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Multivariate Analysis of the Spectroscopic Profile of the Sugar Fraction of Apple Pomace

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ABSTRACT

Pomace, the main by-product of apple juice processing, contains all the fruit's compounds such as minerals, sugars, fibers, enzymes and secondary metabolites after bioprocessing. Dried pomace from four apple varieties (Gala, Fuji, Catarina and Joaquina) was used to extract fructose, glucose, and sucrose, the main soluble sugars contained in apple pomace, to verify the possible use of the sugar fractions. The concentrated sugars were characterized by NMR and FTIR. The partial least squares method (PLS) applied to the NMR and FTIR spectra revealed large amounts of fructose, glucose, and sucrose, without the presence of others compounds. Principal component analysis (PCA) discriminated the studied apple varieties, with a 99% level of significance, as function of the amount of each sugar in the respective extracts.

Key words: Apple pomace, NMR, FTIR, multivariate analysis

INTRODUCTION

The main components of apple pomace are water, sugars and malic acid, which give apple products their predominantly sweet and sour flavors. Trace amounts of phenolic compounds suffice to give apple-derived products their distinctive sourness and astringency, which usually enhance their flavor, such as that of cider (Downing 1989; Devrajan et al. 2004). Apple pomace represents 20 to 40% of the raw material and it is the by-product of apple juice processing. Pomace usually contains 80% of moisture, a high level of sugar and a low pH (3-4), which explains its chemical and biological instability and the environmental impact it causes when discarded without prior treatment (Wang et al. 2007). Due to its composition, several uses have been proposed for pomace, including bioprocessing for ethanol production, physical processing for fiber extraction, and aroma extraction. The sugar content, which may represent 30 to 40% of dried pomace, may be extracted and characterized according to its simple sugars: fructose, glucose and sucrose, which, converted into inverted sugar, has high sweetening value and a low glycemic index. The functional appeal of the sweetener would be its fructose-rich fraction.

Several studies have focused on the quantification of sugars (Blanco-Gomis et al. 1998), organic acids (Fuleki et al. 1995) and phenolic compounds (Price et al. 1999) in apples. Most of these studies involved separating the liquid phase from the fruit, followed by the chromatographic analyses. The official method for measuring the content of reducing sugars in the foods, according to the

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Association of Official Analytical Chemists (AOAC), is by polarimetry of sucrose. However, this method is fairly restricted due to the interference caused by the optical activity of many other substances that are potentially present (Meade, Chen 1977).

Most of the classical methods of sugar quantification are based on the Fehling reaction in alkaline media. Some authors also use the reaction with phenol in a strongly acid medium to produce chromogenic compounds for spectrophotometric monitoring. These methods involve titrimetry (EDTA, Lane-Enyon, Luff-Schoorl), gravimetry (Musson-Walker), and spectrophotometry (Carbazole, DNS, Antron sulfuric acid, phenol-sulfuric acid, Somogyi-Nelson). Among the physical methods, the one most commonly used for the analysis is refractometry (Brix scale) due to its ability to quantify the amount of total soluble solids indiscriminately in a sample (Neto, 2006).

Chromatographic methods are used to identify and quantify the compounds after the hydrolysis. Sugars, organic acids and phenolic compounds can also be analyzed nondestructively by the Nuclear Magnetic Resonance (NMR) spectroscopy. In fact, this type of instrumental analysis has become increasingly popular due to its high selectivity and its ability to simultaneously detect numerous chemical compounds in a single spectrum. NMR spectroscopy coupled with principal component analysis (PCA) has been used to identify the origin of wines (Košir and Kidriˇ 2002), discriminate different kinds of beer (Duarte et al. 2002), determine the amount of fatty acids in the whole seeds (Prestes et al. 2007), detect adulteration in olive oil (Šmejkalová and Piccolo 2010), determine vegetable quality standards (Hills and Clark 2003), infer plant disease levels (Prestes et al. 2009), determine the total soluble solids in the fruits (Keener et al. 1997), and to study food (Košir and Kidri 2002) and fruit juice (Eads and Bryant 1986) compositions in general. Additionally, it is a very interesting tool for comparing the natural and synthetic samples. Recent studies have used NMR coupled with chemometric techniques to characterize and classify the products according to their origin, quality or variety (Fragaki et al. 2005).

