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Optimisation of a new headspace mass spectrometry instrument Discrimination of different geographical origin olive oils

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Abstract

A fast head-space analysis instrument, constituted by an automatic sample introduction system directly coupled to a mass detector without performing any chromatographic separation, was assembled. A suitable and original response was computed to optimise, by experimental design, the measured signals for discrimination purposes. The volatile fractions of 105 extra virgin olive oils coming from five different Mediterranean areas were analysed. The rough information collected by this system was unravelled and explained by well-known chemometrical techniques of display (principal component analysis), feature selection (stepwise linear discriminant analysis) and classification (linear discriminant analysis). The 93.4% of samples resulted to be correctly classified and the 90.5% correctly predicted by cross-validation procedure, whilst the 80.0% of an external test set, created to full validate the classification rule, were correctly assigned.

Keywords: Headspace–mass spectrometry (HS–MS); Olive oil; Experimental design; Chemometrics

1. Introduction

Foodstuffs are characterised by the presence of numerous chemical species, many of them responsible of their flavours. Up to date, sensory analysis performed by expert panels and several analytical techniques, both with and without previous separation procedures, were used to assess food aroma. Many authors reported correlation studies between human and chemical sensorial analysis [1–4]. Unlike traditional electronic noses which are based on sensors, the mass spectrometry technology is far better, as already reported in literature [5–8]. Using a mass spectrometer as detector many features such as selectivity, sensitivity, stability, reproducibility and rapidity are improved.

A new instrument to perform head-space (HS) analysis was assembled in our laboratory, to quickly analyse volatile compounds, directly coupling a sample introduction system to a mass detector (MS) without performing any chromatographic separation. Multivariate statistical techniques are able to extract information from the unique broad instrumental signal ("spectral fingerprints") composed by the complex mixture of volatiles distinguishing among samples with different odours without identification and/or quantification of each chemical specie.

The instrument is a headspace analyser based on a quadrupole mass spectrometer (head space–mass spectrometry: HS–MS), which includes: a head-space auto-sampler, an injection system, an interface specifically built and assembled by Abreg (Abreg s.r.l. Alessandria, Italy) and a quadrupole mass spectrometer. As far as software is concerned, we implemented the interface to PARVUS [9] by ourselves.

The aim of this research was to build a cheaper, more flexible and easy to update apparatus compared to the commercially available ones [10–13].

Regarding food flavour studies, the organoleptic characteristics of virgin olive oils, by means of a panel test, are used to define the "commercial" quality [14] and to assign the Protected Designation of Origin (PDO). Recently, several investigations to evaluate virgin olive oil sensory attributes were

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performed by using different kind of analytical procedures [1,15–17]. Thus, both to test the instrumental performance and to optimise the instrumental parameters, several extra virgin (e.v.) olive oils coming from different geographical areas, Spain, Greece, Tunisia, Liguria (North Italian region) and Apulia (South Italian region), were analysed.

This dataset was properly designed in order to characterise and to distinguish Ligurian e.v. olive oils, from other Mediterranean e.v. ones, by a simple (no sample preparation) and fast analysis. The peculiar organoleptic features of one Ligurian PDO ("Riviera Ligure - Riviera dei Fiori" e.v. olive oils) are clearly defined in PDO regulation [18]. These olive oils, in which cultivar (cv.) Taggiasca prevails, are characterised by a delicate, sweet, slightly pungent and green aroma, but sometimes, by sensorial analysis, they may be undistinguished from olive oils coming from different geographical areas [19].

2. Experimental

2.1. Samples

Three different e.v. olive oil datasets were used to optimise and to test the instrumental performance. All of the analysed samples pertained to the e.v. olive oil commercial class, according to European Commission (EU) regulations [14] and they were produced from olives of various cultivars harvested in 2003.

2.1.1. Dataset A

It consisted of 23 e.v. olive oils: 10 samples coming from Liguria, 5 Greek samples, 4 Spanish samples and 4 commercial samples (without any declared geographical origin).

2.1.2. Dataset B

It consisted of 27 e.v. olive oils from different Mediterranean areas: 9 samples coming from Liguria, 7 samples from South-Italy, 3 samples from Greece, 2 samples from Spain and 6 samples from Tunisia.

These two datasets were used to the instrumental set up.

2.1.3. Dataset C

It contained one hundred and five samples of e.v. olive oil from different Mediterranean countries. The samples were divided as follow: 30 samples coming from West Liguria in which cv. Taggiasca prevails, 30 samples from Apulia in which both cv. Ogliarola and Coratina prevail, 15 samples from Laconia and Crete (Greece) in which both cv. Koroneika and Athinoia prevail, 15 samples from Spain in which cv. Arbequina prevails and 15 samples from Tunisia in which cv. Chemlali prevails.

The samples were stored in hermetically sealed topaz glass flask in a cold dark place to avoid losses or oxidation of the volatile fraction.

