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Detection of pressed hazelnut oil in virgin olive oil by analysis of polar components: improvement and validation of the method

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Abstract

An improved method for the detection of pressed hazelnut oil in admixtures with virgin olive oil by analysis of polar components is described. The method, which is based on the SPE-based isolation of the polar fraction followed by RP-HPLC analysis with UV detection, is able to detect virgin olive oil adulterated with pressed hazelnut oil at levels as low as 5% with accuracy (90.0±4.2% recovery of internal standard), good reproducibility (4.7% RSD) and linearity (R^2: 0.9982 over the 5–40% adulteration range). An international ring-test of the developed method highlighted its capability as 80% of the samples were, on average, correctly identified despite the fact that no training samples were provided to the participating laboratories. However, the large variability in marker components among the pressed hazelnut oils examined prevents the use of the method for quantification of the level of adulteration.

Keywords: Hazelnut oil; Olive oil; Polar components; HPLC; Method validation; Ring test

1. Introduction

Adulteration of olive (*Olea europaea*) oil with hazelnut (*Corylus* spp.) oil has been a serious problem for regulatory agencies, oil suppliers and consumers. This fraudulent practice, arising due to the difference in economic value between the two oils, causes an estimated loss of 4 million euros per year for countries in the European Union (EU Research Committee, 2001).

Detection of hazelnut oil in admixtures with olive oil has always been very difficult to confirm using conventional approaches, especially at adulteration levels below 20%, due to the similarity of the two oils in parameters such as fatty acid and sterol content (Parcerisa, Richardson, Rafecas, Codony, & Boatella, 1998).

Alternative approaches that have been investigated for the detection of pressed hazelnut oil in olive oil include the analysis of filbertone (*E*)-5 methylhept-2-en-4-one) by online LC–GC (Blanch, Caja, León, & Herraiz, 2000; Caja, del Castillo, Herraiz, & Blanch, 1999; del Castillo, Caja, Herraiz, & Blanch, 1998), assessment of sterols and triacylglycerols by high-field ^{1}H NMR and GC (Mannina, Patumi, Fiordiponti, Emanuele, & Segre, 1999) and the use of FT-IR spectroscopy (Ozen & Mauer, 2002). The analysis of filbertone appears to be a very promising method. The other techniques however are likely to be ineffective at lower adulteration levels, considering the natural variation in oil composition of samples from different countries or grown under different conditions.

Another approach is the use of non-volatile marker components present in the polar fraction of hazelnut oils to detect adulteration (Gordon, Covell, & Kirsch, 2001). This is a simple technique based on the RP-HPLC analysis of the polar fraction isolated from adulterated oils and has been successfully used to detect low level (2.5%) admixtures using a marker component found to be present in the polar extracts of hazelnut oils but absent in the same olive oil fractions (Gordon et al., 2001).

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The objective of this work was to develop this method for widespread use for the detection of pressed hazelnut oil in virgin olive oil. This was achieved by improving the extraction and chromatographic analysis of the polar fraction followed by validation of the established protocol using intra- and inter-laboratory parameters as well as an international ring test.

2. Materials and methods

2.1. Oils

Virgin olive and pressed hazelnut oils were obtained from commercial and non-commercial (local mills) sources.

2.2. Reagents, reference compounds and analytical materials

All solvents used were HPLC grade (BDH, Poole, England). Reference compounds used during the various experiments included tyrosol (Fluka Chemicals, Dorset, England), gallic, protocatechuic, homoprotocatechuic, vanillic, caffeic, ellagic, ferrulic, syringic, *trans*-cinnamic, *o*- and *p*-coumaric, *p*-hydroxyphenylacetic and *p*-hydroxybenzoic acids (Sigma-Aldrich Ltd, Dorset, England). SPE was carried out using LC-Diol cartridges (500 mg of sorbent, 60 ml tube volume) (Sigma-Aldrich Ltd, Dorset, England).

2.3. Polar fraction isolation

The procedure followed was similar to that described by Mateos et al. (2001) with a few additional steps. A sample of oil (50.0±1.0 g) was filtered and spiked (1 ml) with a solution of syringic acid in methanol (0.15 mg/ml). The solvent was evaporated (40 °C) and the oil was dissolved in hexane (100 ml). An SPE cartridge was then placed under vacuum (approx. 10 psi pressure) and was conditioned with methanol followed by hexane (120 ml each). The hexane-diluted oil was then passed through the cartridge in three aliquots (approx. 60 ml each). Each time an aliquot was passed through, the cartridge was washed with hexane (60 ml) and the polar fraction was eluted into a clean flask using methanol (180 ml) (re-conditioning with 120 ml hexane followed to prepare the cartridge for the next aliquot). The methanolic fractions were combined, evaporated under vacuum (40 °C) to a volume of about 50 ml and extracted with hexane (3×25 ml). The hexane fraction was discarded and the polar fraction was evaporated (40 °C) to a yellow residue (using a 5 ml pear-shaped flask). Methanol/water (1:1) solution (500 μl) and hexane (2 ml) were added and the flask was vortexed (approx. 30 s). The two layers were allowed to separate (5–60 min) and the methanol/water layer (15–20 μl) was subjected to RP-HPLC analysis.

