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Metabolic profiling using direct infusion electrospray ionisation mass spectrometry for the characterisation of olive oils

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There is a continuing need for improved methods for assessing the adulteration of foodstuffs. We report some highly encouraging data, where we have developed direct infusion electrospray ionisation mass spectrometry (ESI-MS) together with chemometrics as a novel, rapid (1 min per sample) and powerful technique to elucidate key metabolite differences in vegetable and nut oils. Principal components analysis of these ESI-MS spectra show that the reproducibility of this approach is high and that olive oil can be discriminated from oils which are commonly used as adulterants. These adulterants include refined hazelnut oil, which is particularly challenging given its chemical similarity to olive oils.

Introduction

Olive oil is becoming increasingly popular due to its potential health benefits.[1] Extra virgin or virgin olive oils are particularly expensive and this may present the opportunity for unscrupulous producers to stretch their merchandise by adulterating it with lower grade seed or nut oils,[2–7] including peanut and hazelnut. The adulteration of olive oil with hazelnut oil is a sophisticated type of adulteration because hazelnut oil is chemically very similar to olive oil,[8,9] particularly when the hazelnut oil is refined. Moreover, this problem has also been recently highlighted by the UK FSA (formerly MAFF) (reported in Food Authenticity Programme—contractors' review workshop—olive oil authenticity—11 October, 2000).

Thus there is a continuing requirement for rapid, accurate and preferably automated methods to characterize foodstuffs and in particular for determining whether a particular food has the provenance claimed for it or whether it has been adulterated with or substituted by a lower-grade material. Moreover, it is desirable that new methods that are developed do not depend on expert analysis and potentially subjective opinion, or require complex mathematical transformations that do not permit an easy understanding of the relevant (bio)chemistry. Ideal techniques for rapid screening of foodstuffs for adulteration would include those which require minimal sample preparation, permit the automatic analysis of many serial samples with negligible reagent costs, allow their rapid characterisation against a stable database, are easy to use and can be operated under the control of a PC. With recent developments in analytical instrumentation, these requirements are being fulfilled by physico-chemical spectroscopic methods, often referred to as 'whole-organism fingerprinting'[10,11] and more recently 'metabolic fingerprinting'.[12] Metabolic fingerprinting is particularly attractive because due to there being no chromatographic separation, they measure with great rapidity the totality (within the constraints of the analytical tool used) of the low molecular weight chemicals in a given biological sample.[13]

There have been a number of very exciting advances in spectroscopic analysis of olive oils, several of which have stemmed from significant advances in data handling.[14] During this period we were the first to apply artificial neural networks (ANNs), a modern 'supervised' learning technique, to effect the successful identification of biological samples from their pyrolysis mass spectra.[15,16] This study, which was performed double-blind, permitted the rapid and exquisitely sensitive assessment of the adulteration of extra virgin olive oils with various seed oils, a task which previously was labour intensive and difficult. The use of pyrolysis-MS has been investigated by several groups within the food analysis community[17,18] as is the application of a cohort of other metabolic fingerprinting methods for the analysis of the authenticity or provenance of olive oil, including NMR,[19,20] FT-IR,[21,22] Raman,[23,24] and MS either *via* GC[25] or headspace.[7] However, whilst pyrolysis-MS is exceptionally useful for rapid, whole-food fingerprinting, it does have three major disadvantages: (i) the highest m/z value reproducibly attainable is very small (only m/z 200), (ii) the *in vacuo* thermal degradation step means that essentially all information on the structure or identity of the molecules producing the pyrolysate is lost, and (iii) long-term (> 30 days) reproducibility still presents a major problem. Recently there has been an explosion of interest in the use of soft ionisation mass spectrometry (MS) methods such as matrix-assisted laser desorption ionization (MALDI)[26,27] and electrospray ionisation (ESI)[28,29] for the analysis of biomacromolecules, as well as of small molecules. Such mass spectrometric methods are now essential tools in proteomics,[30,31] metabolomics[32,33] and functional genomics[30,34] because softer ionisation MS allows fine structural information to be obtained directly from the biomolecules.