2.2. Apparatus

The head-space autosampler (HT200H, HTA: hi-tech applications, Brescia, Italy) contains a tray with 40 slots and a six position oven to generate the head-space (150 °C max), having the possibility to shake (orbital shaking) or not the samples, accepting vials of 10 or 20 mL (glass vials for headspace analysis crimped by 20 mm TFE/Silicone septa).

The syringe (2.5 or 5 mL), whose temperature is controlled by the autosampler, samples the headspace and injects it directly into the ionisation chamber of the mass spectrometer, using nitrogen as cleaning inert gas and helium as carrier gas.

The Injector (OPTIC 3 from ATAS GL, Veldhoven, The Netherlands), incorporates temperature and gas flow control for sample introduction. It is very versatile since techniques such as thermal desorption, large volume and offline injection can be easily employed as well as on-column methods, split and splitless methods. Heating rates up to 16 °C s^{-1} can be attained and, optionally, it could offer rapid cooling using liquid nitrogen.

The direct interface (Abreg, Alessandria, Italy) between the injector and the detector is ensured by a transfer line, which consists of a 15 cm long empty retention gap placed in an oven with a temperature controller. The transfer line directly enters into the mass spectrometer (HP 5973 MSD, Agilent Technologies).

Data analysis was performed using as standalone software the package PARVUS [9] on a Pentium 4 computer, which controls the whole system.

2.3. Procedure

Aliquots of 10 mL of each sample were introduced in the 20 mL headspace-vials and closed hermetically. The autosampler took each vial from the 40 space carrousel and placed it into the oven. In the optimised conditions, the samples were heated at 40 °C for 30 min in order to create a homogeneous headspace. The needle (heated at 60 °C) of the autosampler syringe, once entered the vial and filled the syringe, injected 1 mL of headspace sample directly into the injector liner (heated at 250 °C) with a split ratio of 0.6 mL/9 mL (1:15). The volatiles went directly to the mass detector through a transfer line heated at 230 °C. The carrier gas was helium at a flow rate of 0.6 mL min^{-1}. Spectral analysis time per sample was 2 min. Mass spectra were recorded by electronic impact ionisation at 70 eV, the range of mass-to-charge ratio (m/z) used was 45–250. Ion source and mass quadrupole temperatures were 230 and 150 °C, respectively.

All volatiles arrive to the detector at the same time, since there is not chromatographic separation. Thus, a total ion current (TIC) signal, which can be considered as a "spectral fingerprint" of the oil sample (Fig. 1), is generated from simultaneous ionisation and fragmentation of the mixture of introduced molecules.

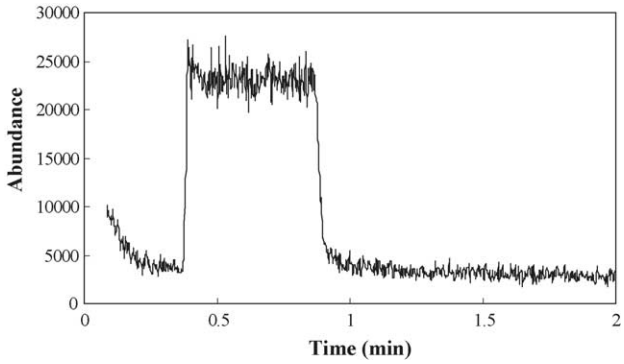

Fig. 1. Spectral fingerprint of an olive oil sample.

2.4. Data analysis

The "spectral fingerprint" of each sample, is obtained from a data table ($\mathbf{T}_{731,206}$) which is composed by as many rows as scans (731 scans) and as many columns as *m*/*z* (206) recorded during the data acquisition time (2 min). It corresponds to an output column vector ($\mathbf{C}_{731,1}$) formed by the row sum of intensities (abundances).

The row output vector ($\mathbf{R}_{1,206}$) is obtained from $\mathbf{T}_{731,206}$, and it corresponds to the column sum of intensities (abundances). This vector was used, as an object, to build the final data matrix ($\mathbf{M}_{samples,206}$) for data analysis.

$\mathbf{M}_{samples,206}$ was composed by as many rows as samples (objects) and 206 columns (variables) (Fig. 2) and it was submitted to a row profile pre-treatment, to correct the irreproducibility of the headspace sampler and sensitivity changes [20]. As display technique, principal component analysis (PCA) was performed on the autoscaled data. As classification tool, linear discriminant analysis (LDA) [21] was used, after applying the stepwise LDA feature selection technique.

3. Results and discussion

3.1. Instrument performance improvement

3.1.1. Repeatability studies

The multivariate repeatability for some influent headspace analysis parameters was assessed: extraction temperature (40–80 °C), extraction shaking condition (presence–absence), and injection split ratio (split/splitless). It was made by replicating six times the analysis of two olive oils of different geographical origin (Spain and Italy). Fig. 3 displays the score plot obtained by PCA of the autoscaled data regarding extraction temperature. As Fig. 3 shows, the most important variability is due to the geographical origin (the first principal component explained 81.8% of total variance) while a less important variability is due to the studied temperatures (the second principal component explained 10.5% of total variance). There was not evident effect on repeatability. Analogously, the other two parameters (shaking and injection split ratio) were studied and neither significant variation of repeatability was found.

