Validation of methodology for simultaneous determination of synthetic dyes in alcoholic beverages by capillary electrophoresis

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Abstract

In this work a method of analysis for synthetic dyes was developed using capillary electrophoresis in alcoholic beverages. The analyses were carried out with fused silica capillary, with 73 cm effective length, at 35 °C, buffer phosphate solution of 10 mmol/L with sodium dodecyl sulphate 10 mmol/L, pH 11, and +25 kV of voltage. For dye analyses, three wavelengths in the visible region were used for the qualitative and quantitative determination of the 11 synthetic dyes allowed in Brazil: 450, 525 and 625 nm for the yellow, red and blue dyes, respectively. The detection limits varied from 0.4 to 2.5 μg/mL and the quantification limits varied from 1.3 to 7.1 μg/mL. The average recovery was 92.6 and 104.0% at two levels of concentration. Repeatability for standards and spiked sample showed that the calculated values were greater than the observed values, demonstrating the precision of the method. The proposed and validated method was used to analyze some alcoholic beverage samples, consisting of 12 red wines, 9 coolers, 6 aromatized spirits, 7 bitters, 3 cocktails and 8 liquors from different Brazilian manufacturers. The results showed the coolers, bitters and red wines did not have synthetic dyes, but dyes were found in six of the eight analyzed liquor samples. In all the samples of cocktails and spirits, the presences of dyes were observed. No analyzed sample exceeded the limit established by Brazilian legislation (maximum 30 mg/100 mL).

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Keywords: Synthetic dyes; Validation; Capillary electrophoresis

1. Introduction

Many methods, using different analytical techniques, have been used to determine synthetic dyes. Spectrophotometric identification methods, based on computational calculations and linear regression quantification, can be found among these techniques [1,2]. However, the most commonly used techniques apply the classic chromatographic methods, such as paper [3,4], thin plate [5,6] and open column chromatography [7,8]. Nevertheless, quantitative analyses using these techniques are very slow and produced data with low accuracy and precision, although the simplicity of the equipment used is indiscernible.

One of the most used techniques for the analysis of synthetic dyes, considered by many researchers as being the most important, is high-performance liquid chromatography (HPLC). This is due to the great development of the equipment and column fillings which makes them more efficient, thus making HPLC a very attractive technique for the control of these additives in foods and beverages [9–16].

In the last few years, capillary electrophoresis (CE) has also been pointed out as a very satisfactory technique for the simultaneous determination of different compounds, among them synthetic dyes [17,18]. This new technique has presented more versatility and simplicity than the other separation techniques used above, in terms of lower costs, greater column resistance, shorter analysis time and less sample volume [17,19,22].

Nowadays, electrophoresis is a generic name given to a series of separation techniques that involve the application of an electric field in a capillary filled with a buffer solution [23]. Micellar electrokinetic chromatography (MEKC), for example, was initially created for the resolution of neutral compounds which cannot be separated using simple capillary electrophoresis.
by zone, where the separation principle is based on the presence of charges. Lately, however, surfactants have been used to solve separation problems, not only for neutral compounds, but also for electrically charged compounds, such as synthetic dyes [18,17,23,24].

The separation conditions involve the use of electrolytes containing levels of surfactants, such as sodium dodecyl sulphate (SDS), above a determined concentration called critical micellar concentration (CMC), where the molecules of the surfactant begin to aggregate forming micelles. The separation is based on the partition of the molecules between the micellar phase and the running buffer. The SDS micelles are negatively charged and migrate against the electroosmotic flow (EOF). However, the EOF is strong enough to force the micelles to go through the detector. Positively charged species are retarded by the association with negatively charged micelles. Neutral molecules or with low polarity have a participation between the micellar and buffer solution phases and have intermediate mobility, while negatively charged molecules are repelled by the micelles [23]. The separations are carried out in pHs where there is a reasonable EOF, in general with pH higher than 7 [25].

The synthetic dyes used in foods and beverages are predominantly azo and triphenylmethane compounds. They contain carboxyl and sulphonic groups, apart from hydroxyls, which enable the formation of negatively charged ions in basic pH values [26], making CE an ideal tool for the analysis of these compounds. Despite this, there are a few developed methods for dye analysis and little data on the performance of the methodology used on these compounds, therefore, making not only the development, but also the validation of the methodology applied in these techniques, essential.

An analytic method can be evaluated in many different ways, which will depend on the objective of the analysis. To develop, evaluate and validate an analytical method many procedures are required, and the most important ones are accuracy, precision, specificity, and sensitivity [27–35].

