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Controlling the Quality of Grape Juice adulterated by Apple Juice using ESI(-)FT-ICR Mass Spectrometry

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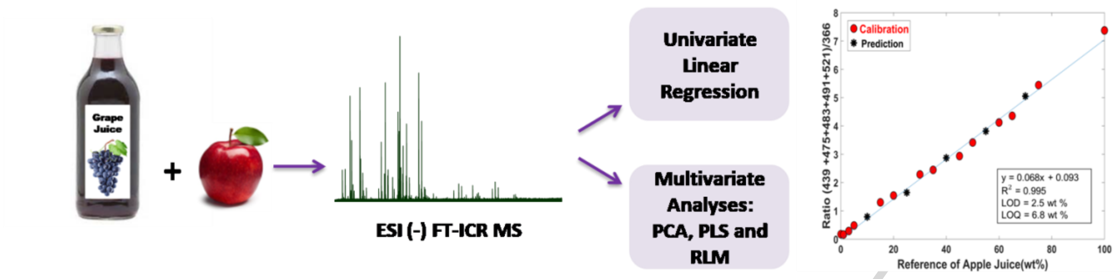
Graphical Abstract



Abstract

Food quality control is undoubtedly a process of great importance. The technological development of the last years has increased the food production and consumption, thus generating, an enormous demand for the food quality control. Grape juice is a very tasty and high quality nutraceutical drink, being widely consumed worldwide. This product has a high commercial value, making it source of several types of fraud, such as other juices' addition, like apple juice. Through negative-ion mode electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, it was possible to detect and quantify apple juice in integral grape juice. In this study, we used the univariate linear regression model using mixtures of integral grape juice with various concentrations of integral apple juice (0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 100 wt%). The model showed good accuracy. In addition, eleven commercial samples of grape juice were tested, and in two of them, apple juice was detected at ≈ 25 wt%.

Keywords: adulteration, grape juice, apple juice, mass spectrometry.

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1. Introduction

Integral grape juice presents excellent nutraceutical qualities and it has a great variety of health beneficial substances, like flavonoids and tannins.[1] Brazilian legislation determines that grape juice is an unfermented and undiluted beverage obtained from the edible part of the grape (*Vitis*ssp.) through an appropriate technological process.[2] So, it is not allowed to add any substance to the juice, such as water, sugars or other fruit juices.

As grape juice is a high commercial value product, it is susceptible to many ways of adulteration such as: water', sugars' or fruits juices of less commercial value' addition. In the latter, a good example is the apple juice addition into integral grape juice.[3-5]

Apple is a fruit of pleasant flavor and rich in pectin, a gelling agent and natural thickener that prevents juice phases' separation.[6,7] Thus, apple juice's addition is of difficult detection and can mask water or other additives' addition to the grape juice.

Grape juice adulteration control methodologies through apple juice addition are rare. Nowadays, one of the most used methods for fraud detection in juices is based in isotope ratio mass spectrometry.[4,8,9] However, this method is the most used to detect adulterants like corn or cane' sugars (via C_4 of fixation of CO_2), and it is not very effective in the differentiation of sugars originating from grape and apple natural juices, in which, they have a closer percentage of carbon-13.[4] In this case, they showed a similar CO_2 fixation mechanism (via C_3).[4,8,9] The detection of two markers of apple juice (phlorizin e sorbitol) was used for the apple juice identification and quantification into the integral grape juice,[3] but, due to the sample complexity, which may undergo diverse influences such as maturation stage, cultivar type and climatic variations; a greater number of markers ($n > 2$) is ever necessary to detect and quantify the

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adulteration in a more reliable way. Therefore, the development of analytical methodologies to detect and quantify adulterations in grape juice is a challenge.

