Analytical Methods

Detection of roasted and ground coffee adulteration by HPLC by amperometric and by post-column derivatization UV–Vis detection

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Abstract

Coffee is one of the most consumed beverages in the world. Due to its commercial importance, the detection of impurities and foreign matters has been a constant concern in fraud verification, especially because it is difficult to perceive adulterations with the naked eye in samples of roasted and ground coffee. In Brazil, the most common additions are roasted materials, such as husks, sticks, corn, wheat middling, soybean, and more recently – acai palm seeds.

The performance and correlation of two chromatographic methods, HPLC–HPAEC-PAD and post-column derivatization HPLC–UV–Vis, were compared for carbohydrate analysis in coffee samples.

To verify the correlation between the two methods, the principal component analysis for the same mix of triticale and acai seeds in different proportions with coffee was employed. The performance for detecting adulterations in roasted and ground coffee of the two methods was compared.

1. Introduction

Coffee is one of the most valued basic products, constituting the second major commodity just after oil (ICO, 2012; Nabais, Carrott, Carrott, Luz, & Ortiz, 2008). According to the International Coffee Organization – ICO (2012), the total coffee production in crop year 2011/2012 was about 131.3 million bags (each bag weighing 60 kg), with approximately 33.1% produced in Brazil.

The economic importance of coffee makes it clear that studies related to its composition, quality evaluation, and fraud detection appear to be of greatest significance (Moreira, Trugo, & de Maria, 1997), setting up marketing requirements, especially in an increasingly globalized market, which demands effective product quality control (ABIC, 2012a; ISO, 1995).

The detection of impurities and mixes in coffee is a constant concern, especially in relation to the product quality assurance. A mix, intentional or not, of foreign materials to the product, usually of low-cost, which alter the product quality and can cause damages to consumers, particularly those of economic nature, is considered fraud (Assad, Sano, Correa, Rodrigues, & Cunha, 2002). According to the ISO 3509: Coffee and its products – vocabulary – The International Organization for Standardization, defines “impurities” as any foreign matter, which may be found in coffee like: wood, twigs, husks (or straw), and whole cherries (ISO, 1989). In Brazil, the most frequently substances reported by the literature, added to coffee are: husks and sticks, corn, barley, wheat middling, brown sugar, and soybean (Assad et al., 2002); rye, triticale, and acai may also be added to this list (ABIC, 2012b). According to Bernal, Toribio, Del Alamo, and Del Nozal (1996), the individual determination of carbohydrates has gained significant importance not only for providing compositional information on samples, but also for assisting in the identification of adulterants.

The carbohydrate profile studies, carried out by Blanc, Davis, Viani, and Parchet (1989) for hundreds of samples of commercial soluble coffees using HPLC with UV–Vis detection, enabled to verify the addition of coffee husk extracts at concentrations above 25%. In this study, the concentration of free and total carbohydrates made it possible to evidence frauds by the determination of intentional contamination with coffee husk and ligneous material (sticks) that had caused an increase in the content of mannitol, xylene, glucose, and fructose, as well as to distinguish pure products from adulterated ones by verifying the adulterant nature (Nogueira & Lago, 2009). For roasted and ground coffee the total carbohydrates content are still scarce in the literature (Garcia et al., 2009).

Methods for the liquid chromatographic analysis of carbohydrates often have employed columns of amino-bonded silica-based or of metal-loaded cation-exchange polymer-based. These columns have the advantage of not requiring regeneration after every run. However, columns of metal-loaded cation-exchange require heating presenting low resolution, with restrictions on pH range and
in use of organic solvents (Dionex, 2012). Although, according to Lanças (2004) mobile phases of liquid chromatography represent a powerful tool for manipulation the analyte retention and selectivity, but in this case usually precludes the use of gradients and often requires stringent sample cleanup prior to injection.

For the ion exchange mechanism the main factors influencing the separation are: selectivity of counter ion, solvent, pH and flow (Lanças, 2004). As the pH is main factor for the analyte become completely ionized, it should be adjusted to two units above the \( pK_a \) (for the acid) and two units below the \( pK_a \) (for the basis). If the analyte is in its ionized form, it will be retained by the strongly anion (SAX) stationary phase. Elution is then done, by adjusting the pH of the mobile phase at two units above the \( pK_a \), which will increase the unionized form and will allows the elution of the exchange stationary phase, promoting regeneration of the column (Lanças, 2004).

Analyses of carbohydrates are also difficult to do, due to their structural diversities. The hydroxyl groups of carbohydrates are partially ionized under highly alkaline conditions to form oxoanions, and thus can be separated by the anion-exchange mechanism (Inoue, Kitahara, Aikawa, Arai, & Masuda-Hanada, 2011). Currently, the high performance anion-exchange chromatography (HPAEC), takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH, using a strong anion-exchange stationary phase with electrochemical detection (ED), as a high sensitive detection method for carbohydrates, without the need for prior derivatization (Dionex, 2012). However, a limited number of sorbents are commercially available: on the electrostatically latex-coated pellicular polymeric-based anion-exchange, and in macroporous poly(styrene–divinylbenzene) with trimethylammonium group. An anion-exchange stationary phase prepared from polystyrene-based copolymer and diamine has been reported for separation of aldopentoses and aldohexoses (Inoue et al., 2011).