The main aim of this study was to investigate the ability of direct infusion electrospray ionisation mass spectrometry, that is to say MS without prior chromatographic separation, for its ability to produce information rich and informative mass spectra from extra virgin and virgin olive oil and common substances (corn, soya, sunflower, peanut and hazelnut oils) that are used to stretch or adulterate this foodstuff.

Materials and methods

Oils

In the first experiment designed to optimise the cone voltage on the MS, nine different oils were analysed by direct infusion ESI-MS (with the code for the plots in parenthesis): corn (C), grapestone oil (G), husk oil (H), olive oil (O), extra virgin olive oil (V), peanut oil (P), soya oil (S), sunflower oil (F) and a mixed oil (M) comprising peanut, sunflower and soya oils.

In the second experiment four oils were analysed by direct infusion ESI-MS-MS. The oils analysed were refined olive oil (O), refined hazelnut oil (H), unrefined peanut oil (P) and sunflower oil (S).

All oils were supplied by G. Bianchi and were stored at 4 °C. Prior to analysis these oils were allowed to come up to room temperature (~ 20 °C) for 24 h.

ESI-MS

All oils were analysed by diluting them 1000-fold in 60% dichloromethane (CH_2Cl_2): 40% 10 mM ammonium acetate (NH_4OAc) in methanol (CH_3OH). All data were collected in the positive ion mode (ES^+). The samples were loaded into a 100 μl volume Hamilton gastight 7000 series syringe and introduced directly to a Micromass LCT™ or Micromass QTOF™ ESI-MS(-MS) (Wythenshawe, Manchester, UK) using a Harvard

