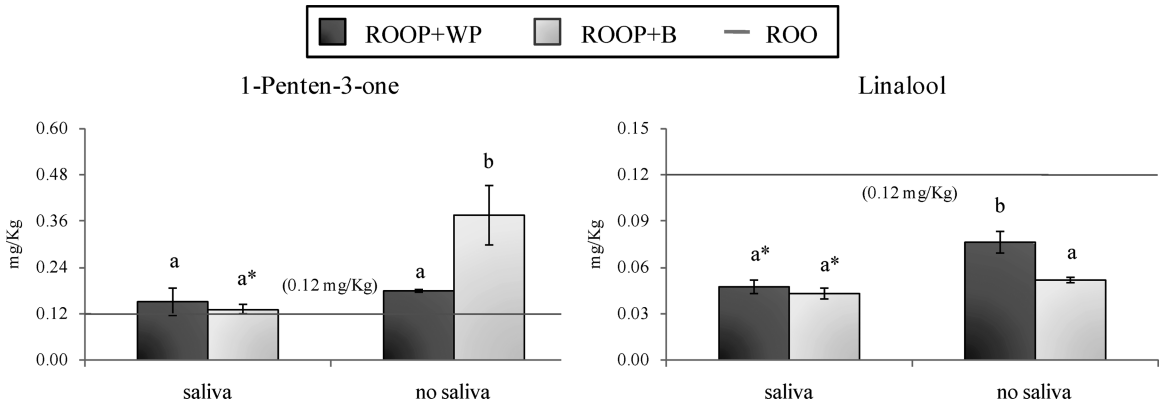

Figure 3. Headspace concentration of 1-penten-3-one and linalool in a refined olive oil model system with the addition of EVOO biophenols (ROOP) in the presence (WP) and absence (B) of whey protein and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between WP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

Table 3. Results of Multifactor Analysis of Variance for Volatile Compounds of Olive Oil in Water Emulsion Differing in Whey Protein Addition and Saliva Treatments

	whey proteins			saliva			whey proteins × saliva		
compound	*F* ratio	*p* value	effect	*F* ratio	*p* value	effect	*F* ratio	*p* value	effect
				Esters					
ethyl isobutyrate	117.219	<0.0001	−	68.745	<0.0001	−	79.146	<0.0001	yes
ethyl butyrate	100.671	<0.0001	−	65.589	<0.0001	−	80.540	<0.0001	yes
ethyl 2-methylbutyrate	165.816	<0.0001	−	100.211	<0.0001	−	117.384	<0.0001	yes
hexyl acetate	0.243	0.635		11.016	0.011	−	2.127	0.183	
cis-3-hexenyl acetate	0.088	0.774		8.771	0.018	−	3.416	0.102	
				Aldehydes					
hexanal	63.465	<0.0001	−	84.982	<0.0001	−	27.894	0.0001	yes
trans-2-hexenal	5.130	0.053		7.883	0.023	−	7.096	0.029	yes
trans-2-pentenal	20.362	0.002	+	29.733	0.001	−	9.511	0.015	yes
				C_6-Alcohols					
1-hexanol	9.979	0.013	+	0.472	0.511		0.891	0.373	
cis-3-hexen-1-ol	0.002	0.962		11.613	0.009	−	3.090	0.117	
				Others					
linalool	28.796	0.001	+	53.019	<0.0001	−	14.292	0.005	yes
1-penten-3-one	28.731	0.001	−	30.581	0.001	−	19.805	0.002	yes

showed a lower release than *trans*-2-pentenal (log $P_{o/w}$ = 1.9), while *trans*-2-hexenal, characterized by an intermediate log $P_{o/w}$ value (1.58), showed an intermediate release. It is important to note that, among the aldehydes, only hexanal showed a higher retronasal release with respect to the orthonasal simulation assay of only refined olive oil (ROO without addition of saliva and WP). This behavior was also previously reported for fresh sunflower oil-in-water emulsions analyzed by a mouth model system and related to hexanal generation by lipid oxidation reactions.[17]