Today, near-infrared and mid-infrared spectroscopy (NIRS and MIRS, respectively) are widely employed in the quantitative and qualitative analyses of the fruits. NIRS has been used to evaluate the quality of apples in terms of fiNMRess, soluble solids content (Peirs et al. 2001), and variety (Leemans et al. 2003), and to detect the bruising (Xing et al. 2003). MIRS coupled to Fourier transform infrared spectroscopy (FTIR) is a very important tool for determining acid and sugar in apple juice, and can be used to detect the adulterants. FTIR has been used successfully to detect the organic acids, sugar and soluble solids and to determine the fruit fiNMRess (Lammertyn et al. 1998). Similar studies have been performed with the Attenuated Total Reflectance (ATR) infrared spectroscopy. NMR and IR methods are analytical tools that allow for the simultaneous and nondestructive measurement of multiple parameters, enabling multiple analyses of the same sample.

A current practice is to combine the data obtained through the spectroscopic analyses using multivariate tools, enabling the evaluation of numerous properties, taking into account their correlations and allowing for interferences to be made from the dataset at a known level of significance (Nascimento 2007). In this work, the natural sugar fractions obtained from the pomace of four apple cultivars were characterized by the NMR and FTIR and analyzed using the multivariate tools, i.e., principal component analysis (PCA) and partial least squares (PLS) regression.

MATERIALS

The materials used here were the samples of Gala, Fuji, Joaquina and Catarina apple cultivars from the 2007-2008 crop, Dowex and Amberlite ion exchange resins, and enzymes for the quantification of sucrose, fructose and glucose.

METHODS

Extraction of the sugar fraction

Apple pomace, the by-product of juice processing on a laboratory scale, was immersed in water (1 kg/1 L) for 5 min, centrifuged at 800 g for 20 min (Arno NCRA centrifuge, 10.5 kg) and then dried in an adiabatic oven under a hot air flow of 50 to 100ºC. The dried pomace was ground, sieved and stored at room temperature. The total soluble solids were extracted extensively with water (Queji 2008) using 50 g of solid pomace and 700mL of water. The contents were then filtered

twice through the filter paper to separate the solids. The extract was cooled and the water-soluble polysaccharides were removed by filtration after cold precipitation (-18°C) with 66% ethanol (1 vol. extract/2 vols. ethanol). This mixture was held at this temperature for 2 h to stabilize and flocculate the pectic substances, which were then removed by filtration and centrifugation. The resulting filtrate containing the soluble sugars was reduced to a small volume by low-temperature, low-pressure evaporation, eliminating all the added alcohol. The sugar fraction was then chromatographically purified in ion exchange columns, 26 cm length x 2.5 cm i.d., using Dowex 50WX8 and Amberlite IRA 420 ion-exchange resins. The purified sugar solution was concentrated to approximately 70º Brix under low pressure and temperature (45°C).

Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR spectra were obtained using a high-resolution Bruker AVANCE-400 MHz NMR spectrometer operating at 9.4 T, frequency of 400 MHz for ^{1}H. Analysis were done in a 5.0 mm coil with 90º pulse, acquisition points at 64 K and a spectral window of 10 ppm, performing 16 cumulative scans. The 90º pulse width was 7.5 μs, acquisition time was 7.5 s and recycle delay were 10 s long, totalizing 17.5 s. These conditions was used in other work (Prestes et al. 2012). For the *in vitro* analysis, the sugar fraction was dissolved in D_2O (600 μL), containing dimethyl-d6 sulfoxide (DMSO-d6) as internal standard and 2,2,3,3-D4-sodium trimethylsilylpropionate tetra deuterated (TMSP-d4) as chemical shift reference. DMSO was left in a dissecator for 24 h prior to analysis in order to remove the excess of water. The solution was prepared in a volumetric flask by diluting both the standards and the samples and adding 60 μL of DMSO-d6, 0.03 mg of TMSP-d4, and D_2O excipient to complete 10 mL.