According to Inoue et al. (2011) separation of \( \alpha \)-aldopentoses (\( \alpha \)-arabinose and \( \alpha \)-xylose – Fig. 1) and \( \alpha \)-aldohexoses (\( \alpha \)-glucose; \( \alpha \)-mannose and \( \alpha \)-galactose) gradually increased in an almost linear manner with the decreasing concentration of the NaOH eluent from 100 to 30 mmol L\(^{-1}\), and below 30 mmol L\(^{-1}\), the retention time ratios steeply increased around 20 mmol L\(^{-1}\) NaOH (pH 12.3), corresponding to the \( pK_a \) values of the aldoses. These results indicate that the dissociated aldoses strongly interact with the quaternary nitrogen atom of the stationary phase, than the competitive hydroxide ions in the eluent. In contrast, at low NaOH concentrations (from 30 to 10 mmol L\(^{-1}\)), were reasonably retained as follows: \( \alpha \)-mannose (\( pK_a \) 12.08) > \( \alpha \)-glucose (\( pK_a \) 12.28) > \( \alpha \)-galactose (\( pK_a \) 12.35).

It is well known that the anomeric hydroxy group of the pyranose form is more acid, that the other hydroxy groups. However, the ionization of the hydroxy groups other than the anomeric one is possible. Koizumi et al. (1992) concluded in his study of positional isomers of methyl ethers of \( \alpha \)-glucose, that the acidity of the monosaccharide is in the following order: 1-OH > 2-OH > 3-OH > 4-OH > 6-OH. Since the individual hydroxy groups of the monosaccharides reveal the different \( pK_a \) values, the ionization of the hydroxy groups other than anomeric one, probably play important roles during elution. So, besides the \( pK_a \) values additional factors for the elution characteristics of carbohydrates should be considered. The aldoses exist as an equilibrium between the pyranoses and furanoses; the percentage composition of the cyclic forms of monosaccharides is given in Table 1.

Usually, in aqueous solutions, aldopentoses and aldohexoses exist primarily in the six-membered pyranose form. But, it is noteworthy that aldoses possessing higher percentage of furanose composition are retained strongly at low NaOH concentrations. This suggested that strong binding ability of fructose with an anion exchange column may be due to their furanose form. These results suggest that the elution behaviour of the aldoses, would probably correlate not only with the \( pK_a \) values, but also with the furanoses forms (Inoue et al., 2011).

In addition, refractive index (RI) and low-wavelength UV detection methods are sensitive to eluent and sample matrix components. These analytical methods require attention to sample solubility and sample concentration (Dionex, 2012). Post-column derivatization is required in HPLC-UV–Vis systems for generating necessary photometrically-active derivatives, since carbohydrates do not possess any conjugated \( \pi \)-bonds, and therefore, they are not directly detectable (Pauli, Cristiano, & Nixdorf, 2011). Despite its simplicity, and considering that in most laboratories HPLC is coupled with UV–Vis detection, the UV–Vis technique has the disadvantage of non-detection of mannitol and the difficulty in quantifying xylose due to its low concentration in coffee (Coutinho, 2003). However, this technique has demonstrated its applicability as a method for initial screening to identify possible adulterants for coffee, despite their low resolution, according to reference values established by AFCASOLE (Pauli et al., 2011).

Unlike the HPLC-UV–Vis method, the ion-exchange chromatographic method, using a strong anion-exchange column coupled with an electrochemical detector and applying pulsed amperometry – high-performance anion-exchange chromatography with...
pulsed amperometric detection (HPAEC-PAD) – has become the ISO 11292 standardized methodology (ISO, 1995) for the determination of free and total carbohydrates found in soluble coffees.

Pulsed amperometry permits detection of carbohydrates with excellent signal-to-noise ratios down to approximately 10 picomol without requiring derivatization. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. At high pH, carbohydrates are electrocatalytically oxidized at the surface of the gold electrode by application of a positive potential. The current generated is proportional to the carbohydrate concentration, and therefore carbohydrates can be detected and quantified. The products of this oxidation reaction also poison the surface of the electrode, which means that it has to be cleaned between measurements. This is accomplished by first raising the potential to a level sufficient to oxidize the gold surface. This cause desorption of the carbohydrate oxidation products. The electrode potential is then lowered to reduce the electrode surface back to gold (Dionex, 2012).

The association of analytical techniques using design, with principal component analysis (Barros Neto, Scarminio, & Bruns, 2003), has been increasingly applied, facilitating the establishing of correlations between various raw materials, based on their chromatographic profiles (Garcia et al., 2009).

This study aims to evaluate the performance and correlation between two different chromatographic systems: HPLC–HPAEC-PAD and post-column derivatization HPLC–UV–Vis, applied for carbohydrate determination (method ISO 11292), following simplex-centroid design, to verify the ability to distinguish a mixture of triticale and acai in arabica coffee.