The two studied alcohols (1-hexenol and *cis*-3-hexen-1-ol) showed similar behavior: when WP was not added to the system, saliva caused a decrease in headspace release (Figure 2). When WP was present, no significant difference was observed in aroma release between the system with distilled water and those with human saliva. As discussed for ethyl esters, this phenomenon occurs because of the interaction between mucin and whey proteins which limits their action on alcohols. Therefore, this finally results in a higher release of these compounds. The retronasal release of 1-hexanol in the system with whey proteins was dramatically higher with respect to the orthonasal simulation assay of only refined olive oil (ROO) and significantly higher with respect to the buffer system. This so-called *salting-out* effect, accompanied by a decrease of about the same magnitude in hexanal headspace concentration, was reported by other authors[44] and was attributed to an enzymatic conversion of these two compounds. In Figure 3 the headspace release of 1-penten-3-one and linanool was shown. The retronasal concentration of 1-penten-3-one was significantly higher when distilled water was used instead of human saliva, but only in the buffer system solution. In fact, no statistical difference was observed between the buffer solution and saliva in the system with whey proteins. This result suggests that the presence of whey proteins has a strong effect over the headspace release of some aroma compounds, resulting in a retention effect similar to that caused by human saliva. Whey proteins seem to have stronger binding effects on retronasal concentration than 1-penten-3-one, due to possible chemical binding, as reported for 2-nonanone, a similar hydrophobic volatile compound.[45] In contrast, in the system with saliva, this phenomenon was not observed, probably because the binding sites of the volatile compound interacting with proteins are not available for further reaction with other proteins such as mucin. In fact, when whey proteins are added to such a system,

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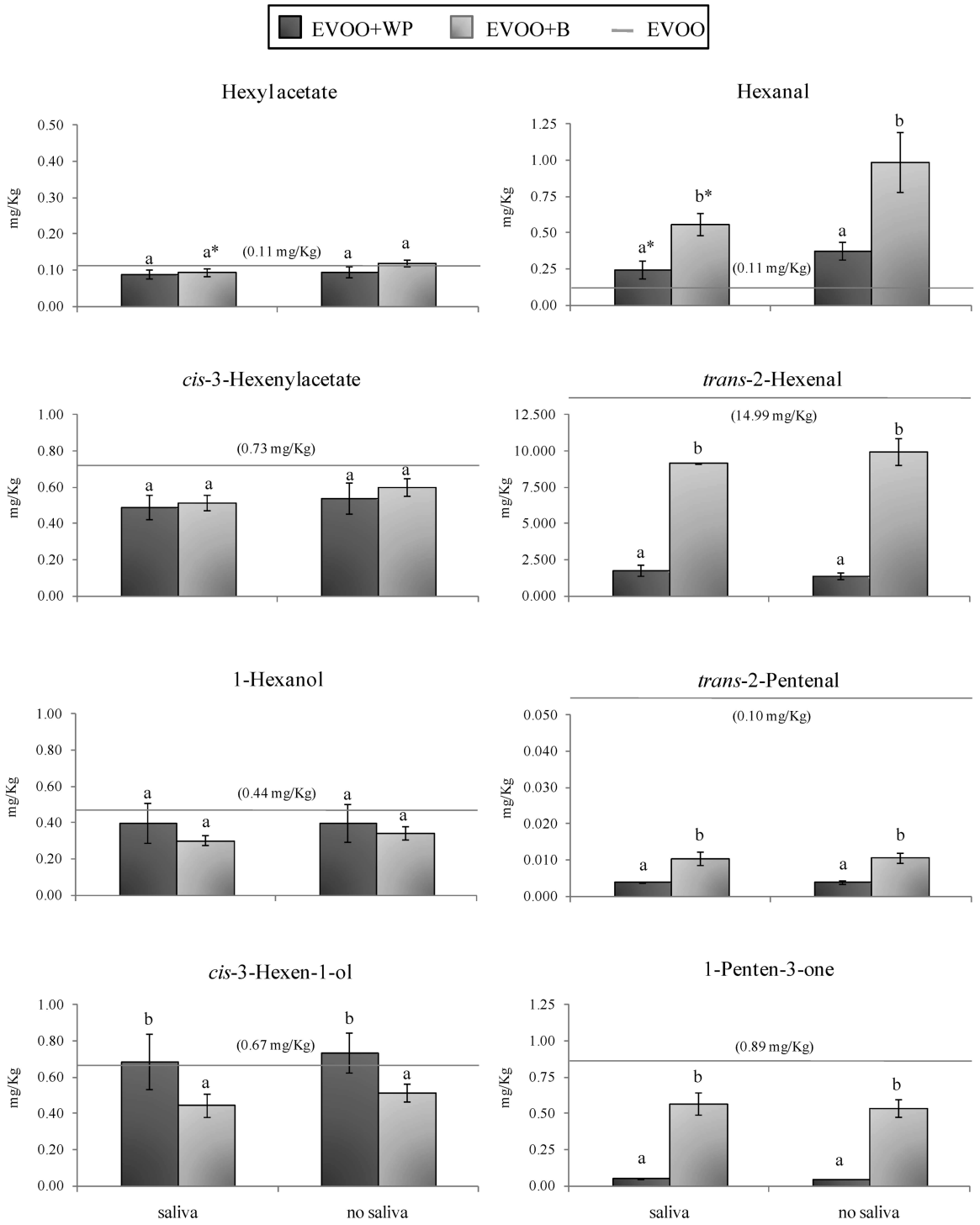