2.4. RP-HPLC analysis

HPLC analysis was carried out on a Dionex Summit quaternary pump system (Dionex Corp., Camberley, England) equipped with an ASI-100 autosampler and a PDA-100 photodiode array detector. The column used was from Kromasil (100-5C18, 3.2 mm i.d × 250 mm; 5 μm particle size) fitted with a guard column (50 mm) of an identical phase. Elution was performed at 30 °C using a 0.490 ml/min flow rate. A mixture of water/glacial acetic acid (97:3, v/v) (solvent A) and methanol/acetonitrile (50:50, v/v) (solvent B) was used as a mobile phase. The composition of the mobile phase changed as follows: isocratic for 15 min (95% A–5% B) then gradient (100% B over 25 min, maintained for 15 min, then back to 5% B over 20 min. Data acquisition was carried out primarily at 293 nm but also at 240, 280 and 335 nm. Quantitation was performed at 280 nm (general absorption wavelength for phenolics) based on syringic acid (results were expressed as syringic acid equivalents as response factors for individual components were not determined).

2.5. HPLC system tests

Injection repeatability was determined by the repeated injection (n = 5) of the polar fractions from two pressed hazelnut oils, Turkish and American, varying in concentration of marker components. Polar fraction isolation and analysis conditions were as described in Sections 2.3 and 2.4.

Detector performance and HETP of the HPLC column were determined by analysing (as per Section 2.4) solutions of syringic and caffeic acids of varying concentrations (4.9–12.1 mg/ml for syringic, 4.1–10.2 mg/ml for caffeic in 1:1 methanol/water).

2.6. Ring test

Scientists from the University of Helsinki (Finland), University of Porto (Portugal), Prague Institute of Chemical Technology (Czech Republic), Reading Scientific Services Ltd (UK), University of Castilla-La Mancha (Spain) and the Aristotle University of Thessaloniki (Greece) participated in the ring test.

3. Results and discussion

The polar fraction of a wide range of virgin olive and pressed hazelnut oils was isolated and analysed in order to establish suitable components that could be used as

markers for the detection of admixtures. Fourteen different pressed hazelnut oils and 10 different virgin olive oils prepared from hazelnuts/olives varying in geographical origin and/or date of harvesting were used.

3.1. Extraction/isolation of the polar fraction from oils

The liquid–liquid extraction (LLE) method described previously (Gordon et al., 2001) for the isolation of the polar fraction from oils was found to be not ideal for routine use as it required a large (500 g) sample for the detection of low-level (<10%) adulterated olive oils and was labour intensive.

As a better alternative, a solid phase extraction (SPE)-based technique reported by Mateos et al. (2001) was employed. However, the scale was increased to accommodate the larger sample size needed compared to that of Mateos et al.

The SPE approach was found to be simpler than LLE and reduced the required sample size 10-fold to 50 g of oil without any effects on the chromatographic profile of the polar fraction. This was determined to be the minimum amount of oil required, for an adulteration of 5% to be reliably detected, independently of pressed hazelnut oil used (with the exception of a Spanish oil, Section 3.4).

3.2. RP-HPLC analysis

HPLC analysis was carried out with UV detection primarily at 293 nm. This wavelength was selected as the main wavelength because it enhanced the signal of the marker component and at the same time reduced the intensity of the signal from tyrosol (λ_{max} 277 nm) to ensure a baseline-to-baseline separation of the two peaks.

Several different mobile phase compositions and gradients were tested during the search for optimum HPLC conditions. The very polar nature of the components of interest in the fraction from hazelnut oil dictated the use of a mainly aqueous mobile phase with a low content of organic solvent for satisfactory separation. The HPLC conditions described here appear to be ideal as they provide excellent peak separation for the components of interest with minimal variation in retention time.

