GRASAS Y ACEITES 67 (4)
October–December 2016, e157
ISSN-L: 0017-3495
doi: http://dx.doi.org/10.3989/gya.0329161

A neuroimaging study of pleasant and unpleasant olfactory perceptions of virgin olive oil*

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Submitted: 16 March 2016; Accepted: 25 July 2016

SUMMARY: Functional magnetic resonance imaging (fMRI) has been used to collect information from neurons that receive direct input from olfactory bulbs when subjects smell virgin olive oil. The pleasant aroma of three extra virgin olive oils (*var*. Royal, Arbequina and Picual) and three virgin olive oils with sensory defects (rancid, fusty and winey/vinegary) were presented to 14 subjects while a fMRI scan acquired data from the brain activity. Data were subjected to a two-sample t test analysis, which allows a better interpretation of results particularly when data are studied across different subjects. Most of the activations, which were located in the frontal lobe, are related to the olfactory task regardless of the hedonic component of perception (e.g. Brodmann areas 10, 11). Comparing the samples with pleasant and unpleasant aromas, differences were found at the anterior cingulate gyrus (Brodmann area 32), at the temporal lobe (Brodmann area 38), and inferior frontal gyrus (Brodmann area 47), while intense aromas activated Brodmann area 6. The actual perceptions described by the subjects and the concentration of the odorant compounds in the samples were considered in the interpretation of the results.

KEYWORDS: *Aroma; Brain activation; Brodmann areas; fMRI; Olfaction; Virgin olive oil*

RESUMEN: ***Estudio mediante neuroimagen de percepciones olfativas agradables y desagradables de aceites de oliva virgen.*** La imagen por resonancia magnética funcional (fMRI) ha sido empleada para estudiar la información de la respuesta cerebral producida al estimular las neuronas que participan en el proceso olfatorio tras percibir el aroma del aceite de oliva virgen (AOV). Se utilizó fMRI para la adquisición de los datos de la actividad cerebral de 14 sujetos a los que se presentaron tres aceites de oliva vírgenes de aroma agradable (var. Royal, Arbequina and Picual) y tres aceites de oliva vírgenes con defectos sensoriales (rancio, atrojado, avinado/avinagrado). Los datos se sometieron a una prueba t para observar diferencias entre dos grupos, la cual permite una mejor interpretación de los resultados, particularmente cuando los datos se estudian a través de diferentes sujetos. La mayoría de las activaciones, que se localizaron en lóbulos frontales, se relacionaron con la tarea olfatoria independientemente de la componente hedónica de la percepción (por ejemplo, áreas Brodmann 10, 11). Al comparar las muestras con aromas agradables y desagradables, se encontraron diferencias significativas en el giro cingulado anterior (área Brodmann 32), el lóbulo temporal (área Brodmann 38) y el giro frontal inferior (área Brodmann 47), mientras que los aromas más intensos activaron el área Brodmann 6. En la interpretación de los resultados se tuvo en cuenta tanto la percepción descrita por los sujetos como las concentraciones de los compuestos volátiles en las muestras.

PALABRAS CLAVE: *Aceite de oliva virgen; Activación cerebral; Áreas Brodmann; Aroma; fMRI; Olfacción*

Citation/Cómo citar este artículo: Vivancos J, Tena N, Morales MT, Aparicio R, García-González DL. 2016. A neuroimaging study of pleasant and unpleasant olfactory perceptions of virgin olive oil. *Grasas Aceites* **67**, e157. doi: http://dx.doi.org/10.3989/gya.0329161.

*This manuscript was presented in the I Workshop INSA-UB "**The Universe of the Olive Oil**", in November 2015.

1. INTRODUCTION

The standard methodology for determining the sensory quality of virgin olive oil (VOO) is the sensory assessment by a normalized methodology, the so-called panel test (IOC, 2015). However, results of the sensory assessment are questioned by members of the olive oil sector because of its poor repeatability when oils are submitted to different panel tests that sometimes result in the classification of the same olive oil indistinctly as extra-virgin (without sensory defects) or virgin (with slight defects) according the panel that evaluates it. Studies have been published on the variability of the panels when evaluating virgin olive oils (García-González *et al.*, 2007).

Volatile compounds are directly responsible for aroma ("I smell therefore there are volatiles") and they can produce desirable or undesirable sensory perceptions, later expressed by panelists as sensory descriptors. Thus, the 'Gordian knot' is the connection between volatiles and sensory descriptors that have to be expressed through standard panel tests, which are questioned by many members of the virgin olive oil sector.

The solution should come from a chemical analysis of volatile compounds. In order to obtain a full understanding of sensory quality from the composition of volatile compounds, a better knowledge of the olfaction process is needed, including the processes that occur after olfactory receptor activations. Gaining knowledge on this aspect entails studying the activity of the neurons that receive direct input from the olfactory bulbs responsible for the perception of virgin olive oil aroma. This approach would mean to explore the cognitive process related to olfaction tasks by using the complex aromas of different virgin olive oils as study material.

The high spatial and temporal resolution of functional magnetic resonance imaging (fMRI) has significantly contributed to the understanding of visual (Bedny *et al.*, 2010), working memory (Kim *et al.*, 2011) and olfaction tasks (Cerf-Ducastel and Murphy, 2001) among many others. The study of the neural response induced by olfaction involves, however, more difficulty than the study of other cognitive tasks (Qureshy *et al.*, 2000). The high number of variables in the presentation of the odorants (e.g. time, concentration of odorant, carrier gas flow and humidity), together with the heterogeneity of the magnetic field in the primary olfactory cortex (POC) because of the near cerebral bone structures, partially explain this difficulty. Furthermore, the samples may be presented to induce an orthonasal and/or retronasal stimulation (Cerf-Ducastel and Murphy, 2001), which introduces an additional variability source. Therefore, the studies of the neural activities induced by olfaction require the use of an optimized system of odorant delivery to be reproducible throughout all the experiments (Tabert *et al.*, 2007; García-González *et al.*, 2011). In general terms, the functional studies of olfaction have described activations in the primary olfactory cortex (POC), which receives direct input from the olfactory bulb and it comprises piriform cortex, entorhinal cortex and periamygdaloid cortex. Other areas as orbitofrontal cortex, insula, thalamus, hippocampus, cerebellum and occipital lobe, including the visual cortex, have been pointed out as brain anatomic zones that are also involved in olfaction processes probably generating mental visual images for comestibility judgments (Cerf-Ducastel and Murphy, 2001). The activations registered in higher order olfactory areas are commonly robust unlike the activations in the primary olfactory cortex, which are inconsistent in some studies. This inconsistency may be due to artifacts or the habituation effect and it must be considered in the data treatment. In fact, it has been demonstrated that the primary cortex habituates very quickly, and it shows inconsistent activation when the stimulus time (ON period) is long enough (Poellinger *et al.*, 2003). In addition, artifacts can be induced by susceptibility differences between brain tissue and the underlying bone and air, which result in signal loss from orbitofrontal cortices that have strong activations when subjects smell highly aversive odors (Royet *et al.*, 2003).