Figure 4. Headspace concentration of volatile compounds in the EVOO model system in the presence (WP) and absence (B) of whey protein and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between WP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

covalent and irreversible interactions occur, as was also reported for aldehydes.[7,9]

In the case of linalool, its headspace concentration in simulated retronasal aroma was always lower than that of the orthonasal simulation assay of ROO. Particularly, in both the buffer and whey protein systems, saliva caused a significantly lower concentration of linalool with respect to distilled water. In systems without saliva, a higher release of linalool was measured in the headspace in comparison to that for the buffer system when WP was added. In other words, also in this case the salting-out effect was observed when whey proteins were added to the system with distilled water. This behavior was previously reported in the literature to be particularly affected at low pH values for other terpene compounds, such as limonene and myrcene.[46] These compounds have highly hydrophobic properties (log $P_{o/w}$ = 4.38 and 4.17, respectively, EPI Suite v.4.1 software, U.S. Environmental Protection Agency and Syracuse Research Corp.) close to those of linalool (log $P_{o/w}$ =

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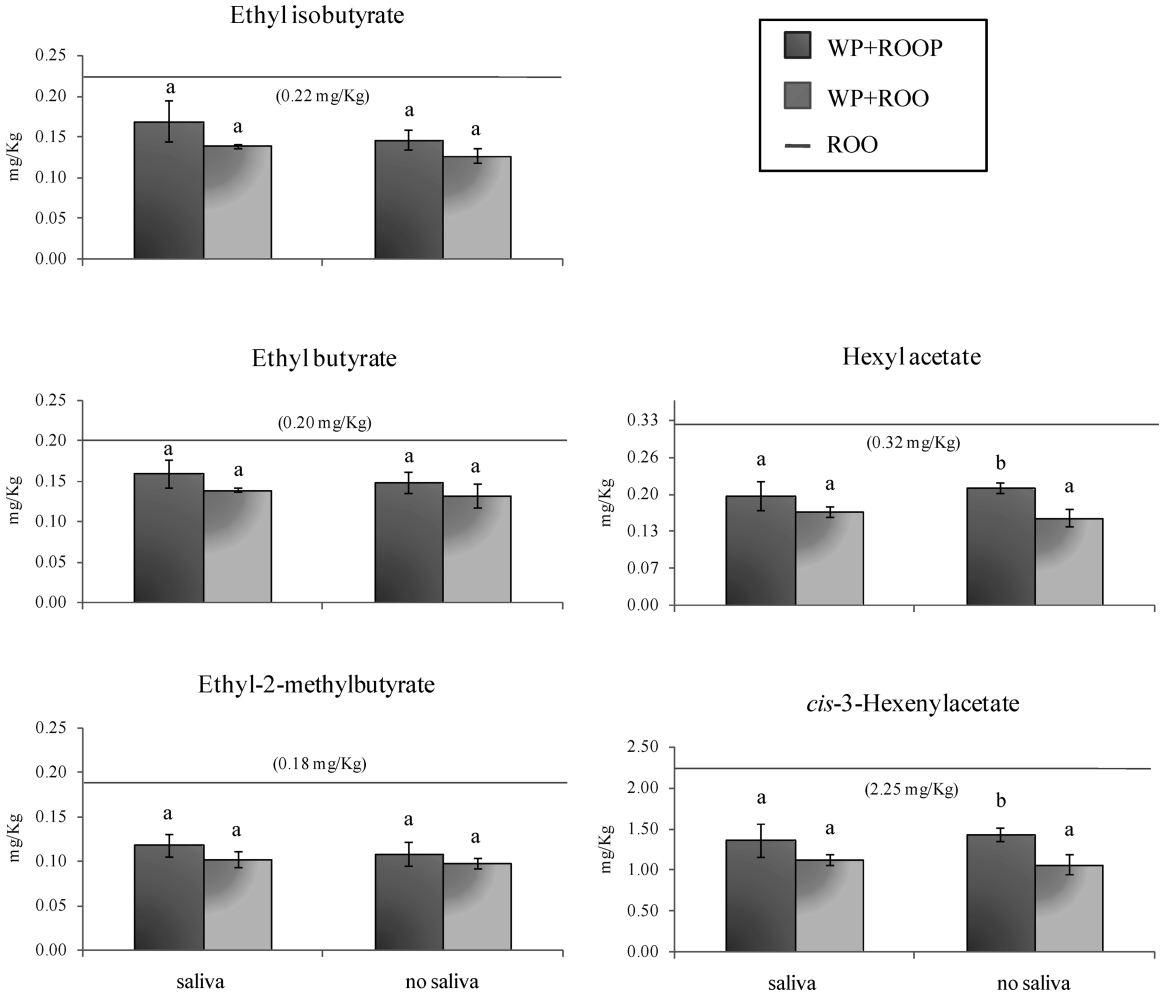

Figure 5. Headspace concentration of ethyl esters and acetates in the refined olive oil model system after interaction with whey protein (WP), in the presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between ROOP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

