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Rapid Quantitative Assessment of the Adulteration of Virgin Olive Oils with Hazelnut Oils Using Raman Spectroscopy and Chemometrics

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The authentication of extra virgin olive oil and its adulteration with lower-priced oils are serious problems in the olive oil industry. In addition to the obvious effect on producer profits, adulteration can also cause severe health and safety problems. A number of techniques, including chromatographic and spectroscopic methods, have recently been employed to assess the purity of olive oils. In this study Raman spectroscopy together with multivariate and evolutionary computational-based methods have been employed to assess the ability of Raman spectroscopy to discriminate between chemically very closely related oils. Additionally, the levels of hazelnut oils used to adulterate extra virgin olive oil were successfully quantified using partial least squares and genetic programming.

KEYWORDS: Raman spectroscopy; olive oil; hazelnut oil; adulteration; quantification; principal component analysis; partial least-squares regression; genetic programming

INTRODUCTION

Authentication includes a wide number of aspects, from adulteration to mislabeling or misrepresentation of the cultivar or country of origin, and it is a crucial matter for the food industry, where raw and elaborated products should undergo strict quality controls and be fully tested for regulatory and health specifications. In particular, authentication is an important issue for the olive oil industry, which has, in the past few years, become popular due to the health benefits of olive oil (*1*, *2*). To increase profits, unscrupulous dealers may be tempted to add lower-priced vegetable or nut oils to fresh extra virgin olive oils, which in addition to being unfair to the consumer, in terms of the cost of this apparent premium commodity, may also cause severe health and safety problems (*3*). Consequently, there is no doubt that the detection of adulteration needs to be addressed in order to ensure the quality of olive oils. Recently, hazelnut oil has been used to adulterate extra virgin olive oil and olive oil due to its great chemical similarity to olive oils; in particular, hazelnut and olive oils have similar triacyglycerol, total sterol, and fatty acid composition. Moreover, due to the fact that the content of a number of compounds in the adulterated oils may be within the limits set for genuine olive oil, it has been reported that such an adulteration seems difficult to detect at low concentration levels (5−20%) (*4*).

Authenticity and adulteration have been extensively monitored using techniques that characterize (qualitative and quantitative) the composition of oils. Most of the work on authentication of edible oils is based on chromatographic techniques, including high-performance liquid chromatography and high-resolution gas chromatography, which have been mainly applied for the quantification of fatty acids, triglycerols, sterols, and hydrocarbons (for references see (*5*)). Recognition of adulterated olive oils can also be performed in some cases by direct analysis of a specific component; however, it has been demonstrated that some adulterations are not detectable if minor components are removed by refining under extreme conditions (*6*). Therefore, classification of various oils and identification and detection of a given adulterant by chromatographic techniques have been complemented by mass spectrometry, including pyrolysis-MS (*7*) and electrospray ionization-MS (*8*), nuclear magnetic resonance spectrometry (*9*−*11*), and vibrational spectroscopy (*12*−*18*). These techniques differ from chromatography in that, rather than separating the components of a given oil sample prior to analysis, the resultant spectrum is considered as an unequivocal "holistic" fingerprint of a given oil. These fingerprint spectra need to be analyzed by chemometric methods such as multivariate statistical analysis and artificial neural networks in order to effect the successful detection of the adulterant(s) (*19*, *20*).

With respect to vibrational spectroscopy, it has been reported that near-infrared, FT-IR and FT-Raman in combination with multivariate analysis could successfully discriminate between different oils (*14*) and also successfully model the composition of binary mixtures of olive oil adulterated with corn, soybean, and raw pomace oils (*13*), non-high-oleic sunflower oil (*21*), or pomace oil (*16*). These reports show the suitability of spectroscopic techniques to discriminate between chemically similar oils and encourage the development of these techniques to study adulteration of olive oils with more chemically similar

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oils. Thus, one of the aims of the present study was to investigate, with aid of Raman spectroscopy, the chemically very similar adulterant refined hazelnut oil mixed with extra virgin olive oil.

