Analytical Methods

High-power gradient diffusion NMR spectroscopy for the rapid assessment of extra-virgin olive oil adulteration

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A R T I C L E   I N F O

Article history:
Received 10 October 2008  
Received in revised form 21 January 2009  
Accepted 21 April 2009

Keywords:
Olive oil
Adulteration
Diffusion NMR spectroscopy
Discriminant analysis

A B S T R A C T

A high gradient diffusion NMR spectroscopy was applied to measure diffusion coefficients (D) of a number of extra-virgin olive, seed, and nut oils in order to ascertain the suitability of this rapid and direct method for discrimination of adulterated olive oils. Minimum adulteration levels that could be detected by changes in D were 10% for sunflower (SuO) and soybean oil (SoO), and 30% for hazelnut (HO) and peanut oil (PO). Qualitative and quantitative prediction of adulteration was achieved by discriminant analysis (DA). The highest prediction accuracy (98–100%) was observed only when two DA models were concomitantly used for sample classification. The first DA model provided recognition of high adulterated EVOO with more than 20% of SuO or SoO, and 30% with PO, whilst the second model could differentiate EVOO adulterated with 10% of SuO or SoO, and more than 30% of HO. The validation test performed with an independent set of randomly adulterated EVOO samples gave 100% classification success. The high accuracy levels together with minimal requirements of sample preparation, and short analyses time, prove the high-power gradient diffusion NMR spectroscopy as an ideal method for rapid screening of adulteration in valuable olive oils.

1. Introduction

High quality food products demanding higher market prices often become subjects of unscrupulous food mislabelling, or increased volume of good quality batches through adulteration with sub-standard procedure. With the increasing awareness of food safety and quality, consumers continuously demand reassurance of food origin and content (Schieber, 2008). Thus, there is a great effort, both from consumers and industrial site, to develop rapid, inexpensive, reliable and non-destructive screening techniques for the determination of food authenticity at any point of the distribution chain (Downey, Kelly, & Rodriguez, 2006; Schieber, 2008).

Recently, major concern was paid to frauds in olive oil industry. Production of olive oil represents about ~3% of the world oils and fat markets, with ~78% of global production taking place in Mediterranean countries (Aparicio & McIntyre, 1998; Rezzi et al., 2005), where oil industry is important in sustaining viability of agricultural economy (Vlachos et al., 2006). Virgin olive oils (VOO), and in particular, extra-virgin olive oils (EVOO), that are produced using solely cold pressing techniques, belong to the most sought after olive oil product on the account of their organoleptic (aroma and taste), antioxidant, and nutritional properties (Aparicio & McIntyre, 1998; Boscou, 1996; Harwood & Yaqoob, 2002). The cultivation of olive trees, harvesting of olive fruits, and extraction of olive oil are hard and time consuming tasks that add to its relatively high commercial price (Fragaki, Spyros, Siragakis, Salivaras, & Dais, 2005). Therefore, attempts to adulterate the high cost VOO with less expensive vegetable oils or low quality olive oils (López-Díez et al., 2003; Vlachos et al., 2006), such as the occurrence of food poisoning in Spain in 1981 (Wesley, Barnes, & McGill, 1995; World Health Organization, 1992).

Authentication and detection of VOO adulteration is based on the comparison of chemical compositions of suspected samples with identified characteristics of olive oils promulgated by the European Union (European Union, 1995, 1996). Despite the fact that composition of VOO differs with various factors such as geographical origin or weather conditions during growths and harvesting (Downey et al., 2006), good results were obtained applying classical analytical methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and pyrolysis mass spectrometry (Aparicio & Aparicio-Ruiz, 2000 and references therein). However, these methods are generally sample destructive and rather unsuitable for oil screening on a large scale,
due to many necessary time-consuming steps required to achieve a complete quantification. Recent research, performed mainly in the last decade, is focused on the determination of adulterants using rapid spectroscopic techniques. Amongst these, Raman (López-Díez et al., 2003), ultraviolet (Aparicio & Aparicio-Ruiz, 2000), near-infrared (Christy, Kasemsuran, Du, & Ozaki, 2004), fluorescence (Pouill, Mousdls, & Georgiou, 2007; Sayago, Garcia-González, Morales, & Aparicio, 2007) and common solution-state NMR spectroscopy (Faulh, Reniero, & Guillou, 2000; Mannina, Sobolev, & Segre, 2003; Sacchi et al., 1996; Vigli, Philippidis, Spyros, & Dais, 2003) were, with some limits, successfully proved as suitable tools for the detection of various adulterants.