Data from fMRI experiments are commonly analyzed with a contrast analysis assuming the hypothesis of a greater activity during a cognitive process compared to the rest state (Bifone *et al.*, 2010). Thus, the stimulus is sequentially presented to a subject (ON period) alternating with a rest time (OFF period) according to a block design. In addition to assuming a lower brain activity in rest periods, another important assumption is that the timing at which the neural responses are registered matches the time specified in the paradigm (Tabert *et al.*, 2007). Furthermore, the stimulus needs to be presented to the individual enough times to get a reliable statistical significance (Cerf-Ducastel and Murphy, 2004).

Despite the extensive studies on functional neuroimaging of olfaction, little is known about the modulation of brain activities in the particular case of complex aromas. Most of the studies are based on the delivery of substances that are strongly odorant, such as geranyl acetate (Rolls *et al.*, 2003), lavender oil (Savic *et al.*, 2000) etc. Few studies have been focused on food aroma produced by a complex mixture of volatile compounds producing a mild olfactory perception (Verhagen and Engelen, 2006). Furthermore, the information on the neural mechanism by which a food aroma results in a pleasant or unpleasant perception depending on the sensory quality is incomplete. In this respect, Royet *et al.* (2003) studied the brain activity of 126 odorants,

some of which being food aromas. These odorants were classified as pleasant and unpleasant and some differences were found in the brain activities stemming from these two groups.

In a previous study we also addressed a study on brain activities when individuals smelled virgin olive oil from different qualities (García-González *et al*., 2011). However, a further step in this study is necessary to examine the different activities by means of a 2nd level analysis (two samples t-test) that consider the variability among different groups of subjects. This study would allow confirming previous results centered on fMRI studies of virgin olive oil aroma (García-González *et al.,* 2011) and would allow obtaining more detailed information between the activations caused by pleasant and unpleasant aromas. Thus, the aim of this paper is to study the brain activity in response of smelling the aroma of virgin olive oils from different sensory qualities and considering different individuals. Assuming the brain activity is different according to the pleasantness and unpleasantness of the aroma, it is expected to find differences in the neuroimages across different quality degrees of a virgin olive oil. The flavor of virgin olive oil is among the most studied from a sensory/chemical perspective (García-González *et al*., 2010), which provides a further advantage in the interpretation of fMRI results in regards to the olfactory perception and the volatile compounds that are responsible for the aroma.

2. MATERIALS AND METHODS

2.1. Samples

Six samples of virgin olive oil (VOO) were selected for the fMRI studies. Three samples of the cultivars Royal, Arbequina and Picual were qualified as extra virgin olive oil with pleasant green-fruity, green-tomato and green-lawn attributes, respectively, according to the sensory assessment methodology for trade standards (IOC, 2015). On the contrary, the other three oils were standards of IOC for VOOs characterized with rancid, fusty and winey/vinegary sensory defects. In addition, several volatile compounds responsible for those sensory defects were diluted in refined olive oil at different percentages and presented to subjects for being smelt while they remained inside the fMRI instrument. Results of heptanal and propanoic acid are displayed in this work.

2.2. Determination of volatile compounds by Solid Phase Microextraction-Gas Chromatography (SPME-GC).

Olive oil samples (2 g) spiked with 2.6 mg/kg of 4-methyl-2-pentanol (internal standard) were placed in a 20 mL glass vial and left for 10 min at 40 °C to allow for the equilibration of the volatiles in the headspace. After the equilibration time, a solid-phase microextraction (SPME) was carried out using a fiber with a stationary phase of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), which was exposed to the headspace for 40 min. When the process was completed, the fiber was inserted into the injector port of a gas chromatograph (GC) and volatiles were thermally desorbed (Tena *et al*., 2007).

The identification of the volatile compounds was first carried out by mass spectrometry. The assessment of the aroma notes and the determination of the recovery factors were carried out as explained in previous works (Tena *et al*., 2007). All standards of volatile compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

2.3. Sensory assessment

The sensory evaluation of olive oil samples was carried out in accordance with the standard method for virgin olive oil sensory assessment (IOC, 2015). A total of 15 mL of each sample was kept in standardized glasses at 29±2 °C for 15 min and then evaluated by five assessors. Assessors were free to qualify VOOs with their own sensory descriptors in addition to those described in the official method (IOC, 2015).

2.4. Presentation of samples for fMRI experiments

A gas-flow olfactometer, which was designed for the presentation of the samples to the subjects, was composed of an air source equipped with a flow meter (Restek Corp., Bellefonte, PA), a PTFE T-connector (Omnifit Ltd., Cambridge, UK), a sample vial of 100 mL, an empty vial of the same volume, a solenoid 24 volt three-way valve (Omnifit Ltd., Cambridge, UK) made mostly of PTFE and PVC, and a nasal mask. The air source, the vials and the valve were connected with PTFE tubes with an internal diameter of 5 mm. The open/close valve periods were automatically controlled by an in-house adjustable valve controller equipped with electronic timer circuits (Cebek S.A., Barcelona, Spain). The valve controller was the only part of the olfactometer (outside the magnetic room) and it was connected to the rest of the system through a RS-232 connector plugged to the room interface electric board. Therefore, the olfactometer within the magnetic room had only a thin wire and a small metallic piece in the solenoid valve as the only metallic parts of the design, the rest being made of PTFE, PVC and glass. Thus, the metallic part and the working voltage (24 V) produced no artifacts in the measurements as was demonstrated during phantom scanning (examining an anthropomorphic object to test the performance of the magnetic resonance imaging system).

The valve that switched between clean and odorant air was placed as close as possible to the nasal mask in order to avoid cross-contamination between functional runs and to ensure an odorless clean carrier gas in the off-periods. All connecting tubes, nuts, ferrules and plugging were made of Teflon.

Fourteen subjects (7 males and 7 females) with ranging in age from 28 to 47 years (mean=34, SD=7) were selected for the fMRI studies. The subjects declared to be habitual consumers of VOO and not to have any neurosurgery or surgery ENT intervention (e.g. adenoidectomy, tonsillectomy, septoplasty, etc) and also declared they did not have asthma, obstructive sleep apnea or obstructive pulmonary diseases. Subjects were instructed to breathe normally and feel familiar with the procedure. The local Research Ethic Committee of San Juan de Dios Hospital approved this study. Each subject participated in the experiments 3 hours after lunch, in a satiated state, for no longer than 45 minutes including 3 functional runs. The stimuli (aromas released from 5 g of sample) were presented to the subject for 3 minutes, alternating with odorless air (Figure 1). The end of the tube releasing the aroma was adjusted to ~2 cm in front of the subject's nose to make sure that the air flow entered in both nostrils at the same rate. Before each fMRI session, the subjects were instructed to breathe normally, avoiding sniffing. A respiratory cycle of approximately 3.0–3.5 s was considered normal and comfortable since it ensured at least 2 inhalations during the period in which the aroma was released. During the scanning, sensors fixed around the thorax measured the number of inhalations and confirmed that the subject did not change their breath pattern as a consequence of the different aroma released. The measurements of the breath cycles guaranteed uniformity in breath patterns across subjects and scan sessions.