It has been argued that Raman spectroscopy is of very limited use in food and biological applications, due to fluorescence of the samples and also to the difficulty of obtaining high-resolution spectra (*22*−*24*). However, relatively recent instrumental advances (e.g. the design of new holographic filters that effectively reject the laser excitation and the development of high-sensitivity photoelectric detectors) have made possible the accurate collection of Raman spectra, by either FT- or dispersive-based Raman spectrometers (*25*).

We believe that Raman spectroscopy may provide the necessary resolution to discriminate between a genuine extra virgin olive oil and an extra virgin olive oil adulterated by hazelnut oil. The Raman analyzer used in the present work was a dispersive instrument employing a near-infrared 780 nm laser and equipped with a fiber-optic sampling station, thus offering remote sampling capabilities that make such a system ideally suited for industrial processes and *in situ* monitoring of olive oil adulteration.

The aim of the present study was to assess the potential of Raman spectroscopy with appropriate chemometric analyses to distinguish between very closely related cultivars of extra virgin olive oils and hazelnut oils. An additional aim was to quantify the level of hazelnut oil−extra virgin olive oil mixtures.

MATERIALS AND METHODS

Oils. All oils used in this work, including a collection of Italian extra virgin olive oil from different regions and cultivars, a hazelnut oil collection, and oils used to prepare olive oil−"adulterant" mixtures were supplied by G.B. Note that adulteration is not just a problem that affects the production of oils in Italy. The present study has investigated a set of Italian oils as a model set, because we know these to be authentic. Details of the oil studied are given in **Table 1**. All oils were stored at 4 °C. Prior to analysis, oils were allowed to stand at room temperature for at least 24 h.

Binary mixtures of extra virgin olive oil−sunflower oil and extra virgin olive oil-refined hazelnut oil were prepared in the concentration range 0−100% of extra virgin olive oil in increments of 5%.

Raman Spectroscopy. Raman spectra were excited with a near-infrared 780 nm laser with the power at the sampling point typically at 20 mW; backscattering radiation was collected by a Renishaw System 100 instrument (Renishaw plc, New Mills, Wotton-under-Edge, Gloucestershire GL12 8JR, U.K.) (*25*, *26*). Each oil was pipetted into a 4 mL Supelco vial (Supelco Park, Bellfonte, PA). The vial was placed into a pre-fixed sample holder such that the laser was focused into the center of the vial (12 mm from the collection lens), as detailed elsewhere (*27*). Samples were analyzed in quadruplicate. Spectra were collected for 2 min over the spectral range 1000−3000 wavenumbers (cm^{-1}) at a resolution of about 6 cm^{-1}. Calibration was periodically checked by recording the position of known Raman lines of a silicon wafer (520 cm^{-1}), and the wavenumber accuracy was estimated to be ±1 cm^{-1}. All data were exported from the GRAMs WiRE software used to control the spectrometer into Matlab (The Mathworks, Inc. Natick, MA) for data analysis.

Data Analysis. The measured signal for each oil sample has two contributions, Raman scattering and fluorescence, and the latter was subtracted as follows: the baseline of each spectrum was approximated by a fifth-order polynomial, and this polynomial contribution was subtracted from each raw spectrum, resulting in flat baseline spectra. Spectral preprocessing also included data denoising using a five-point moving mean filter and was normalized to the highest peak, which for all oils occurred at 1427.0 cm^{-1}; this band was assigned to the scissoring-bending mode of $-CH_2$ groups (*16*).

Data presented in this work (generated by Raman spectroscopy) consist of the results of the observations on a number of samples with many different variables (Raman shifts). Each variable may be regarded as constituting a different dimension, such that if there are *n* variables, each object may be said to reside at a unique position in an abstract entity referred to as an *n*-dimensional hyperspace. Thus, the data generated by Raman spectroscopy has a multidimensional character, or what has often been called a hyperspectral nature (*28*−*31*). Analysis of data with such characteristics is generally analyzed by multivariate analysis methods. Principal component analysis (PCA) (*32*, *33*) is a well-known method used to reduce the dimensionality of the data and was used to assess the ability of Raman data to discriminate between different oil samples according to either their origin or their composition. PCA was performed in Matlab using the NIPALS algorithm (*34*). As mentioned above, the spectra were collected in quadruplicate; thus, repeatability of the spectra was assessed by calculating, for an oil sample and in the first two principal components' space, the mean distance to the group center, which was typically by $(7.47 \pm 0.78) \times 10^{-4}$.