3.1.2. Screening design

Since the instrumental parameters influence the experimental response all together and not one by one, only by multivariate experimental design, a complete optimisation could be achieved. For this reason, a Plackett–Burman de-

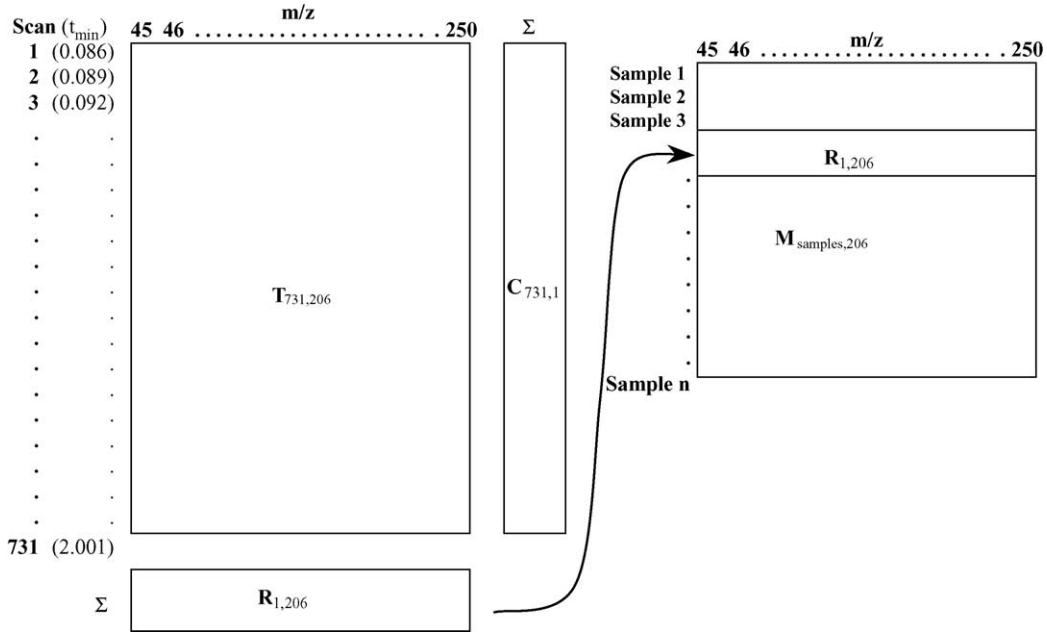

Fig. 2. Scheme of data table ($\mathbf{T}_{731,206}$), spectral fingerprint ($\mathbf{C}_{731,1}$), row output vector ($\mathbf{R}_{1,206}$) and data matrix ($\mathbf{M}_{samples,206}$).

