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## Food Chemistry

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# A screening method based on UV–Visible spectroscopy and multivariate analysis to assess addition of filler juices and water to pomegranate juices

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## ARTICLE INFO

Article history:  
Available online xxxx

Keywords:  
Pomegranate juice  
Filler juices  
Dilution  
Antiradical scavenging activity  
UV–VIS spectroscopy  
Design of experiments  
Multivariate analysis

## ABSTRACT

Consumer demand for pomegranate juice has considerably grown, during the last years, for its potential health benefits. Since it is an expensive functional food, cheaper fruit juices addition (i.e., grape and apple juices) or its simple dilution, or polyphenols subtraction are deceptively used.

At present, time-consuming analyses are used to control the quality of this product. Furthermore these analyses are expensive and require well-trained analysts. Thus, the purpose of this study was to propose a high-speed and easy-to-use shortcut. Based on UV–VIS spectroscopy and chemometrics, a screening method is proposed to quickly screening some common fillers of pomegranate juice that could decrease the antiradical scavenging capacity of pure products. The analytical method was applied to laboratory prepared juices, to commercial juices and to representative experimental mixtures at different levels of water and filler juices. The outcomes were evaluated by means of multivariate exploratory analysis. The results indicate that the proposed strategy can be a useful screening tool to assess addition of filler juices and water to pomegranate juices.

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## 1. Introduction

In these last years, interest in functional foods is increasing in almost all industrialised countries (Bech-Larsen & Scholderer, 2007; Siró, Kápolna, Kápolna, & Lugasi, 2008).

In particular, fruit juices – and, among them, pomegranate (PG) juices – are considered a good source of phenolic compounds with strong antioxidant activity (Faria & Calhau, 2010), whose consumption could improve cardiovascular health and inhibit the proliferation of many cancers (Braga et al., 2005; Malik et al., 2005; Noda, Kaneyuki, Mori, & Packer, 2002; Vidal & Fallarero, 2003).

Nowadays, there is universal agreement that the presence of a highly constant group of six anthocyanins together with punicalagins characterises polyphenols in PG (Zhang et al., 2009). PG contains a significantly high level of powerful antioxidants ellagitannins such as ellagic acid, punicalagin and punicalin (Lansky & Newman, 2007; Visioli & Hagen, 2007), as well as anthocyanins (i.e., delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides) (Hernandes, Melgerajo, Tomas-Barberan, & Artes, 1999; Zhang et al., 2009) responsible for its red–purple colour.

Ellagitannins found in the outer part of the fruit are largely responsible for the antioxidant activity of PG. Among these ellagit-

annins, punicalagin (punicalagin anomers A and B) is responsible for over 50% of the antioxidant activity of PG (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000) and its content can vary from 1500 to 1900 mg/L depending on the cultivar of pomegranate, juice processing and storage methods (Gil et al., 2000). Punicalagin is the most characteristic compound of PG and it is found most exclusively in PG (Tzulker et al., 2007).

Several studies have even shown that authentic PG contain much more antioxidant compounds than other common fruit juices and beverages (Gil et al., 2000).

Due to these characteristics the demand for PG has increased significantly in the last years (Fischer, Carle, & Kammerer, 2011). Consequently adulterations have become of concern.

The most common way for adulteration is either a simple dilution or an addition of another foreign juice, such as grape, apple, sour cherry and strawberry to PG (Zhang et al., 2009). Another potential fraud in this field could be the extraction of polyphenols in order to subtract these great value compounds to be used for other purposes. The effects of this potential practise may be considered equivalent to a dilution of polyphenols.

Another important concern related with such types of adulterations may involve health risk, i.e., undeclared juices could contain potential allergens (Besler, Steinhart, & Paschke, 2001; Marzban et al., 2009).

For these reasons, analytical methods able to verify authenticity of fruit juices are of great importance. Vardin, Tay, Ozen, and Mauer (2008) published (Vardin et al., 2008) an analytical method

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based on FTIR spectroscopy to authenticate PG. In 2009, an algorithm was developed (Zhang et al., 2009) employing a panel of different analytical techniques to assess the complete chemical profile of PG (i.e., polyphenols, sugar, organic acids, amino acids and potassium profiles). Stable isotope dilution liquid chromatography–tandem mass spectrometry was used to detect citric, malic, quinic and tartaric acid in fruit juices to discriminate real PG and adulteration with grape juices and apple juices (Ehling & Cole, 2011).

