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Olive oil adulterated with hazelnut oils: simulation to identify possible risks to allergic consumers

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3 **Olive oil adulterated with hazelnut oils: simulation to identify possible**
4 **risks to allergic consumers**
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ABSTRACT

To be labelled as “extra-virgin”, olive oil should be cold-pressed and may not contain refined oil or oil from other oleaginous seeds or nuts (Reg. EC 1531/2001). Adulteration of extra virgin olive oil (EVOO) with hazelnut oil (HAO) is a serious concern both for oil suppliers and consumers. The high degree of similarity between the two fats complicates the detection of low percentage of HAO in EVOO. Many analytical approaches have been developed during last years to trace HAO in EVOO, principally based on chromatographic analyses, differential scanning calorimetry and nuclear magnetic resonance. Adulteration of EVOO with HAO may introduce hazelnut-derived allergens. The aim of this work was to analyse the protein and allergen content of EVOO intentionally spiked with raw cold-pressed HAO or solvent-extracted HAO. SDS-PAGE analysis confirmed the presence of hazelnut proteins in solvent-extracted HAO with molecular masses ranging from 10-60 kDa. In contrast, cold-pressed HAO did not show traces of protein. In spiked EVOO, solvent-extracted HAO was still detected at 1% contamination level. Several bands on SDS-PAGE migrated at apparent molecular masses coinciding with known allergens like Cor a 1 (~17 kDa), Cor a 2 (~14 kDa), Cor a 8 (~12 kDa), oleosin (~17 kDa) and Cor a 9 (~60kDa). MALDI-TOF MS analysis confirmed the presence of two oleosin isoforms and of Cor a 9. Immunoblotting demonstrated that an allergic patient with known reactivity to Cor a 1 and Cor a 2 indeed recognized a 17 kDa band in solvent-extracted HAO. In conclusion, we have shown that adulteration of extra virgin olive oil with solvent extracted hazelnut oil can be traced by simple SDS-PAGE analysis, and that such practice introduces a potential risk for hazelnut allergic patients.

Keywords:- Hidden allergens, olive oil, hazelnut oil, oleosins.

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Introduction

Due to its nutritional and commercial value, the consumption of olive oil has increased considerably over recent years, representing 15.2% of the total oil utilization in the European Union (Firestone 1985). Production of olive oil represents about 3% of the world oils and fat markets, with 78% of global production taking place in Mediterranean countries, where oil industry is important in sustaining viability of agricultural economy. The cultivation, harvesting and extraction of olive oil is a hard and time-consuming task that adds to its relatively high commercial price. Olive oil is either classified as “virgin olive oil” (i.e. cold-pressed olive oil produced by the use of physical means without chemical treatment), as “olive oil” (i.e. a mixture of virgin and refined oil) or “refined oil” (i.e. olive oil chemically treated to neutralize strong taste and in particular acidity) (Reg. EC 1531/2001). Oils labelled as “extra-virgin olive oil” (EVOO) must not contain any refined olive oil; oils labelled as “olive oil” must not contain oil from other oleaginous nuts or seeds. To increase profits, unscrupulous dealers may be tempted to add lower-priced vegetable or hazelnut oils to fresh extra virgin olive oils. This fraudulent practice, in addition to being unfair to the consumer in terms of the cost (Chiavaro 2008), may also cause severe health and safety problems such as the occurrence of Spanish toxic oil syndrome in 1981 (Gelpi 2002, Blanch 1998).

Hazelnut oil (HAO), obtained from *Corylus avellana* L. seeds, is often used in extra-virgin olive oil (EVOO) adulteration, because of the similar chemical composition with respect to both major and minor compounds (Vichi 2001). The high degree of similarity complicates the detection of HAO in EVOO, especially at concentrations below 20% (Parcerisa 1998; Ozen 2002). The seriousness of the problem has been aggravated during the last twenty years. In 1993/94 1,829,000 tonnes of olive oil were consumed world wide. In 1995 it has been reported that quantities of Turkish hazelnut oil was illegally introduced into the European community, without proper declaration to customs and excise, in order to adulterate olive oil (Aparicio 2004). The loss to European consumers was estimated at 40 million euros due to hazelnut oil being three times as cheap as olive oil. In 1997 the European Commission, Unit for the Coordination of Fraud Prevention