The stimulation paradigm was of 9 s of stimulus (ON period for smelling aroma) followed by 51 s of odorless air (OFF period smelling odorless air), the whole ON/OFF period being repeated three times (three minutes). The selection of a short ON period of 9 seconds avoids the habituation of the primary olfactory cortex (POC), which decreases the neural activity of the subjects (Poellinger *et al.*, 2001). The stimuli and odorless air was delivered with humidified air. After each fMRI scanning the subject was asked to name the odor and describe the intensity and pleasantness/unpleasantness by means of two structured scales ranging from 1 (low intensity/highly pleasant) to 9 points (high intensity/highly unpleasant). The answers were considered to include the samples within the pleasant or unpleasant aroma group. The samples were presented to each subject four times, always in different sessions. Each session included a blank sample (sample vial was kept empty) to control the artifacts and activations that were not due to olfactory stimulation (e.g. activations due to sniffing).

2.5. Acquisition of functional images by fMRI

The fMRI images were acquired on a General Electric 1.5 Tesla Signa Infinity with Excite technology (General Electric Medical Systems, Madrid, Spain), equipped with echo planar capabilities (EPI) using an eight-channel head coil.

An independent sequence of T2 axial scans were acquired at the beginning of each session to rule out brain abnormalities. The functional images were acquired with a gradient-echo T2*-weighted echo-planar images (EPIs) with blood oxygen level-dependent (BOLD) contrast. Imaging parameters were as follows: repetition time (TR)=3000 ms, echo time (TE)=35 ms, flip angle=90°. Five dummy scans were acquired prior to acquiring the functional images to ensure a steady state before the data were collected.

Spatial resolution was set by a 64×64 voxel matrix covering a 250×250 mm^2 field of view

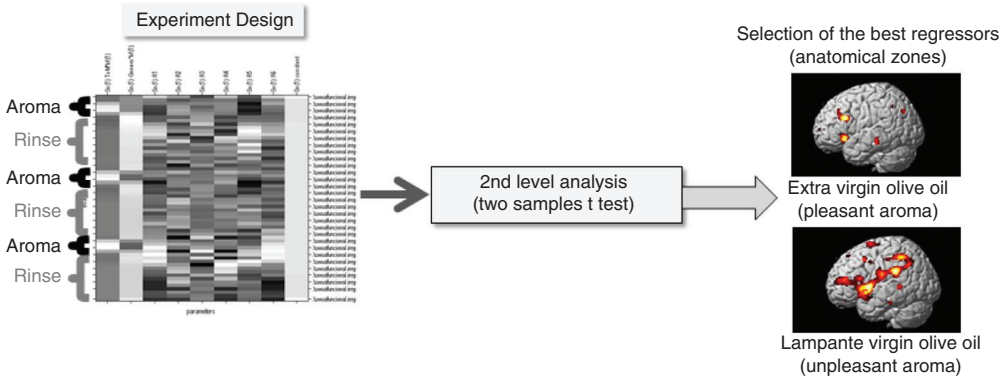

FIGURE 1. Scheme of the procedure followed to present aroma and odorless air (rinse) in an established paradigm, and the subsequent two-sample t-test to select the best regressors.

(FOV), slice thickness 4 mm with no gap, and in-plane resolution 3.91×3.91 mm^2. In each volume, 38 slices were acquired covering the whole brain with an anterior commissure to posterior commissure (AC – PC) slice orientation. High-resolution (0.94×0.94×1.00 mm) coronal T1-weighted anatomical scans were acquired after functional scanning. The images were co-registered to the functional EPI, normalized, and averaged across subjects to aid in localization.

2.6. Image pre-processing and statistical analysis

The fMRI images were processed with SPM8 (Wellcome Department of Cognitive Neurology, London). Functional and structural images were reoriented in the same direction before pre-processing. Afterwards, the images were subjected to a slice timing correction to obtain identical time points for all slices of a given volume. Then, images were realigned for motion correction and they were co-registered to align functional and structural data. After smoothing the resulting images, they were normalized to Montreal Neurological Institute (MNI) template, which approximates the space defined by Talairach and Tournoux (1988). The normalization process included the re-slicing of the structural and functional images into 91 slices with isotropic voxel dimensions (2 mm). The normalized images were smoothed and submitted to statistical analysis.

Activations were studied with the statistical tool based on general linear models available in SPM8 to find brain areas with significant correlations with the stimuli (olive oil aroma–air contrast). The task-induced effect was identified with linear contrast of the parameter estimates by applying one-sample t tests, using $p<0.05$, Z threshold of ~2.0, and minimum activated cluster size of 2 mm^3. The values at each control point (voxel) for each contrast resulted in a statistical parametric map of the corresponding t-statistic. These contrast maps were submitted to a second level inter-subject analysis by means of a group analysis (two-sample t test), which allows corrections for multiple comparisons (Figure 1). The matrix of voxel values was then imported to Statistica 6.0 (Statsoft, Tulsa, OK) for applying the multivariate statistical procedure of cluster analysis to visualize the differences among samples according to their Z score maps extracted from the contrast analysis.

The voxel values were projected onto the structural images, which were previously normalized into the MNI space, to identify the anatomical areas associated with each activation. The representative voxel groups were interpreted according to the anatomical zone, verified by the MNI coordinates and the functional meaning of the activations (Brodmann areas, henceforth BA).

3. RESULTS

The samples under study were subjected to a sensory assessment to determine the median of defects (Md) –unpleasant aroma- and the median of the fruity sensory attribute (Mf) –pleasant aroma-, thereby certifying their inclusion within the categories of extra virgin olive oil (EVOO) and lampante virgin olive oils (LVOO). Three EVOOs were qualified with a median of fruity attribute of 4–4.5 with a relative standard deviation (% RSD) of 6.9%. The three LVOOs, qualified with sensory defects (rancid, fusty and winey/vinegary), were qualified, as expected, with a high value of Md (2.9, 4.0, 7.7 respectively) with a % RSD of 12.9%.