3.38). This difference was not detected when human saliva was used, and this result was explained by the interaction between salivary components and whey proteins, as previously discussed.

In order to better understand the influence of saliva and whey proteins, as well as the interactions among these factors on the retronasal aroma release in olive oil, a multifactorial ANOVA analysis was carried out. Table 3 shows the *F* ratio, *p* value, and direction of the effect of the factors on the average relative recoveries of the aroma compounds. The *F* ratio represents the quotient between the variability due to the effect considered and the residual variance. The higher the *F* ratio value, the more marked the effect of that factor with reference to a variable at $p < 0.05$. The sign of the effect shows the direction of the influence. Whey proteins seem to have a negative effect on the aroma release in the case of ethyl esters, hexanal, and 1-penten-3-one, responsible for fruit, green, and pungent odors, respectively (Table 1). In contrast, they showed a positive effect on *trans*-2-pentenal (green), 1-hexanol (fruit), and linalool (floral). Saliva showed a negative effect on all volatile compound concentrations, except for 1-hexanol. The interaction between the two factors took place for the majority of the volatile compounds, except for both acetates and alcohols. The results herein described suggest that whey proteins affect the aroma release of VOO but their interaction with saliva has an antagonistic effect on ester, acetate, and alcohol release while being synergistic for aldehydes and ketones. This different effect may imply a higher perception of fruity notes of VOO and a lower perception of green notes when VOO and dairy products are paired, if confirmed by further sensory studies.