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2
3 (UCLAF), estimated that more than 20,680 tonnes of hazelnut oil was used for producing 103,400
4
5 tonnes of adulterated olive oil (UCLAF 1997; European Union Research Committee 2001).
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8 Many analytical approaches have been developed during last years in order to detect HAO in
9
10 EVOO, principally based either on chromatographic analyses of free and esterified sterols, or on
11
12 the detection of other minor secondary compounds, like filbertone ((*E*)-5 methylhept-2-en-4-one)
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14 (Parcerisa 2000; Flores 2006a; Flores 2006b). Other advanced approaches such as differential
15
16 scanning calorimetry, nuclear magnetic resonance and isotopic ratio coupled with mass
17
18 spectrometry were also described (Chiavaro 2008; Sacchi 1997; Remaud 1997). Until today, no
19
20 validated PCR-based methods for identifying fraudulently spiked HAO in EVOO have been
21
22 reported.
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26
27 HAO, like other edible oils, is largely considered a “protein-free” food but solvent-assisted
28
29 extraction for “industrial” oil can potentially (and probably) influence the presence of residual
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31 hazelnut proteins, as has been shown for other nuts and legume oil preparations (Crevel 2000).
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33 Some evidence highlighted that peanut oil (as well as the soybean oil) can not always be
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35 considered as a safe product for peanut-allergic patients (Hourihane 1997; Crevel 2000; Ring &
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37 Möhrenschrager 2007). Apart from a recent paper focused on the use of a biosensor (Bremer
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39 2009), no specific data are available for protein content of either raw or refined HAO.
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44
45 Hazelnut allergy is often the result of primary sensitization to birch pollen, more in particular to the
46
47 major birch pollen allergens Bet v 1 and Bet v 2. Cross-reactivity of IgE antibodies to their
48
49 homologues Cor a 1 and Cor a 2 in hazelnut is at the basis of hazelnut allergy (Hirschwehr 1992).
50
51 Other hazelnut proteins that can elicit an allergic response are non-pollen related seed storage
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53 proteins, such as Cor a 11 (7S globulin) (Lauer 2004) and Cor a 9 (11S globulin) (Beyer 2002) and
54
55 the non-specific lipid transfer protein (nsLTP) Cor a 8 (Schocker 2003). More recently two oleosin
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57 isoforms, now designated Cor a 12 and Cor a 13, were identified as new allergens in hazelnut
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59 (Akkerdaas 2006). Moreover, we very recently isolated and cloned 2S albumin from hazelnut
60
(Zuidmeer 2009; GenBank FJ358504; release date: 01-NOV-2009) and demonstrated its

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2
3 allergenicity. This allergen is now listed by the IUIS Allergen Nomenclature Committee as Cor a 14
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5 (<http://www.allergen.org/Allergen.aspx>).
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10 The problem of so-called “hidden allergens” in food is an important issue for the assessment of
11 food safety. Hidden ingredients in food can either originate from cross-contamination during food
12 processing, or from fraudulent use of non-declared ingredients in food (Steinman 1996).
13
14 Fraudulent introduction of “hidden allergens” is a more serious risk for sensitized consumers
15 because the producer wants the adulteration to go unnoticed. Cases of death for anaphylactic
16 shock in children, adolescents, and adults after ingestion of foods able to trigger an allergic
17 reaction in sensitive individuals have been reported. These deaths are often caused by the
18 presence of “hidden” ingredients, sometimes not even declared. Peanuts and nuts are particularly
19 implicated in this kind of severe responses, provoking asthma crisis in sensitive patients
20 (Sampson 1992).
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33 The lipophilic protein fraction containing oleosins and non-specific lipid transfer proteins (nsLTPs),
34 considered allergens in several foods (Ballmer-Weber 2002; Fernandez-Rivas 2002; Pons 2002;
35 Schocker 2003; Fernandez-Rivas 2006), could be present as hidden residual ingredient either in
36 HAO, and, consequently, in adulterated EVOO. The potentially severe risk that such products
37 entail for hazelnut-allergic people has thus far been underexposed.
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46 The first aim of the present study was to evaluate by SDS-PAGE the presence of residual protein
47 in EVOO spiked with hazelnut oil obtained by cold-pressing or solvent-extraction and subsequently
48 to prove through the use of mass spectrometry the eventual existence of relevant hazelnut
49 allergens. Secondly our goal was, using immunoblotting, to establish if serum IgE of some
50 hazelnut allergic patients with known reactivity both to Cor a 1 and oleosin confirm the presence of
51 allergens in order to understand whether there is actually a real concern for sensitized people.
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Materials & Methods

Samples

Five commercial samples of raw HAO (cold-pressed) and six commercial ones of olive oil (three labelled “extra virgin olive oil” EVOO (cold-pressed) and three “olive oil” OO) were purchased in local stores (Piedmont, Italy). Soremartec (Alba, Italy) kindly provided a series of six cold-pressed HAO (both three from un-roasted and three from roasted hazelnut seeds, *Corylus avellana* L.). Solvent-extracted HAO was obtained from raw hazelnuts (“Nocciola Piemonte” PGI certified from Piedmont, Italy) using the following procedure. Before oil extraction, 20 g of hazelnuts were crushed in mortar using liquid nitrogen and subsequently transferred into the extractor paper vessel. Extraction was carried out in a semi-automated extractor (Büchi Extraction System B.811) for 9 hours using dichloromethane as solvent. Oil samples were filtered using a 0,4 µm syringe filter.