To obtain the ^{1}H NMR spectra, 300 μL of the sugar fractions from all the four varieties were used, and the samples and standards were previously dissolved in 600 μL of the solutions. The spectra were processed by the spectrometer's software by adjusting the phase and baseline of each spectrum. NMR chemical shifts were adjusted by using TMSP-d4 peak as reference. The sugar standards and samples were analyzed quantitatively using the same program, by merging two duplets according to α and β glucose (5.21 and 4.63 ppm, respectively), setting boundaries at 5.24 to 5.19 ppm and at 4.66 to 4.60 ppm, respectively. For sucrose, the boundaries were 5.42 and 5.38 ppm for the duplet at 5.39 ppm; for fructose, the boundaries were 4.01 to 3.96 ppm.

Fourier Transform Infrared (FTIR)

The infrared spectra were recorded by a Nicolet 4700 spectrophotometer and each sample was subjected to 32 scans for sugar quantification, using a resolution of 2 cm^{-1} within a range of 4000-400 cm^{-1}. The sugar fraction was prepared in solid pellet form, using 40 mg of KBr and 0.03 mg of sample obtained from each apple cultivar. Standard sugar pellets were added in proportions of 1.00; 1.50; 2.00 and 2.50% with KBr, and their spectra were used to establish the calibration curve for the quantification of each sugar in the samples' sugar fractions.

Multivariate Analysis

The four varieties were correlated by PCA with their respective concentrations of each sugar, and quantified by integrating the peaks in NMR and the relative absorbance in FTIR. This procedure evaluates the similarity between the different varieties and the effect of each sugar in the sugar fraction sample. InfoMetrix Pirouette 4.0 software was used for the preliminary program, autoscale processing, and data processing.

Calibration graphs using the partial least squares (PLS) were established with the data obtained from the FTIR spectra of the three standard sugars (glucose, fructose and sucrose) at known concentrations. The model's calibration and prediction performance was validated by a crossed design of internal and preliminary mean-centered processing. The NMR and FTIR data correlations were analyzed by the matrix determined by the quantitative information of the NMR–measured spectra of the sugars in the range specified as dependent, and that of FTIR as the independent variable. Modeling was also performed with Infometrix Pirouette 4.0 software. Each matrix was analyzed and some spectral regions containing little or no information were excluded.

RESULTS AND DISCUSSION

Analysis of the sugar fraction by high resolution NMR

The composition of each sugar fraction (Gala, Fuji, Catarina and Joaquina) was analyzed by

high-resolution NMR spectroscopy. Figure 1(a) shows the spectrum of the sugar fraction from cv. Gala. When the 5.44 to 4.13 ppm region was expanded (Fig. 1 b), a pair of duplets became visible at 5.21 and 4.63 ppm, corresponding to hydrogen in position 1 from α and β; glucose. Another pair of duplets is visible at 5.39 and 4.20 ppm, which was attributed to hydrogen in position 1 from unities of glucose and 2 of fructose from the structure of a sucrose molecule.