This work discusses the potential of a high-power pulsed-field gradient NMR probe (Diff30) in enabling a direct observation of diffusion coefficient of viscous samples, and detecting adulterated EVOO oils. The suitability of this nondestructive, and rapid spectroscopic technique was evaluated on binary mixtures of olive oil adulterated with soybean, sunflower, peanut and hazelnut oil, all belonging to the most commonly found adulterants in VOO (Guimet, Ferré, & Boqué, 2005; Pouill et al., 2007). Discrimination of authentic and adulterated EVOO was assessed by chemometric approach, in particular, by discriminant analysis (DA).

2. Materials and methods

2.1. Materials

Twenty five extra-virgin olive oil (EVOO) samples of Leccino (L1–L6), Frantoio (F1–F6), Pendolino (P1–P7) and Coratina (C1–C6), obtained from the territory around Salerno, Italy, were kindly provided by the local producer “De Falco”. Sunflower (SuO), soybean (SoO), hazelnut (HO), and peanut oil (PO) were purchased from local distributors. The authentic EVOO samples and the same ones but adulterated with 5%, 10%, 20%, 30% and 50% of SuO, SoO, PO and HO, reaching a total volume of 1 ml, were transferred into 5 mm NMR tubes, placed in the NMR probe, and left to equilibrate at 24.5 °C for 15 min prior to diffusion measurements.

2.2. Diffusion NMR measurements

Pulsed field gradient diffusion NMR measurement was carried out on a Bruker Wide-Bore Avance 300 MHz spectrometer, equipped with a 5 mm inverse Bruker high-power diffusion Diff30 probe yielding a maximum Z gradient strength of 350 G/cm. To avoid probe heating and control sample temperature, the probe was continuously cooled by flowing water at a constant temperature of 24.5 °C. Diffusion spectra (accumulation of four scans) were obtained using a spin-echo pulse sequence with a gradient pulse of 5 ms, diffusion time of 120 ms and gradient amplitude ranging from 11 to 140 G/cm in 128 increments. The total experimental time was 20 min per sample. Diffusion coefficients were calculated from the monoeponential decay dependence of attenuation of signal intensity on the gradient amplitude using Bruker Topspin 1.3 software. All measurements were performed in triplicate. A standard calibration sample (D2O, D = 1.872 × 10−10 at 25 °C) was run prior to every set of experiments to ensure consistency between datasets.

2.3. Statistical analysis

All data were expressed as the means of at least three replications. Analysis of variance (ANOVA) was performed applying Duncan’s multiple comparisons test to determine statistically significant differences at P ≤ 0.05 confidence level. For discrimi-
nant analysis (DA), all analysed samples were employed as training set in order to establish classification rules. The predictive ability of DA model was validated by a supplementary data set consisting of 15 randomly adulterated EVOO samples (Table 2). The statistical data processing was performed by XLSTAT 7.5.2 (Addinsoft SARL, Paris, France).

3. Results and discussion

3.1. 1H NMR analysis

The main constituents of olive oil are variously substituted esters of glycerol and saturated (palmitic, stearic) or unsaturated (oleic, linoleic, linolenic) fatty acids (Sacchi, Addeo, & Paolillo, 1997). Due to the chemical similarity of these triglyceride esters, clusters of co-resonating signals are typically observed in 1H NMR spectrum. This is reflected in Fig. 1 that shows 1H NMR spectra of extra-virgin olive oil (EVOO) acquired with (a) Diff30 and (b) BBI (indirect) probe at 300 and 400 MHz, respectively. Although some resolution is lost when acquiring a spectrum with the Diff30 probe, the number of resonating clusters and their relative intensities are comparable to those observed by common 1H NMR acquisition. The signal assignment of olive oil samples is well recognised and for the main signals is given in Table S1 in Supplementary data (Sacchi et al., 1996). Commonly reported resonance frequency of linolenyl chain, occurring at around 0.95 ppm, observable only upon enlargement of high resolution 1H NMR spectra, was not detected with the Diff30 probe.