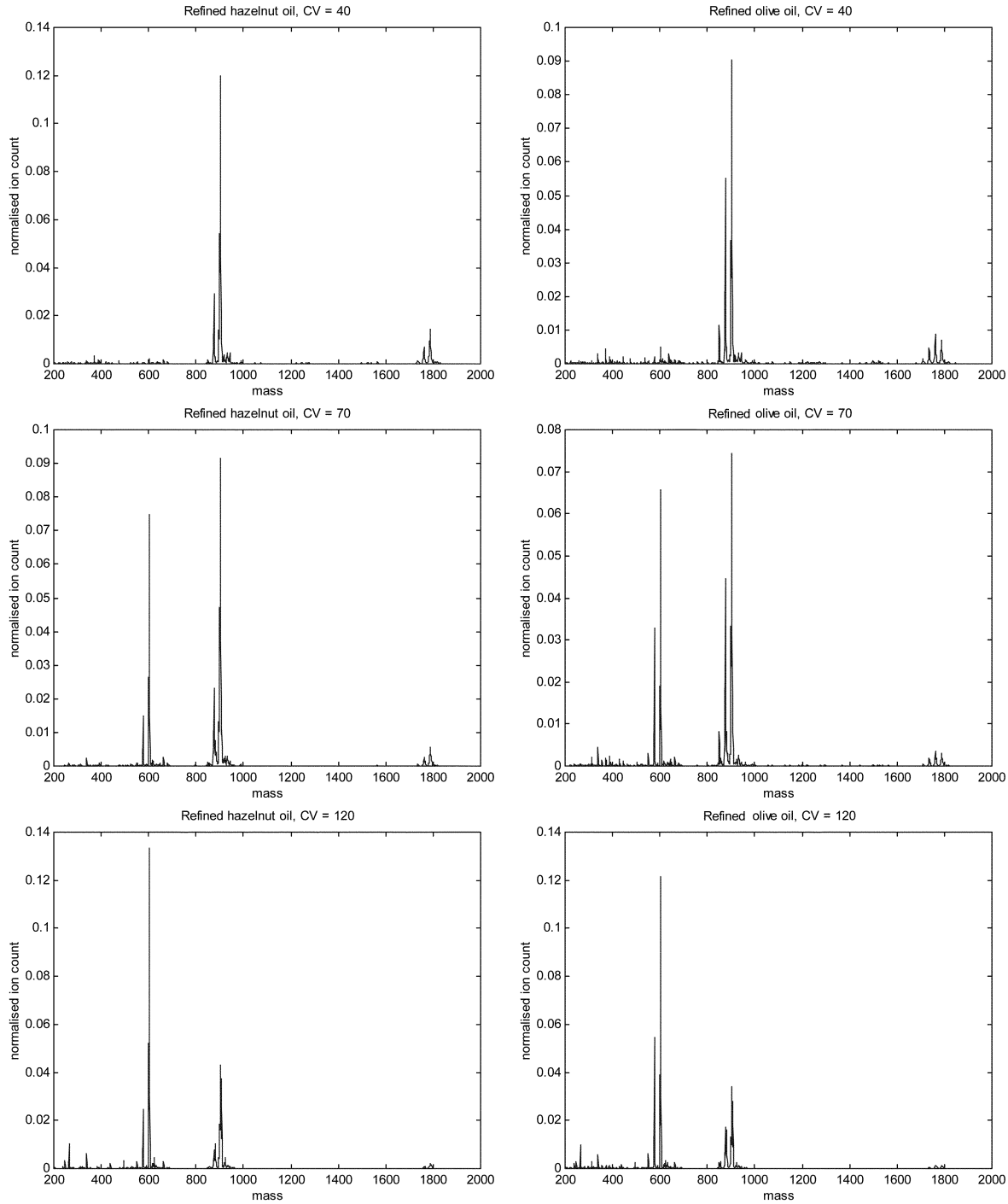

Fig. 1 Typical ESI-MS spectra from the refined hazelnut and olive oils collected at the three cone voltages of 40 V, 70 V and 120 V.

Apparatus Pump 11 (Reno, NV) operating at a flow rate of 5 μl min^{-1} as detailed elsewhere.[35,36] Samples were collected for 1 min, the typical ion count was 10^7 ions and data were collected from *m*/*z* 60–2000; the resolution of the spectra was set to 1/16 of a Dalton. To optimize the spectra three sample cone voltage (SCV) were used: 40 V, 70 V and 120 V. The capillary voltage was set to 3000 V and the extraction cone voltage was 50 V, the source and desolvation temperatures were both 60 °C, and the desolvation and nebuliser gas flow rates were ~650 l h^{-1} and ~90 l h^{-1}, respectively.

For ESI-MS-MS investigations on a Micromass QTOF™, argon was employed as the collision gas, the collision energy was set at 30–60 eV, and both the quadrupole (Q) and TOF analyzers were employed. The SCV was 40 V, and other conditions were the same as for the MS analysis detailed above.

Explanatory cluster analysis

All data were exported from MassLynx™ software provided by the manufacturers and imported at unit mass resolution into Matlab version 5.3 (The MathWorks, Inc., Natick, MA) which runs under Microsoft Windows NT on an IBM-compatible PC. To account for sample size differences prior to analysis all spectra were normalised to total ion count ($\Sigma = 1$). Principal components analysis (PCA)[37] was used to perform exploratory cluster analysis and is a well known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. Matlab was employed to perform PCA according to the NIPALS algorithm.[38] The first five principal components (PCs) were extracted: these typically accounted for >95% of the overall variance. The Euclidean distances between the replicate centres in this PCA space were used to construct a similarity measure, with the Gower similarity coefficient S_G,[39] and these distance measures were then processed by an agglomerative clustering algorithm to construct a dendrogram.[40]