Mass spectrometry (MS) techniques such Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) allows the identification of chemical constituents in complex organic mixtures.[10-13] More aligned with the propositions of this paper, the electrospray ionization (ESI) is a soft ionization technique whose main purpose is "picking up" ions directly from sample solution to the gas phase environment of mass spectrometers.[14] The coupling of ESI source with ultra-high resolution mass spectrometer, such as FT-ICR MS, provides the highest available mass, mass resolving power and mass accuracy (lowest mass error) when compared to other types of mass instruments.[15] It enables the analysis on a molecular level without prior extraction or separation steps.[15,16] ESI-FT-ICR MS has been applied in metabolomics studies, and it has proved to be an excellent approach to control the quality of fruits. High-resolution MS enables the separation and detection of thousands of ions produced by specific compounds. This technique provides accurate important information such as the elemental formula ($C_cH_hN_nO_o$), double bound equivalent (DBE), isotopologue profile, and chemical connectivity (from collision induced dissociation (CID) experiments).[10-13]. However, there are some limitations regarding unambiguous identification of isomers what in some cases can be overcome by performing ESI-MS/MS. Generally, the main advantage of ESI(±)FT-ICR MS technique, in relation to gas chromatography mass spectrometry, for instance, is related to fact of to provide information on molecular level of the molecules with wider molecular weight range (M_w from 200 to 3000 Da) without the need of pre-separation step (the analysis is done via direct infusion of ESI source), being therefore, able to identify thousands of compounds. Besides, the analysis time is fast (t ~ 30 s). On the other hand, the GC-MS provides

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information of low molecular weight molecules (< 500 Da), where the molecules need to have thermal stability.[17]

In this work, a methodology to detect and quantify apple juice into grape juice commercial samples using direct injection by negative-ion mode electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI(-)FT-ICR MS) combined to an univariate calibration method and Principal Component Analysis (PCA). Posteriorly, the developed method was tested in the analysis of 11 grape juice commercial samples.

2. Materials and Methods

2.1 Samples

Mixtures of integral grape juice and integral apple juice were prepared, in triplicate, in the following concentrations of apple juice: 0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 100 wt%. Moreover, eleven brands of integral grape juice bought in local supermarket were analyzed in triplicate: Casa da Uva®, Serra da Uva®, Aurora®, Sunny Day®, Salton®, Natural One®, Campo Largo®, Garibaldi®, UvaSó®, Mito®and Campo Largo Uva Leve®.The juices were randomly named as Grape Juice 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11.

2.2 ESI(-)FT-ICR MS analysis

For analysis in the ESI(-)FT-ICR MS (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany), 2.5 µL of juice and 23 µg of deuterated glucose D-Glucose-1,2,3,4,5,6,6-d7 (SIGMA) were added to 975 µL of methanol. The extracts were injected in triplicate directly into the ESI(-) source using a syringe with 2.0 µL h^{-1} flow. The mass spectra were obtained into a mass range *m/z* 150-1500. The ESI source

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conditions were: nebulizer gas pressure of 2 bar, capillary voltage of 3.8 kV and capillary transfer temperature of 200°C. The ions accumulation time was of 0.01 s. Each spectrum was acquired through the accumulation of 16 scans. The mass spectra were obtained with high resolution (16 M) providing unequivocal molecular formulas for the single charged molecular ions ($C_cH_hN_nO_oS_s$ species). The mass spectra were acquired and processed using the *Compass Data Analysis software* (Bruker Daltonics®, Bremen, Germany). The compounds' structural formulas were obtained through *ChemSpider database software* (www.chemspider.com).

For MS/MS experiments, the quadrupole window was closed in a range of 1 Da. The collision energy was of 7 V and the collision gas used was argon. In order to increase the amount of ions in the ICR cell (FTMS analyzer), the ions accumulation time in the hexapole was of 0.02 s. Each spectrum was acquired through the accumulation of 16 scans with a time domain of 16M (mega-point).

2.3 Univariate Calibration

For the construction of the univariate calibration curve, the values of the sum of ratio of the signals intensities of five markers present in apple juice (*m/z* 439, 475, 483, 491 and 521) were used versus the intensity of the internal standard signals, the dopant D-Glucose-1,2,3,4,5,6,6-d7 (*m/z* 366), added at the concentration of 23.53 µg mL^{-1}.[11] A total of fourteen points were used to construct the calibration curve (0, 1, 3, 5, 15, 20, 30, 35, 45, 50, 60, 65, 75 and 100 wt% of apple juice), where five of them were used to evaluate the method accuracy (10, 25, 40, 55 and 70 wt% of apple juice).The detection (LOD) and quantification (LOQ) limits were calculated using the **equations 1 and 2**:[18]

$$LOD = 2t_{1-\alpha,\gamma}\,\delta_0\,\frac{4\gamma}{4\gamma-1}\quad \gamma \leq 25 \qquad \textbf{(1)}$$

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$$LOQ = 10.\delta_0 \qquad \textbf{(2)}$$

Where t is the student t, γ is the degree of freedom, δ_0 is the standard deviation of the calibration samples, and α is the confidence level.