Partial least-squares (PLS) regression (*35*) is a quantitative spectral decomposition technique generally used for predictive linear modeling and was employed to build a model to determine the concentration of the adulterant oil(s) in the oil mixtures studied. PLS was performed as detailed in (*36*) following the computations given in (*35*).

Although PLS is a popular method for the quantitative analysis of biological systems, it is arguable whether PLS is capable of clearly determining which are the crucial spectral features that the PLS routine has employed to build prediction models. In this respect evolutionary computational-based methods have recently been used (e.g. see (*37*, *38*)) as a tool not only to determine the relationship between the spectra and a particular property but also to identify the features of a given spectra that are relevant for discriminatory purposes. Consequently, further data analysis was performed by genetic programming (GP) (*37*, *39*, *40*)). GP is part of the "so-called" evolutionary computational methods, which are based on the concepts of Darwinian selection to generate and to optimize a desired computational function or mathematical expression to produce explanatory rules. GP was performed using the genomic computing software Gmax-bio (Aber Genomic Computing, Aberystwyth, U.K.). Details on the Gmax-bio software can be found elsewhere (*41*). The default parameter settings for population

Table 1. Extra Virgin Olive Oils Analyzed

label	cultivar	locality	region
D1	Dritta	Bucchianico (CH)	Abruzzo
D2	Dritta	Bucchianico (CH)	Abruzzo
D3	Dritta	Pianella (PE)	Abruzzo
D4	Dritta	Pianella (PE)	Abruzzo
D5	Dritta	Spoltore (PE)	Abruzzo
D6	Dritta	Spoltore (PE)	Abruzzo
F7	Frantoio	Arpino (FR)	Lazio
F8	Frantoio	Canino (VT)	Lazio
F9	Frantoio	Citta' S. Angelo (PE)	Abruzzo
F10	Frantoio	Citta' S. Angelo (PE)	Abruzzo
F11	Frantoio	Saludecio (RN)	Emilia Romagna
F12	Frantoio	Saludecio (RN)	Emilia Romagna
G13	Gentile di Chieti	Atessa (CH)	Abruzzo
G14	Gentile di Chieti	Atessa (CH)	Abruzzo
G15	Gentile di Chieti	Guardiagrele (CH)	Abruzzo
G16	Gentile di Chieti	Guardiagrele (CH)	Abruzzo
G17	Gentile di Chieti	Scernì (CR)	Abruzzo
G18	Gentile di Chieti	Scernì (CH)	Abruzzo
L19	Leccino	Campli (TE)	Abruzzo
L20	Leccino	Campli (TE)	Abruzzo
L21	Leccino	Citta' S. Angelo (PE)	Abruzzo
L22	Leccino	Citta' S. Angelo (PE)	Abruzzo
L23	Leccino	Corropoli (TE)	Abruzzo
L24	Leccino	Corropoli (TE)	Abruzzo
N25	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
N26	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
N27	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
N28	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
N29	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
N30	Nera di Gonnos	Gonnosfanadiga (CA)	Sardegna
OO[a]	Leccino	Citta' S. Angelo	Abruzzo

[a] Used in the admixtures.