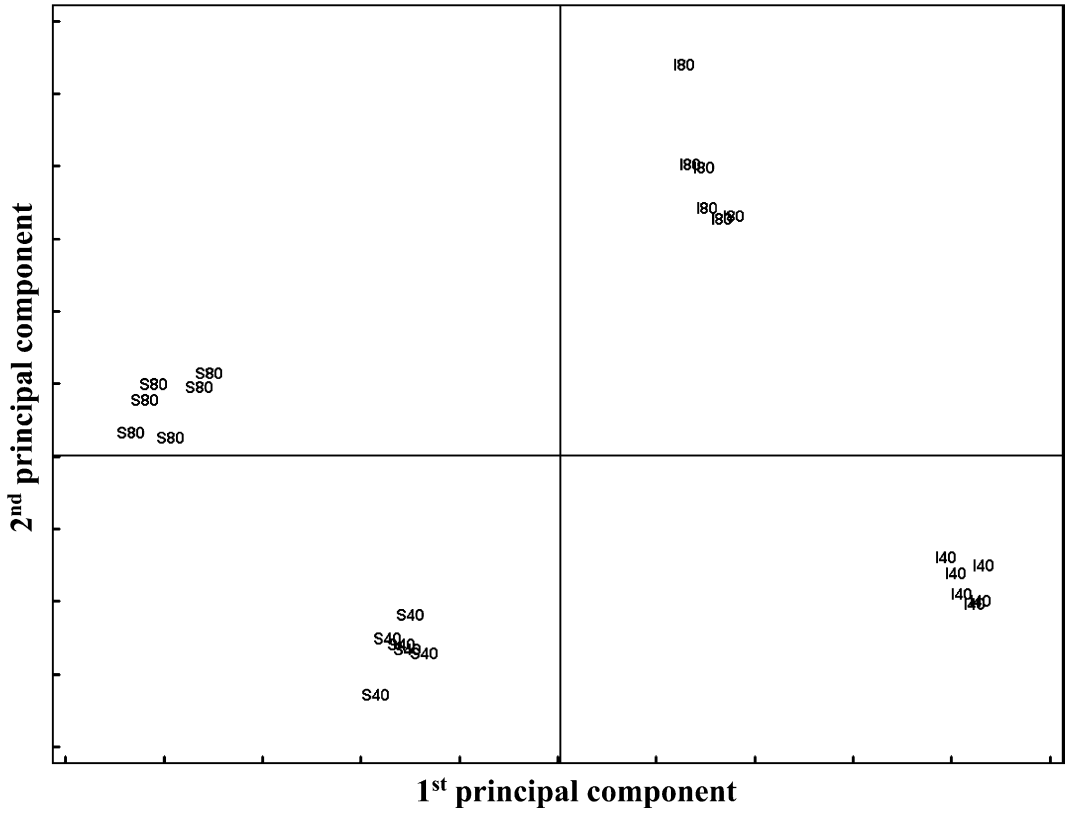

Fig. 3. Score plot of replications of one Spanish (S) and one Italian (I) oils on the first two principal components. Extraction temperatures at 40 °C (S40 and I40) and 80 °C (S80 and I80).

sign (PB) [22] was applied for the screening of the influent parameters.

From preliminary investigation, it was found that seven experimental variables could influence the response performance (Table 1) and so, the PB design (two levels design) required eight experiments. The two tested levels for each parameter are reported in Table 1.

Particularly, as far as incubation time is concerned, the lower level was set at 5 min, because it is difficult to generate a reproducible headspace in a shorter time. The higher level was set at 60 min, limiting time for a fast methodology. The lower value of incubation temperature was set at 40 °C, because this is the minimum temperature reachable by the autosampler oven without using a cryogenic gas. Moreover, this temperature allowed an easier comparison with results in literature, which have been usually obtained after odour extraction at 38–40 °C, close to human body's temperature. One hundred and thirty degrees centigrade are a little bit higher

Table 1
The Plackett–Burman experimental matrix, responses and parameter effects

Experiment	Autosampler parameters			Injection parameters				Response
	Extraction time (min)	Extraction temperature (°C)	Shaking[a]	Split ratio	Flow ramp[b]	Temperature ramp[c]	Injection volume (mL)	
1	60 (+)	40 (−)	Present (+)	1:15 (−)	Absent (−)	Present (+)	1 (+)	1.93
2	5 (−)	130 (+)	Absent (−)	1:100 (+)	Absent (−)	Present (+)	1 (+)	1.27
3	5 (−)	40 (−)	Present (+)	1:100 (+)	Present (+)	Absent (−)	1 (+)	1.36
4	60 (+)	130 (+)	Present (+)	1:100 (+)	Absent (−)	Absent (−)	0.1 (−)	0.88
5	5 (−)	130 (+)	Present (+)	1:15 (−)	Present (+)	Present (+)	0.1 (−)	0.65
6	60 (+)	130 (+)	Absent (−)	1:15 (−)	Present (+)	Absent (−)	1 (+)	1.25
7	60 (+)	40 (−)	Absent (−)	1:100 (+)	Present (+)	Present (+)	0.1 (−)	0.91
8	5 (−)	40 (−)	Absent (−)	1:15 (−)	Absent (−)	Absent (−)	0.1 (−)	1.22
Effect	+0.06	−0.17	+0.02	−0.08	−0.14	+0.01	+0.27	

[a] Present: orbital shaking every 20 s. Absent: not shaking.
[b] Present: from 0.6 to 1.5 mL min^{-1}; step: 0.01 (mL min^{-1}) s^{-1}. Absent: constant flow.
[c] Present: from 150 to 250 °C; step: 5 °C s^{-1}. Absent: isothermal condition.

than what reported in literature [23–24] for a similar kind of investigation. The possibility of shaking samples (every 20 s) or not shaking at all was tested. A low and high split ratio values were studied: 1:15 and 1:100. Due to the liner volume, the following volumes of injected sample were chosen: 0.1 and 1 mL. The possibility of using a flow ramp during the injection step, from 0.6 to 1.5 mL min^{-1}, or working in a constant flow condition was evaluated. As far as temperature ramp is concerned, both the isothermal condition and the use of a temperature ramp during the injection step (from 150 to 250 °C) were investigated.

To study the effects of the PB design, data set A was used. Thus, the data matrix of 23 rows and 206 columns was submitted to a row profile pre-treatment. Then, PCA was performed on the autoscaled data.

In the space of the first two principal components, the barycentre of Ligurian samples was computed. Afterwards, the Mahalanobis distances (MD) [25] of each sample from this barycentre were calculated. Finally, the ratio between the medians of non Ligurian MD and the Ligurian ones was computed and used as response of each experiment, indicating the discrimination between Ligurian and other samples (Table 1). Median instead of mean was used, in order to decrease the influence of possible anomalous data (outliers). From the response values of the eight experiments, the effects of the studied parameters were evaluated (Table 1).