The objective of the present work is to develop, validate and apply a methodology using MEKC for the analysis of synthetic dyes used as beverage additives.

2. Experimental

2.1. Materials and equipment

2.1.1. Samples

So as to validate the method, a matrix among the beverages was needed, which would present a more complex composition, even with the presence of natural colors. Therefore, red wine was chosen for the tests. After validation, the method was used in different samples of alcoholic beverages, consisting of 6 aromatized spirits, 9 coolers, 3 cocktails, 7 bitters, 8 liquors and 12 red wines from different Brazilian manufactures. Three different lots of each product, in duplicate, were analyzed. Each lot consisted of the contents of two homogenized bottles. The samples were degassed by mechanical agitation and filtered with a cellulose ester, HAWP0013 (Millipore) membrane (0.45 μm pore), with a syringe system.

2.1.2. Standards

The synthetic dye standards, tartrazine (E-102), sunset yellow (E-110), azorubine (E-122), bordeaux S (E-123), ponceau 4R (E-124), eritrosine (E-127), red no 40 (E-129), patent blue V (E-131), indigo carmin (E-132), brilliant blue FCF (E-133) and fast green (E-143) were obtained from Importadora Brastôquio Ltda. All of the standards presented over 85% purity levels. To prepare the stock solution, all of the standards were used together, with a 400 μg/mL concentration of each dye, and from this smaller concentration diluted solutions were prepared. The synthetic dye solutions were prepared using water from the Milli-Q (Millipore) system.

2.1.3. Buffer solution preparation

The buffer solution used in the CE system was prepared with 10 mmol/L phosphate (Na₂HPO₄·12H₂O) and 10 mmol/L SDS, adjusted with NaOH for pH 11 in a 100 mL recipient. The solution was filtered with cellulose ester, HAWP0013 (Millipore), 0.45 μm pore diameter, before being used.

2.1.4. Equipment

For the CE analyses, a Prince Technology capillary electrophoresis series 600, with detector UV/VIS Lambda series 1000, was used. The analyses were carried out with fused silica capillary, with 73 cm effective length, at 35 °C, 10 mmol/L buffer phosphate solution with 10 mmol/L SDS, pH 11, and +25 kV voltage. The samples were introduced into the equipment using a hydrodynamic injection with the application of 35 mbar of pressure for 9 s. The acquisition and treatment of data was carried out through the Prince Technology software program 4880.

2.2. Methods

2.2.1. Analytical methodology

After the filtration and degasification of approximately 200 mL, the samples were introduced into the equipment, using a pressure hydrodynamic system. The synthetic dyes in the CE were separated by a silica capillary filled with a phosphate buffer solution described previously, applying +25 kV voltage to the system, resulting in a 53 μA electrophoretic current. The temperature of the system was maintained at 35 °C with forced ventilation. For the analyses of the dyes, a UV–vis detector was used and three injections were necessary, as the electropherograms were being monitored at different wavelengths, 450 nm for yellow dyes, 525 nm for red dyes, and 625 nm for blue dyes. The identification of the dyes was carried out by comparing the migration times obtained with standards analyzed under the same conditions and by addition of standards to the samples. The quantification of the dyes was carried out through external standardization, with curves constructed with 7 concentration levels, each point being represented by the average of three determinations [36].

A system clean-up, between injections was developed and introduced into the methodology in order to minimize possible interference due to previous sample residue. The best results were obtained with the passage of a sequence of solutions, starting with 1 mol/L NaOH, followed by 0.2 mol/L NaOH and water,
for the elimination of the sodium hydroxide, ending with a phosphate buffer solution. All solutions were left for a time equal to 1 min each and with 1000 mbar of pressure.

2.2.2. Methodology validation

The detection limits were first established using standards, successively diluted to determine the lowest detectable quantity, approximately near which represents the \( S \) sign three times the equipment noise \( R \) \( (3 \times S/R) \). Concentrations near, above or below the detection limit found for each dye standard, were added to the wine. After the addition of the dyes to the wine, the samples were analyzed under the same conditions previously described, and the detection limits were determined. The quantification limits were considered to be the concentrations corresponding to 10 times the sign/noise relation value \( (10 \times S/R) \) for each dye [32,37,38].