3.3. Marker components in the polar fraction from pressed hazelnut oils

Several additional peaks were found to be present in the chromatograms of the polar fraction of pressed hazelnut oils when compared with the same fraction of virgin olive oils (Fig. 1). Two of those peaks (labeled as components '1' and '4' in chromatograms shown here) were found to be present in all 14 pressed hazelnut oils at a higher concentration than most of the other detected constituents of the polar fraction. These peaks were then selected, as a consequence of their presence in the oils, to be used as markers for the detection of adulteration of virgin olive oil by pressed hazelnut oil.

As can be seen from Fig. 1, peak 1 appeared early in the chromatogram (after gallic acid, before hydroxytyrosol) whilst peak 4 eluted (under these conditions) immediately after tyrosol (RRT 1.1).

Examination of the UV spectra obtained from these peaks revealed the presence of two components co-eluting under peak 1. The component at the forward end exhibited a spectrum with $\lambda_{max} = 284.9$ nm (Fig. 2 a) whilst the component at the rear end of peak 1 had $\lambda_{max} = 293.5$ nm. (Fig. 2b). The ratio of the co-eluting components appear to vary between unrefined hazelnut oils (based on comparison of the spectra across the peak). Peak 4 represents a single UV-absorbing component with a simple spectrum exhibiting an λ_{max} of 293.8 nm (Fig. 2c). This is the component reported as a marker compound in the previous study (Gordon et al., 2001).

Several authentic phenolic standards were analysed (under identical HPLC conditions) in order to aid in the identification of the marker components. However, none of the standards used matched the marker components in terms of retention time and UV spectrum. Future work will attempt to further characterize the marker components using techniques such as LC–MS and LC–^{1}H NMR.

3.4. Within-laboratory method validation

Intra-laboratory method validation was performed by examining parameters such as HPLC system suitability as well as method accuracy, reproducibility, linearity and adulteration detection limit. Most of these parameters were established based on the behaviour of the internal standard (syringic acid) as the identities of the marker components were unknown.

3.4.1. Chromatographic system

The suitability of the HPLC system to perform the analysis was assessed by examining injection repeatability, diode array detector performance, column resolution and height equivalent of a theoretical plate.

The injection repeatability values were obtained from the repeated injection of two hazelnut oil polar fractions varying in concentration of marker components. Detector performance was assessed by determining the absorptivity coefficients (peak area/amount injected) of syringic and caffeic acids. This was required in order to address potential differences in detection sensitivity between the various laboratories using the developed method.

Chromatographic performance of the column used was assessed by its resolution (between the peaks for