Even if all these analytical approaches are exhaustive, they are expensive and time-consuming.

On the contrary, fingerprinting instrumental techniques, like UV–VIS and near infrared (NIR) spectroscopy, electronic nose and electronic tongue are fast and they can be used for qualitative analyses applied to authentication problems (Forina, Boggia, & Casale, 2007; Reid, O'Donnell & Downey, 2006; Reid, Woodcock, O'Donnell, Kelly, & Downey, 2005; Urbano, Luque de Castro, Pérez, García-Olmo, & Gómez-Nieto, 2006). Since these techniques provide a non-selective signal, it is necessary the use of appropriate chemometric techniques to interpret them.

The purpose of this research was to develop a rapid and inexpensive screening analytical method able to detect possible non-legal additions of filler juices (such as grape and apple) and water to the more expensive and esteemed PG juice.

Despite of their useful approaches, at present the techniques proposed in the literature and mentioned above still use expensive equipments (Ehling & Cole, 2011; Vardin et al., 2008; Zhang et al., 2009). In this context, ultraviolet–visible (UV–VIS) spectroscopy would represent a simpler and less expensive alternative, at least in a first screening step.

UV–VIS spectroscopy was used recording for each sample the whole UV–VIS spectrum. In this way, each sample analysed was described by a vector of absorbances, which can be considered a fingerprint of that sample.

Since many studies have established that PG have superior antioxidant activity compared to other fruit juices, the antioxidant activity of all the samples was evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method – an easy method commonly implemented for measuring the antioxidant activity of fruit and vegetable juices or extracts (Gil et al., 2000; Tezcan, Gültekin-Özgülven, Diken, Özçelik, & Bedia Erim, 2009). Gil et al. (2000) showed that DPPH method results obtained from PG samples are highly reproducible and comparable to those obtained from other free radical scavenging methods, this one was the only antioxidant activity assay applied.

## 2. Experimental

### 2.1. Raw materials

Pomegranate fruits of unknown cultivar obtained from local market were washed and used to obtain two variants of authentic juices (PA). The first variant of authentic pomegranate juice (variant 1) was prepared in laboratory from fresh fruits using a home juice extractor, in which the pith, carpellary membrane and the arils were juiced. The second one (variant 2) was prepared squeezing the juice just from the manually isolated arils (Table 1).

Then 59 different commercial fruit juices claiming to be authentic were collected in several markets (Italian, French and English retail stores) and analysed (Table 1). They included 14 pomegranate juices (PG), 27 grape juices (21 red grape juices, RG; two white grape juices, WG; four mix grape juices, XG), 11 apple juices (AP) and seven mix fruit juices containing pomegranate juice as one of the ingredients (MP, MG, MO and MR when pomegranate, or grape, or orange or red fruits were cited as the first ingredient, respectively).

**Table 1**

Authentic pomegranate juices (PA = authentic pomegranate) prepared in laboratory in two different ways (V1 = variant1, V2 = variant2). Commercial fruit juices categorised in four classes (PGJ = pomegranate, GPJ = grape, APJ = apple, MXJ = mixed) as declared on the label. For each sample, the radical scavenging activity (RSA) estimated by the DPPH assay (with a 1:10 dilution) is reported.