The PB design showed that the volume of the injected sample had a large positive effect, so it was set to 1 mL. Whereas the extraction temperature and the flow ramp had a negative effect on the instrumental performance, so a low temperature (40 °C) and a constant flow condition should be chosen. The remaining variables did not show an important effect on the response improvement so they could be fixed at the most convenient value: not shaking, short extraction time, split ratio set to 1:15 and isothermal condition. Anyway, extraction temperature and time were submitted to further investigation, since a screening design could not take into account interactions.

Table 2
Two-factor two-level experimental matrix, responses, parameter effects and interaction coefficient

Experiment	Extraction time (min)	Extraction temperature (°C)	Response
1	5 (−)	40 (−)	2.79
2	30 (+)	40 (−)	2.14
3	5 (−)	80 (+)	0.59
4	30 (+)	80 (+)	0.94
5	15 (0)	60 (0)	1.20
6	15 (0)	60 (0)	1.12
Effect	−0.08	−0.85	
Interaction coefficient	0.25		

3.1.3. Full factorial design

A further full factorial design 2^2 [26] with a central point was performed both to evaluate the presence of interactions and to optimise the extraction temperature and time. For this study, the data set B was used. The remaining experimental conditions were set as PB design suggested, or they were set to the most convenient value when low effect was obtained in the PB design.

Two replicates of the experiment in the central point were analysed to evaluate the experimental variance. The six experimental conditions and their relative responses (calculated as already mentioned) are reported in Table 2. The model coefficients were computed, according to a linear model equation,

Table 3
The fragments ions selected by the two criteria of stepwise LDA and their chemical interpretation (alphabetic order)

Fragment[a]	Chemical interpretation	Fragment[b]	Chemical Interpretation
67	1,3,7-Nonatriene, 3-hexen-1-ol acetate, α-copaene, farnesene, hexanal, *trans*-2-hexenal, *trans*-2-hexenol, *trans*-β-ocimene	67	1,3,7-Nonatriene, 3-hexen-1-ol acetate, α-copaene, farnesene, hexanal, *trans*-2-hexenal, *trans*-2-hexenol, *trans*-β-ocimene
92	α-Copaene, benzeneethanol, farnesene, methylsalicylate, *trans*-β-ocimene, toluene	92	α-Copaene, benzeneethanol, farnesene, methylsalicylate, *trans*-β-ocimene, toluene
79	1,3,7-Nonatriene, α-copaene, farnesene, *trans*-2-hexenal, *trans*-β-ocimene	149	α-Copaene, eremophilene, farnesene, muurolene
72	Hexanal, nonanal, *trans*-2-hexenol	94	1,3,7-Nonatriene, eremophilene, farnesene, limonene, *trans*-β-ocimene
69	1,3,7-Nonatriene, farnesene, nonanal, *trans*-2-hexenal	76	α-Copaene, farnesene, naphthalene, *trans*-2-hexenal, *trans*-β-ocimene
76	α-Copaene, farnesene, naphthalene, *trans*-2-hexenal, *trans*-β-ocimene	55	Hexanal, *trans*-2-hexenal, *trans*-2-hexenol
201	Not interpreted	61	*cis*-3-Hexenylacetate, *n*-hexylacetate, *trans*-2-hexenal
100	1-Pente-3-olo, cis-3-hexenol, heptane, hexanal, nonanal, *trans*-2-hexenal, *trans*-2-hexenol	74	3-Hexenolacetate, *cis*-2-hexenol, *cis*-3-hexenylacetate, *n*-hexylacetate, *trans*-2-hexenal, *trans*-2-hexenol
70	*cis*-2-Hexenol, *cis*-3-hexenal, *cis*-3-hexenol, hexanol, nonanal, *trans*-2-hexenal		
74	3-Hexenolacetate, *cis*-2-hexenol, *cis*-3-hexenylacetate, *n*-hexylacetate, *trans*-2-hexenal, *trans*-2-hexenol		

[a] Stepwise LDA by minimum Wilks Lambda criterion.
[b] Stepwise LDA by selection of the variables producing the greatest Mahalanobis distance between the category Liguria and the closest one.

and only the extraction temperature resulted to have a significant negative effect (90% confidence level), while the incubation time and their interaction resulted not to be influent.

3.2. Multivariate data analysis

In the optimised conditions, described above (Section 2.3.), samples of dataset C were analysed at least twice, trying to differentiate the geographical origin of Mediterranean e.v. olive oils: a total of 218 samples were analysed. A row profile pre-treatment was applied on the $\mathbf{M}_{218,206}$ data matrix, composed by 218 rows (data set C samples) and 206 columns (*m*/*z* values), to correct the drift sensitivity [27].