Results and discussion

ESI-MS spectra were acquired at the three different SCVs of 40 V, 70 V and 120 V. Typical spectra from refined hazelnut and olive oils collected at the three SCVs are shown in Fig. 1. The spectra from both of these oils using a low SCV of 40 V are dominated by an analyte with *m*/*z* 903, including a few adjacent peaks, and this was observed for all the oils (data not shown). At the higher SCV of 120 V there was a shift in dominance and the base peak was now *m*/*z* 603, again with some adjacent analyte ions. A SCV between these (70 V) produced a roughly equal amount of *m*/*z* 603 and *m*/*z* 903. This marked influence of SCV on the spectral information obtained from complex biosystems has been observed by us previously.[35] While shifts in charge-state distributions of proteins have been seen by altering the SCV,[41,42] it is more likely that we are observing increased ion dissociation.[43] To prove this further, ESI-MS-MS was conducted using a low SCV, and the peak at 903 was selected for analysis. The tandem MS of this selected ion is shown in Fig. 2, where the major daughter ions observed are *m*/*z* 603 and *m*/*z* 265. It is evident therefore that at higher SCVs the analyte at *m*/*z* 903 dissociates into *m*/*z* 603. By its molecular weight alone the *m*/*z* 903 analyte can be tentatively identified as the ammonium adduct of triolein ($C_{57}H_{104}O_6$), which is reported as the major triglyceride in olive oil, comprising 43.5%.[1] After controlled fragmentation this is confirmed further since the peak at 265 is the acylium ion ($C_{17}H_{33}C{\equiv}O^+$) of the oleic acid fatty acyl substituent of this triglyceride, and the peak at *m*/*z* 603 is $[M + NH_4^+ - (C_{18}H_{34}O_2)]^+$. The adjacent peaks to the ammonium adduct of triolein can be identified as triglycerides comprising mixed fatty acids (code used below in parenthesis) of palmitic (P), oleic (O), stearic (S), and linoleic (L) acids. The next most prominent peak is *m*/*z* 877 which is the ammonium adduct of POO, which is the second most abundant triglyceride in olive oil (18.4%),[1] also seen at lower ion abundances are $[OOL + NH_4]^+$ at *m*/*z* 901, $[POL + NH_4]^+$ at *m*/*z* 875, and $[PPO + NH_4]^+$ at *m*/*z* 851.

From the above it would seem prudent to concentrate on analysing the oil samples at low SCVs to avoid in-source