| Sample name          | Declared composition  | Category | % Reduction of DPPH |
|----------------------|-----------------------|----------|---------------------|
| PA01_V1 <sup>a</sup> | Authentic pomegranate | 1        | 24.3                |
| PA02_V1 <sup>a</sup> | Authentic pomegranate | 1        | 23.2                |
| PA03_V2              | Authentic pomegranate | 1        | 27.3                |
| PA04_V2              | Authentic pomegranate | 1        | 28.4                |
| PG1 <sup>a</sup>     | Pomegranate           | 1        | 20.2                |
| PG2 <sup>a</sup>     | Pomegranate           | 1        | 19.6                |
| PG3 <sup>a</sup>     | Pomegranate           | 1        | 26.8                |
| PG4 <sup>a</sup>     | Pomegranate           | 1        | 21.6                |
| PG5 <sup>a</sup>     | Pomegranate           | 1        | 24.0                |
| PG6 <sup>a</sup>     | Pomegranate           | 1        | 14.0                |
| PG7                  | Pomegranate           | 1        | 27.4                |
| PG8 <sup>a</sup>     | Pomegranate           | 1        | 16.2                |
| PG9                  | Pomegranate           | 1        | 41.2                |
| PG10 <sup>a</sup>    | Pomegranate           | 1        | 13.4                |
| PG11 <sup>a</sup>    | Pomegranate           | 1        | 18.4                |
| PG12 <sup>a</sup>    | Pomegranate           | 1        | 23.6                |
| PG13 <sup>a</sup>    | Pomegranate           | 1        | 25.7                |
| PG14 <sup>a</sup>    | Pomegranate           | 1        | 27.9                |
| RG1                  | Red grape             | 2        | 17.8                |
| XG2                  | White and red grape   | 2        | 11.9                |
| RG3                  | Red grape             | 2        | 19.0                |
| RG4                  | Red grape             | 2        | 23.0                |
| RG5                  | Red grape             | 2        | 21.7                |
| RG6                  | Red grape             | 2        | 15.6                |
| RG7                  | Red grape             | 2        | 18.0                |
| WG8                  | White grape           | 2        | 21.0                |
| RG9                  | Red grape             | 2        | 28.6                |
| RG10                 | Red grape             | 2        | 40.0                |
| RG11                 | Red grape             | 2        | 49.9                |
| RG12                 | Red grape             | 2        | 30.5                |
| RG13                 | Red grape             | 2        | 31.1                |
| RG14                 | Red grape             | 2        | 26.0                |
| RG15                 | Red grape             | 2        | 42.0                |
| RG16                 | Red grape             | 2        | 34.0                |
| WG17                 | White grape           | 2        | 18.8                |
| RG18                 | Red grape             | 2        | 34.3                |
| RG19                 | Red grape             | 2        | 32.1                |
| RG20                 | Red grape             | 2        | 29.7                |
| RG21                 | Red grape             | 2        | 30.6                |
| RG22                 | Red grape             | 2        | 11.7                |
| RG23                 | Red grape             | 2        | 18.0                |
| RG24                 | Red grape             | 2        | 30.6                |
| XG25                 | White and red grape   | 2        | 44.7                |
| XG26                 | White and red grape   | 2        | 8.2                 |
| XG27                 | White and red grape   | 2        | 26.1                |
| AP1                  | Apple                 | 3        | 12.0                |
| AP2                  | Apple                 | 3        | 7.7                 |
| AP3                  | Apple                 | 3        | 16.2                |
| AP4                  | Apple                 | 3        | 20.8                |
| AP5                  | Apple                 | 3        | 10.2                |
| AP6                  | Apple                 | 3        | 3.0                 |
| AP7                  | Apple                 | 3        | 3.8                 |
| AP8                  | Apple                 | 3        | 1.9                 |
| AP9                  | Apple                 | 3        | 6.7                 |
| AP10                 | Apple                 | 3        | 6.8                 |
| AP11                 | Apple                 | 3        | 2.9                 |
| Test set             |                       |          |                     |
| MG1                  | Mixture               |          | 41.7                |
| MR2                  | Mixture               |          | 21.2                |
| MO3                  | Mixture               |          | 25.8                |
| MP4                  | Mixture               |          | 51.6                |
| MP5 <sup>a</sup>     | Mixture               |          | 14.7                |
| MP6                  | Mixture               |          | 38.8                |
| MP7                  | Mixture               |          | 7.7                 |

<sup>a</sup> 1:100 dilution.

As far as the commercial pure juices were concerned, all the companies claimed on the packages that none of them contained extra undeclared added juices.

The samples studied were chosen on the basis of commercial availability so that they were representative of the various commercial typologies diversified for ingredients (i.e., fresh and concentrated ingredients), geographical origin, price, type of certification (i.e., organic agriculture) and retailing chain.

## 2.2. Chemicals

All chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland) and from Sigma–Aldrich (Steinheim, Germany).

## 2.3. UV–VIS

Absorption spectra in the ultraviolet and visible regions were obtained in the range 190–1100 nm using an Agilent 8453 spectrophotometer with 1 nm resolution. The cells were rectangular quartz cuvettes with 0.1 cm path length. MilliQ water was used as a blank. The samples were centrifuged at 5000 rpm for 15 min before being analysed. For each sample juice the spectrum was collected at room temperature in duplicate and the results were averaged.

## 2.4. Radical scavenging activity (RSA)

The radical scavenging activity (RSA) was determined by the DPPH assay (Brand-Williams, Cuvelier, & Berset, 1995) with slight modifications. Samples were appropriately diluted in the ratio of 1:10 – or 1:100, in the case for samples with elevated absorption at the wavelength of interest – with water, as reported in Table 1.