The original dataset was divided in two subsets by Kennard–Stone algorithm [28]: one group was used to build the chemometric rules (*training set*: 168 objects) and the other one was used to validate them (*external test set*: 50 objects, representing the 20% of objects of each category). By this algorithm, the objects having the largest distance in the multivariate space were assigned to the test set in order to have a severe validation of the classification rules. Obviously, this criterion penalizes the external test set results.

To visualise and rationalise the information of the analytical results, PCA was performed on the autoscaled data. The first PCs did not show a significant differentiation among geographical origin and the LDA results showed a poor predictive ability. It was probably due to the large number of fragments revealed by the spectral fingerprints. Actually, many of the 206 measured variables could be not informative and noisy, so it was necessary to select the relevant *m*/*z* values. Thus, in order to improve the prediction results, the stepwise LDA method of feature selection was applied using two different criteria: (a) minimum Wilks Lambda [29] and (b) selection

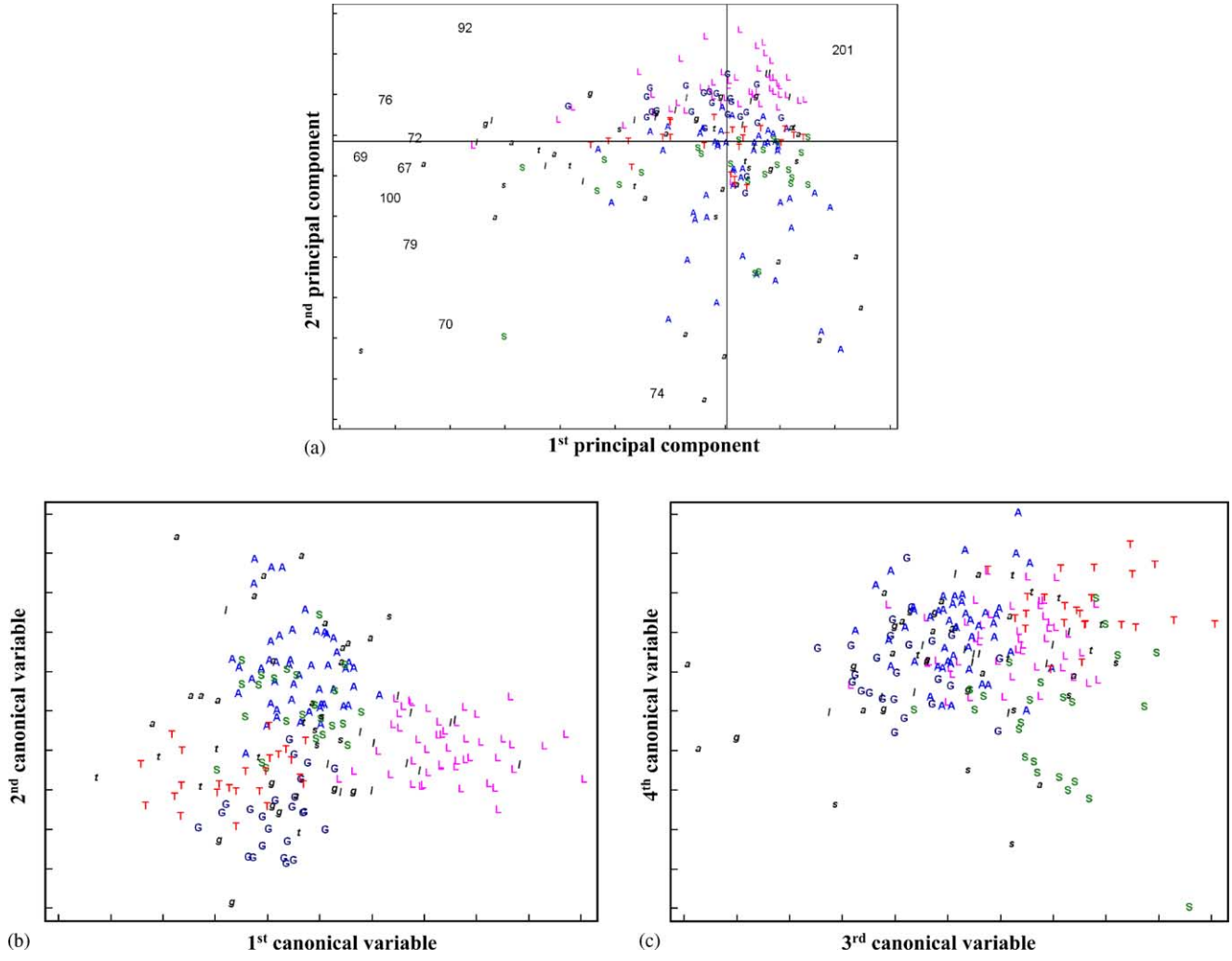

Fig. 4. Wilks Lamba criterion (case a): Samples are shown by a class symbol: L (Liguria) in pink, S (Spain) in green, G (Greece) in dark blue, A (Apulia) in blue and T (Tunisia) in red. External test set samples are represented by the same symbols in lowercase and black. (a) Score and loading plot on the first two principal components of the 10 selected variables. Loadings are represented by their *m*/*z* value in black and bigger font. (b) Projection on the first two canonical variables of LDA. Two objects of the test set are outside the plot. (c) Projection on the third–fourth canonical variables of LDA.