The volatiles quantified in the samples are presented in Table 1, together with the odor activity value (OAV) and sensory descriptors. The OAV of each compound, expressed as the ratio between the concentration and the odor threshold, revealed that 20 volatiles contribute to EVOOs with pleasant aromas (OAV>1). Volatiles with high OAVs that contribute to pleasant sensory attributes – like fruity, sweet and green - are 3-methyl-butanal, pentanal, 1-penten-3-one, hexanal, E-2-hexenal and Z-3-hexen-1-ol while volatiles contributing with unpleasant aromas are some aldehydes such as hexanal (responsible for a fatty perception at a high concentration) (Morales *et al.*, 2005), heptanal, octanal, E-2-heptenal, nonanal, E-2-decenal and E-2-undecenal, together with some alcohols (e.g. 2-butanol), ketones (e.g. 2-octanone) and all of the organic acids.

The most significant volatiles contributing to the unpleasant aroma depend on the kind of defect. Thus, high concentrations of aldehydes were particularly remarkable in the rancid LVOO, as a consequence of oxidation (Morales *et al.*, 2005): hexanal, heptanal, E-2-heptenal, octanal, nonanal, 2,4-hexadienal, E-2-decenal and E-2-undecenal. The rancid LVOO also showed the highest concentrations of hexanoic and octanoic acids. The winey/vinegary LVOO showed a high concentration of acetic acid and ethyl acetate. The fusty LVOO was characterized with a high concentration of some aldehydes such as nonanal and, overall, for the high concentration of acids such as butanoic, pentanoic, hexanoic and octanoic acids. The high concentration of all these compounds responsible for sensory defects explained that no panellist disagreed in the description of the defects, and the samples were considered to be adequate as representative of unpleasant aromas in defective virgin olive oils for the fMRI study. Thus, it was expected that all the subjects smelling these off-flavors could identify the associated negative perceptions in contrast to pleasant aromas provided by extra virgin olive oil.

The aromas of the virgin olive oils were presented to the subjects during the fMRI experiments. The results of the two-sample t test study allow

TABLE 1. Concentration of volatile compounds (mg/kg) quantified in virgin olive oils of the categories extra virgin (EVOO) and lampante (LVOO) qualified with sensory defects (rancid, fusty and winey/vinegary), together with the odor activity value (OAV) and the sensory descriptors.

Volatile compounds	Trr[a]	Kovats index	LVOO Mean ±STD	OAV (LVOO)	EVOO Mean ± STD	OAV (EVOO)	Sensory descriptors
Octane	0.19	800	16.27±5.59	17.31	0.55±0.31	0.59	Alkane, solvent
Methyl acetate	0.20	828	0.92±0.41	4.60	0.27±0.12	1.35	Sweet, ethereal
E-2-Octene	0.23	830	0.20±0.11	0.02	tr[b]	--	Plastic
Butanal	0.25	832	tr[b]	--	0.09±0.04	0.60	Green, pungent
Ethyl acetate	0.26	892	17.57±16.41	18.69	0.51±0.12	0.54	Sticky, sweet, aromatic
3-Methyl butanal	0.29	910	tr[b]	--	0.04±0.03	7.41	Sweet, fruity, ripe fruit, almond
Ethanol	0.31	932	6.71±2.16	0.22	36.80±26.54	1.23	Sweet, alcohol, apple,
Ethyl propionate	0.35	950	0.72±0.40	7.20	0.09±0.04	0.90	Fruity, strawberry, apple, sweet
Pentanal	0.38	969	1.34±0.66	5.58	1.15±0.95	4.79	Oily, wood, bitter, almond
4-Methyl-2-pentanone	0.44	980	tr[b]	--	0.24±0.24	0.80	Fruity, strawberry, sweet
1-Penten-3-one	0.47	1016	tr[b]	--	0.28±0.16	400.00	Pungent, mustard
2-Butanol	0.50	1024	1.81±0.99	12.07	0.07±0.03	0.47	Winey
Hexanal	0.67	1074	10.88±6.85	13.60	2.14±0.19	2.68	Oily, fatty, green, green apple, lawn
2-Methyl-1-propanol	0.72	1099	tr[b]	--	0.02±0.01	0.02	Solvent, penetrating, wine, butter
E-2-Pentenal	0.82	1131	3.19±1.53	10.63	0.50±0.16	1.67	Harsh green, apple, tomato, pungent
1-Butanol	0.92	1145	0.02±0.01		0.03±0.01	--	Sickly sweet, oily, medicine
2-Heptanona	1.02	1170	1.15±1.03	3.83	tr[b]	--	Watered earth, soap, cinnamon
Heptanal	1.03	1174	6.87±6.27	13.74	0.16±0.08	0.32	Greasy. rancid
Limoneno	1.09	1201	0.08±0.04	--	0.03±0.02		Citrus, mint
3-Methyl-1-butanol	1.13	1213	0.20±0.17	2.00	0.18±0.11	1.80	Whiskey, woody, burnt, unpleasant, sweet
E-2-Hexenal	1.14	1216	2.07±0.70	4.93	25.49±18.01	60.69	Bitter almond, fruity, green
3-Octanone	1.26	1244	0.84±0.61	--	1.77±0.77	--	Grass, mould, green, butter
Hexyl acetate	1.29	1274	0.11±0.07	0.11	0.02±0.01	0.02	Sweet, fruity, apple, green grass
2-Octanone	1.35	1279	1.90±1.58	3.80	0.05±0.03	0.10	Mould, over ripe, fruity,
Octanal	1.36	1280	44.91±44.16	140.34	0.37±0.22	1.16	Greasy, soap, fatty
E-2-Heptenal	1.46	1282	18.21±17.41	3642.00	2.61±1.44	522.00	Soap, greasy, almond, pungent
2-Heptanol	1.48	1288	0.45±0.42	45.00	0.43±0.27	43.00	Mushroom, earthy, sweet,
Z-2-Pentenol	1.50	1320	0.02±0.01	0.08	0.09±0.04	0.36	Banana
6-Methyl-5-hepten-3-one	1.51	1347	0.42±0.21	0.42	0.07±0.04	0.07	Fruity, green, grass, pungent
Hexanol	1.57	1357	0.54±0.24	1.35	3.58±1.31	8.95	Fruity, sweet, aromatic
E-3-Hexen-1-ol	1.61	1366	0.05±0.03	0.05	0.02±0.01	0.02	Green lawn
Z-3-Hexen-1-ol	1.65	1378	0.54±0.51	0.49	2.49±1.50	2.26	Banana, fresh, green lawn
Nonanal	1.66	1385	481.99±470.76	3213.27	4.78±1.77	31.87	Rancid, fatty, waxy, pungent