Aliquots (250 µl) of the juices to be analysed, already diluted and centrifuged, were transferred into a 10 mL volumetric flask and a daily prepared DPPH<sup>•</sup> mother solution (approximately  $10^{-4}$  M in methanol) was added to the mark. The reaction flask was shaken for 10 s in a vortex apparatus and, then, it was kept in the dark for 30 min. The residual absorbance was measured at 515 nm, at 25 °C. Blank (solution without radical) was measured before each sample and subtracted. The initial DPPH<sup>•</sup> concentration was measured by control samples (without juice), obtained by diluting 250 µl of methanol with the DPPH<sup>•</sup> mother solution in a 10 mL volumetric flask. The RSA of the samples was expressed as the % reduction of DPPH<sup>•</sup> concentration in a DPPH<sup>•</sup> solution exactly  $1.00 \times 10^{-4}$  M and was not dependent on the concentration of the daily DPPH<sup>•</sup> solutions.

$$\text{RSA} = ([\text{DPPH}^{\bullet}]_{\text{control}} - [\text{DPPH}^{\bullet}]_{\text{sample}}) / 10^{-4} \times 100$$

Three replicate analyses were performed for each sample. Results are reported in Table 1.

## 2.5. Experimental design and data analysis

This study can be split into two sequential steps.

As the first step, the possibility to use UV–VIS spectroscopy as a method able to assess the differences among fruit juices was evaluated; peaks characteristic of each fruit were identified looking for markers able to reveal possible adulterations in pomegranate juices.

The second step was focused on the variation induced, on the signals of pomegranate juice, by addition of water or juice of different nature.

### 2.5.1. Exploratory analysis of fruit juice spectra

The UV–VIS spectra of all the juice samples computed as the mean of the two signals acquired for each of them were organised into a data-matrix consisting in how many rows as the number of juice samples and how many columns as the recorded absor-

bances. The number of columns was subsequently reduced to 411 (the absorbances at different wavelengths in the range 250–660 nm) removing two intervals (190–249 nm and 661–1100 nm) in which the signal was saturated or without interesting absorptions.

Principal component analysis (PCA) (Jolliffe, 2002) of the column centred data, was used as unsupervised pattern recognition technique, in order both to visualise the data structure and, to extract useful information from the data.

### 2.5.2. Preliminary study of the differences induced in the pomegranate juice spectra by the addition of a different juice

In order to obtain maximum information at minimum cost, every step of the experimental work was preliminary planned.

Design of experiments (DOE) is a well-established concept for planning informative experiments with real advantages in terms of reduced experimental effort and in terms of increased quality of knowledge. An important DOE application concerns the preparation and modification of mixtures (Leardi, 2009).

At first, ternary mixtures (pomegranate juice/filler juice/water) were prepared as to explore uniformly the space of the three-component mixture, which can be represented as an equilateral triangle, in which the three vertices correspond to the pure components (pure juices or water), the sides to the binary mixtures (at 25%, 50% and 75%) and the internal points to the ternary mixtures (33.3% of each component).

Three different pomegranate juices were taken into account: two authentic juices prepared in laboratory from fresh fruits and one commercial juice with a high value of RSA.

As far as filler juices are concerned, two different grape juices (i.e., a red and a white grape juice, respectively) and one apple juice were taken into account to prepare mixtures as already described.

Subsequently, trying to generalise the observations, the study was extended to a greater number of commercial pomegranate juices, in which adulterations were simulated by adding juices of a different nature. In this case, the experimental domain investigated was reduced, exploring smaller additions of potential filler juices (ranging from 10% to 40%), which can be of greater practical interest. Taking into account that the exploration of all the possible combinations of PG juices and filler juices at the different levels would be impractical, it is worth finding the minimum number of mixtures that is maximally representative of all the variability factors that characterise all the possible combinations. The optimal design techniques select highly representative subsets according to particular criteria. The usual approach is to specify a model, to determine the region of interest, to select the number of runs to be made, to specify the optimality criterion and, finally, to find the subset of designed points from the whole set of candidate points (Montgomery, 2001). D-optimality is the criterion most widely applied for such a purpose (Mitchell, 1974; Zunin et al., 2001).