The five points (10, 25, 40, 55 and 70 wt% of apple juice) were used to calculate the method accuracy from relative error calculation through **equation 3**: [19]

$$\%\,Relative\ Error = \frac{Xlab - Xv}{Xv}\,100 \qquad \textbf{(3)}$$

where $Xlab$ is the concentration of apple juice experimentally found and Xv is the true value.

2.4 Chemometric analyses

The Principal Component Analysis (PCA) was applied to a data matrix constructed from the triplicate of 19 different concentrations of apple juice into grape juice (0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 100 wt%), totalizing 57 samples. The chosen variables were the intensity ratios of each marker (*m/z* 439, 475, 483, 491 and 521) by the intensity of the internal standard D-Glucose-1,2,3,4,5,6,6-d7 (*m/z* 366), in concentration of 23.53 µg/mL, obtaining a total of five variables per sample. The matrix rows were filled with the samples and the columns with the variables. MATLAB 9.0 (R2013a) was used to perform the data preprocessing (centering the values in the mean) and the models' construction. Moreover, another matrix constructed from 11 grape juice commercial samples, also in triplicate, totaling 33 rows, with the same variables and five columns was applied to assimilate the real percentage (wt %) of apple juice in the commercial samples.

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3. Results and Discussion

3.1 ESI(-)FT–ICR MS analysis

ESI(-)FT-ICR mass spectra of all the mixtures containing integral apple and grape juices were acquired, in triplicate, in concentrations varying from 0 to 100 wt% of apple juice doped with glucose-1,2,3,4,5,6,6-d7 at 23.53 µg mL^{-1}. For the calibration model construction, five markers of apple juice, as well as the internal standard signal were used, where their respective molecular formulas (M), DBE values, mass error (in ppm), *m/z* of their fragments and proposed structure are shown in **Table 1**. The compound 1, $[C_{12}H_{16}D_7O_{12}]^-$ ion of *m/z* 366, is identified as adduct composed of a glucose molecule and of the glucose-1,2,3,4,5,6,6-d7. The CID experiment confirms the compound connectivity through the formation of the fragments of *m/z* 186 and 179 that correspond to the glucose and glucose-1,2,3,4,5,6,6-d7 losses, respectively (**Fig. 1Sa, supplementary material**). The compound 2, $[C_{19}H_{20}O_{12} - H]^-$ ion of *m/z* 439, is identified as Maclurin 3-C-glucoside and its fragmentation profile shows fragment of *m/z* 259 due to the hexoside ring loss (**Fig. 1Sb**). The compound 3, ion $[C_{16}H_{28}O_{16} - H]^-$ of *m/z* 475, is identified as a trisaccharide and its fragmentation profile generates the ion of *m/z* 295 due to the loss of a hexoside ring (**Fig. 1Sc**). The compound 4, $[C_{15}H_{28}O_{15} + Cl]^-$ ion of *m/z* 483, is identified as an apple juice marker, however, it is not possible to attribute a chemical structure to this signal from its ESI(-)MS/MS spectrum. The compound 5, $[C_{16}H_{28}O_{17} - H]^-$ ion of *m/z* 491, is identified as an adduct of disaccharide/dihydroxybutanedioic acid, where its fragmentation gave rise the ion of *m/z* 311, related to hexoside ring loss (**Fig. 1Sd**). Lastly, the compound 6, $[C_{18}H_{34}O_{17} - H]^-$ion of *m/z* 521, is identified as an adduct of disaccharide and monosaccharide and its fragmentation profile created the ion of *m/z* 341, produced from the neutral elimination of a 180 Da monosaccharide (**Fig. 1Se**).