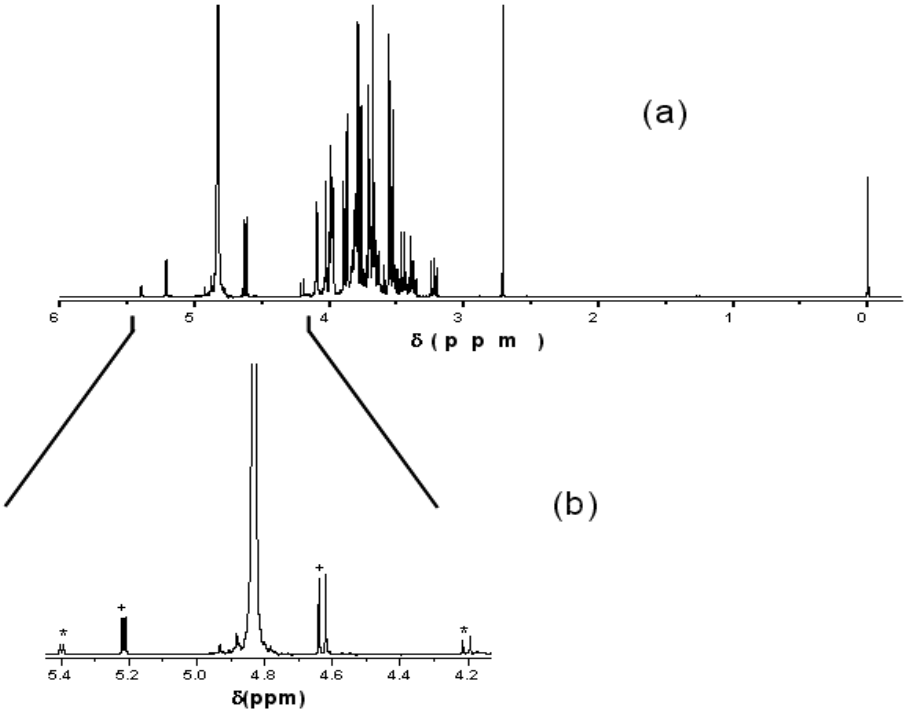


Figure 1 – (a) ^{1}H NMR spectrum in D2O (400 MHz) of the sugar fraction in Gala cv. (b) Expansion of spectrum (a) in the region of 5.44 to 4.13 ppm, showing glucose (+) and sucrose (*) signals.

Figure 2 showed the profile of the Gala cultivar spectrum from 4.15 to 3.16 ppm, whose peaks corresponded to the soluble sugars glucose, fructose and sucrose. This spectrum showed glucose signals at 3.75, 3.73, 3.72, and 3.71 ppm and from 3.51 to 3.19 ppm. Fructose signals were in the intervals of 4.11 to 4.08, 4.01 to 3.85, 3.78 to 3.75, 3.72 to 3.69, 3.68 to 3.65, and 3.62 to 3.51 ppm, and the peaks were located at 3.77 and 3.76 ppm. Sucrose showed the peaks at 4.05, 4.03, 4.01, 3.74 and 3.63 ppm, as well as signals from 3.85 to 3.78 ppm. The peak intensities indicated that the highest concentration of sugar found in this specific apple variety was fructose. The three peaks corresponding to all the sugars from 3.2 to 5.4 ppm have already been found in cider (Berregi et al. 2003).

Among the three main sugars, fructose showed the highest content in the sugar fraction of all the samples. In addition, no signals corresponding to sorbitol, organic acids, and phenolic compounds were detected, leading to the inference that they were either completely absent from the pomace extracts or in such low concentrations that they were undetectable by the NMR. Assuming the presence of only the three main sugars, the NMR spectra were used to determine their contents in the fraction samples, integrating their respective peaks: 3.20 ppm for glucose, 3.89 ppm for fructose and 4.19 ppm for sucrose. The same methodology was used by Eads and Ni (Eads and Ni 1993) in the fruits and Moitrier and Rinke (Moitrier, Rinke, 2007) in orange juice.

Table 1 shows the amounts quantified by ^{1}H NMR based on the integrated signal corresponding to the invert sugars, mainly glucose and fructose. These amounts were fairly high and were consistent with those reported by Kennedy et al. (1999). However, it was not possible to perform the statistical inferences and the results might be considered as

semi-quantitatives as the samples were not replicated. The four varieties contained similar proportions of glucose, fructose and sucrose, but the Gala cultivar showed more desirable features, such as a sweeter flavor, low caloric value, unquestionable bioactivity and low glycemic index, consistently showing the highest quantity of sugars. Glucose ranked second in terms of simple sugar concentration, especially in the Catarina cv. The characteristics of these sugars and their specific positions in the NMR spectrum have already been reported by Eads and Bryant (1986), who stated that these sugars existed in different concentrations depending on the variety, processing, and maturation level.