The experimental plan was thus defined through a D-optimal design, applied to a matrix of candidate points obtained by all the possible combinations of seven different PG juices (PG2, PG3, PG4, PG7, PG8, PG9, PG10) and seven filler juices (two red-grape juices: RG7, RG23; one mix grape juice: XG25; one white-grape juice: WG8; three apple juices: AP1, AP6, AP7, see Table 1).

## 3. Results and discussion

From examination of the spectral profiles recorded for the fruit juices under study (Fig. 1), it is possible to notice that pomegranate juices, both authentic and commercial ones, are characterised by strong absorptions in the range 250–300 nm and by a band in the range 370–379 nm whose intensity varies from juice to juice.

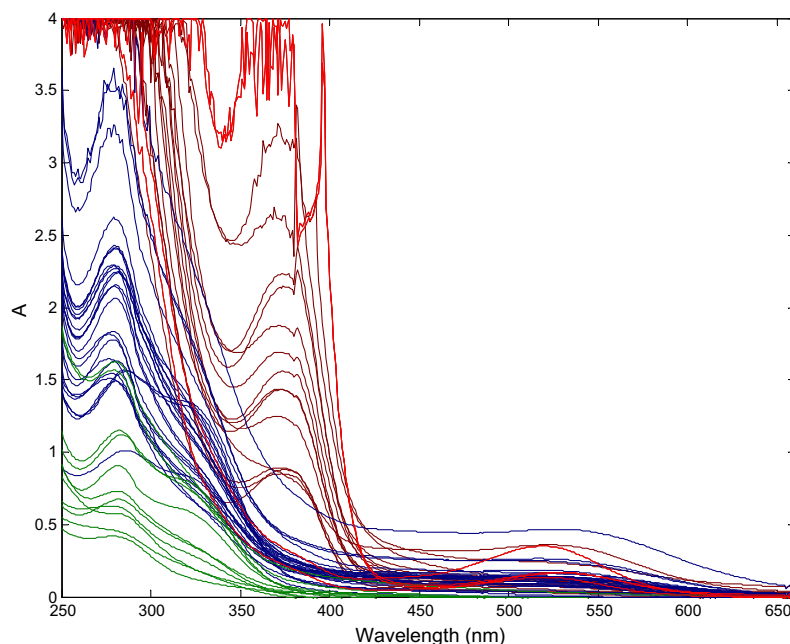


**Fig. 1.** UV–VIS spectral profiles of fruit juice samples. Red lines for authentic lab prepared pomegranate juices (PA), ginger lines for commercial pomegranate juices, blue line for commercial grape juices and green lines for commercial apple juices.

Even if both grape and apple juices did not present any absorptions in the region around 370–379 nm, it is not possible to exclude *a priori* the presence of informative features, therefore the whole spectrum of each sample was taken into account as a fingerprint.

PCA was performed as preliminary data examination. The sample scores and the variable loadings on PCs 1 and 2 – that explain 96.8% of the total variance – are shown in Fig. 2. A satisfactory separation among the different juice categories is achieved on the first two PCs. In particular, pomegranate juices, authentic and commercial ones, are well separated from all the other juices. The spectral variables having the greatest importance (loading value) on PC 1 are represented by the region around 250–300 nm. All the juices of pomegranate present high absorptions in this region, while apple juices show weak absorptions. Both the 1st and the 2nd PC contribute to differentiate pomegranate juices from grape juices. PC 2 is influenced principally by absorptions around 360–380 nm (see loadings), whose intensity not only contribute to separate pomegranate juices from grape juices, but differentiate pomegranate juices among them too. Examining the authentic pomegranate juices, whose scores are the farther apart in the pomegranate juice category, it is possible to notice that samples obtained from the whole fruit (variant 1) have stronger absorptions around 360–380 nm compared to the authentic ones obtained squeezing just the isolated arils (variant 2). Probably, as already reported in literature (Faria et al., 2010), the use of the arils alone, instead of the whole fruit (variant 1) to make juice, has an enormous impact on the polyphenol content and consequently on the antioxidant capacity of the juice, as the % reduction of DPPH confirms (Table 1).

As far as the test set (mix juices) is concerned, only two out of the seven samples have scores close to the PG area on the first two PCs. For both of these samples (MP05 and MP06), PG was the main ingredient declared on the label and their RSA values are quite high (see Table 1).