A recovery test with standards added in red wine was carried out in duplicate, at two different concentration levels [36]. The repeatability was determined from 10 duplicates of synthetic dyes standard solutions and those solutions were added to the red wine. It was calculated according to Caulcutt and Boddy [35], using the following formula:

\[
R = t(2\sigma)^{1/2}
\]

where \( t \approx 2.26 \) (according to Caulcutt and Boddy, Table B); \( R \) the repetition, with 95% of significance; \( \sigma \) estimated standard deviation.

3. Results and discussion

Table 1 presents values obtained for the limits of detection and quantification for 11 synthetic dyes added to the wine. The values are comparable to those presented in literature [18,24], and have been shown to be inferior to those presented by Frazier et al. [17].

Sample quantification has been carried out by external calibration. The linear regression for synthetic dyes at a 0.5–400 \( \mu g/mL \) concentration. Through the correlation coefficient \( (r) \), good linearity can be observed in the working range adopted with values beside 0.9941 to 0.9999.

The average recovery (Table 2), for 11 dyes analyzed, was 92.6 and 104.0\%, at two levels of concentration in the red wine. The values of repeatability are presented in Table 3. These data showed a good recovery, in the levels here analysed. The recovery percentages found in this work are higher than those by Graichen [39] and Graichen and Molitor [8], but similar to the recovery found by Prado and Godoy [32] and Ashkenazi et al. [40] although these authors used the HPLC. Only Nevado et al. [18] carried through recovery tests for synthetic dyes in CE, and observed slightly superior levels to the ones presented in Table 4.

Based on mathematical calculations, so that the methodology can present good levels of repeatability, it is necessary that the difference between the observed values (standard deviation) must be smaller than the values calculated by equation \( R = t(2\sigma)^{1/2} \) [35]. It can be observed from Table 4 that the methodology presented showed good repeatability levels with smaller average values than the calculated ones (values). No repeatability values for methods using CE for synthetic dyes were found in works revised (Table 3).

Fig. 1 shows synthetic dye standard electropherograms added to the wine samples. The electropherograms in the three wavelengths presented in this work were overlapped for better visualization.

Even though the migration times seemed higher to those presented in literature [18], few are the works that use mixtures with more than six dyes [17,18,20,21]. In the methodology presented here, a mixture of 11 dyes was used, that is, all of the synthetic dyes allowed by the Brazilian legislation. Therefore, analysis time is expected to be higher, due to the greater number of compounds analyzed.

### Table 1
Limits of detection (LOD) and limits of quantification (LOQ) determined in the wine with added standards

<table>
<thead>
<tr>
<th>Dyes (µg/mL)</th>
<th>E-102</th>
<th>E-110</th>
<th>E-122</th>
<th>E-123</th>
<th>E-124</th>
<th>E-127</th>
<th>E-129</th>
<th>E-131</th>
<th>E-132</th>
<th>E-133</th>
<th>E-143</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (3 × S/R)</td>
<td>2.0</td>
<td>2.5</td>
<td>1.3</td>
<td>0.8</td>
<td>0.8</td>
<td>0.5</td>
<td>1.5</td>
<td>0.4</td>
<td>1.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>LOQ (10 × S/R)</td>
<td>6.6</td>
<td>8.3</td>
<td>4.3</td>
<td>2.7</td>
<td>2.7</td>
<td>1.7</td>
<td>5.0</td>
<td>1.3</td>
<td>5.0</td>
<td>2.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Means of 10 determinations.

### Table 2
Recovery of the standards added to the wine in two different concentration levels

<table>
<thead>
<tr>
<th>Levels</th>
<th>Dyes</th>
<th>E-102</th>
<th>E-110</th>
<th>E-122</th>
<th>E-123</th>
<th>E-124</th>
<th>E-127</th>
<th>E-129</th>
<th>E-131</th>
<th>E-132</th>
<th>E-133</th>
<th>E-143</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (µg/mL)</td>
<td>24.9</td>
<td>25.9</td>
<td>25.7</td>
<td>25.2</td>
<td>24.8</td>
<td>25.2</td>
<td>25.1</td>
<td>24.4</td>
<td>25.7</td>
<td>25.1</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>100</td>
<td>104</td>
<td>103</td>
<td>101</td>
<td>99</td>
<td>101</td>
<td>100</td>
<td>98</td>
<td>103</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>II (µg/mL)</td>
<td>50.4</td>
<td>52.0</td>
<td>51.9</td>
<td>50.5</td>
<td>49.7</td>
<td>50.4</td>
<td>46.2</td>
<td>51.0</td>
<td>49.8</td>
<td>50.9</td>
<td>50.9</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>101</td>
<td>104</td>
<td>104</td>
<td>101</td>
<td>99</td>
<td>101</td>
<td>93</td>
<td>102</td>
<td>100</td>
<td>102</td>
<td>102</td>
<td></td>
</tr>
</tbody>
</table>

I and II are the two different concentration levels (µg/mL).