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Table 1. Chemical species proposed from ESI(-)FT-ICR MS.

	m/z	**Molecular Formula $[M - H]^-$**	**DBE**	**Error (ppm)**	**MS/MS**	**ChemicalIdentification**
1	366.16357	$C_{12}H_{16}D_7O_{12}$	1	-0.36	186; 179	Adduct of glucose and deuterated glucose
2	439.08612	$C_{19}H_{19}O_{12}$	10	4.74	259	Maclurin 3-C-glucoside
3	475.13074	$C_{16}H_{27}O_{16}$	3	-0.60	295	Trisaccharide
4	483.11235	$C_{15}H_{28}ClO_{15}$	1	-0.27	324; 295	-
5	491.12571	$C_{16}H_{27}O_{17}$	3	-0.69	311	Adduct of disaccharide/ dihydroxybutanedioic acid
6	521.17270	$C_{18}H_{33}O_{17}$	2	-0.73	341	Adduct of disaccharide and monosaccharide

It is possible to observe in the ESI(-)FT-ICR mass spectra that the signals intensities of the deuterated glucose (values highlighted in red) decrease while the intensities of the signals corresponding to the natural markers (values highlighted in blue) increase in function of the apple juice concentration (**Figure 1**).

The juices glycidic profile is an important parameter to evaluate possible adulterations of other fruits that can be added as juices and that possess inferior commercial value. Although various factors as maturation stage and harvest season, which affect the fruits sugars contents, in general, there is constancy in the relation between the contents of the different sugars of each fruit.[20] Previous works have shown that grape fruit presents high content of the glucose' and fructose'monosaccharides in relation to the low content of the disaccharide sucrose, whereas, apple fruitshows the disaccharides/monosaccharides ratio higher than the grape fruit.[20,21] Analyzing the ESI(-) mass spectra, it was possible to notice that with the increase of the apple juice content, there was an increase of the signals intensities in

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the region of *m/z* 400-600 in which predominates the presence of the disaccharides (**Figure 1**).

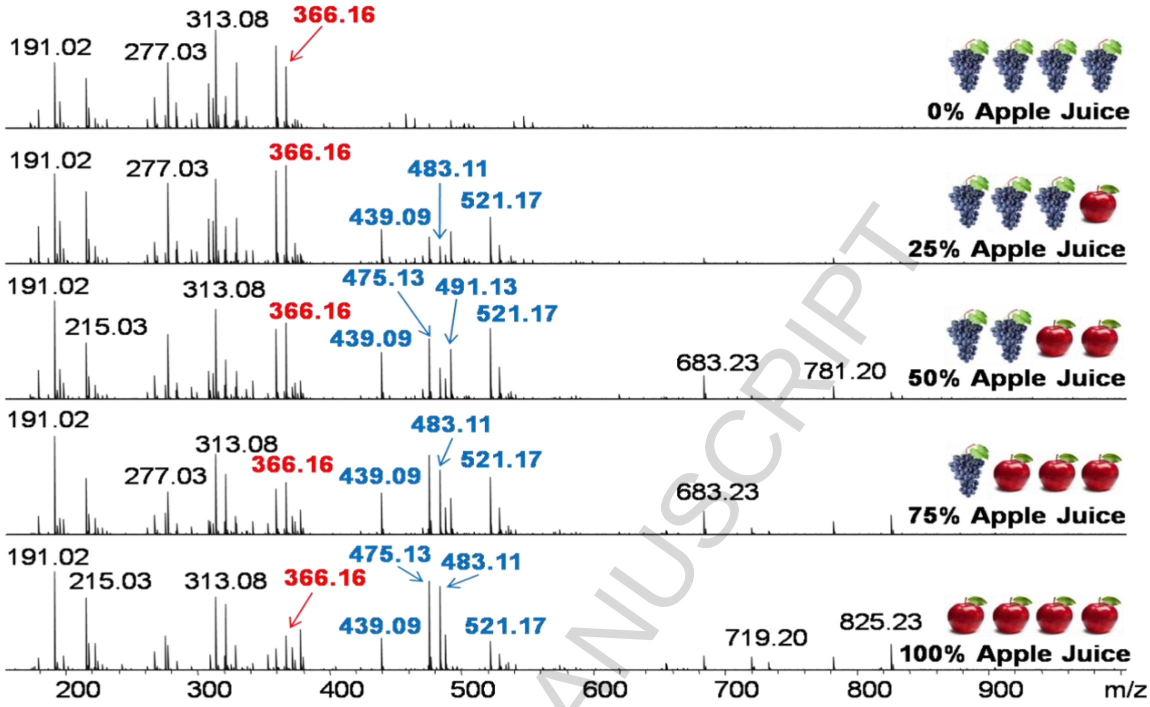

Figure 1. ESI(-)FT-ICR mass spectra of grape/apple juices mixtures at 100/0, 75/25, 50/50, 25/75 and 0/100 wt%.