For a chemical interpretation of the loadings, the possible origin of the absorptions was tentatively investigated. In particular, the spectral regions around 250–300 and 360–380 nm, which are respectively characterised by high PC1 and PC 2 loading values,

could be related to ellagitannins unique of *Punica* botanical gender (i.e., punicalagin isomers, ellagic acid, etc.) and to a pattern of anthocyanins, typical of pomegranate (i.e., delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides) (Gil et al., 2000).

In the second phase of the study, 11 mixtures (at different concentration levels) were prepared and analysed for all the cases studied, as described in the Section 2.

The spectra of these mixtures were acquired and the data obtained were analysed by PCA. Fig. 3 reports the score and loading plot on the first two PCs for the case of mixtures of commercial PG juice, RG juice, and water. Observing the plot, it is worth noticing that the scores of the samples, reproduce almost exactly the typical triangular pattern of ternary mixtures. This means that the UV–VIS spectra do contain information relatable to the adulteration of PG with grape juice or water. On the first principal component it is possible to observe primarily the dilution with water, on the second instead, the addition of grape juice.

Moreover, from the analysis of the loadings, once again it is possible to highlight that the variables around 380 nm are particularly important in the separation among the different mixture compositions. An identical trend is evident for all of the other cases (fresh squeezed or commercial pomegranate juice/red grape juice/water; fresh squeezed or commercial pomegranate juice/white grape juice/water, fresh squeezed or commercial pomegranate juice/apple juice/water).

As previously reported, the investigation was extended to a more general case study in which seven different commercial PG juices and seven different filler juices (A) were considered.

In order to reduce the number of experiments, a D-optimal design was performed.

The variables taken into account are two qualitative variables (PG and A, both at seven levels) and three quantitative variables at four levels each (i.e., % w/w of PG: 90, 80, 70, 60; % w/w of A: 40, 30, 20, 10; % w/w of water: 30, 20, 10, 0), which overall define 10 points on the mixture triangle scheme.

The choice algorithm was applied to the candidate matrix, whose rows are 490, i.e., all of the possible combinations. Using such a matrix of candidate experiments and having defined the