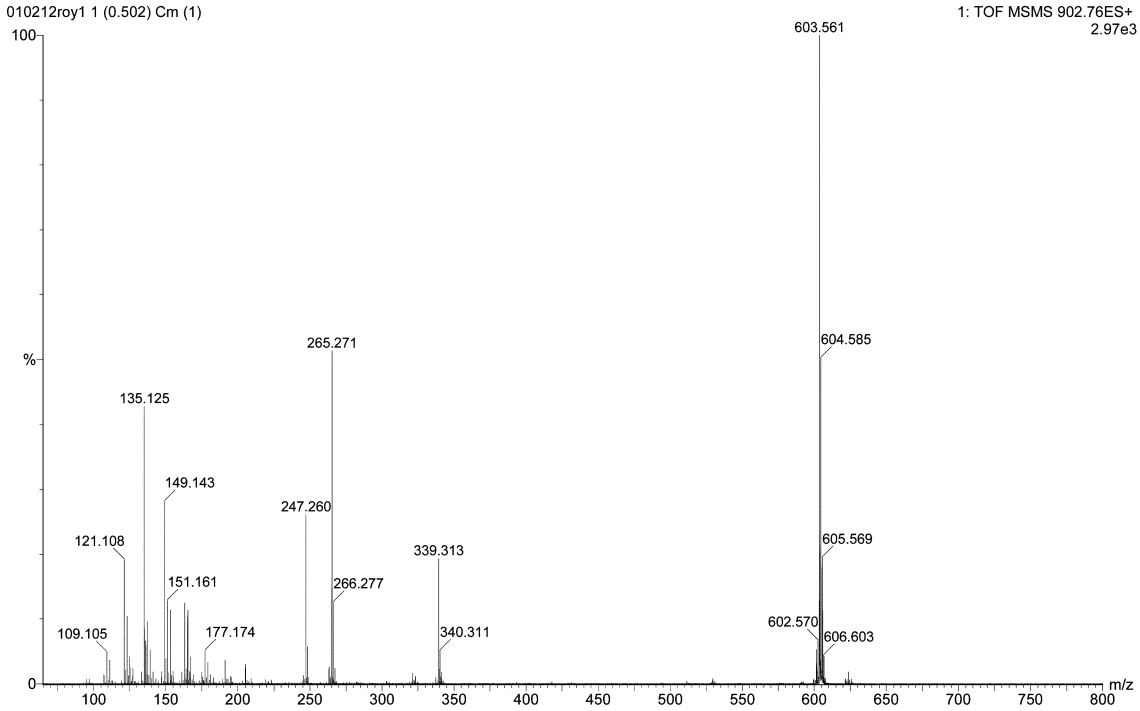

Fig. 2 ESI-MS-MS on the dominant peak with *m*/*z* 903 observed in hazelnut and olive oils.

fragmentation. It is also evident from the spectra in Fig. 1 that direct infusion ESI-MS has allowed us to collect ES^+ mass spectra in a very short time and with an extremely simple and convenient method of sample preparation using dichloromethane, ammonium acetate, methanol as the solvent. The obvious question is therefore 'Do these ESI-MS spectra contain enough information to characterise and discriminate each of the different oils?'

Nine different oils including corn, grapestone, husk, olive, extra virgin olive, peanut, soya, sunflower oils and a mixed oil comprising peanut, sunflower and soya oils were analysed as describe above using the three different cone voltages. Analysis of these data was by simple cluster analysis using hierarchical clustering[44,45] of the first 5 PCs, extracted as described above, and resulted in the dendrogram shown in Fig. 3. It is clear from this that the main differences in the spectra result from the different cone voltages used. The spectra generated using SCVs of 40 V and 70 V are more similar to one another than either is to an SCV of 120 V. Within the highest SCV there are two main groups: the first cluster comprises all the olive oil samples (extra virgin, refined, and husk) grouped together with peanut oil, and the second cluster corn, grapestone, soya, sunflower oils and the mixed oil. This split is reflected at the two lower SCVs. For the cluster comprising the olive oil samples and peanut oil, this is sub-grouped according to the SCV employed and not the different oils. By contrast, sunflower oil, corn oil and the mixed oil sample are recovered together irrespective of the SCV used, whilst soya and grapestone cluster together according to SCV. It is therefore evident that there is sufficient information in the ESI-MS spectra to characterise each of the different oils, but that it would be prudent to use a single SCV for collection of all the spectra.

To investigate the ability of direct infusion ESI-MS to generate information-rich mass spectra further, we analysed refined hazelnut oil and refined olive oil because the adulteration of olive oil with hazelnut oil is difficult to detect as both oils are chemically very similar, and particularly when the hazelnut oil is refined.[8,9] To 'benchmark' the spectra, in terms of spectral difference, two other oils were included and these were peanut oil and sunflower oil. All analyses were carried out at the lower SCV of 40 V to minimise fragmentation. The resulting ordination plot from PCA on these oils is shown in Fig. 4. The first 2 PCs are shown which account for 74.8% and 20.3% of the variance respectively (total = 95.1%). It can be seen even from this simple PCA that clear separation in PCA space is seen between all the oils and that *refined* hazelnut oil is clearly separated from *refined* olive oil, and that this difference accounts for 20% of the total explained variance. Moreover, that the replicates effectively superimpose this demonstrates that the reproducibility of the direct infusion ESI-MS method is excellent in this short term study. Further work is necessary to ascertain the long term reproducibility of this approach.

The spectra shown in Fig. 1 are dominated by an ammonium adduct of the oleic acid containing triglyceride (analyte with *m*/*z* 903), and in order to illustrate further that these spectra are in fact very information rich with metabolites in lower concentrations, the region between mass 65 and 800 is magnified for both oils (cone voltage = 40 V) in Fig. 5. This allows one to observe any differences between hazelnut oil and olive oil. It is clear from these spectra that once the influence of the base-peak on the scaling of the spectra is removed that analytes that are either in lower abundance than the triglycerides or do not ionise so efficiently can be easily observed. Note that these spectra, whilst not wholly qualitatively different, do vary significantly in the abundance of the analytes present and that it is these quantitative differences that are being used in PCA that allows hazelnut oil to be clearly separated from olive oil. In order to confirm this further, PCA was recalculated omitting the triglyceride contributions to the mass spectra. The triglyceride peaks from *m*/*z* 860–940, doubly charge triglycerides above *m*/*z* 1700, and fragments ions from *m*/*z* 540–700 were removed prior to PCA, and no appreciable difference in the PCA plot was seen with that reported in Fig. 4 (data not shown).