TABLE 1. *(Continued)*

Volatile compounds	Trr[a]	Kovats index	LVOO Mean ±STD	OAV (LVOO)	EVOO Mean ± STD	OAV (EVOO)	Sensory descriptors
2,4-Hexadienal	1.68	1391	60.01±37.00	222.26	15.60±7.92	57.78	Fresh, green, floral, citric
Acetic acid	1.73	1450	35.09±23.21	70.18	3.69±1.95	7.38	Sour, vinegary
Propanoic acid	2.05	1533	1.91±1.62	2.65	0.32±0.13	0.44	Pungent, sour, rancid
2,4-Octadienal	2.09	1555	0.92±0.63	0.92	0.05±0.03	0.05	Green, seaweed
2-Methylpropanoic acid	2.12	1563	8.13±7.14	--	tr[b]	--	Rancid, buttery, cheese
Butanoic acid	2.26	1628	26.57±23.51	40.88	1.43±1.24	2.20	Rancid, fusty, cheese
E-2-Decenal	2.28	1651	38.00±36.55	3800.00	1.82±1.14	182.00	Tallow, painty, fishy, fatty
Pentanoic acid	2.50	1720	14.71±8.57	24.52	0.25±0.13	0.42	Rancid, unpleasant, pungent
E-2-Undecenal	2.52	1760	457.81±436.19	109.00	tr[b]	--	Soap, grease, green
Hexanoic acid	2.73	1829	113.40±102.36	162.00	1.98±0.69	2.83	Rancid, sour, sharp
Heptanoic acid	2.94	1990	1.42±1.15	14.20	0.03±0.01	0.30	Rancid, fatty
Octanoic acid	3.15	2083	122.43±80.94	40.81	3.27±0.90	0.59	Rancid, cheese, oily, fatty
Nonanoic acid	3.35	2202	0.86±0.45	17.31	0.12±0.04	1.35	Grease, green

Note: [a]relative retention time; [b]trace levels

comparing human brain areas that are activated in an inter-subject study (Figure 2), unlike a 1st level study, which is more appropriate for intra-subject studies (García-González *et al*., 2011). Table 2 shows several brain regions that were activated in the course of the experiments and they are split into left and right hemispheres because of their different locations (x y z MNI coordinates: mean±standard error of the mean) and the lateralization of the olfactory system. The activated regions (Table 2) included the piriform cortex, the amygdala, the entorhinal cortex, the parahippocampal gyrus, the orbitofrontal cortex (OFC), the insula, the cingulate gyrus and the cerebellum. Some of these areas are illustrated in Figure 2 as areas highlighted in the contrast analysis of pleasant vs. unpleasant aromas. The first three regions may be considered as parts of the primary olfactory cortex as they receive direct projections from the olfactory bulb.

4. DISCUSSION

A great number of previous studies on olfaction report strong activation in the heterogeneous orbitofrontal cortex (García-Falgueras *et al*., 2006) that we have also found with the bilateral activations of inferior and middle frontal gyrus (Table 2). Thus, Figure 3 shows the MRI signal of the orbitofrontal cortex averaged across 14 subjects after stimulation with virgin olive oil aroma. Also other regions are profusely described in the literature and found in this study such as the cerebellum, the insula and the cingulate gyrus (Poellinger *et al*., 2001). In contrast, the activations of the amygdala, the entorhinal, parahippocampal gyrus, middle temporal gyrus, precentral gyrus and posterior cingulated gyrus have scarcely been reported in olfactory studies, and these regions were activated by the odor of virgin olive oils.

The bilateral activation of the orbitofrontal cortex was found in different parts (inferior and middle frontal gyrus) either in the left or the right hemisphere. The lateralization of orbitofrontal activation in this study seems to relate to the place on a hedonic scale of the two kinds of samples that are placed at opposing extremes of the scale: pleasant and appreciated odors of EVOOs at the top of the scale, and high intensity of defective sensory descriptors of LVOOs at the bottom although the activation was more robust in the left hemisphere in the case of oils qualified with unpleasant aromas, which explains the activation observed in this region when comparing pleasant and unpleasant aroma smelled by subjects (Figure 2). This result agrees with Royet *et al*. (2003), who found a left-dominant network involved in the hedonic valence. Furthermore, Royet *et al*. (2004) also reported that the right orbitofrontal cortex is much more activated by familiar odors, which was also observed for the selected subjects who were

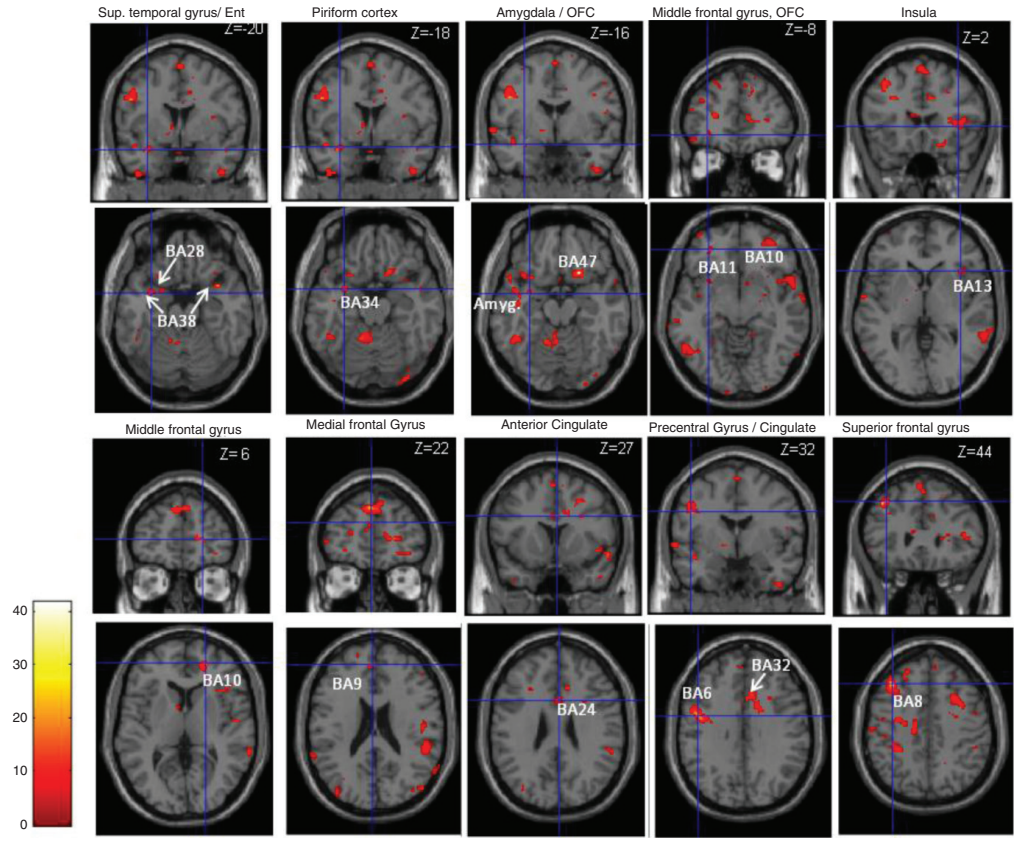

FIGURE 2. fMRI Scans illustrating the main activations (results from two-sample t test $p<0.05$) when comparing the response to the smelling of pleasant and unpleasant aromas in extra virgin olive oil. Note: Ent, entorhinal; Z, value of MNI coordinate of Z-axis.