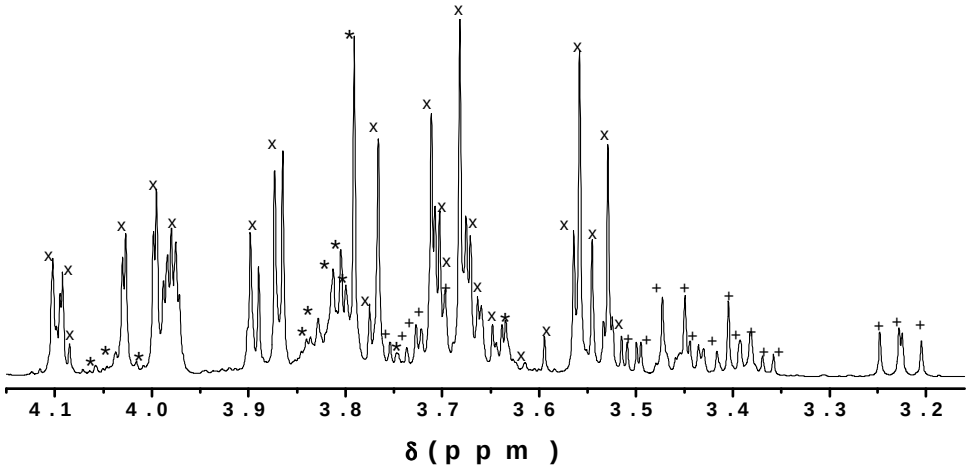


Figure 2 – Expansion of the 4.15 to 3.16 ppm region with the peaks corresponding to glucose (+), fructose (x) and sucrose (*).

Table 1 – Relative concentrations of simple sugar fractions (glucose, fructose, and sucrose) in the Gala, Fuji, Catarina and Joaquina cultivars quantified by NMR.

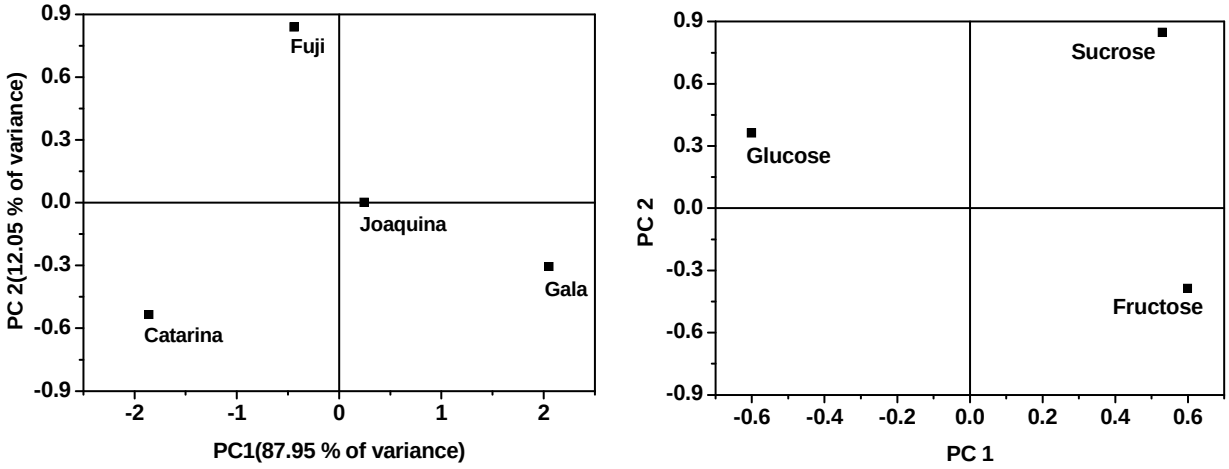
Variety	Glucose (%)	Fructose (%)	Sucrose (%)
Catarina	30.5	66.6	2.9
Joaquina	27.4	69.5	3.0
Fuji	29.5	67.5	3.0
Gala	24.1	72.9	3.0