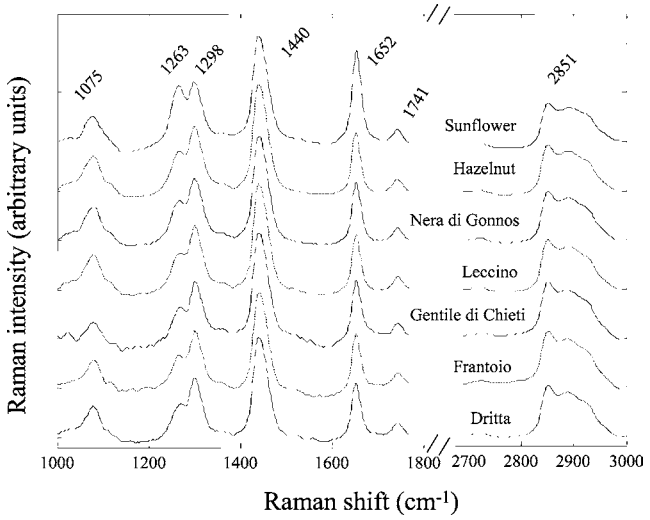

Figure 1. Typical Raman spectra from five different olive oils, hazelnut oil, and sunflower oil. Band assignments are as in (*16*): 1075 cm^{-1}, C–C stretching, group $–(CH_2)_n–$; 1263 cm^{-1}, =C–H bending, group *cis*-RHC=CHR; 1298 cm^{-1}, bending (twisting), group $–CH_2$; 1440 cm^{-1}, C–H bending (scissoring), group $–CH_2$; 1652 cm^{-1}, C=C stretching, group *cis*-RHC=CHR; 1741 cm^{-1}, C=O stretching, group RC=OOR; 2851 cm^{-1}, C–H stretching (sym), group CH_2.

size (1000) mutation, and recombination rates were used throughout. The operators that were used were as follows: +, −, /, *, 0.1, 1, 3, 5, rand, $\log_{10}$, 10^x, $\sqrt{}$, tanh.

RESULTS AND DISCUSSION

Figure 1 shows typical Stokes Raman spectra of extra virgin olive oil from the five cultivars considered here: Dritta, Frantoio, Gentile di Chieti, Leccino, and Nera di Gonnos. Also shown in **Figure 1** are the labels of the major peaks in the Raman spectra which are mainly due to the vibrations of chemical bonds coming from triglycerides, and these are in good agreement with previously published results from FT-Raman spectroscopy (e.g. (*16*)). As can be seen, the spectral characteristics of these oils are very similar, with the exception of the shift in the peak at 1652.0 cm^{-1} in the sunflower oil to 1653.8 cm^{-1}. The similarity of the spectra indicate the significant chemical similarity between the oils, and this is consistent with the fact that fatty acids may account for the 98% of the content of an olive oil (*5*). Despite these common spectral features, variations in the chemistry between these oils are expected to arise from several factors (*7*), including cultivar, soil, climate, storage conditions of the fruit or nut, and extraction processes.

The first experiment involved the spectral characterization of a selection of 30 extra virgin olive oils from five different Italian cultivars (see **Table 1**), and in order to assess geographical origin as well as botanical origin, samples were chosen so that oils from the same cultivar came from three different localities. To assess the natural variation in these 30 olive oils as measured from their Raman spectra, the unsupervised learning method of PCA was employed. While the plot representing the first two principal components (**Figure 2**) shows that the cultivars are not recovered into five separate clusters, it is significant that the replicate spectra did cluster together, indicating the reproducibility of the Raman approach. In addition, it is notable that the oils from the Sardinian region appear to be separated from the rest, which all come from the Italian peninsula. Most research concerned with the olive tree generally agrees that the genetic properties of a given oil are more important in determining the oil's molecular composition, rather