Effect of Whey Protein Addition on EVOO Aroma Release. In the present section of our study a real sample of extra virgin olive oil was tested. In order to compare this system (EVOO cv. Ravece) and the model systems (refined olive oil added with selected aroma compounds), a quantification of volatile compounds was performed. EVOO (Figure 4) generally showed a slightly different behavior in comparison to the "simulated VOO" (Figures 1−3). Ethyl esters and linalool were not found in the EVOO headspace, mainly being present at a trace level. At low levels, ethyl esters contribute to the olive oil flavor,[31] while at higher levels they are associated with the fusty defect of virgin olive oil.[47] Linalool, as well as all terpene compounds, could depend on their variety.[48] Generally, headspace releases of volatile compounds were similar among their chemical classes: e.g., the releases of aldehydes and ketones were lowered by the presence of WP, with no effects or slight effects of saliva addition. For acetate compounds, no

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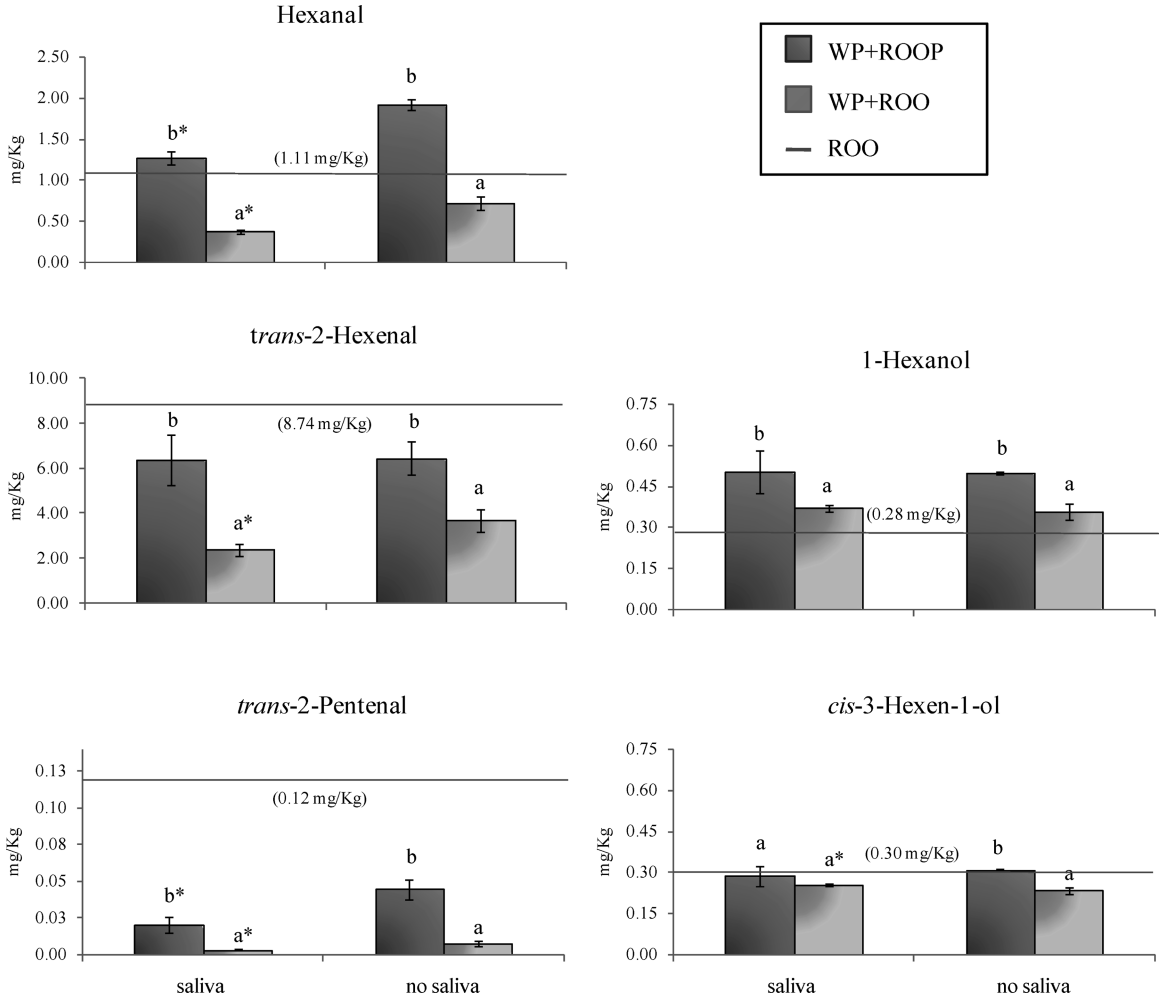