3.2 Univariate Calibration

A calibration curve was obtained using the grape:apple juices mixtures in concentrations from 0 to 100 wt% of apple juice. Fourteen points were used to construct the calibration model, where five of them (10, 25, 40, 55 and 70 wt% of apple juice) were used to check the method accuracy. The analytical curve shows linearity with $R^2 = 0.995$ (**Figure 2**). The LOD and LOQ values were of 3 wt% and 7 wt%, respectively. The method accuracy was measured using the five points chosen for test. The test samples with concentration 10, 25, 40, 55 and 70 wt% of apple juice presents mean relative errors of -1.7, -9.7, -1.8, -3.8 and 0.9%, respectively. For example, the mixture containing 10 wt% of apple juice can vary up to ± 1.7% of its true value. The best

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accuracy was obtained for samples whose concentrations were of 10, 40, 70 wt%, in which, the measured samples varied less than the true value while the samples of 25 and 55 wt% concentrations had higher variations in their predictions. Nevertheless, all variations were lower than 10 wt% in relation to the actual content, which means a good accuracy of model.

Eleven commercial grape juices samples were applied to linear regression and the result is expressed in **Table 2**. Nine samples did not show apple juice content or presented values below the LOD (< 3 wt%) while two samples remaining (Grape juice 9 and Grape juice 11) show apple juice contents of 24 ± 1 wt%.

The univariate model showed linear, having R^2 of 0.9950, and with relative error of 4.8%. For low apple juice concentrations (< 10 wt%), the model presented lower LOD (3 wt%) and LOQ (7 wt%) values, producing RMSEp = 0.7. The advantage of the univariate model lies in the fact that the calculations employed are less complex, already consolidated in the field of analytical chemistry and available in many statistical software packages.

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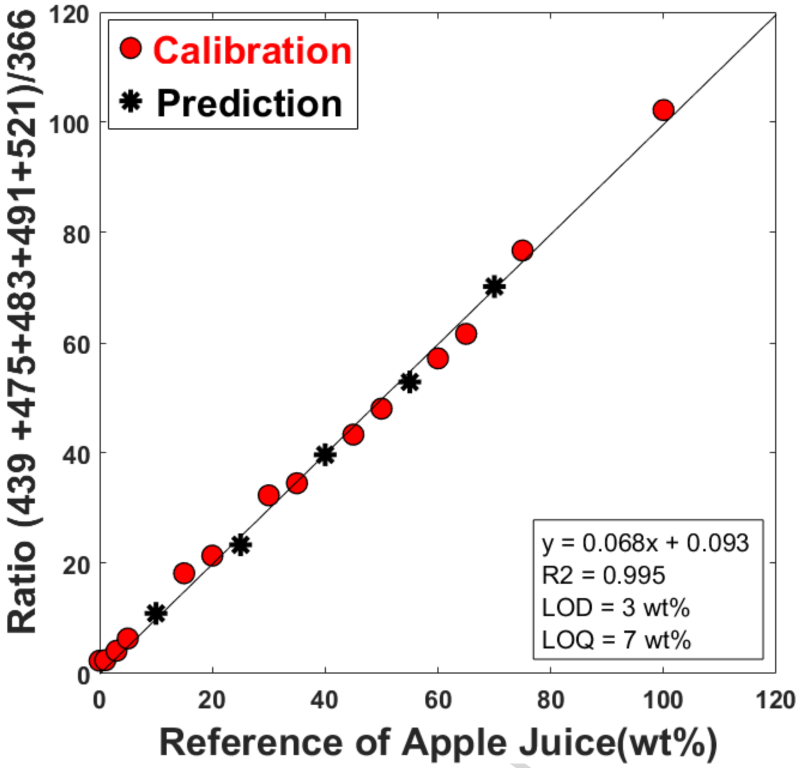

Figure 2. Calibration curve constructed from a univariate model for quantification of apple juice in grape juice.

Table 2. Content of apple juice in commercial grape juices.