NMR Principal Component Analysis (PCA)

Figure 3 shows the PCA results for the sugars in the four samples quantified by the NMR and listed in Table 1. Figure 3a showed the scores and Figure 3b showed the loads, with 99.99% of the variance explained and the continuous and categorical variables shown separately. Figure 3b shows the distributions of the three standard sugars with good separation and comparable load values. A comparison of the loadings and score plots indicated that the Fuji variety behaved differently from the other varieties, presenting a higher influence from glucose and sucrose. The Catarina cultivar had highest glucose content, whereas the Joaquina and Gala varieties contained highest amounts of fructose. These results clearly demonstrated that any specific sugar concentration was strongly dependent on the apple variety. This analysis is important because it enables the choice of a suitable apple variety based on the desired sugar fraction.

FTIR analysis

Figure 4 shows the spectrum of the sugar fraction obtained from the Gala cv. The spectra of the other three sugar fractions were very similar, showing comparable glucose, fructose and sucrose compositions. However, it should be noted that the presence of several compounds in a mixture might increase the possibility of overlapping between the absorption signals in the IR spectrum (Tozetto et al 2007).



(a) (b)

Figure 3 – PCA of the NMR data of the categorical and continuous variables: (a) Scores, (b) loads.

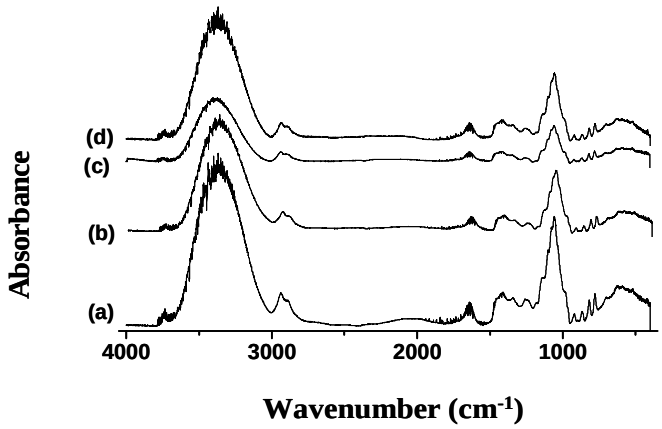


Figure 4 – FTIR spectrum of sugar fraction obtained from the apple varieties: (a) Gala, (b) Joaquina, (c) Fuji, and (d) Catarina.

FTIR Partial Least Squares (PLS) Regression

Associating the evaluation of peak distributions in some fragments of the IR spectra with chemometric tools is essential to understand all the information yielded by the experiment (Cocchi et al. 2005; Hopke 2003; Kateman and Buydens 1993). Thus, due to the need to identify the characteristic peaks for each sugar, the spectra were obtained from patterns at different concentrations in order to provide a standard curve for each sugar. Because the standard curve for sucrose in four concentrations was still unsatisfactory, two more dots were added.

The spectra of the standard sugars were also used to quantify the sugars found by the PLS in the sugar fraction of the four cultivars in order to design the calibration models for each sugar (Fig. 5). It showed strong correlation between the FTIR data of glucose (Fig. 5a), r_{val}= 0.99, fructose (Fig. 5b), r_{val}= 0.91 and sucrose (Fig. 5c), r_{val}=0.95.

The models obtained were used to predict the content of each sugar in the sugar fractions of the four apple varieties. The FTIR spectra of each sample were used as independent variables and the sugar concentrations as dependent variables. Fructose showed the highest concentration, followed by the other sugars. This result was consistent with the proportion of sugars in apple pomace reported by Queji (2008) and observed in the ^{1}H NMR analysis.