In conclusion, we have shown that information-rich ESI-MS spectra from vegetable and nut oils can be generated without the need for chromatographic separation using direct infusion sample presentation. PCA of these metabolic profiles show that the reproducibility of this ESI-MS approach is high and that olive oil can be clearly separated from oils which are commonly used as adulterants, including refined hazelnut oil. Future work will be to assess this approach for investigating the varying composition of oils due to their cultivar, regional origin and preparatory processes used in oil production.

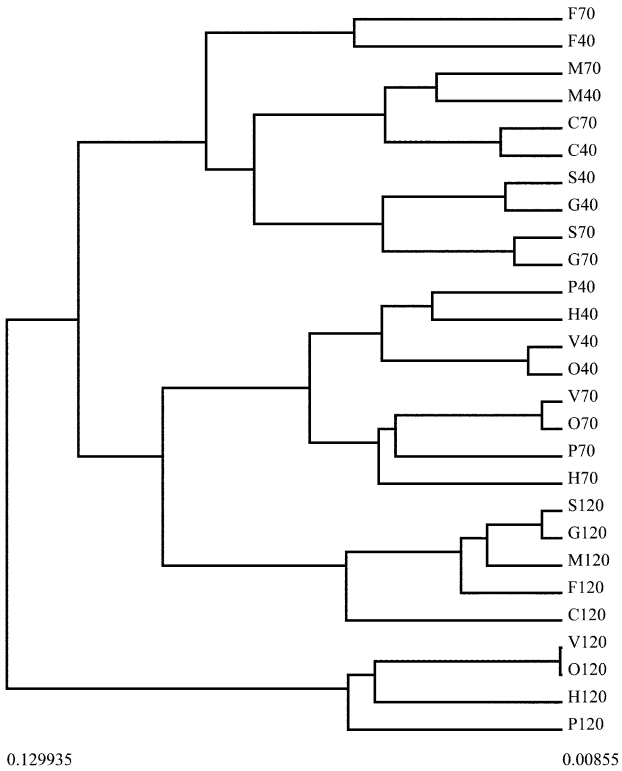

Fig. 3 Dendrogram showing the relationships between the oils analysed in the first experiment, and the effect of different cone voltages used. The code refers to the type of oil analysed (corn (C), grapestone oil (G), husk oil (H), olive oil (O), extra virgin olive oil (V), peanut oil (P), soya soil (S), sunflower oil (F) and a mixed oil (M) comprising peanut, sunflower and soya oils) and sample cone voltage (40 V, 70 V and 120 V) used.

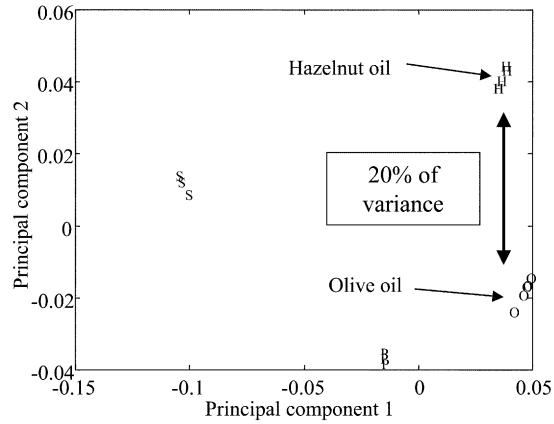

Fig. 4 PCA on ESI-MS oils from the second experiment with a sample cone voltage of 40 V. The first 2 PCs are shown which account for 74.8% and 20.3% of the variance respectively (total = 95.1%). Code; refined olive oil (O), refined hazelnut oil (H), unrefined peanut oil (P) and sunflower oil (S).