2. Materials and methods

2.1. Preparation of samples of arabica coffee and adulterants (triticale and acai)

The samples of arabica coffee, triticale, and acai seeds were provided by the Agronomic Institute of Parana (Londrina, Parana State, Brazil). The samples were roasted and ground to achieve a colour similar to that of commercial roasted and ground coffee, presenting a medium roast.

For the adulterant study, sampling followed the simplex-centroid experimental design, represented by an equilateral triangle, with a total of 10 different compositions coded from 1 to 10. The vertices of which, corresponded to the pure matrices. The edges corresponded to the binary mixes of the same proportion; the central point – to the ternary mix with equal proportions; and the three axial points – to the proportions 4:1:1, 1:4:1, 1:1:4. All samples of arabica coffee-triticale-acai were prepared in duplicate for both systems, except for the central point, that samples were prepared in triplicate. The preparation was given by weighing different proportions of the matrices in order to always reach on a dry weight basis 0.3000 g for the analysis by HPLC–HPAEC-PAD, and 0.2000 g for the analysis by post-column derivatization reaction HPLC–UV–Vis. In sequence, samples with the respective weights, according to each method, were hydrolyzed, by transferring to a 500 mL Erlenmeyer with screw-cap, with adding 50 mL of 1.00 mol L\(^{-1}\) hydrochloric acid, and by placing in a water bath thermostated at 85 °C for 180 min, stirring every 30 min manually. After, the solution was cooled down with tap water until room temperature, filtered with a blue-stripe pleated paper into a 100 mL volumetric flask that was completing up to the mark with ultrapure water. An aliquot of 10.0 mL of the solution was passed through a C18 cartridge (Sep Pak, Waters) preconditioned with methanol and water, and in a 0.22 μm nylon membrane (Millipore), collecting the filtrate in vials that were injected into the respective chromatographic systems. For the HPLC–UV–Vis system the extracted samples were neutralized, prior to the injection, due to the narrow range of pH tolerance of the Aminex column. Each aliquot of the mixture of samples (weighed and extracted) in duplicate or triplicate, were then randomly analyzed, by the HPAEC-PAD and by HPLC–UV–Vis (mean values are shown in Table 2). The standards and samples were injected randomly to avoid any tendency of systematic error in the data throughout the day.

For the principal component analysis, the SPSS 18 software (Softonic, Spain) was used.

2.2. Reagents and standards

Sodium hydroxide (50% solution; Fisher, USA and Isotol, Brazil) and hydrochloric acid (p.a grade; F. MAIA, Brazil) were used as solvents for the mobile phase extraction and preparation steps. All water used for the preparation of standards and solutions was purified and filtered with a Milli-Q® system (Millipore, Milford, MA, USA). The mobile phases were degassed with nitrogen prior to use (99.99973% purity cylinder from LINDE, Brazil, with 2nd-stage regulator from Inpagás).

The standards used were: \(\alpha\)-m-anal, \(\alpha\)-arabinose, \(\alpha\)-galactose, \(\alpha\)-glucose, \(\alpha\)-xylose, \(\alpha\)-mannose, \(\alpha\)-fructose, all from Merck (Darmstadt, Germany). Due to high hygroscopicity of carbohydrates, the standards were stored in a glass desiccator under vacuum over phosphorus pentoxide (Merck, Darmstadt, Germany) and utilized only after one week desiccation.

2.3. Standard solutions

2.3.1. HPLC–HPAEC-PAD analysis

For the preparation of the carbohydrate standard stock mix solution, 0.0030 g of mannitol, 0.0300 g of arabinose, 0.1200 g of galactose, 0.0450 g of glucose, 0.0120 g of xylose, 0.0900 g of mannose, and 0.0450 g of fructose were weighed, added to a 100.0 mL volumetric flask and made up to the mark with ultrapure water. The solution was sonicated in an ultrasonic bath for 10 min (Garcia et al., 2009).

The identification and quantification of the carbohydrates were performed on the basis of retention times of components eluted from the column, comparing them the retention times of the components with known concentrations of individual external standards, and by co-chromatography.

<table>
<thead>
<tr>
<th>Aldose</th>
<th>(\phi_{\text{K}}) (25 °C)</th>
<th>Temperature (°C)</th>
<th>Pyranose (\alpha) (%)</th>
<th>Pyranose (\beta) (%)</th>
<th>Furanose (\alpha) (%)</th>
<th>Furanose (\beta) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>12.34</td>
<td>31</td>
<td>60</td>
<td>35.5</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>Xylose</td>
<td>12.15</td>
<td>31</td>
<td>36.5</td>
<td>63</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glucose</td>
<td>12.28</td>
<td>31</td>
<td>38</td>
<td>62</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>Mannose</td>
<td>12.08</td>
<td>44</td>
<td>64.9</td>
<td>34.2</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>12.35</td>
<td>31</td>
<td>30</td>
<td>64</td>
<td>2.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>
For the carbohydrate quantification in the samples, a 10% (v/v) mix of analytical standards was injected into ultrapure water. This standard mix corresponded to the following concentrations in relation to 0.3000 g of sample: 0.10% (w/w) of mannitol, 1.00% (w/w) of arabinose, 4.00% (w/w) of galactose, 1.50% (w/w) of glucose, 0.40% (w/w) of xylose, 3.00% (w/w) of mannose, and 1.50% (w/w) of fructose.