TABLE 2. Regions of interest (ROI) activated during the fMRI experiments. Values correspond to mean±standard error of the mean (mean±SEM)

ROI	H[a]	x[b]	y[b]	z[b]
Inferior frontal gyrus	R	26.8±1.0	30.0±5.0	−16.4±2.8
	L	−29.2±5.9	32.0±5.5	−14.4±4.0
Middle frontal gyrus	R	38.7±5.9	33.5±9.3	16.0±10.8
	L	−33.4±4.2	44.0±2.8	5.2±9.0
Superior frontal gyrus	R	23.3±4.8	39.3±10.5	27.3±19.0
	L	−30.0±0.0	52.0±10.0	19.0±21.0
Inferior temporal gyrus	R	51.5±12.3	−24.0±26.9	−5.0±32.8
	L	−50.0±4.0	−10.0±4.0	−24.0±2.0
Middle temporal gyrus	R	54.3±5.2	−31.7±10.6	−8.0±9.4
	L	−52.0±4.5	−1.5±4.6	−22.5±4.1
Superior temporal gyrus	R	44.7±8.7	3.3±2.9	−15.3±10.9
	L	−52.0±6.4	13.3±20.7	−4.7±16.2
Amygdala	R	18.0±0.0	−3.0±0.0	−17.0±0.0
	L	−6.0±0.0	0	−22.0±0.0
Parahippocampal gyrus	R	26.0±10.0	−10.0±8.0	−16.5±0.5
	L	−18.7±3.5	−11.7±4.3	−19.7±1.5

TABLE 2. *(Continued)*

ROI	H[a]	x[b]	y[b]	z[b]
Insula	R	37.5±2.5	8.5±10.5	7.0±1.0
	L	−40.0±3.1	8.0±8.3	5.3±1.8
Cingulate gyrus	R	3.7±1.2	7.7±16.1	30.7±3.7
	L	−2.0±0.0	−1.0±19.0	30.0±4.0
Anterior Cingulate	R	0	24.0±0.0	−4.0±0.0
Posterior Cingulate	R	4±0.0	−50±0.0	10±0.0
	L	−3.0±0.6	−44.0±2.3	22.5±2.6
Frontal Lobe (sub-gyral)	R	42.0±0.0	42.0±0.0	−2.0±0.0
Hyppothalamus	R	6.0±0.0	−2.0±0.0	−8.0±0.0
Thalamus	R	19.0±1.0	−18.0±4.0	9.0±3.0
	L	−9.0±3.0	−23.0±3.0	13.0±3.0
Precentral gyrus	R	47.0±7.0	−11.0±9.0	52.0±10.0
	L	−44.0±0.0	0	6.0±0.0
Postcentral gyrus	R	54.0±6.1	−18.0±1.2	44.0±8.0
	L	−57.3±5.7	−6.0±9.0	36.3±10.8
Occipital gyri	R	14.4±4.4	−86.0±1.9	0.0±3.4
	L	−14.7±6.8	−84.0±9.2	1.3±7.1
Entorhinal cortex	R	26.0±6.0	−6.0±10.0	−28.0±2.0
	L	−26.0±0.0	−12.0±0.0	−30.0±0.0
Frontal opperculum	R	54.5±0.5	33.0±3.0	2.5±0.5
	L	−52.0±0.0	38.0±0.0	4.0±0.0
Suppramarginal gyrus	R	43.3±6.4	−52.7±1.8	45.3±6.8
	L	−42.0±10.0	−41.0±7.0	−41.0±7.0
Precuneus	R	26.0±0.0	48.0±0.0	42.0±0.0
	L	−15.0±8.4	−67.0±3.1	33.5±4.2
Cerebellum, declive	R	25.3±8.4	−73.3±4.7	−20.0±2.0
	L	−8.0±2.0	−70.0±3.1	−18.0±1.2
Cerebellum, culmen	R	32.0±6.1	−56.0±7.0	−25.3±0.7
	L	−4.0±0.0	−50.0±0.0	−6.0±0.0
Cerebellum, pyramis	L	−29.0±13.0	−67.0±1.0	−30.0±2.0
Cerebellum, uvula	R	25.0±3.0	−75.0±7.0	−24.0±0.0
	L	−19.0±11.0	−76.0±12.0	−24.0±0.0

Note: [a] H, hemisphere, R, right hemisphere, L, left hemisphere; [b] x, y, z, MNI spatial coordinates.

habitual consumers of EVOOs. These authors also associated the activation of the left amygdala with the emotional processing of olfactory stimuli from unpleasant odors (Royet *et al*., 2003) which agrees with a strong left orbitofrontal activation at the individual level when subjects smell aversive odorants (rancid, fusty and winey/vinegary virgin olive oils).

In all the fMRI experiences, high activation values were registered on orbitofrontal, frontal and temporal lobes, in the BA 10, 11 and 20 (Table 3). Bilateral activations on BA 10 and 11 were associated with the olfaction process itself (García-Falgueras *et al.,* 2006), which explains their activation in response to both pleasant and unpleasant samples. The activation of these areas in the orbitofrontal cortex is justified by the projection received by the olfactory primary cortex (Royet *et al*., 1999). In particular, Zatorre *et al*. (1992) found a high activation of right orbitofrontal cortex at BA 11 during olfaction. Royet *et al*. (1999) also observed an increment in cerebral blood flow at BA 11 (right medial frontal gyrus) during the smelling of a large set of odorants, and this area was clearly associated with the familiarity of the odors. The activation of this area by familiar odors would explain the high intensities observed in all cases, where subjects were regular consumers

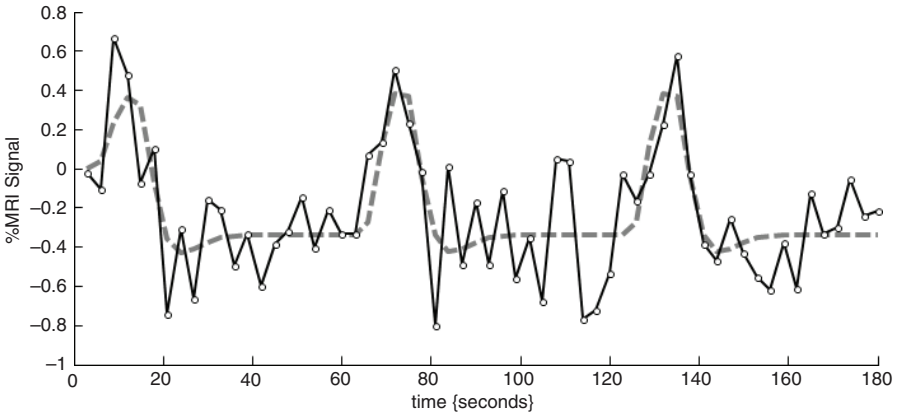

FIGURE 3. Activation of the right orbitofrontal cortex averaged across 14 subjects. The dotted line points out the stimulation paradigm with extra virgin olive oil aroma.