Preparation of reference olive oil sample spiked with different percentage of hazelnut oil

Solvent-extracted hazelnut oil was added to olive oil aliquots (commercial sample of EVOO) at 0.5, 1, 2, 3, 4 and 5% (v/v).

Extraction of protein from oils

Diethylether (100 mL) was added to 100 g of oil (after gently mixing of the bottle, in order to obtain a homogeneous surfactant phase mixed with the oil dreg typical of EVOO) and the mixture was maintained at 18 °C for 30 min. 20% w/v trichloroacetic acid (100 mL), previously cooled at 4 °C, were added and the mixture was transferred in a separating funnel. The trichloroacetic acid extraction step was repeated three times. The three aqueous fractions were collected and washed twice with diethylether (2 x 100 ml) to remove all the lipid material. The aqueous fraction was partially concentrated using a rotary evaporator at < 30 °C and centrifuged at 20,800 g for 20 minutes at 4 °C. The supernatant containing trichloroacetic acid was then discarded. The pellet was washed twice with acetone (2 x 10 ml) to remove all trichloroacetic acid traces, and dried in a vacuum oven for 2 h at room temperature. The dried pellet, obtained as an average of three

1
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3 separate extractions, was quantified as mg/albumin equivalent according to Bradford 1976. The
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5 pellet was stored at -20 °C.
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8 9 10 *SDS-PAGE*

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12 Extracted proteins were dissolved in Laemmli sample buffer without 2-mercaptoethanol (Laemmli
13
14 1970) and separated, 10 ug of protein per lane, by sodium dodecyl sulphate polyacrylamide gel
15
16 electrophoresis SDS-PAGE (Mini-PROTEAN II Cell, Bio-Rad, Richmond, CA) with 12% acrylamide
17
18 resolving and 4% acrylamide stacking gel. Following electrophoresis, the gel was fixed in 7%
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20 acetic acid and 40% methanol for 1 h, then stained with brilliant blue G-colloidal (Sigma Chemical
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22 Co., Milan, Italy) for 1 h, and destained with 25% methanol for 48 hours (Laemmli 1970). In the
23
24 preliminary phase of work in addition to Coomassie Blue also Silver staining procedure was
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26 applied (Switzer 1979).
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31 32 *Patient sera*

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34 For the immunoblotting, one in-house reference serum with known IgE-reactivity to Cor a 1 and
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36 profilin was used. In addition, 4 patient sera with a positive clinical history to hazelnut were used.
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38 Known specific IgE (sIgE) are listed (IU/ml). 1: Hazelnut (8.8), Cor a 1 (7.2), Cor a 2 (0.3), oleosin
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40 (0.1) 2: Hazelnut (8.1), Cor a 1 (0.3), oleosin (21.9) 3: Hazelnut (15), Cor a 14 (15.6), oleosin (0.8)
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42 4: Hazelnut (2.1), Cor a 1 (0.9), Cor a 8 (0.2) and oleosin (7.1). Total IgE content has not been
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44 determined.
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50 51 *SDS-PAGE/immunoblotting*

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53 Proteins were separated by SDS-PAGE (NuPAGE® 4-12% Bis-Tris gel, protein: 10 µg/cm) and
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55 western blotting was performed by transferring the proteins semi-dry to nitrocellulose on a
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57 Novablot electrophoretic transfer apparatus, according to the protocol of the manufacturer
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59 (Invitrogen, Carlsbad, California, USA). After blocking with PBS/5% ELK/0.02% Tween-20 for a
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minimum of 10 minutes, the blots were cut into strips and incubated overnight with 150 µl human
serum, 1:3.000 (v/v) in-house polyclonal rabbit antisera (to hazelnut extract (extracted at neutral

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3 and low pH), Cor a 8 or natural hazelnut oleosin) or 1:30.000 (v/v) in-house monoclonal mouse
4 antiserum (to the major birch pollen allergen Bet v 1, shown to be cross-reactive to Cor a 1) in 3 ml
5 of PBS/0.02% Tween-20/0.5% ELK. After washing 5x (PBS/0.02% Tween-20), blots were
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7 of PBS/0.02% Tween-20/0.5% ELK. After washing 5x (PBS/0.02% Tween-20), blots were
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9 incubated (4hrs) with 1:30.000 (v/v) HRP-labelled goat anti-human IgE (KPL, Gaithersburg, MD,
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11 USA) and washed as before. Detection was performed with ECL according to the protocol of the
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13 manufacturer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were exposed to X-ray
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15 film (Fuji Super RX, Fujifilm coop., Tokyo, Japan).
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20 *MALDI-TOF/TOF MS analysis of protein extracts*