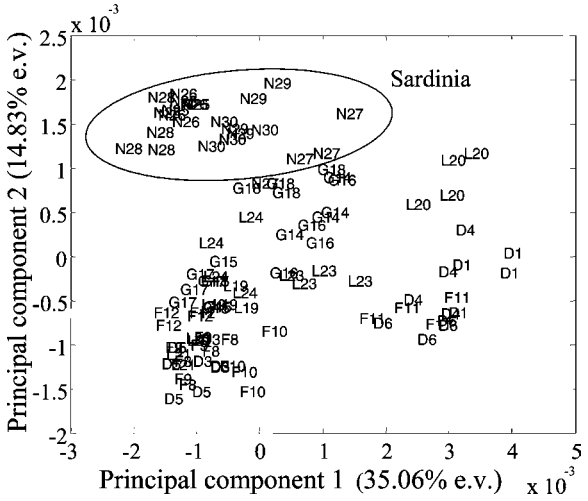

Figure 2. PCA plot for the Italian extra virgin olive oil collection. Principal components 1 and 2 are shown; these account for the 49.89% of the total explained variance (e.v.). The variance shown by each component is given in parentheses in the axis labeling. Note that the Sardinian oils appear to cluster together (indicated by the ellipse), separating from the oils coming from the Italian peninsula. The ellipse is drawn as a visual guide.

Table 2. Hazelnut Oils Used in This Study[a]

label	cultivar	region/country
H1	Mortarella	Avellino Campania
H2	Moltalbano Nostrale	Sicily
H3	Tonda Gentile Romana	Lazio
H4	Tonda Gentile Delle Langhe	Piemonte
H5	Tonda Di Giffoni	Salerno Campania
H6	common hazelnut oil	Avellino Campania
H7	Tonda Gentile Delle Langhe	Piemonte
H8	common hazelnut oil	Turkey
H9	hazelnut pomace oil	Turkey
HZ[b]	refined hazelnut oil	Turkey

[a] All oils obtained from unroasted nuts. [b] Oil used in the admixtures.

than the soil and climatic conditions. However, this result suggests otherwise, and although the cultivar of the individual olive oils is known, these cannot be assumed to be isogenic, which coupled with the differing soil, climate, and storage conditions may suggest that the chemical composition of each of the oils is in fact unique.

Next, a collection of hazelnut oils was analyzed as detailed above (**Table 2**). PCA on the Raman spectra from these nut oils does show some separation according to geographical origin (**Figure 3**). The oils from Turkey (H8 and H9) are clearly separated in the first PC from the other oils, and the hazelnut pomace oil (H9) can be separated from the hazelnut oil (H8). Moreover, it is possible to separate the oils from the Italian peninsula (H1, H3−H7) from the single hazelnut oil from Sicily (H2). To assess the similarity between the hazelnut oils and the extra virgin olive oils, the Raman spectra of a subset of the extra virgin olive oil collection together with the hazelnut collection were collected and analyzed by PCA. The subset of extra virgin olive oil consisted of two samples from each cultivar, whereas the whole hazelnut collection (H1−H9) was used in this experiment. The resultant ordination plot is shown in **Figure 4**. This figure shows that the hazelnut oils are recovered together in a tight cluster, while the virgin olive oils are separated from the hazelnut oils. Since the Raman spectra are determined by the chemical composition of the oils, the result plotted in **Figure 4** seems to indicate that the hazelnut oils show

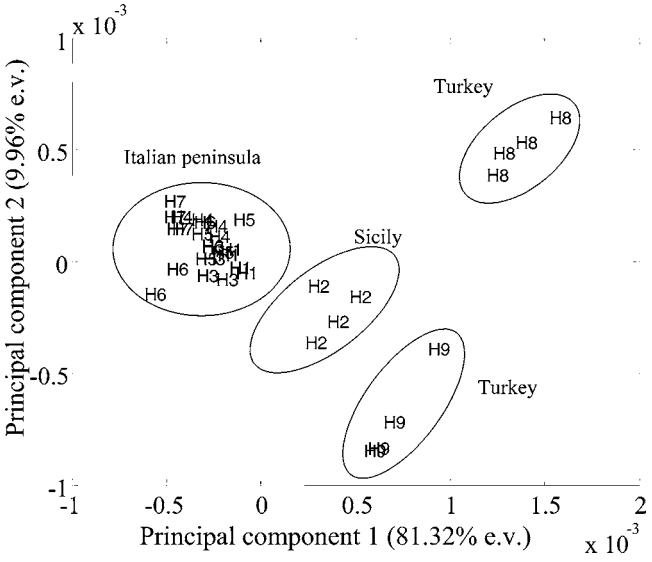

Figure 3. PCA plot for the hazelnut oil and olive oil collection. The two principal components account for the 72.29% of the total explained variance. The variance shown by each component is given in parentheses in the axis labeling. Note that the oils which come from the Italian peninsula appear to cluster together, separating from the oils coming form Sicily and Turkey. Ellipses are drawn as visual guides.