Grape Juice	% Apple Juice Linear Regression
1	NQ
2	NQ
3	NQ
4	NQ
5	NQ
6	NQ
7	NQ
8	NQ
9	24 ± 1
10	NQ
11	24 ± 1

* NQ = value below the limit of quantification

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3.3 Chemometric analysis

A PCA model was constructed from ESI(-)FT-ICR MS data of 19 grape and apple juice mixtures whereupon the two principal components, PC1 and PC2, explain together 98.92 % of the total variance (**Figure 3**). The purpose is to select regions related to the apple juice addition in grape juice. It was possible to verify an apple juice concentration increase tendency from the positive quadrant to the negative quadrant of PC1, in other words, the further to the left of the negative quadrant of PC1, the greater the adulteration by apple juice. It is important to verify that even with addition of small quantities of adulterant; there was an evident separation between the samples (from 0 to 10wt% of apple juice) from PCA model constructed.

The mass spectra for eleven commercial grape juice samples were applied to the PCA model. Of all of them, only the samples Grape juice 9 and Grape juice 11 appear shifted on the negative direction of PC1. These samples appear in a region close to the calibration samples with a content of ≈ 25 wt% of apple juice. These results are consistent with the values obtained in the univariate model constructed.

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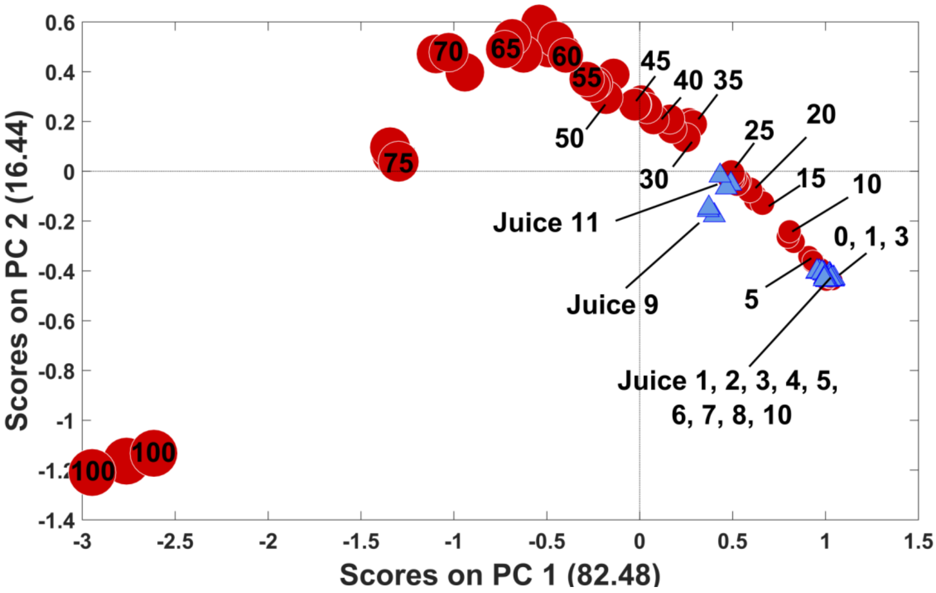

Figure 3. PCA analysis containing scores plots for the grape juice samples adulterated with different contents of apple juice. The size of the marker is proportional to the content of adulterant in the samples.

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The variables more related to apple juice adulteration in grape juice can be observed from the loadings graph **(Figure 4)**, in which the abscissas axis represents the variables (*m/z*) and the ordinates axis correspond to the loadings of each variable. The abundance of loading values indicates the importance of each variable in the model. For PC1 loadings graph **(Figure 4a),** all the five variables has inverse influence in the model, that is, at PC1<0 region. The *m/z* 475.13 variable (loading = -0.7027) is the most important while the *m/z* 491.13 (loading = -0.1231) was the least significant. The PC2 loadings graph **(Figure 4b)** show that the *m/z* 475.13 (loading = -0.1041) and 483.11 (loading = -0.4776) variables inversely influence the model, PC2<0 region, whereas *m/z*439.09 (loading = +0.2342), 491.13 (loading = +0.5921) and 521.17 (loading = +0.5963) has influenced in PC2>0 region. The most significant variables at PC2 were of *m/z* 491.13 and 521.17. Analyzing PC1 and PC2 loadings graphs in concomitance **(Figure 4)**, it is observed that the most important variables are *m/z* 475.1, 491.13 and 521.17, that correspond to disaccharides molecules that possess higher intensities in the ESI(-)FT-ICR mass spectra, as illustrated in **Figure 1**.