TABLE 3. Summary of the most relevant brain activations determined by fMRI during olfaction of virgin olive oils with pleasant and unpleasant aromas

BA[a]	Comment	ROI[b] /MNI Coordinates (*x y z*)
6	Activated in most unpleasant samples and some fragrant pleasant oils	Frontal lobe, middle and frontal gyrus/(42 4 50)
9	Activated in most samples	Prefrontal cortex, middle frontal gyrus/(-54 22 24)
10	Activated in most samples	Frontal lobe, superior frontal gyrus/(30 60 12)
11	More active in unpleasant aromas	Frontal lobe, inferior frontal gyrus/(-22 34 -22)
13	Occasionally activated, mostly in unpleasant oils	Insula/(32 18 10)
20	Activated in both pleasant and unpleasant aromas	Temporal lobe, inferior temporal gyrus/(60 -38 -23)
24, 32, 33	Much more activated in unpleasant aromas The voxels of three areas are commonly overlapped	Limbic lobe, cingulate gyrus/(-2 18 38)
38	Activated in unpleasant aromas	Temporal lobe, superior temporal gyrus/(-40 16 -28)
40	Activated in both pleasant and unpleasant aromas	Parietal lobe, inferior parietal lobule/(-50 -38 28)
47	Bilateral, mostly shown in unpleasant samples	Frontal lobe, inferior frontal gyrus/(-20 16 -16)

Note: Z-score within 1.99–5.22; [a]Brodmann area; [b]Region of interest.

of virgin olive oils. The activation of BA 11 was more intense in most of the unpleasant aromas, probably because of the relation of this area as a consequence of unexpected outcome and emotional arousal (Yamasaki *et al*., 2002), which would explain the observed activation of this area when pleasant and unpleasant aromas were compared with a two-sample t test (Figure 2). This higher activation in unpleasant aromas is ultimately related to the occurrence of volatile compounds that normally are not present in good quality virgin olive oil (Table 1). These results reveal the importance of the orbitofrontal cortex in higher-order olfactory processing, supported by the association between physiological damages in this zone and anomalies in odor perception and identification (Zatorre *et al*., 1992).

Activation at the inferior temporal lobe (BA20) was also found in pleasant and unpleasant aromas. This activation was bilateral in some odors, while only one of the sides (right side) was activated in the others. The activation of this area was related to recognition and working memory tasks (Cutting *et al*., 2006), probably associated with the consumption habits of the subjects. It is important to note that subjects were asked to name the odors after the fMRI experiments, so it may cause the activation of those areas related to memory retrieval and naming. On the other hand, although fusty and winey/vinegary aromas might be unknown odors

to the subjects, the rancid attribute is among the most commonly known unpleasant aromas. Thus, people are usually familiarized with a rancid odor produced by a rise in hexanal, nonanal and other aldehydes originating from oxidation (Table 1), and this defect may evoke negative memories for them.

The importance of memory integration and recognition tasks in the olfaction process was also pointed out by the maximum responses in pleasant odors. In the olfaction of pleasant aromas of virgin olive oils characterized by positive green and fruity aromas - due to the presence of volatiles as E-2-hexenal, hexanal at low concentration, and Z-3-hexen-1-ol, among many others - the maximum responses were mostly localized at the inferior frontal gyrus (BA 47) (Table 3, Figure 2). The activation of this area is also related to the recognition of familiar odors (Savic and Berglund, 2004; Royet *et al*., 1999). On the contrary, the maximum responses recorded for unpleasant odors (rancid, fusty and winey/vinegary virgin olive oils) were localized in other foci. Among them, in most samples the maximal response corresponded to inferior parietal lobule in the assigned BA 40, which is bilaterally activated. This area is activated during aversive feelings (Uher *et al*., 2005), which would explain its activation during the smelling of off-flavors in defective virgin olive oil. No activation of this area was observed when smelling good quality virgin olive oils where these compounds were not present or they were present at low concentration (e.g. acids). On the other hand, the lack of activation of the brain areas related to familiar odors may be involved in the cognitive process of unpleasant aromas since neophobia appears to be a key factor in hedonic responses (Rouby and Bensafi, 2002). Thus, Royet *et al*. (1999) found a high correlation between familiarity and pleasantness in the smelling of odors.

Other areas related to negative feelings were activated in most of the unpleasant aromas, with no activation in pleasant samples. Thus, superior temporal gyrus was activated in BA 38, in the left side or bilaterally (Table 3, Figure 2). The location of this area explains that it participates in emotion processing (Pelletier *et al*., 2003). In fact, this activation could point out aversion or dislike, according to previous studies that have related this area with negative emotions (Eugéne *et al*., 2003). Kosslyn *et al*. (1996) also observed more activation in left middle temporal gyrus when perceiving aversive stimuli in comparison to neutral stimuli.

Intense activations were also found in the anterior cingulate gyrus when virgin olive oils with off-flavors were presented to the subjects. The activated voxels comprised the Brodmman areas numbered 24, 32 and 33 (Figure 2). The activations in the anterior cingulate gyrus are related to familiar odors (Royet *et al*., 1999). As these areas are within the limbic system; they are associated with emotions (Britton *et al*., 2006). A previous work has shown that the activation of anterior cingulate may depend on whether the emotion is social or non-social. Thus, Britton *et al*. (2006) observed activations of anterior cingulate in negative emotions when they were affected by a social component (e.g. sadness). Nevertheless, the effect of the social dimension on negative and positive emotions seems not to be clear and it requires further studies. The interaction between sociality and emotion makes the cingulate area more difficult to interpret. Concerning olfaction, activation in the anterior cingulate is detected during odor naming, which involves a semantic work (Qureshy *et al*., 2000) and during the smelling of familiar odors (Royet *et al*., 1999). However, the activation in this area observed during the presentation of defective virgin olive oils seems to be related to the averse, negative emotions produced by those volatiles that were present in defective oils at high concentrations (Table 1), rather than familiarity of odors, since no activation was observed in most of normal virgin olive oils. In particular, some areas in the anterior cingulate gyrus (e.g. BA 32) are clearly involved in negative emotions produced by aversive olfactory stimuli. Thus, Fulbright *et al*. (1998) detected significant activation in BA 32 (lateralized to left) in response to a compound responsible for an unpleasant aroma (isovaleraldehyde). The activation of BA 32 may be strongly affected by emotion since it is a zone richly connected with the limbic system (Fulbright *et al*., 1998), which could explain something which is not distinctly associated with off-flavor. The effect of emotion and familiarity of odors in the activation of brain areas involved in olfaction explains the difficulty in interpreting the hedonic response to odors (Rouby and Bensafi, 2002). In order to check the hypothesis that the activation of the cingulate area in the BA 32 is due to an unpleasant perception, two volatile compounds characterized with undesirable aromas, heptanal and propanoic acid were presented to the subjects. These compounds were diluted in odorless refined oil up to a concentration of 100 mg/kg. Both volatiles are produced during the oxidation and fermentation processes and they are commonly present in virgin olive oils with sensory defects (Morales *et al*., 2005). The concentrations quantified in the defective samples for these compounds were higher than their odor thresholds (OAV>1, Table 1) and, in consequence, they were contributors to the aroma perceived by the panelists. In particular, the contribution of heptanal to the aroma is significant in rancid oils, where the saturated aldehydes originated during oxidation are mainly responsible for the rancid and fatty attributes (Morales *et al*., 2005). On the other hand, propanoic acid was a volatile marker of the fusty sensory defect and it is also typically found in