22 For "in gel" reduction and trypsin digestion, seven bands, chosen for their intensity, were cut
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24 out (fig. 1), reduced with 100 mM DTT (56 °C, 1h), alkylated by adding iodoacetamide to a
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26 final concentration of 200 mM, and incubated in the dark at room temperature for 45 min. The
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28 proteins were then "in gel" digested with trypsin (Promega, USA), as previously described
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30 (Lauer 2004). For MALDI-TOF mass spectrometry, 0.5 µl of each peptide mixture was applied
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32 to a target disk and allowed to air-dry. Subsequently, 0.5 µl of matrix solution (1% w/v α-
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34 cyano-4hydroxycinnamic acid in 30% acetonitrile, 0.1% TFA) were applied to the dried sample
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36 and again allowed to dry. Spectra were obtained using a Bruker Autoflex II MALDI-TOF/TOF
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38 spectrometer (Bruker Daltonik, Germany). The MS data were searched against UniProt
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40 database with Mascot search engine (<http://www.matrixscience.com>) following the peptide
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42 mass fingerprinting (PMF) method (Pappin 1993). Mass tolerance of 30 ppm, hydrolysis with
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44 trypsin and two missed cleavages, carbamidomethylation of cystein as fixed modification,
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46 oxidation of methionine as variable modification were specified, no post-traslational
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48 modification were included in the search. To confirm the protein identification, the most intense
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50 signal of each spectrum underwent to MS/MS analysis. The MS/MS spectra were
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52 automatically analysed by the Flex Analysis software (Bruker Daltonik, Germany).
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Results and Discussion

Several studies reporting the authenticity assessment of olive oil based on analytical techniques

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3 have been carried out (Ruiz del Castillo 1998; Crevel 2000; Parcerisa 2000; Vichi 2001; Baeten
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5 2005; Flores 2006a). Our aim was to evaluate the isolation and concentration of putative residual
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7 protein from EVOO, OO and HAO; then estimating by SDS-PAGE the presence of residual protein
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9 in EVOO spiked with hazelnut oil. Retail samples of EVOO, OO and cold-pressed HAO were used.
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11 In addition, samples of HAO were obtained by solvent extraction with dichloromethane. A method
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13 for isolating and concentrating protein based on diethylether and trichloroacetic acid was used on
14
15 all these samples. Both in case of EVOO and cold-pressed HAO visible solid residue (dreg) at the
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17 bottom of the bottle was carefully mixed with the oil prior to protein isolation, in order to obtain a
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19 representative extraction of the "whole" protein residue from the oil sample. OO did not contain
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21 such residue. After the extraction, the oil was filtered avoiding any other kind of refining process.
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25 We chose not to consider any additional refining steps following filtration (e.g. deacidification,
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27 deodorisation, etc.), to allow a clear comparison of residual protein using two extraction
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29 procedures, i.e. solvent and cold-pressed extraction. Moreover solvent-extracted HAO is very likely
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31 to be used in fraudulent adulteration of EVOO as it is a simple and cheap one step procedure. Any
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33 additional refining steps would make the process of adulteration financially less attractive. The
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35 extraction method used in our laboratory (accelerated solvent extraction) is comparable to the
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37 industrial-solvent extraction of seed oils, allowing us to use a good model-system to evaluate the
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39 residual presence of proteins secondary to the disruption of tissue.
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44 Solvent-extracted hazelnut oil proved to be the only one of the analyzed samples, subjected to this
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46 procedure of extraction-concentration, to have made a protein precipitate (160 mg/albumin
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48 equivalent from 100 g of oil); cold-pressed HAO and olive oil did not showed the presence of
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50 protein. Only the solvent-extracted procedure is therefore able to extract proteins from hazelnut.
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52 We have also increased by three times the initial amount of cold-pressed oil (300 g) and tried a
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54 SDS-PAGE separation followed by a very sensitive silver staining technique with a detection level
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56 down to the 0.3-10 ng level (Switzer 1979). Even using this technique we were unable to visualize
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58 any band. Considering these results, regard to the gel staining, we have chosen to apply the
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60 protocol of Colloidal Coomassie Brilliant Blue (G-250), more sensitive (ca. 30 ng per band) than

