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Detection of refined olive oil adulteration with refined hazelnut oil by employing NMR spectroscopy and multivariate statistical analysis

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ABSTRACT

NMR spectroscopy was employed for the detection of adulteration of refined olive oil with refined hazelnut oil. Fatty acids and iodine number were determined by ^{1}H NMR, whereas ^{31}P NMR was used for the quantification of minor compounds including phenolic compounds, diacylglycerols, sterols, and free fatty acids (free acidity). Classification of the refined oils based on their fatty acids content and the concentration of their minor compounds was achieved by using the forward stepwise canonical discriminant analysis (CDA) and the classification binary trees (CBTs). Both methods provided good discrimination between the refined hazelnut and olive oils. Different admixtures of refined olive oils with refined hazelnut oils were prepared and analyzed by ^{1}H NMR and ^{31}P NMR spectroscopy. Subsequent application of CDA to the NMR data allowed the detection of the presence of refined hazelnut oils in refined olive oils at percentages higher than 5%. Application of the non-linear classification method of the binary trees offered better possibilities of measuring adulteration of the refined olive oils at a lower limit of detection than that obtained by the CDA method.

1. Introduction

Numerous attempts have been made in the past to detect virgin olive oil adulteration with seed oils and particularly with hazelnut oil [1–23]. Although no official analytical method has been adopted by the European Commission and other international institutions for the detection of hazelnut oil, several methods using chromatographic and spectroscopic techniques have been proposed to distinguish extra virgin or virgin olive oil from hazelnut oil. Several of these methodologies detect virgin olive oil adulteration with crude hazelnut oil on the basis of chemical compounds that are present in one oil but not in the other oil. For instance, filbertone, which was considered as a hazelnut oil marker [1], had been proposed to detect olive oil fraud [1–4], whereas other researchers suggested to determining chlorophylls [5], tocopherols [6], volatile compounds [7,8] and phenolic compounds [9,10]. Detection of virgin olive oil with refined hazelnut oil (RHzO) presents increasing difficulties because the filbertone marker and other minor components in hazelnut oil are removed upon refinement [3,4], and in addition the triacylglycerol profile of both oils is very similar. Chromatography has been employed to distinguish RHzO in virgin olive oil on the basis of triacylglycerol [11,12] and/or sterol [11–13] profiles. Several spectroscopic techniques in combination with multivariate statistical methods were successful in classifying edible oils and detect the presence of RHzO with little or great success [14–21]. The real problem, however, is the detection of RHzO in refined olive oil (ROO), since their minor components, and in particular their glyceridic composition, were considered to be very similar. Recent applications of FT MIR and FT-Raman and fluorescence spectroscopy [22,23] allowed detection of ROO adulteration with RHzO at concentrations >8%.

NMR spectroscopy is one of the most promising spectroscopic techniques for the analysis of complex systems, such as food matrices. It has been proven to be very successful in the analysis of olive oil and in combination with multivariate statistical methods was able to classify edible oils and detect olive oil adulteration with seed oils and olive oils of inferior quality [15,16,20,24–26]. It made possible the detection of the presence of refined hazelnut oil in virgin olive oil at concentrations 5–10% [14–16,20]. ^{1}H and ^{13}C NMR spectroscopy shows a number of advantages relative to other analytical techniques; it is fast (less than 5 min are required to record an one-dimensional ^{1}H NMR spectrum of an olive oil sample in chloroform-d); it needs no calibration with internal standards or separation of the various components prior to the analysis; it shows remarkable selectivity, inasmuch it identifies unknown compounds at a molecular level; it gives a wealth of information in a single experiment; it is quantitative with excellent repeatability and reproducibility. Disadvantages of NMR spectroscopy may be considered its low sensitivity compared with chromatographic

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and mass spectrometry techniques and the high cost of the analysis, since the NMR spectrometers are quite expensive. Nevertheless, sensitivity problems have been overcome lately by using strong magnetic fields produced by super-conductive selenoids and properly designed probes, especially cryogenic probes, whereas the screening of a large number of samples in a short period of time may compensate the high cost of the analysis. The same advantages shares the NMR spectroscopy of the phosphorus-31 nucleus, although derivatization with phosphorus reagent is needed prior to the analysis, since apart from phospholipids, compounds in olive oil do not contain phosphorus atoms in their molecules. This method is based on the replacement of the acidic hydrogens of the hydroxyl and carboxyl groups of olive oil constituents with the phosphorus reagent 2-chloro-4,4,5,5-tetramethyphospholane and the use of ^{31}P NMR spectroscopy to identify the phosphitylated compounds [27]. This facile magnetic resonance method supplements ^{1}H and ^{13}C NMR techniques, especially in cases where severe overlapped signals in ^{1}H NMR spectra or long relaxation times of the insensitive ^{13}C nuclei render the analysis a difficult task.

The aim of the present study was to assess the potential of ^{1}H and ^{31}P NMR spectroscopy to detect ROO adulteration with RHzO at low concentration. This goal will be achieved upon application to the NMR data two statistical methods, namely, forward stepwise canonical discriminant analysis (CDA) and classification binary trees (CBTs).