Figure 6. Headspace concentration of aldehydes and C_6-alcohols in the refined olive oil model system after interaction with whey protein (WP), in the presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between ROOP and blank samples (ROO). Asterisks indicate significant differences between saliva/no saliva runs.

dramatic differences were observed. Alcohols had a slight tendency to higher headspace release when WP was added, and also in this case saliva showed no statistical differences. Therefore, it is possible to state that WP affects the VOO aroma release more than our model systems simulating VOO, while human saliva, in contrast, showed an opposite trend. In contrast to the model systems, the release of volatile compounds seems to be more in accordance with the chemical class they belong to. These differences are probably due to the chemical complexity of virgin olive oil in terms of both volatile and nonvolatile compounds. This complexity leads to a higher competition in the interaction among volatile compounds, human saliva, and WP which can affect their partition and release. In addition, the nature of the phenolic compounds and their physicochemical properties affect the dispersion properties and the oxidative stability of olive oil O/W emulsified systems.[49] Further studies to deepen the possible specific effect of individual phenolic compounds on aroma release are needed.

Effect of EVOO Biophenol Addition on Aroma Release in the Presence of Whey Proteins. In the previous section an oil-in-water emulsion was studied, by considering a pairing between VOO and WP. It is known that VOO is naturally rich in phenolic compounds, which are important for human health. They are probably also involved in the *in vivo* aroma release during consumption of this food paired with other food products. For this reason, the specific effect of VOO biophenol presence was studied by comparing a system with refined olive oil and refined olive oil added with olive oil biophenols. All of the model systems were made to interact with whey proteins. As shown in Figure 5, no statistical difference was obtained for ethyl esters between the system with phenolic compounds and the blank (absence of phenolics). Biophenols did not influence the release of either ethyl esters or acetate compounds in the presence of saliva. However, in the system with distilled water a significantly higher level of acetate compounds was found in ROOP in comparison to that for ROO. This phenomenon is probably due to a weak salting-out effect, as occurs for wine polyphenols.[50] In contrast, in the presence of saliva this level decreases due to the possible action of the carboxylesterases, which would be able to hydrolyze these aroma compounds.[32] In all of the systems, however, a lower release of volatile compounds was measured in simulated retronasal assays with respect to the orthonasal simulation assays of ROO.