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3 Coomassie Brilliant Blue (R-250) (ca. 100 ng per band), and fully compatible with mass
4 spectrometry analysis (Neuhoff 1985). We found that the sensitivity of this staining technique was
5 suitable for the purpose of this study.
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11 Protein isolation from EVOO, OO and cold-pressed HAO did not result in any detectable bands on
12 SDS-PAGE (fig 1). The same analysis of solvent-extracted HAO clearly demonstrated the
13 presence of different proteins with apparent molecular masses ranging from 10 to 60 kDa (fig.1).
14 The visible solid residue in EVOO and cold-pressed HAO can most likely be explained by the
15 presence of phenolics and fibre compounds. Solvent-extracted HAO probably showed clear
16 presence of residual proteins, because the solvent more efficiently extracted protein from hazelnut
17 seeds as well as disaggregated residual protein-coated oil bodies. As already known, the use of
18 different solvents in Soxhlet extracting method is currently chosen to lead the lipid extraction at
19 inter-cellular or intra-cellular level. Hence, we justify the presence of proteins in solvent-extracted
20 and micro-filtered HAO, according to the "technological" differences occurring among the different
21 oils employed in our analysis.
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37 Concerning the EVOO samples spiked with solvent-extracted HAO, a conventional SDS-PAGE
38 was able to show two bands down to 1% (v/v) solvent-extracted HAO. Hazelnut oil was added to
39 olive oil aliquots at 0.5, 1, 2, 3, 4 and 5% (v/v) corresponding to 0.8, 1.6, 3.2, 4.8, 8 mg of hazelnut
40 protein respectively (fig. 2). This result highlights how this simple method could be considered a
41 good tool for the discrimination of HAO adulterated EVOO's, even though more advanced
42 analytical chemical techniques were previously suggested (Ruiz del Castillo 1998; Baeten 2005;
43 Flores 2006a; Chiavaro 2008). In our case, the sensitivity was higher than those observed
44 monitoring the presence of filbertone using an on-line hyphenated HPLC-GC technique, allowing
45 the detection of adulterated olive oil with 5-10% of virgin and refined hazelnut oils, respectively
46 (Flores 2006b). A restriction of our suggested technique could be that the presence of the hazelnut
47 proteins should be correlated only with some categories of HAO (solvent-extracted), as clearly
48 shown in this study. When we applied the method to some commercial samples of HAO
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3 (undeclared whether cold-pressed or refined), no protein contamination in this set of commercial
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5 samples was detected, pointing towards the need of a more accurate and enlarged study in this
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7 field.
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11 The detection of protein in adulterated oils is a major concern for allergic consumers. The
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13 allergenicity of different oils processed by different methods was previously investigated, namely in
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15 peanut (*Arachis hypogaea* L.) and soybean (*Glycine max* L.) oils (Crevel 2000). The presence of
16
17 hazelnut protein in HAO was demonstrated in several studies on nut oils; hazelnut gourmet oil was
18
19 reported to contain up to 62.0 ± 1.1 μg of protein on 1 ml of oil (Teuber 1997). To date the possible
20
21 allergenicity of nut oils is still under discussion (Peeters 2004); the understanding of this safety
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23 issue is clearly impacted by different oil extraction and refining processes. The detection of
24
25 residual hazelnut protein in adulterated olive oil (as well as the qualitative description and
26
27 identification) was never deeply investigated. Two isoforms of oleosin with molecular masses of
28
29 16.7 and 14.7 kDa have recently been identified as new hazelnut allergens (Akkerdaas 2006).
30
31 Oleosins isolated from seed- or nut-derived oilbodies have been reported to be contaminated with
32
33 small but significant quantities of albumins and globulins (Millichip 1996). Albumins and globulins in
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35 seeds and nuts are major sources of allergenicity. Taken together, oil extraction from hazelnuts
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37 may result in residual protein content with a high likelihood of including allergens.
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45 Considering the presence of allergenic proteins in solvent-extracted HAO, we aimed at identifying
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47 proteins using MALDI-TOF MS. This analysis clearly demonstrated the presence of two oleosin
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49 isoforms (UniProt ref. Q84T91, Q84T21) (fig. 1, bands 1 and 2), newly described by Akkerdaas
50
51 (http://expasy.org/uniprot/Q84T91_CORAV; http://expasy.org/uniprot/Q84T21_CORAV) and of two
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53 bands both containing 11S globulin-like protein (UniProt ref. Q8W1C2) (fig. 1, bands 3 and 4)
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55 (Beyer 2002). Fifteen peptides, belonged to the 11S globulin-like protein, in band 4 whereas only
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57 nine in band 3 probably because the different amount of the protein in the two bands. The
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59 presence of 11S globulin-like protein in two distinct bands is not surprising and it is probably due to
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the post-translational modifications occurring on the protein and responsible for the slight