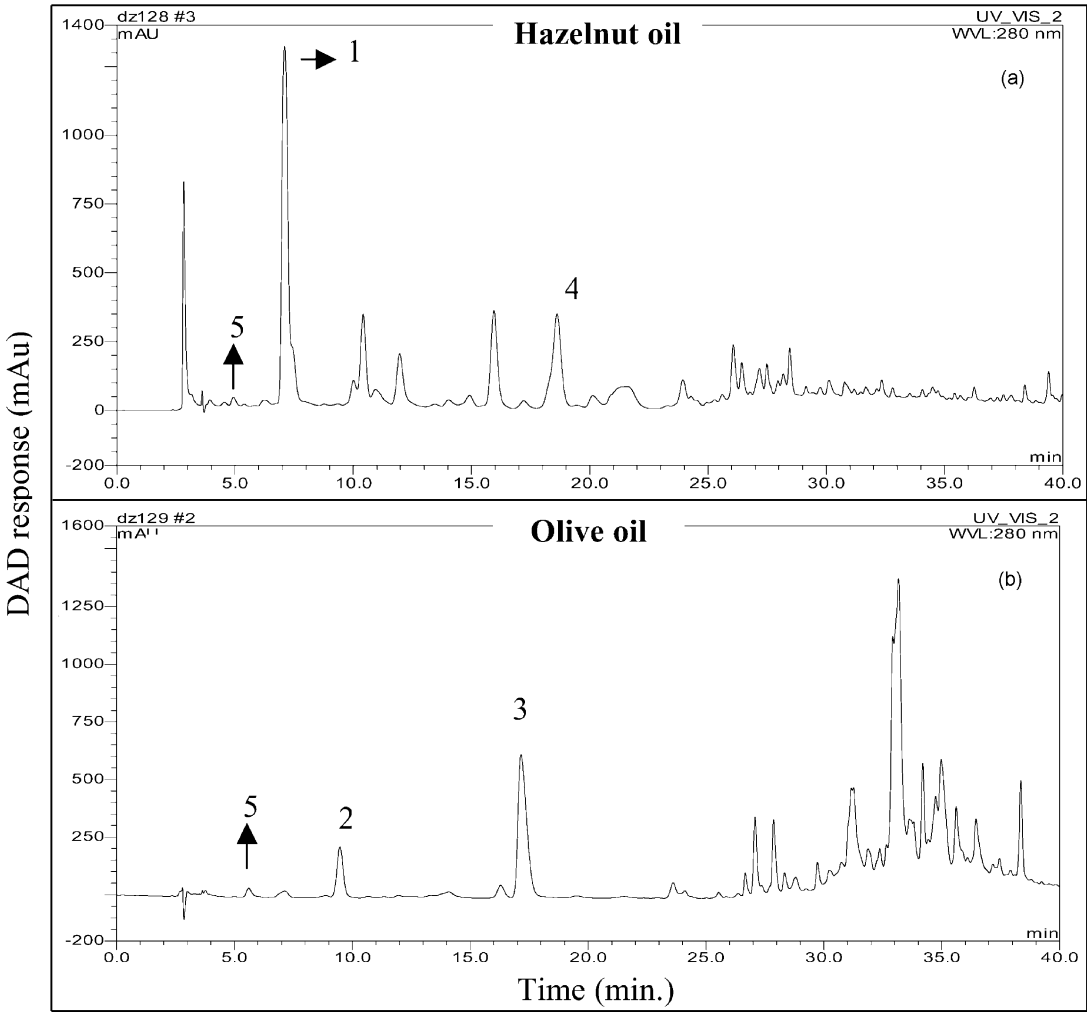

Fig. 1. RP-HPLC traces ($\lambda = 280$ nm) of a polar fraction from (a) pressed commercial hazelnut oil (Anglia Oils Ltd.) and (b) extra virgin commercial olive oil (Philippo Berrio). Peaks: component 1 (1), hydroxytyrosol (2), tyrosol (3), component 4 (4), gallic acid (5).

marker component 4 and tyrosol) and the height equivalent of a theoretical plate (HETP) (for syringic and caffeic acids, five different concentrations used). As Fig. 3 shows, an acceptable (Dolan & Snyder, 1989) resolution of 1.87 between tyrosol and the peak for marker component 4 was obtained using the developed conditions. The HETP of the column used was also found to be satisfactory under the developed conditions (Table 1).

3.4.2. Overall-method parameters

Analytical parameters such as accuracy, reproducibility, linearity and adulteration detection limit were examined in order to assess the overall validity of the developed method.

Recovery of the internal standard (syringic acid, added at 3 mg kg^{-1}) after analysis of samples from all hazelnut oils available was found to be $90.0 \pm 4.2\%$ (mean $\pm$ SD, $n = 14$). In the same experiment the reproducibility of the method, determined by the area of the peak corresponding to syringic acid, was 4.7% (RSD). An identical value was obtained from the averaging of the RSD's resulting from the determination of the total phenolics found in the polar fractions of 14 unrefined hazelnut oils.

Adulteration detection limit and method linearity were assessed by preparing blends (0, 5, 10, 20 and 40%) of extra virgin olive with pressed Turkish hazelnut oil. This hazelnut oil was used because it was found to contain the least amount of marker components of all hazelnut oils examined (with the exception of a Spanish oil which contained marker components from trace to undetectable amounts and thus could not be used). Results showed that detection of adulteration was possible even at 5% by using marker component 4 (identity of the marker confirmed by its UV spectrum) (Fig. 4).

The developed method was found to be linear over the 0–40% adulteration range (r^2: 0.9982) (based on peak area of component 4 using Turkish hazelnut oil) (Fig. 5).