Inspection of 1H NMR spectra, acquired with the Diff30 probe (Fig. 2) revealed that the composition of PO and HO did not significantly differ from that of EVOO. Conversely, significantly increased signal intensities can be noticed for peaks 1 and 4 in the spectrum of SuO and SoO. Peak 1 is attributed to the olefinic protons of all unsaturated fatty acids, whilst increased amount of peak 4 usually reflects larger amount of linoleic but at the same time smaller content of oleic acid (Vigli et al., 2003). The concentration of olefinic protons detected below peak 1 is one of the important input parameters for the estimation of the degree of oil unsaturation, known as iodine value (Sacchi et al., 1996). In fact, VOO having lower iodine value than SuO and SoO, are characterised by high amounts of monounsaturated fatty acid, such as oleic acid, and low content of polyunsaturated fatty acids, that include linoleic and linolenic acid (Vigli et al., 2003). The increased intensities of peak 1, and particularly of peak 4, were also noticeable for mixtures of EVOO containing more than 20% of SuO and SoO adulterants (data not shown).

3.2. Diffusion analysis

The mean values and standard deviations of diffusion coefficients (D) of all vegetable oils are summarised in Table 1. Diffusion values of signals 2, 4 and 6 are not reported since these peaks became undetectable under the optimised conditions for the applied NMR spin-echo sequence. This is noticeable in Fig. S1 in Supplementary data that reports 1D diffusion profiles of vegetable oils. With exception of peak 7, that was weighted by a maximum of
15% error (Table 1), standard deviations (SD) of less than 1% were observed for the triplicate diffusion measurements of SuO, SoO, PO and HO (Table 1), thereby indicating an excellent method reproducibility. Interestingly, low variability of diffusion coefficients for peaks 1, 3, 5, 8 and 9 was also noted for the 25 different EVOO samples (Table 1). The larger deviation observed for peak 7 was caused by the low signal/noise ratio of this resonance in the 1D diffusion spectra (Fig. S1 in Supplementary data).

The mean values for $D$ (Table 1) further give information about dissimilarities in physical properties, namely viscosity, of seed and nut oils as compared to EVOO. The most similar diffusion behaviour to EVOO was noted for HO, whereby, except for signal 7, only a slight increase in $D$ was observed for the remaining signals. The $D$ value of signal 7 was also the only one greatly reduced in the case of PO, for which a small decrease in diffusion was otherwise noted. For these reasons, $D$ calculated for signal 7 was later used as an input for statistical analysis, though it was weighted by a larger statistical error than similar coefficients observed for remaining signals. Unlike nut oils, a substantial difference in diffusion behaviour was noted for SuO and SoO. In comparison to EVOO, the SuO and SoO oils showed significantly larger diffusion for signals 3, 5, 8, and 9, and a smaller but still greatly varied diffusion coefficient for signal 7. In addition, signal 1 became undetectable in diffusion spectra of SuO and SoO under the experimental set-up conditions optimised for EVOO oils (Fig. S1 in Supplementary data).

3.3. Discriminant analysis

Dissimilarities of $D$ reported in Table 1 suggested that high-power gradient diffusion NMR spectroscopy might be able to detect the presence of SuO, SoO, PO and HO in EVOO. To assess this possibility, diffusion spectra were collected for a set of 25 binary mixtures, each containing EVOO diluted with 5%, 10%, 20%, 30% and 50% of adulterant seed and nut oils. The obtained diffusion data were used as inputs for discriminant analysis (DA), a statistical technique applied for supervised pattern recognition. The main
DA task is to find the best linear discriminant functions of a set of variables, which reproduce, as far as it is possible, *a priori* grouping of the considered cases. The set of linear functions that are used to build a model for samples of known classes are then in turn used as predictors in order to determine group membership of anonymous samples (Brereton, 2003).

Preliminary DA analyses (DA plot not shown) performed on diffusion data of EVOO adulterated with HO indicated a strong overlay of authentic olive samples with samples adulterated up to 20%. Similar finding was observed for PO adulteration. In both cases, only the more largely adulterated samples were well separated from EVOO samples. The SuO- and SoO-diluted olive oil samples showed close grouping with EVOO for adulteration of 5%, acceptable separation for 10% adulteration, and well distinctive separation for adulteration larger than 20%. However, there was a strong overlay amongst groups adulterated with SuO and SoO. These findings indicate that the high-power gradient diffusion NMR technique was not sensitive enough to recognise adulterations lower than 20% in case of HO and PO, and 5% in case of SuO and SoO. Nor it was able to distinguish whether the adulteration was caused by SuO or SoO. To overcome this limitation in the development of a universal DA model, oil samples were divided into seven predefined groups. One group comprised all authentic EVOO; SuO and SoO adulterated EVOO samples were grouped together into four classes according to the adulteration percentage: 10%, 20%, 30% and 50%; and the last two predefined classes were formed by EVOO samples containing 30–50% of HO, and 30–50% of PO.