### Table 2

<table>
<thead>
<tr>
<th>Systems</th>
<th>Sample composition</th>
<th>Carbohydrates</th>
<th>Arabinose</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
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<tr>
<td><strong>HPLC–HPAEC-PAD</strong></td>
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</tr>
<tr>
<td>PC100</td>
<td>1.72 ± 0.30</td>
<td>5.59 ± 0.58</td>
<td>0.24 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>7.96 ± 0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT100</td>
<td>1.36 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>30.92 ± 1.49</td>
<td>1.90 ± 0.11</td>
<td>0.45 ± 0.05</td>
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<tr>
<td>PA100</td>
<td>0.15 ± 0.00</td>
<td>0.74 ± 0.01</td>
<td>0.31 ± 0.00</td>
<td>0.68 ± 0.06</td>
<td>14.57 ± 0.74</td>
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</tr>
<tr>
<td>PC50A50</td>
<td>1.00 ± 0.08</td>
<td>3.69 ± 0.22</td>
<td>0.26 ± 0.01</td>
<td>0.39 ± 0.06</td>
<td>10.50 ± 1.63</td>
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<tr>
<td>PC50T50</td>
<td>1.35 ± 0.16</td>
<td>3.18 ± 0.35</td>
<td>10.46 ± 0.17</td>
<td>0.94 ± 0.01</td>
<td>4.22 ± 0.15</td>
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<tr>
<td>PA50T50</td>
<td>0.71 ± 0.06</td>
<td>0.43 ± 0.01</td>
<td>11.83 ± 0.07</td>
<td>1.19 ± 0.04</td>
<td>8.22 ± 1.07</td>
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<td>PC34T33A33</td>
<td>1.01 ± 0.05</td>
<td>2.66 ± 0.19</td>
<td>8.81 ± 1.06</td>
<td>0.87 ± 0.03</td>
<td>7.79 ± 0.90</td>
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<tr>
<td>PC66T17A17</td>
<td>1.25 ± 0.08</td>
<td>3.94 ± 0.13</td>
<td>5.66 ± 0.40</td>
<td>0.43 ± 0.02</td>
<td>7.66 ± 1.03</td>
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<tr>
<td>PT66C17A17</td>
<td>1.07 ± 0.02</td>
<td>1.41 ± 0.01</td>
<td>21.09 ± 1.58</td>
<td>1.30 ± 0.01</td>
<td>4.32 ± 0.15</td>
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<tr>
<td>PA66C17T17</td>
<td>0.62 ± 0.01</td>
<td>1.70 ± 0.01</td>
<td>5.96 ± 0.09</td>
<td>0.75 ± 0.06</td>
<td>9.28 ± 0.47</td>
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<tr>
<td><strong>HPLC-UV–Vis</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>UC100</td>
<td>1.36 ± 0.03</td>
<td>5.62 ± 0.04</td>
<td>0.40 ± 0.03</td>
<td>0.11 ± 0.01</td>
<td>7.79 ± 0.27</td>
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<tr>
<td>UT100</td>
<td>1.12 ± 0.02</td>
<td>0.43 ± 0.00</td>
<td>29.89 ± 1.02</td>
<td>1.81 ± 0.11</td>
<td>0.40 ± 0.01</td>
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<tr>
<td>UA100</td>
<td>n.d.(b)</td>
<td>0.49 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.52 ± 0.03</td>
<td>14.90 ± 0.52</td>
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</tr>
<tr>
<td>UC50A50</td>
<td>0.66 ± 0.01</td>
<td>2.09 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>10.17 ± 0.37</td>
<td></td>
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</tr>
<tr>
<td>UC50T50</td>
<td>0.91 ± 0.02</td>
<td>1.56 ± 0.01</td>
<td>10.45 ± 0.35</td>
<td>0.63 ± 0.04</td>
<td>2.98 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA50T50</td>
<td>0.54 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>13.09 ± 0.47</td>
<td>1.07 ± 0.07</td>
<td>6.46 ± 0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UC34T33A33</td>
<td>0.81 ± 0.01</td>
<td>1.32 ± 0.01</td>
<td>7.64 ± 0.22</td>
<td>0.60 ± 0.03</td>
<td>6.45 ± 0.19</td>
<td></td>
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</tr>
<tr>
<td>UC66T17A17</td>
<td>1.09 ± 0.02</td>
<td>2.33 ± 0.02</td>
<td>3.92 ± 0.13</td>
<td>0.24 ± 0.01</td>
<td>5.90 ± 0.21</td>
<td></td>
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</tr>
<tr>
<td>UT66C17A17</td>
<td>1.09 ± 0.02</td>
<td>1.43 ± 0.01</td>
<td>20.07 ± 0.72</td>
<td>1.15 ± 0.07</td>
<td>4.44 ± 0.16</td>
<td></td>
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</tr>
<tr>
<td>UA66C17T17</td>
<td>0.48 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>4.40 ± 0.15</td>
<td>0.48 ± 0.03</td>
<td>9.84 ± 0.39</td>
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</tr>
</tbody>
</table>

n.d., not detected. Different lower case letters in the same column like “a” and “b”, represent significant difference at 5% (p < 0.05) between carbohydrates contents using the same method. Different upper case letters “A” and “B” in the same column, represent significant difference (p < 0.05) between the amounts of carbohydrates, obtained for the same sample by comparing the two methods of analysis.