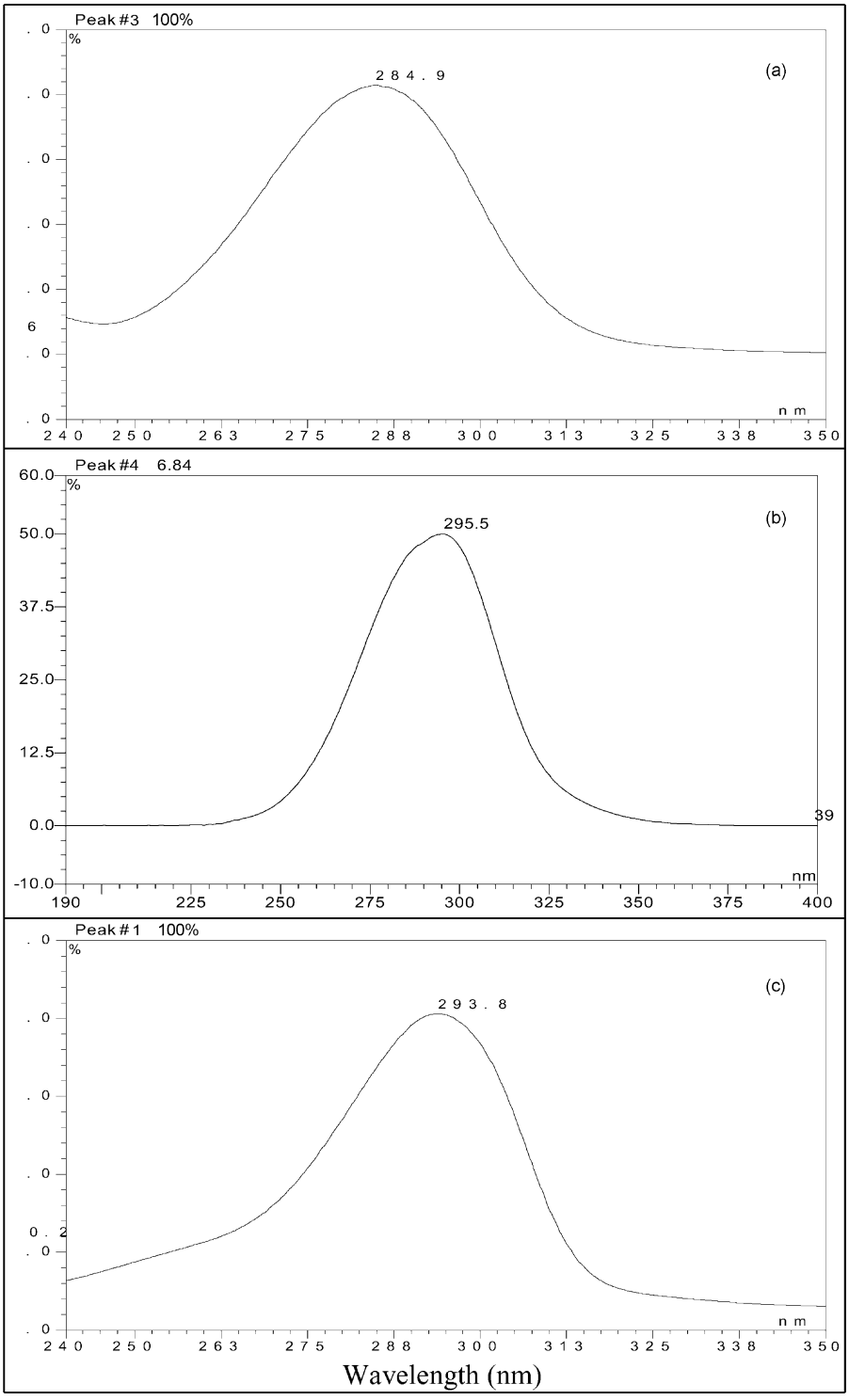

Fig. 2. UV spectra of components under peak 1 (a,b) and 4 (c) (obtained under the conditions described in Section 2).

3.5. Ring-testing of the developed method

Six independent laboratories were used to ring-test the developed method. Six commercially available virgin olive oils were mixed with two unrefined hazelnut oils at The University of Reading to provide ten samples containing 0, 5, 10 and 20% hazelnut oil. The samples were then divided into sets (10 samples/set), coded and sent to the participating laboratories (one set/lab) together with detailed protocols and instructions. The laboratories were asked to determine which samples were adulterated based on the presence of marker component 4 and report their assignments in writing. To simplify interpretation of the chromatograms and correct