More interesting results were obtained for aldehydes (Figure 6). In these systems higher release of 1-hexanol was measured in simulated retronasal assays with respect to the orthonasal simulation assay of only ROO. For hexanal, this occurs only for model systems with phenolic compound addition. A significant

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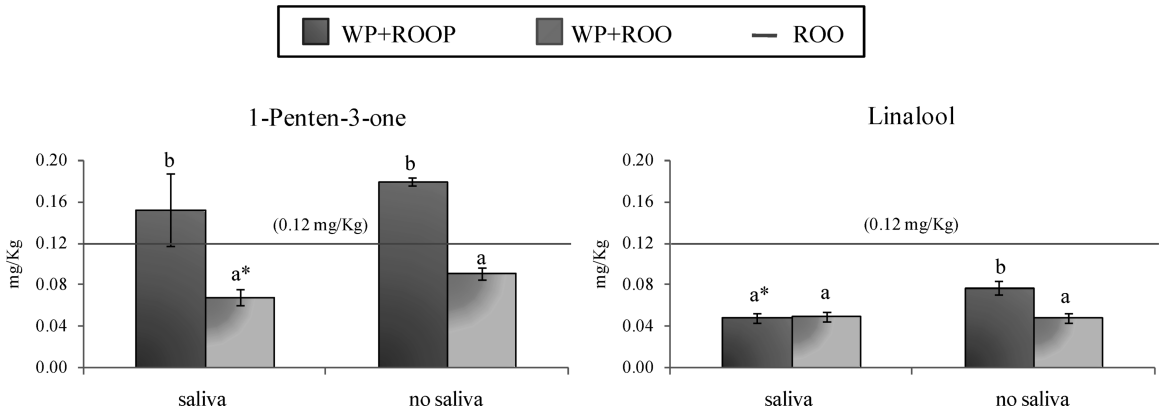

Figure 7. Headspace concentration of 1-penten-3-one and linalool in the refined olive oil model system after interaction with whey protein (WP), in the presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva addition. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences ($p < 0.05$) between ROOP and blank samples (ROO). Asterisks indicate significant differences between saliva/no saliva runs.

Table 4. Results of Multifactor Analysis of Variance for Volatile Compounds of Olive Oil in Water Emulsion Differing in Phenolic Compound Addition and Saliva Treatments

	phenolic compound			saliva			phenolic compound × saliva		
compound	*F* ratio	*p* value	effect	*F* ratio	*p* value	effect	*F* ratio	*p* value	effect
				Esters					
ethyl isobutyrate	0.119	0.739		4.260	0.073		0.377	0.556	
ethyl butyrate	0.268	0.619		1.425	0.267		0.058	0.816	
ethyl 2-methylbutyrate	0.043	0.840		1.281	0.291		0.251	0.630	
hexyl acetate	7.068	0.029	+	0.065	0.805		1.960	0.199	
cis-3-hexenyl acetate	4.594	0.064	+	0.006	0.941		0.812	0.394	
				Aldehydes					
hexanal	146.648	<0.0001	+	161.752	<0.0001	−	14.387	0.005	yes
trans-2-hexenal	1.394	0.272		2.615	0.145		2.095	0.186	
trans-2-pentenal	47.657	0.000	+	30.042	0.001	−	14.633	0.005	yes
				C_6-Alcohols					
1-hexanol	3.759	0.088	+	0.159	0.701		0.022	0.885	
cis-3-hexen-1-ol	10.501	0.012	+	0.007	0.937		3.475	0.099	
				Others					
linalool	38.807	0.000	+	21.722	0.002	−	25.323	0.001	yes
1-penten-3-one	7.745	0.024	+	5.518	0.047	−	0.035	0.855	

effect of addition of both saliva and biophenols was observed. In particular, saliva seems to decrease the headspace release of aldehydes, while the presence of phenolic compounds seems to enhance their headspace release. For *trans*-2-hexenal, the concentration was always significantly higher when biophenols were added, but for this compound no difference was observed between distilled water and saliva, in the system with biophenols. This result strongly suggests that biophenols had a salting-out effect, for a possible interaction between phenolic compounds and WP. In fact, in these model systems the addition of WP is always responsible for both irreversible covalent and hydrophobic bonds with these volatile compounds,[8] as previously discussed. When VOO biophenols were added to the systems, they weakly interacted with whey proteins,[27] with the result that the binding sites may be much less available for hydrophobic interactions with aldehydes. In the model system with saliva addition the release was lower than that for distilled water due to the mucin interaction.[6,39]