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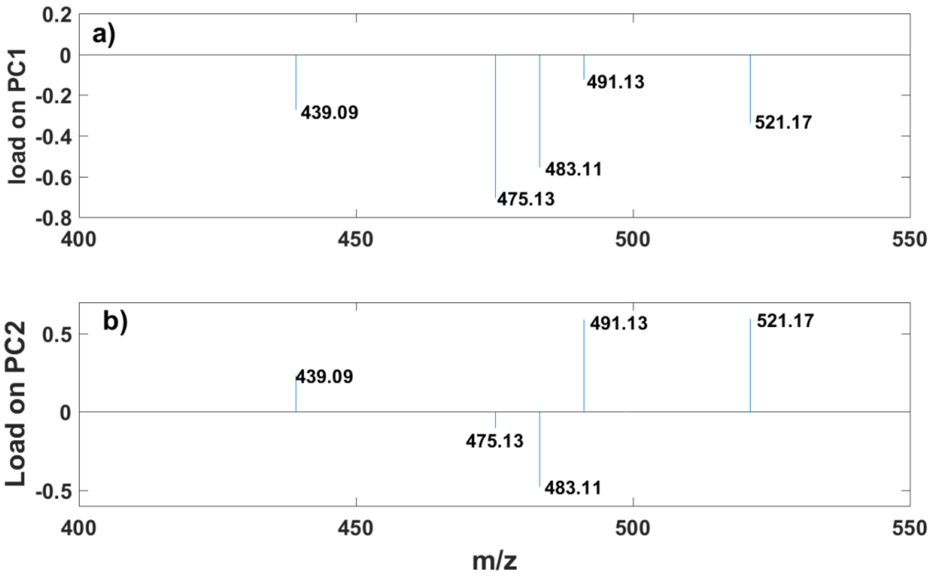

Figure 4. PCA analysis containing loadings plots to PC1 **(a)** and PC2 **(b)** for the grape juice samples adulterated with different contents of apple juice.

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4. Conclusions

The FT-ICR MS technique associated to univariate calibration model showed to be a reliable procedure for the identification and quantification of apple juice into integral grape juice. The PCA, made from the intensities ratio of the five natural markers found in the fruit (*m/z* 439, 475, 483, 491 and 521) by the internal standard intensity (*m/z* 366) provided good separation between the 19 different apple juice contents (wt%) in integral grape juice. Among the studied commercial samples, two (Grape juice 9 and 11) exhibited similar contents of apple juice, about 25 wt%.

5. Acknowledgements

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Conflict of Interest: Author A declares that he has no conflict of interest. Author B declares that she has no conflict of interest. Author C declares that he has no conflict of interest. Author D declares that she has no conflict of interest. Author E declares that she has no conflict of interest. Author F declares that he has no conflict of interest.

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Author G declares that she has no conflict of interest.Author H declares that he has no conflict of interest.

Author contributions: Author Acontributed with the acquisition, analysis, interpretation of data for the work and drafting the work and revising it critically for important intellectual content; Author B, author C and Author D contributed with the acquisition of the data. Author C contributed with interpretation of data for the work and drafting the work and revising it critically for important intellectual content, Authors E and F contributed with interpretation of data for the work and revising it critically for important intellectual content. Author G and Author H contributed with designing the work and drafting the work and revising it critically for important intellectual content. The entire author approved the version to be published.

Institutional Review Board (IRB): Review by the IRB was not required for this study because human subjects were not involved, as per US Department of Health and Human Services guidelines.

Ethical approval: This article does not contain any studies neither with animals nor with humans performed by any of the authors.

Informed consent: not applied.

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Highlight

- Grape juice is a very tasty and high quality nutraceutical drink, being widely consumed worldwide;
- This product has a high commercial value, making it source of several types of fraud;
- ESI-FT-ICR MS coupled to regression models showed good accuracy to quantify apple juice in integral grape juice;
- Eleven commercial samples of grape juice were tested, and in two of them, apple juice was detected;

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