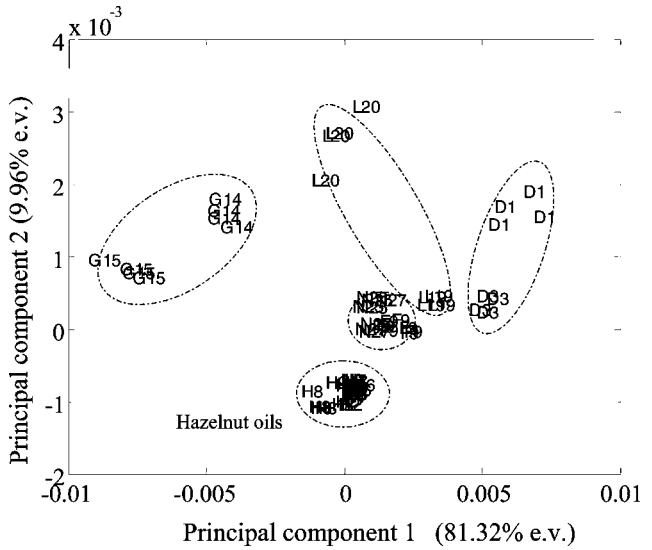

Figure 4. PCA plot for the hazelnut oil collection including some extra virgin olive oils. Principal components 1 and 2 account for the 91.29% of the total explained variance The variance shown by each component is given in parentheses in the axis labeling. Ellipses indicate which oils are hazelnut oils or which extra virgin olive oils come from the same region.

high (chemical) similarity to one another and that the olive oils are more chemically distinct not only from the hazelnut oils but also from one another. Despite differences within virgin olive oils, on closer inspection, with this reduced set from the whole collection, some evidence of clustering according to cultivar can be observed.

The results described above suggest that Raman spectroscopy might be able to detect the presence of hazelnut oil in extra virgin olive oil samples. To assess this possibility, the spectra of 21 binary mixtures (composition range 0−100% of olive oil in 5% steps) were collected in replicate (×4). In the first stage of the analysis PCA was applied to the processed Raman data, and it was found that two PCs accounting for 82% of the total explained variance successfully separate the mixtures according

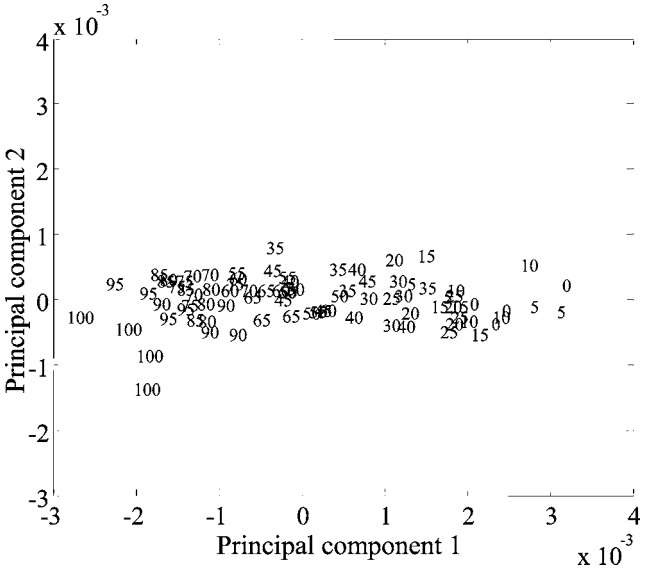

Figure 5. PCA plot for the extra virgin olive oil–hazelnut oil mixtures. As can be seen, Raman data can separate the mixtures according to composition (0–100% hazelnut oils).