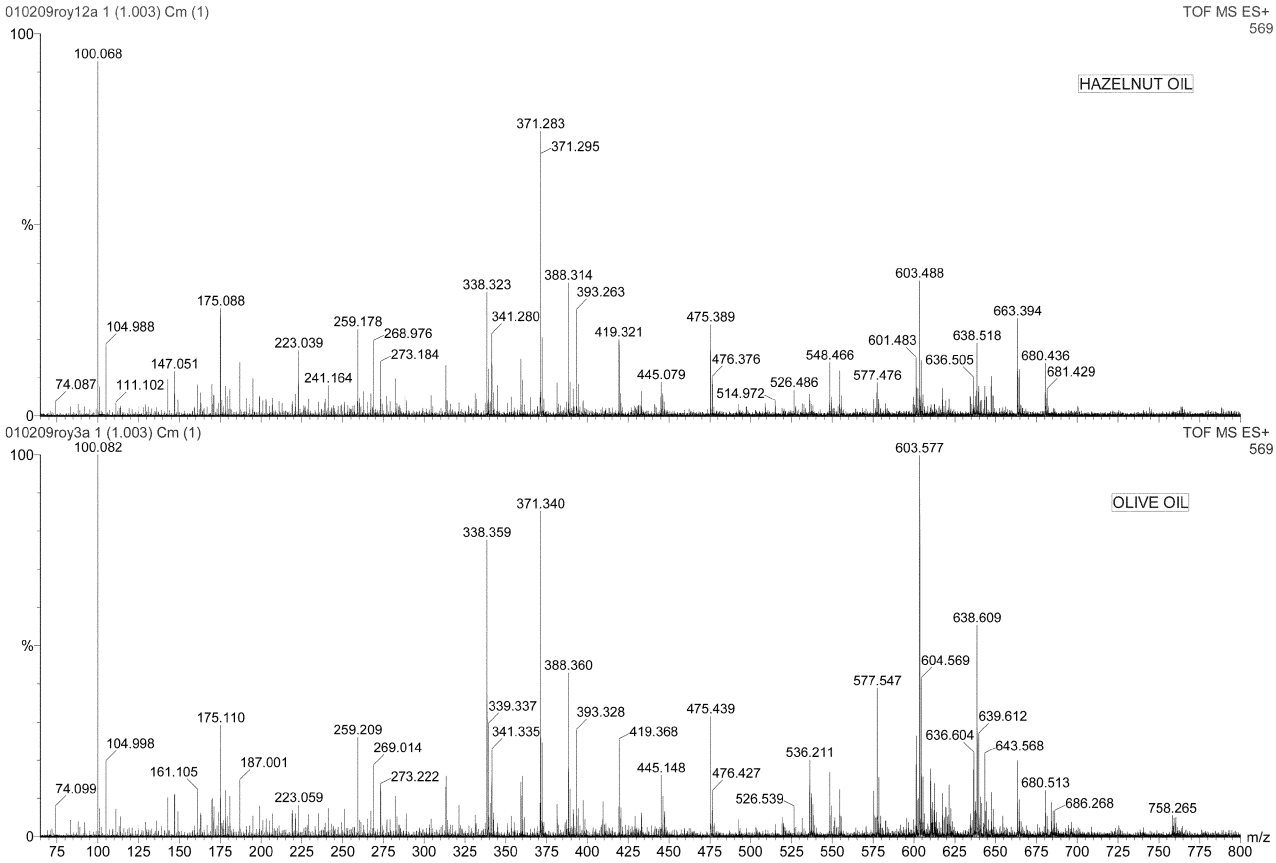

Fig. 5 To observe any differences between hazelnut oil (top) and olive oil (bottom) the region between *m*/*z* 65 and 800 is magnified for both oils (cone voltage = 40 V).

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