2.3.2. Post-column derivatization reaction HPLC-UV–Vis analysis

For the preparation of the carbohydrate standard stock solution, 0.0300 g of glucose, 0.0200 g of xylose, 0.1100 g of galactose, 0.0400 g of arabinose, and 0.0600 g of mannose were weighed, transferred to a 100.0 mL volumetric flask and made up to the mark with ultrapure water. The solution was sonicated in an ultrasonic bath for 5 min (Pauli et al., 2011). The standard was stored in a refrigerator at ~4 °C. This stock solution was diluted to obtain a 25% (v/v) analytical standard, which was injected each quantification day.

2.4. Chromatographic systems

2.4.1. HPLC–HPAEC-PAD

For the chromatographic analysis, an instrumental system was used, which consisted of a PEEK inert liquid chromatograph composed of two Nalgene® bottles for the mobile phase storage; a LC-10Ai inert high-pressure pump (Shimadzu®, Tokyo, Japan); a Research-1367–72 3-way solenoid low-pressure valve with a “lab-made” solenoid valve activation external circuit for eluent exchange; a SIL-Prominence 20A automatic injector (Shimadzu®, Tokyo, Japan); a CarboPac PA1 pre-column and a CarboPac PA1 polystryrene-divinylbenzene-based high-performance anion-exchange resin column (10 μm, 250 mm × 4 mm; Dionex, Sunnyvale, CA, USA); a Waters thermostatic column oven coupled with a 650 CHX temperature controller (Pickering Laboratories); a RS570 current amplifier (Stanford Research System); a dual supply of ±12 V used for activation of an external circuit for electrical signal transmission; an ED-50 electrochemical cell (Dionex, Sunnyvale, CA, USA); an Autolab PGSTAT 30 potentiostat (Eco-Chemie, Utrecht, Netherlands), an Autolab interface (Eco Chemie, Utrecht, Netherlands); a data acquisition and processing system on the basis of Pentium IV working with the GPES (General Purpose Electrochemical System) – Eco software.

For the integration of chromatograms, the INTEGRA software, developed by Professor Dr. Carlos Alberto Paulinetti da Camara (DIA Group, Department of Chemistry, State University of Londrina, Brazil), was employed. It makes use of Eq. (1) to quantify the carbohydrate content (ω), expressed as a percentage of mass, by implementing external standardization:

\[
\omega = \frac{A_{p}V}{A_{m}V_{m}} \times 100
\]

where A – peak area for the individual carbohydrate in the sample; A₀ – peak area for the individual carbohydrate in the standard solution; m – mass of the sample aliquot, expressed on a dry weight basis, in g; m₀ – mass of the carbohydrate in the standard solution, in g; V – volume of the sample, in mL; Vₘ – volume of the standard solution, in mL.
2.5. Chromatographic analyses

For the extraction of the coffee carbohydrates, triticale and acai, the ISO 11292 standardized method (ISO, 1995) was implemented according to the adaptation described above.

The chromatographic conditions used for the two validated HPLC systems are: electrochemical detection HPAEC-PAD: pre-column: CarboPac PA1; column: CarboPac PA1: 250 mm × 4 mm, 10 μm; mobile phase composition: eluent and equilibrium: 1.4 mmol L\(^{-1}\) of NaOH and regeneration: 300.0 mmol L\(^{-1}\) of NaOH; flow rate: 1.0 mL min\(^{-1}\); detection: determination potential: +0.20 V (400 ms), oxidation potential: +0.65 V (200 ms), reduction potential: −0.20 V (400 ms); injection volume: 20.0 μL; column temperature: 28 °C and chromatographic run-time: 72.60 min (Garcia et al., 2009). UV–Vis post-column derivatization: pre-column: SP-1010P; column: Aminex HPX-87P; mobile phase: eluent composition (pump 1) – ABH + NaOH; flow: pump 1: 0.5 mL min\(^{-1}\), pump 2: 0.6 mL min\(^{-1}\); detection: 410 nm; injection volume: 20.0 μL; column temperature: 85 °C, post-column reactor temperature: 100 °C and chromatographic run-time: 25 min (Pauli et al., 2011).

The accuracy for both methods previously cited (HPLC–HPAEC-PAD and HPLC–UV–Vis) was calculated by the recovery rate of analyte, which was done in triplicate, by adding into the sample in proportion of 1:1 (v/v) of standard in low concentration level (50%), medium (100%) and high (150%), according to calibration curve in the dynamic range, calculated by Eq. (2).

\[
\text{rec} (\%) = \frac{C_1 - C_2}{C_3} \times 100
\]  

where, rec (%) = percentage of recovery; \(C_1\) = concentration of analyte in the spiked sample with standard addition; \(C_2\) = concentration of analyte in the original sample without spiked standard; \(C_3\) = concentration of the analyte standard added to the sample spiked.