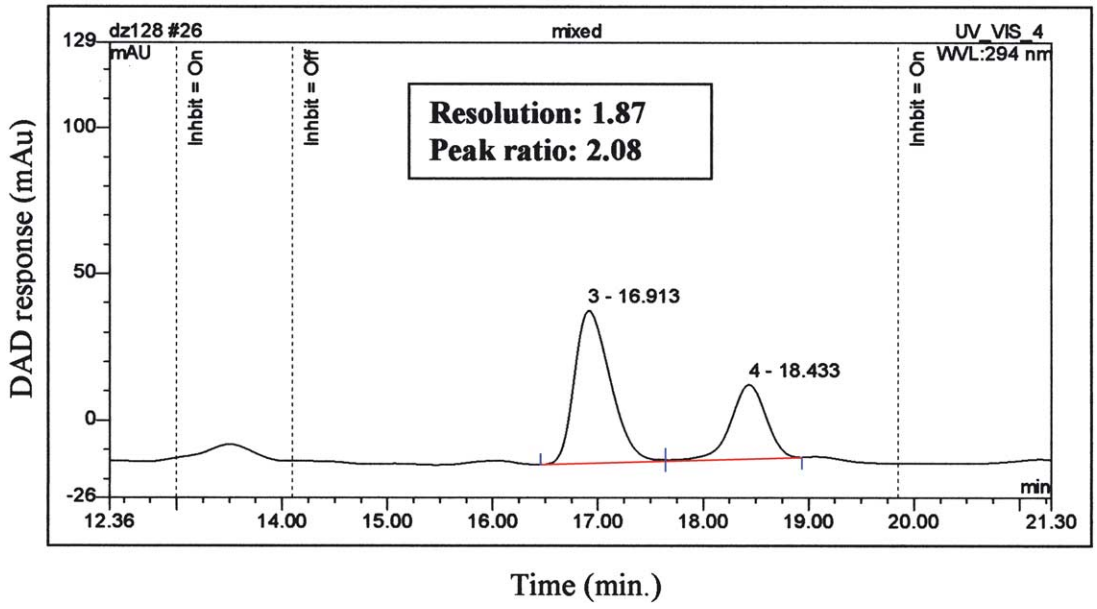

Fig. 3. Resolution between the peak for marker component 4 (rt 18.4 min) and tyrosol (rt 16.9 min). (HPLC trace of a polar fraction from a Greek olive oil adulterated with 40% American hazelnut oil).

Table 1
Suitability of the HPLC system for the detection of olive oil adulteration by hazelnut oil

Oil	Turkish hazelnut oil		American hazelnut oil	
	Comp. 1	Comp. 4	Comp. 1	Comp. 4
Injection reproducibility peak area (n = 5)				
Mean	353.9	153.6	33.1	32.1
SD	3.2	1.3	0.2	0.3
RSD (%)	**0.90**	**0.85**	**0.77**	**0.82**
			Area counts/μg injected[a] ($n = 5$)	
			Syringic acid	Caffeic acid
Diode array detector performance				
Mean			102.4	82.9
SD			1.2	1.4
			HETP (cm^{-1})[b]($n = 5$)	
HPLC column efficiency				
Mean			21510	4047
SD			778	64
RSD (%)			3.6	1.6

[a] In Kromasil® 100-5C_{18} column (25 cm × 3.2 mm i.d).
[b] Determined including the length of the guard column (total length 30 cm).

assignment of samples it was decided that component 1 was not to be used as a marker because it was not known whether the two compounds under the peak would separate under slightly different chromatographic conditions/columns. Also use of component 1 as a marker would disadvantage laboratories with single-wavelength detectors.

Overall, the results obtained from the ring test were mixed. Most laboratories were able to correctly distinguish the adulterated from the non-adulterated samples in most cases (Table 2), but some assignments were incorrect. Only one laboratory was 100% successful in detecting adulteration. Close examination of the chromatograms from all but one of the laboratories showed that the marker components were present in all the adulterated oils and absent in all the controls (Table 2). Most incorrect assignments in Table 2 resulted from the inability of some participants to locate the marker components in a chromatogram as the chromatographic conditions/equipment employed for the analysis differed from those recommended.

The results obtained from one participating laboratory showed inadequate peak resolution (derived from poor chromatography) and the results from this laboratory are omitted from the analysis of the data. Maintenance of acceptable chromatographic standards (such as those described in Section 3.4) is essential if the developed method is to be used to its full capability.

As the developed method is based on UV detection, it is important that adequate sensitivity is available especially during the analysis of low (<10%) level adulterated samples. Sensitivity of UV lamps is known to deteriorate with age and thus periodic monitoring of lamp/detector performance is required. For example, the inability of Lab 1 (Table 2) to detect the low level adulterated samples may be associated with deteriorating detector performance.

The accuracy, reproducibility and linearity of the method (as assessed from the results produced by the independent laboratories) were found to be similar to