Table 4
LDA results: percent of correct classifications and predictions

	Stepwise LDA Wilks			Stepwise LDA Mahalanobis		
	Classification	Internal evaluation set (five CV groups)	External test	Classification	Internal evaluation set (five CV groups)	External test
Liguria	96.3	93.8	76.9 (3/13)	95.8	95.8	84.6 (2/13)
Greece	96.7	95.8	100.0	84.2	83.3	100.0
Apulia	94.2	91.7	76.5 (4/17)	68.3	62.5	52.9 (8/17)
Spain	77.5	66.7	83.3 (1/6)	72.5	62.5	66.7 (2/6)
Tunisia	100.0	100.0	71.4 (2/7)	70.8	58.3	85.7 (1/7)

For the external test set, the number of misclassified samples is indicated between parentheses.

of the variables producing the greatest Mahalanobis distance between a selected category (Liguria) and the closest one [9]. The first criterion does not advantage any category, while the second one benefits the Ligurian samples: the selected features are reported in Table 3, respectively.

3.2.1. Case (a)

The number of selected variables was set to 10. PCA was applied on the reduced data matrix $\mathbf{M}_{218,10}$: the first four PCs explained the 82.3% of total variance. The score and loading plot, on the first–second components is shown in

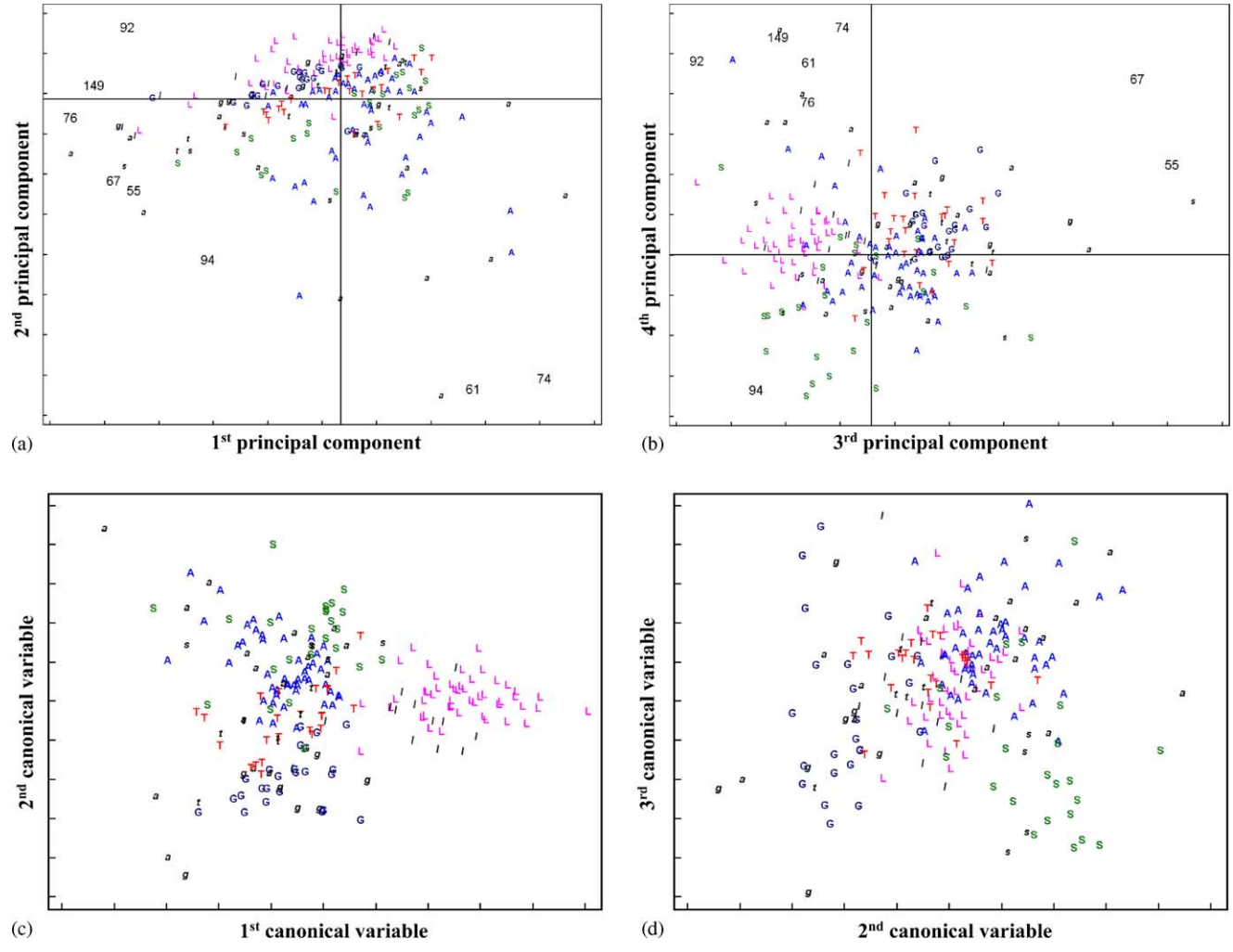

Fig. 5. Mahalanobis criterion (case b): Samples are shown by a class symbol: L (Liguria) in pink, S (Spain) in green, G (Greece) in dark blue, A (Apulia) in blue and T (Tunisia) in red. External test set samples are represented by the same symbols in lowercase and black. (a) Score and loading plot on the first two principal components of the eight selected variables. Loadings are represented by their *m*/*z* value in black and bigger font. (b) Score and loading plot on the third–fourth principal components. Loadings are represented by their *m*/*z* value in black and bigger font. (c) Projection on the first two canonical variables of LDA. Two objects of the test set are outside the plot. (d) Projection on the second–third canonical variables of LDA. Two objects of the test set are outside the plot.

Fig. 4a where only fragment 74 with a high loading value on the second PC permits a partial separation of Liguria category from the others.

The LDA results were highly improved by using the selected variables: as average values, 93.6% of samples were correctly classified, 90.5% of total internal prediction (five cross-validation groups) and 80.0% of total external prediction (external data set) were achieved and they are reported for each category in Table 4. A graphical display of these results is shown in Fig. 4b, where the objects are projected on the first two canonical variables of LDA: the Ligurian samples are well separated, Greek and Tunisian samples show a reasonable separation, while Spanish and Apulian samples are partially overlapped.

In Fig. 4c, where the samples are plotted on the third–fourth canonical variables, the Spanish and Apulian samples are well separated.

3.2.2. Case (b)