Results were expressed as mean recoveries from the low, medium and high concentrations levels.

3. Results and discussion

3.1. Evaluation of separation of carbohydrates

For separation system employing HPLC–UV–Vis post-column derivatization, after testing three columns, we chose to use a divalent cation lead – Aminex HPX-87P, as it had the highest resolution compared to the other two – a divalent column of calcium and the other a monovalent of hydrogen. By being cationic, their use required a higher temperature (85 °C) which discourages the interaction, as can be observed by rapidly eluting peaks, impairing resolution (Fig. 3).

The variation of solvent, flow, pH and ionic strength, to improve the selectivity (Laçãs, 2004) were not feasible in these experiments, since the strength of the mobile phase could not be varied; by the fact of Aminex column does not allow the use of organic solvents. The flow rate could not be reduced to increase interaction, since was already low (0.5 mL min\(^{-1}\)). Adding salt for change the ionic strength favoured the competition with the active sites disadvantaging the interaction between the counter-ion of the stationary phase and the carbohydrates, resulting in a worsening in the resolution between the peaks. In this case also, it was not possible ionize the sample, using a pH two points above of the \(pK_a\) of the carbohydrates (12.08–12.35, Table 1), as recommended by Laçãs (2004), since the pH range of this column is restricted to 5.0–9.0. This justify that the hydrolized sample should be neutralize before analysis. Thus, not as many remaining alternatives to the use of different mobile phases, the ultrapure water were adopted. The neutral medium favours broadening and flattening of chromatographic peaks in the ion exchange mode, helping in the inadequate resolution, observed in the chromatogram of the UV–Vis system with a partial co-elution of arabinose and mannose (peaks 4 and 5, Fig. 3).

Adding to this, for sure the major factor contributing in proportion to the low resolution is the way in which the chemical structures of carbohydrates (aldoses) are in the aqueous medium. Table 1 permits us to see that there are higher proportions of pyranose, compared to furanose, once the cycle of six members is thermodynamically more stable in aqueous medium (Inoue et al., 2011). MUTE rotations of the anomeric carbon also in Table 1, shown that there is a predominance of the alpha pyranose form. This is justified by the hydroxyl group in the alpha configuration is pointing down, while in β form hydroxyl is pointing upwards (Fig. 1), so that the aligning two heteroatoms partially suffer repulsion. It was also observed, from the data of Table 1 that in equilibrium in the aqueous medium, there is predominance to the β form for glucose (62%), xylose (63%) and galactose (64%); while is a predominance of α form for arabinose (60%) and mannose (64%), the form more stable and retained in chromatography. Considering the aldopentoses, the arabinose has a superior retention than the xylose, since it has a higher proportion of furanose (2.5%) against (<1%) respectively, agreeing with the work of Inoue et al. (2011) that suggests that better retention is achieved when higher proportion of furanose is present. We can observe that the chromatographic elution occurs according in increasing order of these proportions, getting out from column, first the β-aldoses, followed by alpha-aldoses that are more stable. This agrees with studies of Inoue et al. (2011) showing that the elution behaviours of the aldoses were probably due to not only the individual \(pK_a\) values, but also the chemical structures of the cyclic aldoses.

In order to improve resolution, the use of other columns as a Shim-pack CLC-NH₂ (Shimadzu, Tokyo, Japan), with separation mechanisms based on reverse phase, normal phase, and ion exchange (Chemalink, 2012) were tested. Although the use of this column led to a good separation to the free carbohydrates – sucrose, glucose and fructose, the same efficiency was not achieved for the seven total carbohydrates analyzed in this work. It is intended to continue the search, in order to find a column that presents a best resolution for the system UV–Vis.

Moreover, the HPAEC column, CarboPac PA1, which is strong anion exchange, with pH range of 0–14, allowed using of basic medium employing NaOH solutions. With this, the carbohydrates molecules are ionized, taking the advantage of its weakly acid character to lead to high selectivity in separation (Dionex, 2012). At the lower basis concentrations (<10 mmol L\(^{-1}\), in this work 1.4 mmol L\(^{-1}\)) peaks are eluted from the column in decreasing order of \(pK_a\) for aldopentoses: α-arabinose (12.34) and α-xylose (12.15); and aldohexoses: α-galactose (12.35), β-glucose (12.28) and β-Mannose (12.08) respectively, according to Table 1 and Fig. 2. The HPAEC allows working at low temperatures (28 °C), with more efficiently in interactions, improving also the resolution between the peaks.

However the HPAEC-PAD, requires a specific instrumentation, and requires skilled manpower with knowledge of electroanalytical for proper operation, demands longer time (72.5 min), with an additional step required for regeneration after each run. On the other hand, UV–Vis analysis proves to be faster (25 min), with equipment available in most laboratories, where its use as a screening methodology in routine, becomes an interesting alternative for quality control.
3.2. Evaluation of mix effects on carbohydrate concentrations

When comparing the chromatograms of the standard mixes of the carbohydrates (A) and the pure matrices of arabica coffee (B), triticale (C), and acai (D), distinct characteristics are observed for both the HPLC–HPAEC-PAD (Fig. 2) and the post-column reaction HPLC–UV–Vis (Fig. 3) chromatographic systems, as demonstrated by the mean values of the concentration of total carbohydrates summarized in Table 2.