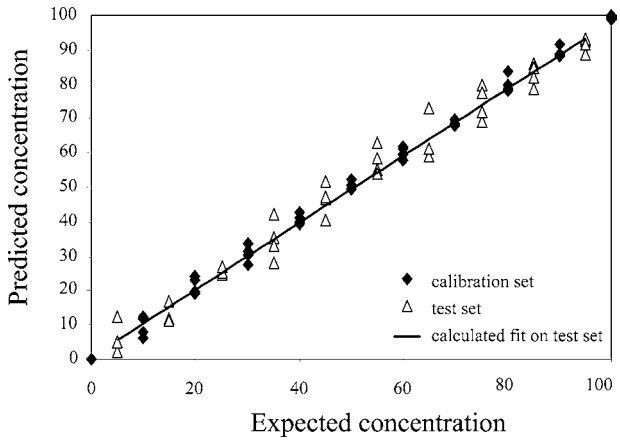

Figure 6. Estimates from PLS versus the true concentration values of hazelnut oil present in an extra virgin olive oil sample. The calculated fit on test set results is $y = 0.974x + 0.756$ and $R^2 = 0.979$. RMSEP values of 4.16 and 0.94 were found for the calibration and validation sets respectively.

to composition (**Figure 5**). This strongly suggests that these Raman spectra contain biochemical information that will allow correlation of pertinent spectral features with the concentration of the adulterant. Therefore, subsequent supervised learning analyses using PLS and GP were used to build a model to quantify the level of hazelnut adulteration in extra virgin olive oil.

The data were split into a training (or calibration) set and a test set. The training set consisted of replicate spectra containing 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% hazelnut oil, while the test set consisted of Raman spectra with 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95% of the adulterant. PLS regression was calibrated with the training set and cross-validated with the test set. It was found that the best model (i.e. lowest prediction error in the test set) occurred when five latent variables were used. The plot of the PLS estimates versus the known concentration (**Figure 6**) shows good predictions for both the training data and the test set. Moreover, close inspection of the model indicates that the composition of the mixture can be successfully predicted in the range of commercial interest, 0−20%. In addition, GPs were also evolved to predict success-

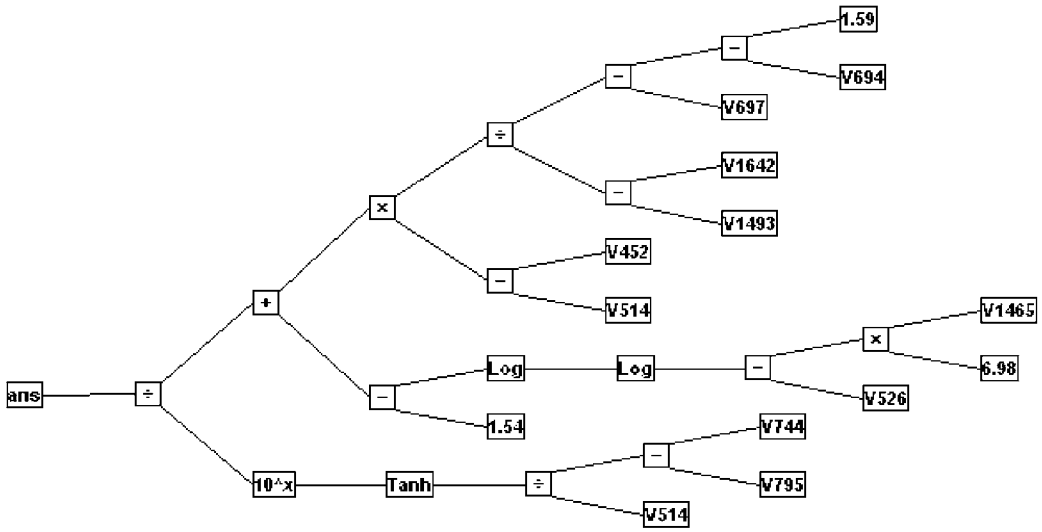

Figure 7. Typical GP rule evolved to model the composition of virgin olive oil and refined hazelnut oil admixtures.