The best results were observed only when two DA models were used concomitantly. These models are shown in Figs. 3 and 4. The first DA model (Fig. 3) involved EVOO samples with an adulteration of 30–50% of PO, and 20%, 30% and 50% of SuO and SoO. The prediction ability of this training set was 98%. There was no prediction error in the classification of EVOO. A 2% error was observed due to the slight overlap between 20% and 30% adulterated samples with SuO and SoO. The second DA model (Fig. 4) is complementary to the first one and indicates the presence of SuO and SoO when as low as 10%, whereas that of HO was detected only when present from 30% to 50%. The prediction ability of the second DA model reached 100%. Therefore, due to the very good predictability of both model training sets, it is expected that the combined use of the two DA models should ensure certain recognition of EVOO adulterated by more than 10% with SuO or SoO, and 30% with PO and HO. Concomitantly, olive oil samples classified by both models as EVOO are to be considered as unadulterated, or possessing smaller amounts of adulterants which are under the detection limits of the high-power gradient diffusion NMR technique, as indicated above.

In order to validate such approach, a set of randomly adulterated EVOO samples was prepared. The list of chosen EVOO and the amounts of added adulterants is shown in Table 2. The observed diffusion data were used as input variables in terms of supplementary observations, and the two DA models were applied to compute the probabilities by which such anonymous samples could be recognised in a specific group. The first DA model was initially employed to evaluate samples with high percentage adulteration (Fig. 3). As it is indicated in Table 2, samples F2, C2, P3, P1, C3, C5 and F3 were recognised as adulterated EVOO samples, whilst the remaining samples were temporarily labelled as belonging to unadulterated EVOO group. However, as discussed earlier, an authentic EVOO can be such only when both models classify it as unadulterated. Therefore, the second DA model (Fig. 4) was applied on the remaining dataset, and this time only four samples out of eight were further confirmed as unadulterated (Table 2). It can be noticed that these four samples (P4, L6, C3 and F1) were the only authentic EVOO amongst the independent test samples (Table 2). Moreover, a group membership was correctly recognised for all the 11 remaining adulterated test samples. Therefore, the validation gave 100% success.

Furthermore, it must be noted that the validation success can be expected to be somewhat reduced by interchanging class assignment of EVOO samples adulterated with 20% and 30% of both SuO and SoO. This may be due to the overlay between these two groups in the training set used to build the DA model (Fig. 3). Nevertheless, such reduction of validation does not have any effect on the recognition of an adulterated EVOO.

### 4. Conclusions

Using a limited number of authentic and adulterated oils, this work demonstrates that a combination of a high-power gradient

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**Fig. 3.** Classification model I as calculated by discriminant analysis from diffusion data. EVOO, PO, SuO, and SoO refer to extra-virgin olive, peanut, sunflower, and soybean oil, respectively.

**Fig. 4.** Classification model II as calculated by discriminant analysis from diffusion data. EVOO, HO, SuO and SoO refer to extra-virgin olive, hazelnut, sunflower, and soybean oil, respectively.
diffusion NMR technique with multivariate classification method can successfully discriminate between authentic and high adulterated EVOO with hazelnut (as low as 30%), peanut (as low as 30%), soybean (as low as 10%) and sunflower oil (as low as 10%).

The main advantage of this approach is the rapid recognition (5–20 min of analysis) of such adulteration without employing any pre-extraction. The greatest classification success was observed by a combined use of two DA models, one of them involving high (more than 20%) adulteration with SuO, SoO and PO, whilst the other one was based on the diffusion data measured at low adulteration levels (10%) for SoO and SuO and high amount for HO (30–50%).

The correct recognition of adulterated EVOO samples was as high as 98%, whilst, concomitantly, the detection accuracy of nonadulterated EVOO was 100%. Such high accuracy levels together with minimal requirements of sample preparation, call for the high-power gradient diffusion NMR spectroscopy as the ideal method for rapid screening of adulteration in olive oils. Only EVOO samples that pass such screening as authentic oils might undergo more costly and time consuming analyses in order to confirm their real authenticity. In addition, future diffusion NMR studies should indicate whether it is possible to apply this technique to detect adulteration of EVOO by an admixture of two and more vegetable oils.

Acknowledgement
This work was supported by the Regione Campania under programme “Legge 5” 2005.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.foodchem.2009.04.088.

References