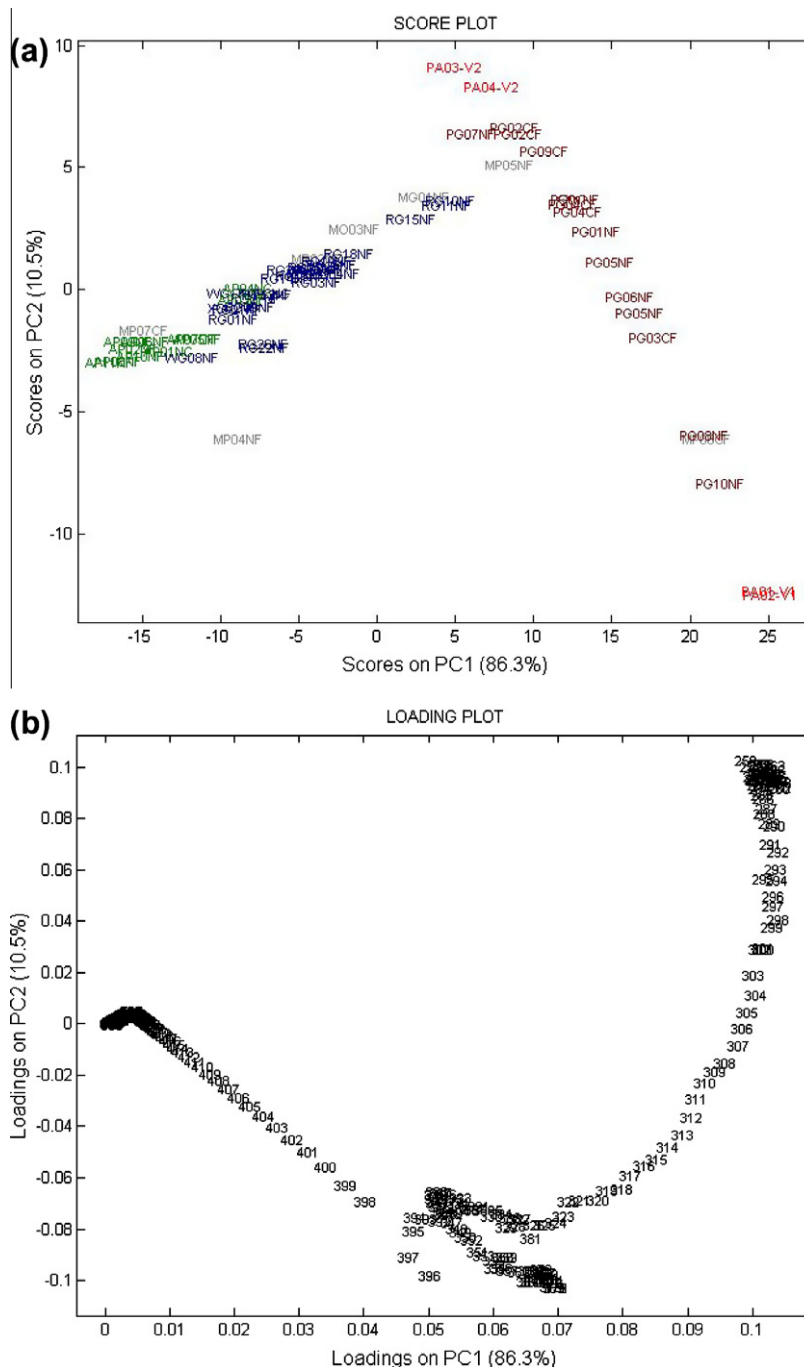


**Fig. 2.** Score and loading PC1/PC2 plot of the juice spectra. Colour of score symbols indicates the nature of the juices: red = authentic lab prepared pomegranate juices (PA); ginger = pomegranate (PG); blue = grape (including red grapes, RG, white grapes, WG, and mix grapes, XG); green = apples (AP); gray = mix fruit juices (MP, MG, MO and MR when pomegranate, or grape, or orange or red fruits were cited as the first ingredient respectively).

maximum number  $n$  of experiments to be performed (between 100 and 150), the D-optimal design strategy allowed to select a subset of experiments with the best compromise between information quality and number of experiments to be performed.

In this case, the optimal solution resulted in a plan of 128 experiments. In addition to these experiments, other 21 experiments, corresponding to the “pure” PG and to its water dilutions at 20% w/w and 40% w/w were explored. Overall, the final experimental plan included 149 experiments.

After acquisition of the spectra on each mixture, data were processed by PCA and the biplot is reported in Fig. 4, where mixtures obtained with different PGs are coloured differently while the

score label corresponds to the amount of PG in the mixture (from 10, corresponding to 100% PG, to 06, corresponding to 60% PG). Examining the biplot, it is possible to observe that the first two PCs are important to explain the addition of both a filler juice and water, similarly to what described before.

In particular, PC 1 singles out a dilution direction while both of the first two PCs single out a filler juice direction. As far as loadings are concerned, the same spectral variables are confirmed as important to distinguish among the different mixtures.

Moreover, the mixture obtained with the two commercial PGs characterised by the lowest RSA values are quite shifted towards the increasing dilution direction.

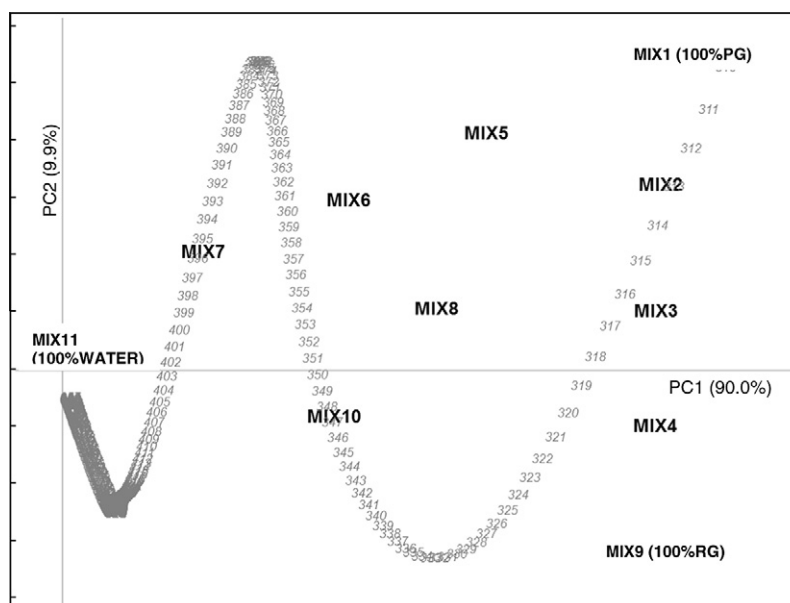


**Fig. 3.** Score and loading PC1/PC2 plot for a mixture composed by commercial PG and RG juices and water. Experiment are coded as follows: MIX1 (100% PG juice), MIX9 (100% RG juice), MIX11 (100% water), MIX3, MIX6 and MIX10 (50% mixtures) MIX2 and MIX5 (75% PG mixtures), MIX4 and MIX7 (25% PG mixtures), and MIX8 (ternary mixture).