2. Experimental

2.1. Refined oils

A collection of 20 samples of refined olive oil were supplied by the olive oil company MINERVA (Athens, Greece). The refined oils were obtained upon refinement of lampante olive oils originated from various locations of Greece. Twenty commercial samples of RHzO were purchased from Henry Lamotte (Bremen, Germany). To detect ROO adulteration, admixtures were prepared by mixing different samples of ROO with different samples of RHzO. The percentage of RHzO in ROO of the adulterated refined olive oils (AOO) varied from 1% to 95% (1%, 2%, 3%, 4%, 5%, 7%, 10%, 12%, 15%, 17%, 20%, 25%, 30%, 50%, 60%, 70%, 80%, 90%, 95%: w/w), The ROO and RHzO samples were selected randomly for the formation of each one of the 19 admixtures. All oil samples and their admixtures were analyzed twice.

2.2. Reagents

Pinacol, triethylamine, phosphorus trichloride, protonated solvents (reagent or analytical grade), and deuterated solvents used in the present study were purchased from Sigma–Aldrich (Athens-Greece). The derivatizing phosphorous reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane used in ^{31}P NMR spectroscopy was synthesized from pinacol and phosphorus trichloride following the method described in the literature [28]. However, to increase the yield of the reaction, we utilized hexane solvent instead of benzene and pyridine instead of triethylamine suggested in the original method. This modification resulted in ~45% yield of the product against 19% obtained with the original method.

2.3. Extraction of minor polar components from refined oil

Phenolic compounds were extracted following the method developed by Montedoro et al. [29] using 35 g of olive oil and a mixture of methanol–water (80:20, v/v). The polyphenol extracts were used immediately for sample preparation and ^{31}P NMR measurements.

2.4. Sample preparation for ^{31}P NMR spectral analysis

A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$ (0.165 μM) and 13.5 mg cyclohexanol (13.47 mM) in 10 ml of a mixture of pyridine and $CDCl_3$ solvents (1.6:1.0, volume ratio) and protected from moisture with 5A molecular sieves. Cyclohexanol was used as an internal standard for quantification purposes. 100–150 mg of each oil was placed in a 5 mm NMR tube. The required volumes of the stock solution (0.4 mL) and the reagent **1** (15 μL) were added. The reaction mixture was left to react for about 15 min at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra.

2.5. NMR experiments

All NMR experiments were conducted on a Bruker AMX500 spectrometer operating at 500.1 and 202.2 MHz for proton and phosphorus-31 nuclei, respectively, at 26 ± 1 °C.

One-dimensional ^{13}P NMR spectra were recorded by employing the inverse gated decoupling technique in order to suppress NOE effects. Typical spectral parameters for quantitative studies using reagent **1** were: 90° pulse width 12.5 μs, sweep width 55 kHz, relaxation delay 25 s, memory size 32 K. To ensure quantitative spectra, the magnitude of the relaxation delay adopted was more than five times the relaxation time (T_1 = 4.6 s) of the phosphitylated cyclohexanol; 32 transients were accumulated for each spectrum. For all FIDs, line broadening of 1 Hz was applied and drift correction was performed prior to Fourier transform. Polynomial fourth-order base-line correction was performed before integration.

One-dimensional high-resolution ^{1}H NMR spectra were acquired with the following acquisition parameters: time domain 32 K; 90° pulse width 9.3 μs; spectral width 12 ppm; relaxation delay 2 s. 16 scans and 8 dummy scans were accumulated. Baseline correction was performed carefully by applying a polynomial fourth-order function in order to achieve a quantitative evaluation of all signals of interest. The spectra were acquired without spinning the NMR tube in order to avoid artificial signals, such as spinning side bands of the first or higher order.

2.6. Statistical methods

Classification by stepwise forward CDA is used to achieve the most discriminative variables for the arrangement of samples in a space of reduced dimensionality in a way that maximizes the distances between the *a priori* formed groups and the independence of the axes of the configuration [30,31]. The *a priori* groups are ROO, RHzO, and the adulterated refined olive oils (AOO). The chemical compounds contributing most to the discrimination of preformed groups are shown by means of the *F-ratio* as a criterion for inclusion or removal of the compound in the forward stepwise CDA mode. *Wilks'* λ and the *F* approximation were used to check the significance and estimate the importance of each compound in CDA analysis. The significance of the representation of the original configuration of the samples by the newly formed discriminant axes was expressed by the last value of *Wilks'* λ. The performance of the method was measured by the percentage of total variance explained by all significant discriminant axes. In a sense, this is a measure of non-linearity and complex interrelations among variables, since CDA is a linear technique. The classificatory efficiency of CDA can be seen in the resulting classification tables which illustrate the group relations of the samples. The ability of the oil samples to be predicted by the rest is another property of CDA, although the percentage of the correctly classified oil samples is a property of the original data rather than an intrinsic feature