Using t-test for compare carbohydrates contents in Table 2, almost all of them were significant at the 5% level (p > 0.05). This indicates that results are significant in general, for the same method and for the 2 different methods. For the same method, differences are demonstrated by the different lower case letters appearing in the results “a”, “b”, ..., and for different method by the upper case letter “A” more frequently for HPLC–HPAEC-PAD method, indicating that the absolute concentrations were higher when compared to HPLC–UV–Vis, denoted most by the upper case letter “B”. This can be also seen in Fig. 4, where the two methods show the same trend, but a small shift occurs in the PCA axes. For significant at 10% (data not shown), almost the differences disappeared, as expected because the coefficient of variation are in average of 7% for all carbohydrates studied. These variations agree with those reported in the literature (Dionex, 2012).

On the other hand, the two methods used (HPLC–HPAEC-PAD and HPLC–UV–Vis) were accurate, considering that showed average recovery rates at low, medium and high concentrations levels, calculated by Eq. (2), remaining within the range 93.90–111.00%. Carbohydrates analyzed in the HPLC–HPAEC-PAD system showed the following recovery rates (%) for: arabinose – 96.22%; galactose – 95.86%; glucose – 94.56%; xylose – 93.90% and mannose – 111.00%. While using HPLC–UV–Vis system with post-column reaction the recovery rates were for: arabinose – 103.49%; galactose – 96.65%; glucose – 96.71%; xylose – 100.71% and mannose – 98.73%.

When using the HPLC–HPAEC-PAD system, the predominance for pure arabica coffee can be seen (Fig. 2B), with the highest concentrations found for galactose (peak 3, 5.59% (w/w)) and mannose (peak 6, 7.96% (w/w)) (Table 2), following the same trend as the post-column derivatization reaction HPLC–UV–Vis system (Fig. 3B) that also exhibits the highest concentrations for galactose (peak 3, 5.62% (w/w)) and mannose (peak 5, 7.79% (w/w)) (Table 2).

Although there are few studies reported in the literature on concentration of total carbohydrates for roasted and ground coffee, taking into account other variations, such as cultivar type, farming and harvest conditions, defects, as well as analytical methodology implemented, the total carbohydrate concentration, presented in this work, have confirmed the same trend as shown in the previous studies performed by Oosterveld, Voragen, and Schols (2003b), Garcia et al. (2009), and Pauli et al. (2011). The concentration values are consistent if the breakdown of cell wall coffee components, reported by Buckeridge, Tiné, Santos, and Lima (2000), Fischer, Reimann, Trovato, and Redgwell (2001), Redgwell, Trovato, Curti, and Fischer (2002) and Oosterveld, Harmsen, Voragen, and Schols (2003a), is considered, with predominance of the polysaccharides arabinogalactan and galactomannan, and in a smaller proportion, xyloglucan.

When observing the chromatogram obtained with the HPLC–HPAEC-PAD system for pure triticale (Fig. 2C), it can be noted the appearance of peak 4, with a mean concentration value of 30.92% (w/w) (Table 2), for glucose – the carbohydrate that discriminates this matrix, since this peak is representative neither for coffee, nor for acai. This behaviour is also observed in the post-column reaction HPLC–UV–Vis system (Fig. 3C), where glucose (peak 1) presents a concentration of 29.89% (w/w) (Table 2).

In the case of acai, it can be seen that for the HPLC–HPAEC-PAD system (Fig. 2D), there is a high concentration of mannose (peak 6) with a content of 14.57% (w/w) (Table 2) for the pure matrix; the same chromatographic profile is observed for the post-column derivatization reaction HPLC–UV–Vis system (Fig. 3D), with a content of mannose (peak 5) equal to 14.90% (w/w) (Table 2). Despite the arabinose (peak 4 of Fig. 3) be within its limit of detection by HPLC–UV–Vis system, its content was lower when compared to concentration obtained by HPLC–HPAEC-PAD, as can be seen in Table 2, and as discussed above. So, by owning a small peak, the fact that the peak is not well resolved in relation to the neighbour mannose (peak 5 of Fig. 3D) in this system, may have affected, and can explaining why it was not detected (Table 2), which probably had been covered by the higher proportion of mannose presented by the acai seed, since in others mixtures arabinose could be quantified. In this case is not considered as critical, since arabinose is not used to characterize the studied matrix of coffee, triticale, and neither the acai seeds.

For assessment in conjunction of the discrimination capacity amongst the samples of arabica coffee, triticale, and acai by variable arabinose, galactose, glucose, xylose, and mannose, the principal component analysis (PCA) was implemented for the simplex-centroid mixture design results of each system (Table 2), represented by Fig. 4.