oils with other sensory defects. During these fMRI experiments all the subjects described the aroma of the dilution of these compounds as unpleasant and an intense activation was observed in BA 32 (Figure 4). A high activation was also detected in BA 38, as it was previously observed in defective virgin olive oil samples and in the two-sample t test study (Figure 2).

In addition to areas that are activated in response to off-flavor, other brain areas located on the medial frontal lobe seem to act as modulators in the cognitive processing of odors although they are not activated consistently in all the samples. Thus, strong odors (both pleasant and unpleasant) elicited an intense activation in BA 6 (Figure 2). In most of cases, the activation of this area was observed in the defective samples (e.g. rancid and fusty oils) and in those good quality samples characterized with an intense green-fruity aroma. Thus, Table 1 shows that 29 volatile compounds in defective samples had OAV>1 compared to only 21 compounds in extra virgin olive oil, which explains the higher intensity in aroma of the defective samples. The activation of this area in the left hemisphere is associated with strong odors, as was reported by Miyanari *et al.* (2007) who did not observe activation when weak odorants were presented to the subjects. Although this area is also activated by other tasks such as object naming (Hirsh *et al.*, 2001), their activation may modulate the pleasantness of the aroma, since the perceived intensity has an important role in rising/diminishing pleasantness.

5. CONCLUSION

The results obtained after presenting the aroma of pleasant and unpleasant VOOs show that the hedonic component of olfactory perception is explained by the combined effect of three groups of brain areas. The first group (BA 9, 10, 11, 13, 20, 47) is activated in both pleasant and unpleasant samples and their activations are clearly related to the olfaction process itself. A second group of BAs (mainly 32 and 38) points out adversity and they are activated in most of the unpleasant samples. Finally, the third group of brain areas would act as modulators of the perception indicating strength of the aroma (BA 6) and familiarity (BA 9, 40, 47).

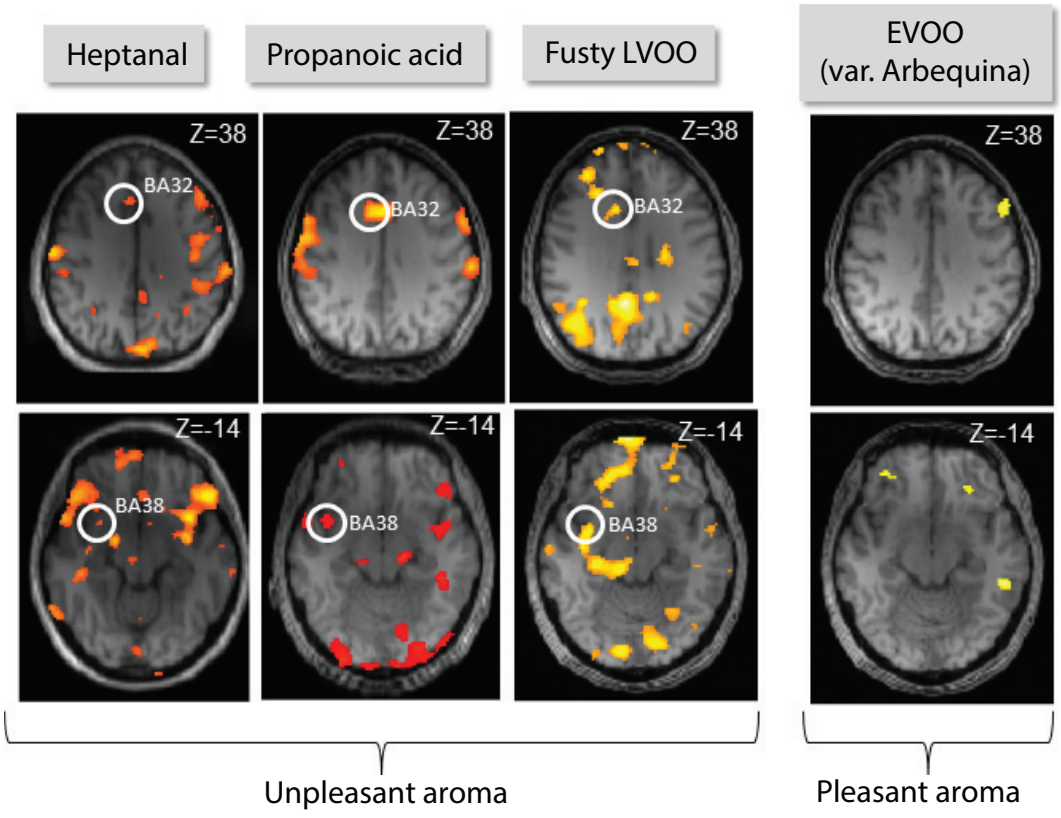

FIGURE 4. Axial activations (p<0.05) in response to the aromas of extra virgin olive oil (EVOO), a lampante virgin olive oil (LVOO) with a rancid defect, and the volatile compounds heptanal and propanoic acid. Brodmann areas (BA) 32 and 38 are marked with circles (Table 3). Note: Z, value of MNI coordinate of the Z-axis.

The results, which are being checked with more individuals and other kinds of virgin olive oils, will help in the building of a complete neuroscientific model to identify problems in panelists evaluating virgin olive oils and to understand consumer preferences for some particular varietal oils, integrating multimodal features of oil perception such as taste (bitterness, pungency, astringency) and color (Verhagen and Engelen, 2006). This procedure is also useful to verify the importance of volatile markers in producing off-flavor and therefore validating their utility as quality markers.

ACKNOWLEDGEMENTS

This work was supported by the State Secretariat for Research, Development and Innovation (Ramón y Cajal Program, AGL2008-01411 and AGL2011-30371-C02-01/02 projects).

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