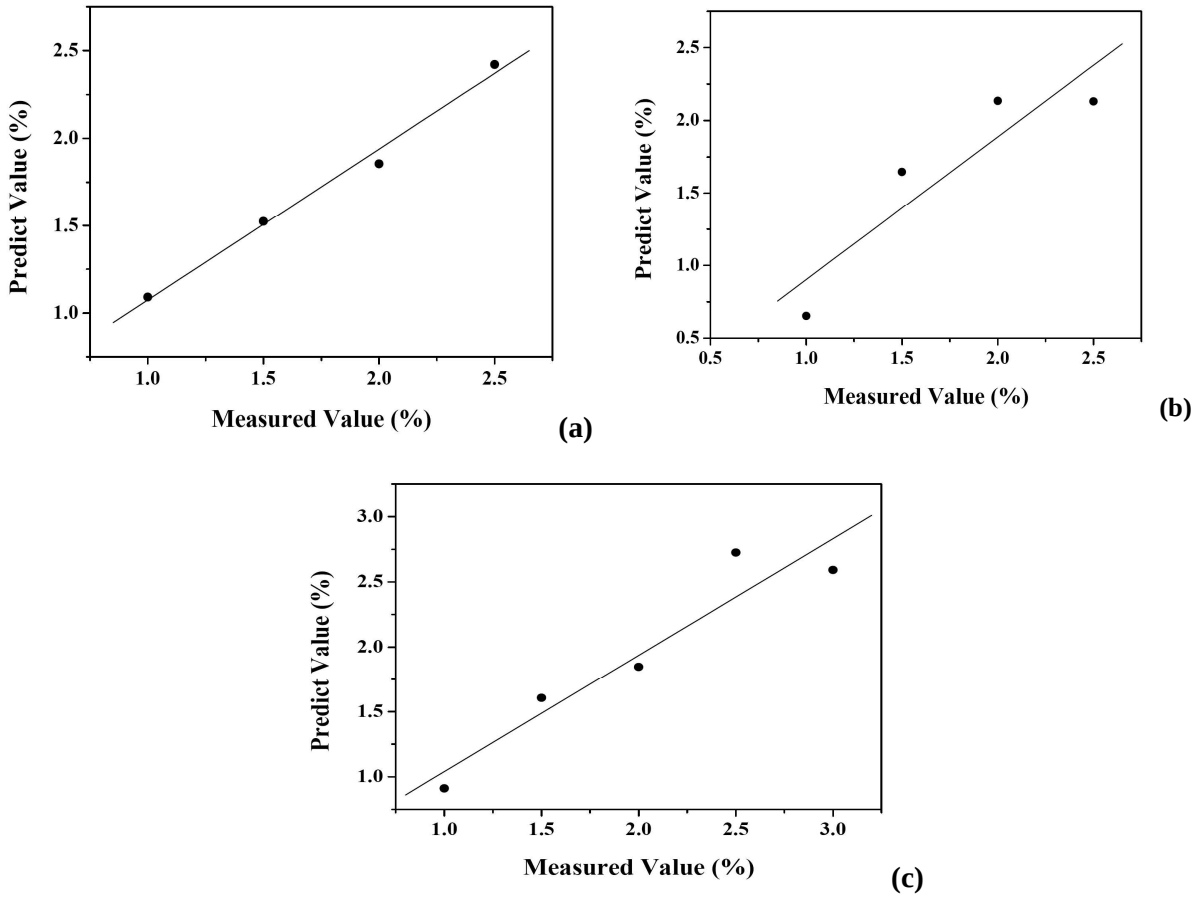


Figure 5 – PLS correlation between the measured and predicted values of glucose (a), fructose (b) and sucrose (c), used to predict the concentration of sugar in the samples.

CONCLUSIONS

The simple sugar fractions of apple pomace were quantified and qualified by the NMR and FTIR spectroscopy associated with the multivariate statistical procedures of PCA and PLS. Instrumental analysis was used to confirm the absence of phenolic compounds and organic acids. The simple sugars extracted could be potentially used as a natural sweetener with a functional appeal due to its high levels of fructose. This use of apple pomace could represent a potentially valuable source of income for the juice processing plants, provided the required chemical and microbiological standards and quality were met.

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