For the HPLC–HPAEC-PAD system, it is observed from Fig. 4A that principal components 1 and 2 together explain 99.00% of the data variance. The analysis of the projection of the variables onto principal components 1 and 2 verified that the most important parameters along the horizontal axis (component 1) with a positive correlation were glucose and xylose, thereby characterizing the adulterant triticale (Fig. 4C), whereas mannose presented a negative correlation along the horizontal axis, characterizing the matrix of the adulterant acai (Fig. 4A and C). Galactose was the most important parameter along the vertical axis (component 2), with a positive correlation, thus characterizing the coffee matrix (Fig. 4A), and the correlations were confirmed by the separation of the pure matrices into distinct groups that can be visualized in Fig. 4C.

When observing Fig. 4B, for the post-column derivatization reaction HPLC–UV–Vis system, it is possible to notice that principal components 1 and 2 together explain 95.90% of the data variance. According the projection of the variables onto principal components 1 and 2 it was verified that the most important parameters along the horizontal axis (component 1) with a positive correlation were glucose and xylose, thereby characterizing the adulterant triticale (Fig. 4C), whereas mannose showed a negative correlation along the horizontal axis, characterizing the matrix of the adulterant acai (Fig. 4B and C). In a similar manner, galactose was found to be the most important parameter along the vertical axis (component 2), with a positive correlation, thus characterizing the coffee matrix (Fig. 4B and C). The separation of the pure matrices into distinct groups can be visualized in Fig. 4C.

Although the carbohydrate concentration values were different for the HPLC–HPAEC-PAD and the post-column reaction HPLC–UV–Vis systems, with lower chromatographic resolution, and explanation of variance for the second method, Fig. 4A and B, demonstrate that there is a great similarity in terms of alignment between the distributions of the carbohydrates, allowing observing correlation with both the adulterants.

The Fig. 4C show the clustered samples of the two systems, where the principal components 1 and 2 together explain 99.00% of the data variance. It can be observed the separation of the pure matrices into distinct groups, as well as the formation of five groups for the matrices containing the mixtures.

Group (1) is affected either by galactose (a characteristic of coffee), or glucose and xylose (characteristic of triticale). Nevertheless,
Fig. 2. Chromatograms of pure carbohydrate matrices using the HPLC–HPAEC-PAD system. (A) Monosaccharide standard – 10% (v/v), (B) arabica coffee – 100%, (C) triticale – 100%, (D) acai – 100%. Peaks: (1) mannitol, (2) arabinose, (3) galactose, (4) glucose, (5) xylose, (6) mannose, and (7) fructose.
as this group presents binary mixtures of coffee and triticale, it is also influenced by the carbohydrate arabinose. In Group (II), there is a predominance of glucose and xylose, being confirmed due to the fact that the ternary mixes show a higher proportion of

Fig. 3. Chromatograms of carbohydrates using the post-column reaction HPLC-UV–VIS system. (A) Monosaccharide standard – 10% (v/v), (B) arabica coffee – 100%; (C) triticale – 100%, and (D) acai – 100%. Peaks: (1) glucose, (2) xylose, (3) galactose, (4) arabinose, and (5) mannose.
triticale. Group (III) has a greater influence on the adulterant acai than on the adulterant triticale, because in both the binary mixes of the two adulterants, and the mixes with a higher proportion of acai, the amount of mannose is more significant than the amounts of glucose and xylose. For Group (IV) with the ternary mix presenting a much higher proportion for the adulterant acai than for the other components, only the influence of the carbohydrate mannose can be observed, making it possible to affirm that there is a direct correlation with this adulterant. And finally, for Group (V), it can be seen that for both the binary mix of coffee and acai, and the ternary mix with a greater proportion of coffee, only the influence of the carbohydrate galactose exists, evidencing the possibility of identifying potential frauds. Considering the results, it is possible to correlate between each other the evaluated systems, because all the parameters followed the same trend.

4. Conclusions

The total carbohydrate analysis performed with the HPLC–HPAEC-PAD and the post-column derivatization reaction HPLC–UV–Vis systems, using the ISO 11292 methodology, was proved effective in determining the concentration of each of the monosaccharides evaluated in roasted and ground coffee and the studied adulterants, triticale and acai, considering the original constituents of different matrices.

From the simplex-centroid experimental design for three components of the arabica coffee-triticale-acai mixes, evaluated for the two chromatographic systems, it was possible to correlate post-column derivatization reaction HPLC–UV–Vis with HPLC–HPAEC-PAD, and the principal component analysis allowed to distinguish the carbohydrates for each of the matrices, showing similar trends. Galactose was a characteristic for the arabica coffee matrix. Glucose and xylose were the predominant carbohydrates in triticale. And finally, mannose characterized the acai matrix at higher concentrations.

The carbohydrate determination by the post-column derivatization reaction HPLC–UV–Vis system, although demonstrating numerically different concentrations, with lower chromatographic resolution, sensitivity, and predictive model fitting, compared to the HPLC–HPAEC-PAD system, was faster and easier operated, and it could be used in most laboratories, considering that they have a UV–Vis detector. Therefore, this system demonstrated a potential to be used for routine screening of adulterants in coffee quality control, since the matrix samples could be grouped and correlated with each distinct carbohydrate. However, for quantification and forecasting by mathematical modelling, the HPLC–HPAEC-PAD technique was shown to be superior, but for that, more expensive, specific and sensitive instrumentation is needed, requiring deeper knowledge in electrochemistry and different precautions from the analyst.
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References