Using this criterion, for data set C, the maximum number of selectable variables was eight. PCA was applied on the reduced data matrix $\mathbf{M}_{218,8}$: the first four PCs explained the 87.8% of total variance. The score and loading plots, both on the first–second components and the third–fourth are reported in Fig. 5a and b, respectively, showing a partial class separation. Fig. 5a shows similar results to those obtained in case (a): Liguria category is separated from the others on the second PC, principally composed by *m*/*z* 74 and 61. In Fig. 5b, mainly, the fourth PC contributes to distinguish Ligurian and Spanish samples from the others: *m*/*z* 92, 94 and 149 are primarily responsible of this separation.

LDA average results were: 79.4% of samples correctly classified, the 74.4% of total internal prediction (five cross-validation groups) and 74.0% of total external prediction (external data set) (Table 4). Even if the average results were worst than those obtained by the Wilks Lambda criterion, as far as Ligurian oils is concerned, results improved.

Fig. 5c and d show the plots on the first two canonical variables and on the second–third canonical variables, respectively. In Fig. 5c, an almost complete separation of Ligurian oils from all the others is emphasized. Fig. 5d puts in evidence a good separation of Spanish and Greek oils.

For a chemical interpretation of the feature selection results, the possible origin of the ten and eight fragments, chosen in cases (a) and (b) respectively, was investigated. Four of the selected *m*/*z* are common in both cases. Many molecules may produce these fragments, but only some of them are typical of olive oil aroma [4]. In Table 3 the fragments and their possible interpretation are reported. Liguria category is rich in 92, 94 and 149 fragment ions, which may be caused by the fragmentation of some terpenoids and aromatic esters. Contemporary, it is poor in 61 and 74 ions whose origin could be attributed to the so-called "C_6 compounds", which mainly form the volatile fraction of olive oils [4], explaining the delicate and light aroma typical of the Ligurian samples.

4. Conclusions

The new HS–MS instrument assembled in our laboratory represents a more versatile and cheaper solution respect to the commercial ones: it uses free-ware software and it is easy to update in each of its hardware component.

It was able to discriminate the different aromas of olive oils coming from several geographical areas with a mean prediction ability of 80.0% after feature selection. The selected features are fragment ions strictly connected with the typical olive oil volatile components and with the related cultivar.

In order to optimise the instrumental parameters, a new multivariate statistical index was used as response in the experimental design. As far as experimental design results are concerned, it is important to underline that the headspace generation temperature was optimum at low values. Indeed, high extraction temperatures might promote oxidation and degradation reactions, whose results could confuse the delicate and light aroma of Ligurian olive oils with those of the other Mediterranean olive oils.

The good results obtained, regarding the geographical discrimination of several e.v. olive oils, seem to be promising for the use of this analytical procedure as a simple quality control tool for foodstuff characterisation.

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