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3 differences of the molecular weight compared to the nominal one reported in Table 1. No specific
4 investigation on post-translational modifications was performed because the MS/MS data were
5 obtained only for one signal and the MS data are unreliable without MS/MS confirmation. Evidence
6 for the presence of other allergens like Cor a 1 or Cor a 8 was not found. A more accurate bi-
7 dimensional analysis should probably be carried out to improve the selectivity of protein fingerprint
8 separation, permitting the clear identification of unknown proteins.
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18 As we detected oleosin and Cor a 9 in hazelnut oil, immunoblotting method was applied in order to
19 confirm the presence of possible allergens testing sera from both patients and sensitized animal
20 (rabbit and mouse). We analyzed sera from four patients with a positive clinical history to hazelnut,
21 and an in-house serum with IgE reactivity to Cor a 1 and 2. The four patient sera (Fig. 3, lanes 12-
22 15) did not show any IgE reactivity, however the in-house serum showed IgE reactivity to a protein
23 with an apparent molecular mass of 17-18 kDa (Fig. 3, lane 1). The main difference between the
24 reference serum and the other sera is that the former has IgE-reactivity to profilin, while the four
25 others do not. Profilin was not one of the proteins recognized in the MalDI-TOF analysis, however,
26 that may be due to the low amounts present in the extract.
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40 Two rabbit antisera against hazelnut extracts confirmed the presence of hazelnut-derived protein
41 (Fig. 3, lanes 4 and 6). According to these preliminary results, although only one in-house serum
42 from an allergic patient has given a positive response in the immunoblot, the initial assumption of a
43 potential risk for the consumer can be considered plausible. The in-house serum is from a patient
44 with known inhalant allergies, however, since this a serum from a retrospective patient, we do not
45 have data on the clinical reactivity to hazelnut. So, although we do know the IgE-reactivity to Cor a
46 1 and 2 on blot and in RAST/CAP experiments, we can't claim the reactivity will lead to clinical
47 reactions on hazelnut. However, the purpose of the paper was to detect allergens in adulterated
48 olive-oil, and for this reason this serum can be used.
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3 The fraudulent adulteration of olive oil with hazelnut oil, already a problem in terms of commercial
4 costs for the consumer, it appears at this point exacerbating those health and food safety related
5 problems with an additional risk associated to its allergenic component. This new argument, even if
6 highlighted by a preliminary study, should certainly be taken into account for the most severe
7 reported cases of allergy in future researches.
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13 14 15 16 **Conclusions**

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18 In conclusion, there is a potential risk for hazelnut-sensitized patients when exposed to fraudulently
19 spiked virgin olive oil, especially in the case of solvent-extracted not-refined HAO. This preliminary
20 observation confirms other previous highlights, concerning the putative presence of allergens in
21 oils from different origin. We can conclude that the method used here represents a step toward the
22 more difficult detection of hazelnut oil in other olive oils enlarging the problems associated with this
23 fraudulent practice that, besides provide an economic damage, introduces a potential health risk
24 for sensitized patients.
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34 35 36 **Acknowledgements**

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Tab. 1: Identification by PMF and MS/MS of proteins selected from the total protein extract from solvent-extracted HAO (used to spike EVOO)

Figure 1: SDS-PAGE of total proteins isolated from solvent-extracted HAO (1) and from EVOO (2) (Colloidal Coomassie Brilliant Blue staining). Numbers highlight the proteins as “putative allergens” and (*) the bands analyzed but not identified.

Figure 2: SDS-PAGE of total protein extract isolated from EVOO, artificially spiked with different percentage of solvent-extracted HAO (from 5 to 0,5%, V/V). The arrow highlights hazelnut oleosins identified by MS/MS analysis.

Figure 3: immunoblotting profiling performed on the total protein fraction isolated from hazelnut oil. Lane 1: Bet v 1/ Bet v 2 - and homologues - recognizing serum; lane 2: Rabbit anti-hazelnut oleosin; lane 4: Rabbit anti-hazelnut; lane 6: Rabbit anti-hazelnut (pH 2.5 performed extraction); lane 8: Rabbit anti-LTP; lane 10: Mouse anti-Bet v 1 (Cor a 1); lanes 3,5,7,9,11: Pre-immune sera (taken previously immunization with the allergen or extract, then before the animal develops specific antibodies); lanes 12-15: Hazelnut positive human sera; lanes LC: Label controls. Spots in lanes 2 and 5 are caused by a phenomenon of background.