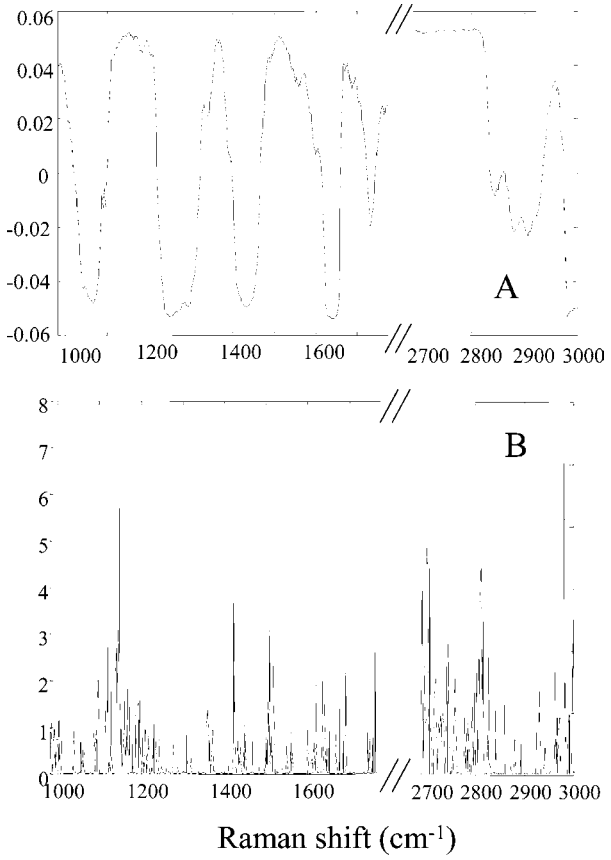

Figure 8. (A) PLS-1 loadings. (B) Frequency plot of the number of times an input (a wavenumber shift) was used in 25 independent GP's, evolved to quantify the level of hazelnut oil spiked into olive oil.

fully the adulterant level in the extra virgin olive oil (**Figure 7** shows a typical GP rule), with an RMSEP value similar to that for the PLS predictions (0.98).

It has been argued that while the quantitative results obtained from PLS are excellent, the models produced are not easily interpreted (*38*). That is, it is not obvious how this method exploits information specifically in terms of the values of the different input variables (i.e. Raman shift, in this case). Thus, the PLS-1 loadings as well as the number of times each input (Raman shift) was selected by GP (from 25 evolved populations) was calculated and plotted against the Raman shift (**Figure 8**). It appears from this figure that no single input variable was selected by either PLS or GP and that both methods used the whole spectra to map from the Raman spectrum to the level of the hazelnut oil adulterant. This finding suggests that in terms of their Raman spectra hazelnut oil and extra virgin olive oil are very similar, and this reflects their very close chemical similarity to one another. This is perhaps not surprising, considering that these spectra are dominated by the triglyceride vibrations, and these appear to mask any subtle spectral differences that may arise from other chemical species. Furthermore, it can be seen from the typical GP tree (**Figure 7**) that the rule is complex, and as a single chemical is not important, the information is spread across the whole Raman spectrum.

CONCLUSION

It has been shown that Raman data can successfully distinguish between closely related extra virgin olive and hazelnut oils and therefore aid in their identification. Moreover, the composition of hazelnut and extra virgin olive oil mixtures could be accurately predicted using either PLS regression or the more sophisticated computational method of GP. In conclusion, Raman spectroscopy, together with appropriate chemometrics, presents itself as a powerful tool for the authentication of extra virgin olive oil; moreover, the method used here represents a step toward the more difficult detection of hazelnut oil in other olive oils: e.g. pomace and lampante olive oils. Further work will include the study of such mixtures as well as different ranges of concentration to accurately determine lowest concentration of hazelnut oil that can be detected by Raman spectroscopy.

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