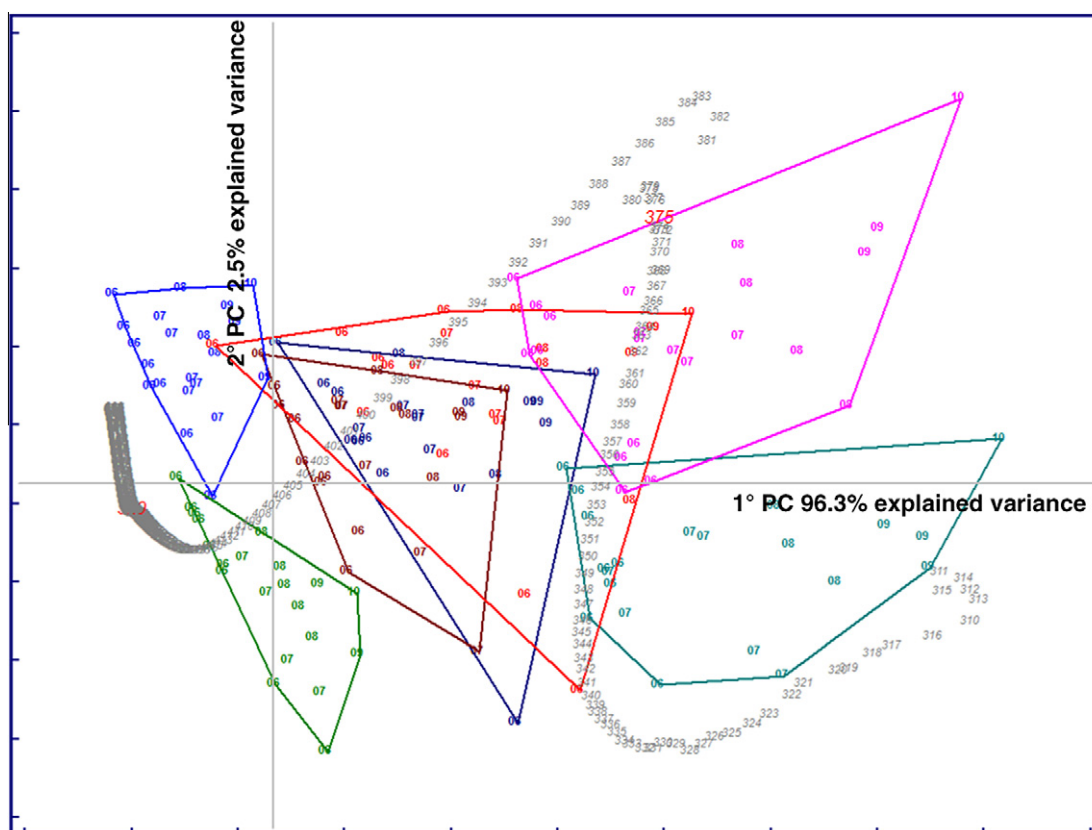


**Fig. 4.** Score and loading PC1/PC2 plot for the 149 experiments planned by the D-optimal design.

#### 4. Conclusions

According to the latest European Directives, the origin of the juice as well as the total quantity of pure juice employed to prepare beverages should be stated clearly. Therefore, reliable detection

methods were of interest to governments, consumers and producers to ensure the authenticity of juice labels.

As far as commercial PGs are concerned, because of their high value, they are subject of fraudulent actions as filler juice addition or dilution. Even if several analytical methods to verify

the authenticity of these juices have been recently published, most of the techniques reported involve many equipments and/or the use of large amounts of solvents, therefore they are both money and time consuming.

The analytical method reported in this study proposes a rapid and inexpensive alternative to screen samples in order to individuate samples that potentially contain undeclared filler juices or an excessive water amount, which could decrease the antiradical scavenging capacity of pure products. This screening approach may allow to detect suspicious samples that deserve further investigations, thus avoiding to extensively analyse a large number of samples.

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