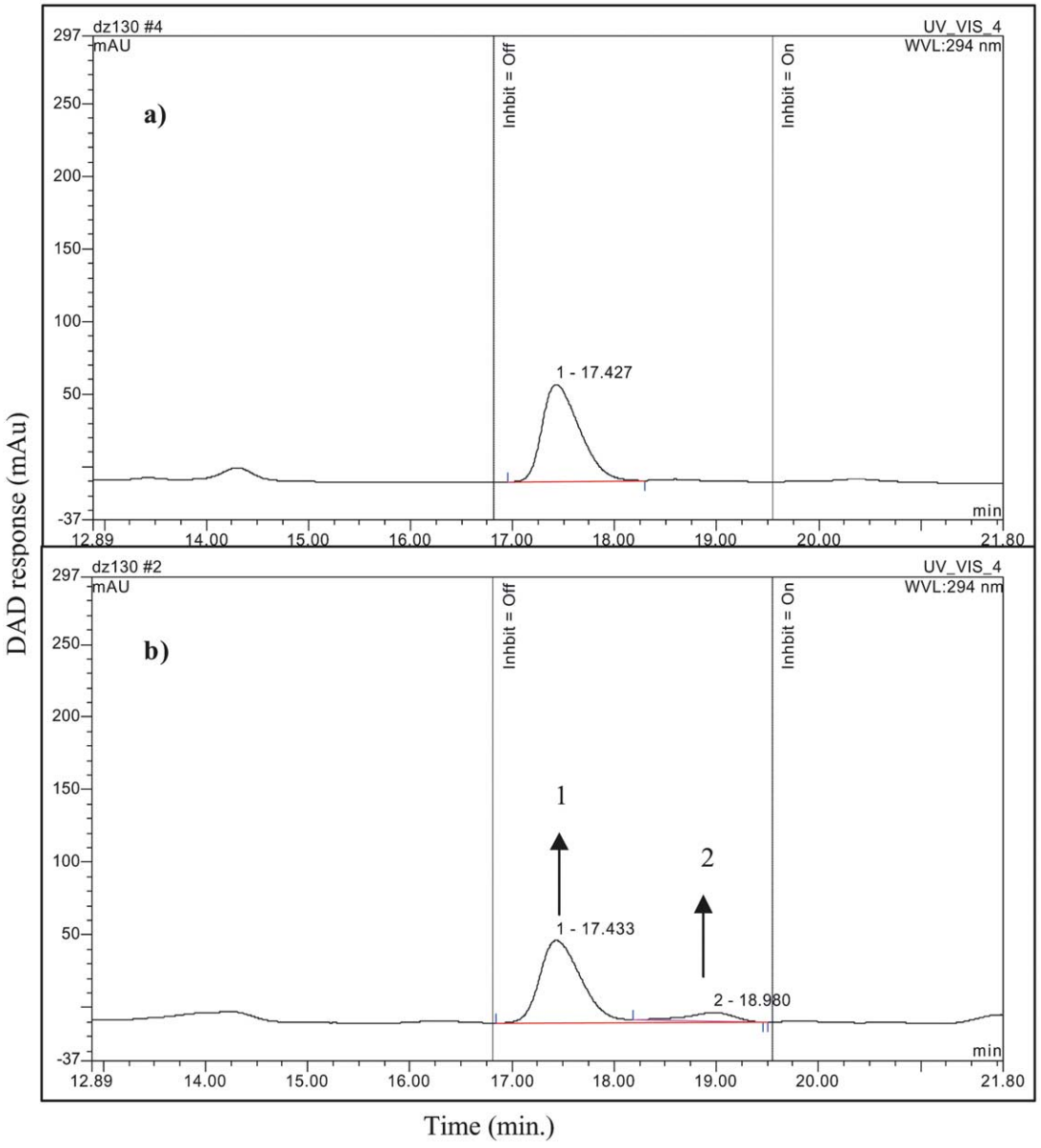

Fig. 4. Comparison of RP-HPLC traces ($\lambda = 293$ nm) of (a) unadulterated Greek extra virgin olive oil and (b) the same oil adulterated at 5% with Turkish pressed hazelnut oil. Peaks: tyrosol (1), component 4 (2).

those obtained at the University of Reading. For example, results from Lab 3 show a 94.0±4.8% recovery of syringic acid with an rsd of 5.5% ($n = 18$) and an R^2 of 0.9964 and 0.9895 based on peak area of component 4 (0–20% adulteration with Turkish and American hazelnut oil, respectively). The similarity in these parameters indicates the robustness of the developed method.

It must be noted that correct assignment of peaks is aided by training with known pure and adulterated samples, since the relative retention times of peaks do vary under different conditions (e.g. column manufacturer) used by the laboratories. Analyst-training will also reduce the time required for sample extraction as some participants in the ring-test noted that the method, and especially the extraction step, was time-consuming (1–2 samples/working day; efficiency of our laboratory reached four samples/working day).