Band	Species	Protein name	UniProt KB/TrEmbl Entry	Nominal MW	Number of peptides	Sequence coverage	MS/MS (sequence)
1	<i>Corylus avellana</i>	Oleosin	Q84T91	14,723 Da	4/47	27%	HPPGADQLDHAR
		Oleosin	Q84T21	16,688 Da			
2	<i>Corylus avellana</i>	Oleosin	Q84T91	14,723 Da	4/84	27%	HPPGADQLDHAR
3	<i>Corylus avellana</i>	11S globulin-like protein	Q8W1C2	59,614 Da	9/86	22%	AESEGFVAVFK
4	<i>Corylus avellana</i>	11S globulin-like protein	Q8W1C2	59,614 Da	15/75	28%	AESEGFVAVFK

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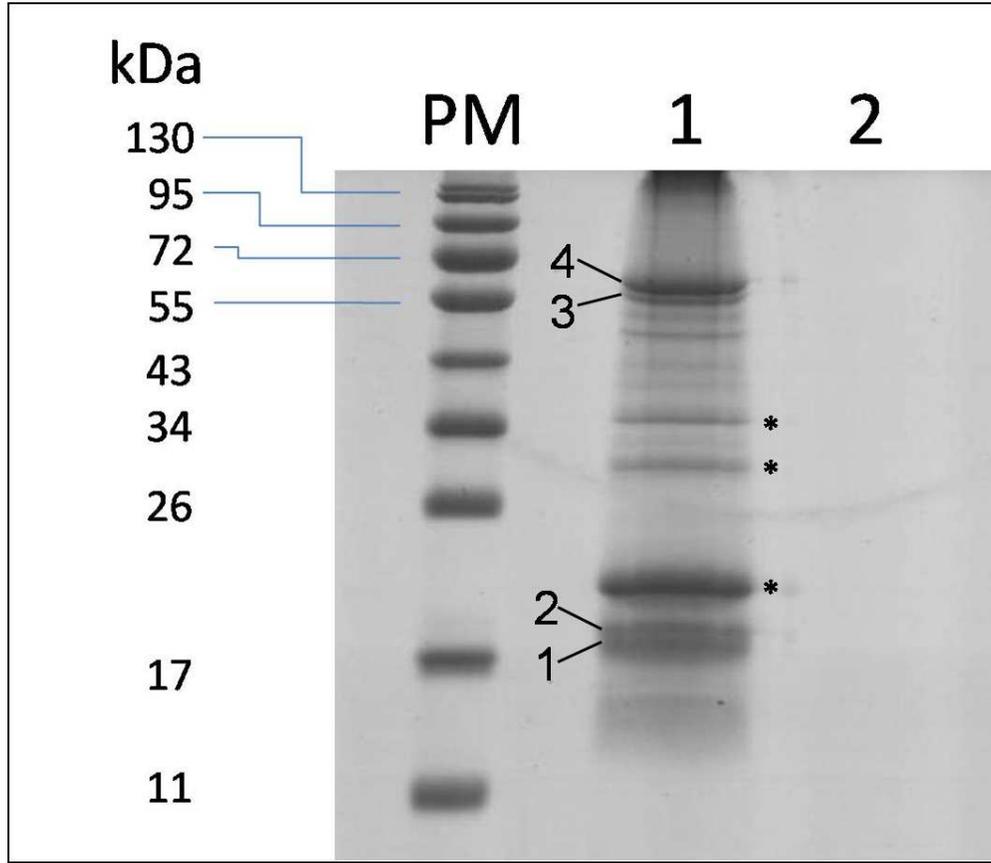


Figure 1: SDS-PAGE of total proteins isolated from solvent-extracted HAO (1) and from EVOO (2) (Colloidal Coomassie Brilliant Blue staining). Numbers highlight the proteins as "putative allergens" and (*) the bands analyzed but not identified.
179x155mm (150 x 150 DPI)