A similar result was obtained for alcohols, which resulted in significantly higher headspace concentration in the system with phenolic compounds with no saliva addition. No difference was obtained between water and human saliva for 1-hexanol, while a significant but weak increase was observed for *cis*-3-hexen-1-ol (Figure 6). As previously discussed, this behavior could be due to a WP−mucin interaction, which leads to significantly higher concentration of alcohols because it limits their action in trapping alcohols, the hydrophobic binding sites no longer being available. Thus, for this class of compounds the presence of saliva seems to be of little or no effect, while the interaction with biophenols was significant, however. The behavior observed for 1-penten-3-one was similar to that reported for aldehydes and 1-hexanol, i.e. higher headspace release when biophenols were present, even more than the orthonasal simulation assay of only refined olive oil (ROO without the addition of saliva and whey proteins) (Figure 7). No significant difference was obtained between distilled water and the saliva system, due to the high variability obtained in this latter case. In general, the binding capacity of proteins increases from alcohols to ketones and to aldehydes.[8] For this reason the salting-out effect of VOO biophenols, inversely correlated to the binding capacity of whey proteins, was higher for aldehyde than for ketones and alcohols. Linalool headspace concentration was affected by the presence of phenolic compounds only with distilled water (Figure 7). This result could be due to a possible

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J. Agric. Food Chem. 2015, 63, 3838−3850

linalool trapping during the interaction between biophenols and salivary constituents. In any case, its decrease was not due to mucin.[39] In order to better understand the influence of saliva and olive oil biophenols, as well as the interactions among these factors on the retronasal aroma release in olive oil, a multifactorial ANOVA analysis was carried out. Table 4 shows the *F* ratio, *p* value, and direction of the effect of the factors on the average relative recoveries of the aroma compounds. The VOO biophenols seem to have a strong positive effect on the aroma release in the case of both acetates and alcohols, hexanal, *trans*-2-pentenal, linalool, and 1-penten-3-one being responsible for fruit, green, floral, and pungent odors (Table 1). Saliva showed a negative effect on hexanal, *trans*-2-pentenal, linalool, and 1-penten-3-one concentrations; these compounds are responsible for green, floral, and pungent odors (Table 1). The interaction between the two factors took place only for hexanal, *trans*-2-pentenal, and linalool. Therefore, our results suggest that VOO biophenols could enhance the extent of VOO aroma release of volatiles related to green, floral, and pungent notes, while saliva negatively affects this release.

In conclusion, our results indicate that whey proteins negatively affected aroma release, whereas the interaction between proteins and human saliva led to a lower effect, particularly for some volatile compounds, such as acetates and alcohols. The presence of VOO biophenols positively affected the extent of aroma release, probably in competition with the binding of volatile compounds by whey proteins, for the polyphenol−protein interaction phenomenon. Therefore, it is suggested that, in the presence of a higher level of biophenols, VOO would probably have a higher retronasal release of green notes. Our results could be useful for the formulation of new functional foods to modulate flavor release and consumer acceptability, as well as in the design of new food products by using milk industry byproducts and olive mill biophenols. In fact, as these products considered to be waste and polluting substances, their use in functional foods could at least partially solve the problem of their disposal and could enhance the nutritional value of foods: for example, in the case of whey protein added to beverages as dietary supplements.

■ ASSOCIATED CONTENT

Supporting Information

Figure detailing the experimental plan applied for the retronasal aroma simulator (RAS) analysis of the refined olive oil model (A) and extra virgin olive oil (B) systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GC, gas chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; SPME, solid-phase microextraction technique; VOO, virgin olive oil; RAS, retronasal aroma simulator; ROO, refined olive oil; ROOP, refined olive oil with added olive oil polyphenols; WP, whey proteins; B, buffer solution; EVOO, extra virgin olive oil

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