3.6. Quantitative detection of hazelnut oil/olive oil admixtures

Analysis of the polar fractions from pressed hazelnut oils revealed marked differences in the level of marker components. As can be seen from Table 3, there is almost a 20-fold difference in amount of component 1 and approximately a 30-fold difference in amount of component 4 between the French and the Turkish hazelnut oil. This means that the developed method cannot be used for the quantitative determination of the

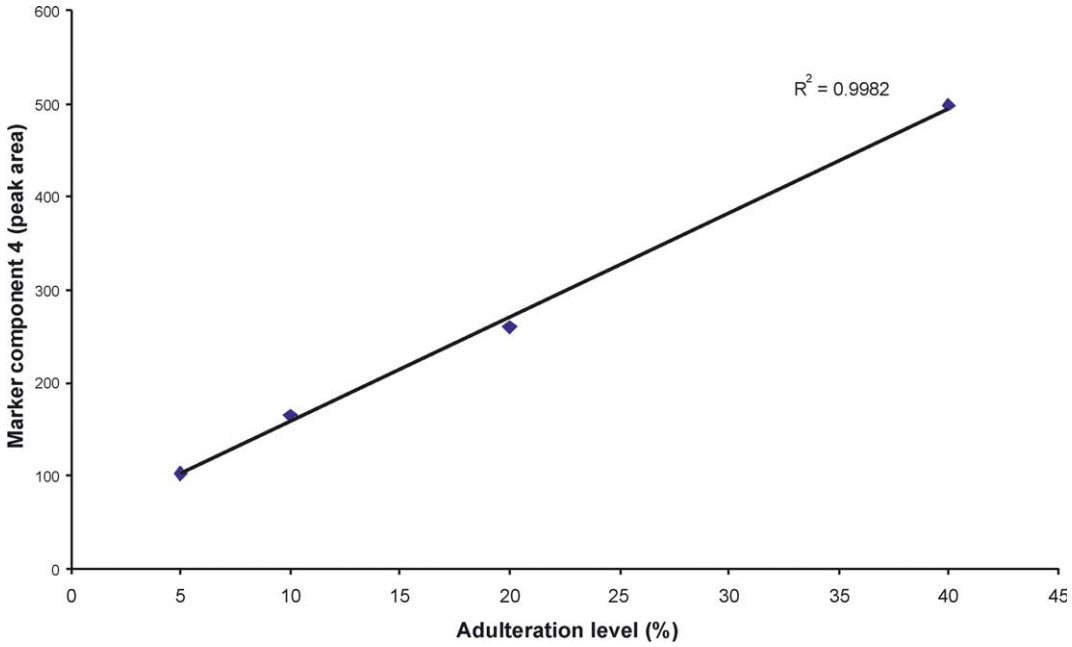

Fig. 5. Peak area of marker component 4 against level of adulteration of extra virgin olive oil with Turkish pressed hazelnut oil.

Table 2
Summary of the results from the independent ring-test. Results from laboratory 6 are not included

Oil sample[a]	Adulteration level (%)	Participants				
		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
		Correct assignment (yes/no)				
Olive oil 1	0	Yes	No (Yes)[b]	Yes	Yes	Yes
Olive oil 2	0	Yes	No (Yes)[b]	Yes	Yes	Yes
Olive oil 3	0	Yes	No (Yes)[b]	Yes	Yes	Yes
Olive oil 4	0	Yes	No (Yes)[b]	No	Yes	No
Olive oil 5/Turkish hazelnut oil	5	No	Yes	Yes	Yes	Yes
Olive oil 3/American hazelnut oil	5	No	Yes	Yes	Yes	Yes
Olive oil 6/Turkish hazelnut oil	10	No	Yes	Yes	Yes	Yes
Olive oil 1/American hazelnut oil	10	Yes	Yes	Yes	Yes	Yes
Olive oil 2/Turkish hazelnut oil	20	Yes	Yes	Yes	Yes	Yes
Olive oil 4/American hazelnut oil	20	Yes	Yes	Yes	Yes	Yes
Number of correct conclusions		**7**	**6 (rising to 10)[b]**	**9**	**10**	**9**

[a] All olive oils used were virgin olive oil samples (produced in Italy, France and Spain).
[b] Assignment by D. Zabaras after examination of the chromatograms.

Table 3
Marker-component content (mean±SD, $n=3$) of various pressed hazelnut oils

Oil source	Amount (mg kg^{-1})[a]	
	Component 1	Component 4
France	6.23±0.09	4.42±0.11
USA	0.71±0.04	0.43±0.05
Turkey	0.37±0.01	0.15±0.02

[a] Syringic acid equivalents at 280 nm.

level of adulteration of virgin olive oil with pressed hazelnut oil.

4. Conclusion

Although not very rapid and not quantitative, the developed method could be used, on its own or in combination with other techniques, for the qualitative detection of adulteration of virgin olive oils adulterated

to low levels (down to 5%) with most pressed hazelnut oils. The method was only 82% successful on average in the ring test with five independent laboratories, but this success rate could be improved by provision of training samples.

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