Only

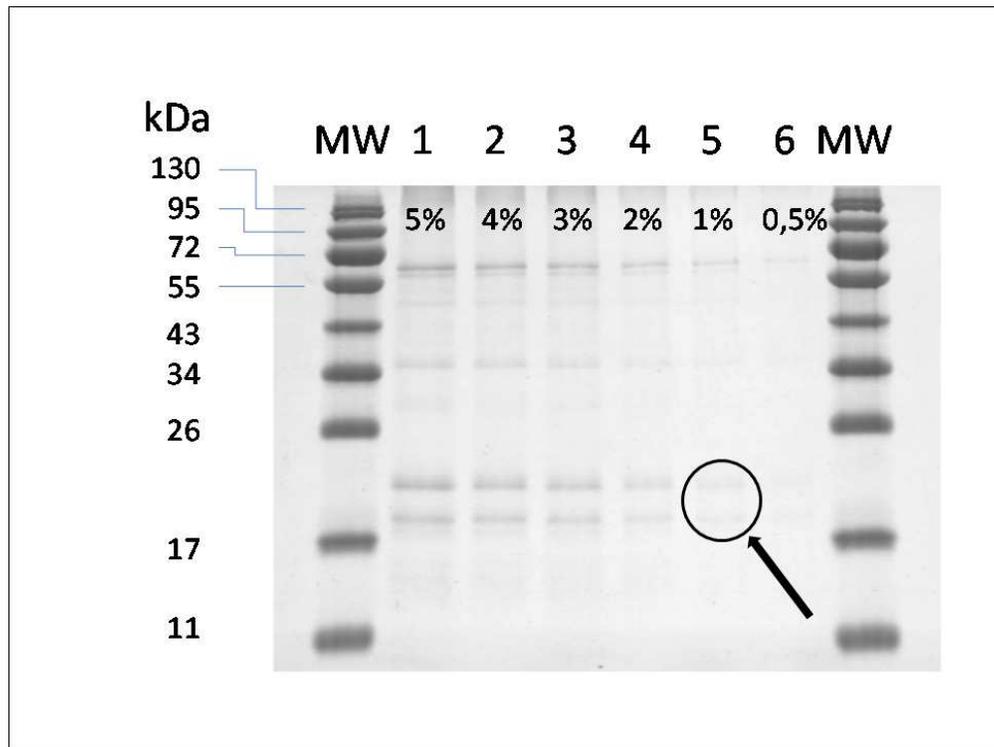


Figure 2: SDS-PAGE of total protein extract isolated from EVOO, artificially spiked with different percentage of solvent-extracted HAO (from 5 to 0,5%, V/V). The arrow highlights hazelnut oleosins identified by MS/MS analysis.
164x123mm (150 x 150 DPI)

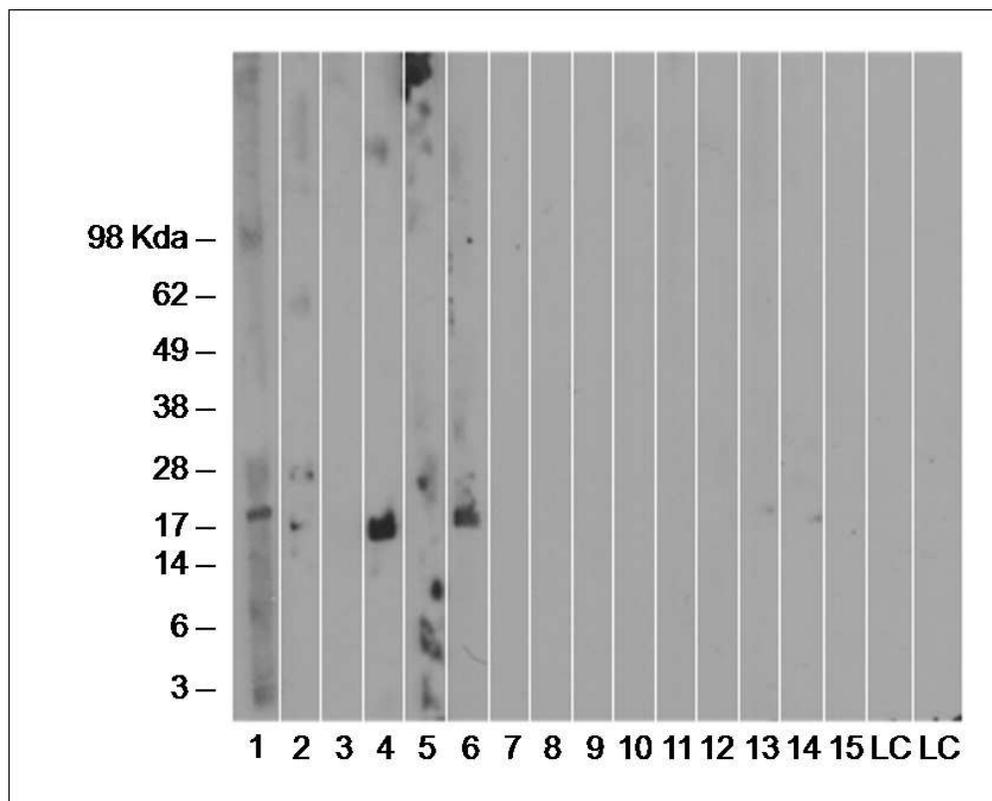


Figure 3: immunoblotting profiling performed on the total protein fraction isolated from hazelnut oil.

Lane 1: Bet v 1/ Bet v 2 - and homologues - recognizing serum; lane 2: Rabbit anti-hazelnut oleosin; lane 4: Rabbit anti-hazelnut; lane 6: Rabbit anti-hazelnut (pH 2.5 performed extraction); lane 8: Rabbit anti-LTP; lane 10: Mouse anti-Bet v 1 (Cor a 1); lanes 3,5,7,9,11: Pre-immune sera (taken previously immunization with the allergen or extract, then before the animal develops specific antibodies); lanes 12-15: Hazelnut positive human sera; lanes LC: Label controls. Spots in lanes 2 and 5 are caused by a phenomenon of background.

142x114mm (150 x 150 DPI)