* Means of duplicate determinations.
Table 3
Repeatability of dyes added in red wine and standard solution

<table>
<thead>
<tr>
<th>Dyes</th>
<th>E-102</th>
<th>E-110</th>
<th>E-122</th>
<th>E-123</th>
<th>E-124</th>
<th>E-127</th>
<th>E-129</th>
<th>E-132</th>
<th>E-133</th>
<th>E-131</th>
<th>E-143</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/mL)a</td>
<td>25.2</td>
<td>25.8</td>
<td>25.5</td>
<td>24.6</td>
<td>25.7</td>
<td>26.4</td>
<td>25.9</td>
<td>25.8</td>
<td>25.4</td>
<td>25.4</td>
<td>25.2</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Repeatability</td>
<td>2.3</td>
<td>2.0</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
<td>2.0</td>
<td>1.8</td>
<td>2.5</td>
<td>1.0</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/mL)a</td>
<td>25.2</td>
<td>26.0</td>
<td>26.0</td>
<td>24.8</td>
<td>25.5</td>
<td>25.5</td>
<td>24.9</td>
<td>25.2</td>
<td>25.8</td>
<td>25.1</td>
<td>25.5</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.7</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.7</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Repeatability</td>
<td>2.7</td>
<td>2.0</td>
<td>1.8</td>
<td>1.0</td>
<td>1.0</td>
<td>1.8</td>
<td>1.4</td>
<td>2.7</td>
<td>1.4</td>
<td>1.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

95% significance.

a Means of 10 determinations.

Table 4
Qualitative and quantitative (µg/mL) composition of synthetic dyes in alcoholic beverages

<table>
<thead>
<tr>
<th>Products</th>
<th>Samples</th>
<th>Dyes (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E-102</td>
</tr>
<tr>
<td>Aromatized spirit</td>
<td>1</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>39.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>33.4 ± 0.7</td>
</tr>
<tr>
<td>Cocktail</td>
<td>24</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>13.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>42.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>36.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>36.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>30.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>54.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>39.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>19.9 ± 0.4</td>
</tr>
<tr>
<td>Liquor</td>
<td>27</td>
<td>16.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>28.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>42.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>42.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>29.7 ± 0.6</td>
</tr>
</tbody>
</table>

Means and estimated standard deviation (n = 6).

Fig. 1. Electropherograms of a wine sample added with synthetic dye standards, solution standards 25 µg/mL, operation conditions: 10 mmol/L buffer phosphate and 10 mmol/L SDS (pH 11); 25 kV and 35 °C.

It was decided to work with the visible region instead of the ultraviolet one, which is used more in literature [17,18,20,21] due to the low number of interference substances presented. Alcoholic beverages, wines and liquors specially, have a great number of compounds that absorb light in the ultraviolet region, therefore tests carried out in the methodology development initial steps showed that the simple change in the absorption region is enough to eliminate these interferences.

The use of phosphate as a running buffer was proven to be very efficient for the separation of synthetic dyes. Although in literature [17,18,37] many buffer solutions can be found, the most common ones being borate, carbonate and phosphate, the use of the first two did not result in a satisfactory separation. The use of pH 11 also proved to be very efficient in separating these compounds enabling an easier migration when a difference in potential is applied [18,37]. At this pH, all the structures were ionized. This occurs due to the presence of sulphonic acid groups in the molecules which dissociate at pH > 4 in sulphonic cations, and carboxylic groups that only dissociate at pH > 7. With the
increase in pH (pH > 10) phenolic groups, present in E-110, E-123 and E-124 dyes for example, also dissociate and improve the separation [26].

Even with an increased pH value, the use of SDS in the buffer solution composition was indispensable for the separation of the synthetic dyes, especially for the red dyes which present great similarity in their structures, where two of them, E-123 and E-124, are position isomers. This separation has been described in other works, where the use of surfactants presented improvements in the separation of these compounds [17,18,24,41].

The clean-up procedure applied in this methodology is very satisfactory and confirmed observations made by Frazier et al. [17] and Nevado et al. [18] for the use of NaOH for the cleaning of the capillaries [17,18].

The quantification data, only for the samples in which the presence of synthetic dyes was detected, are presented in Table 4. Brazilian legislation prohibits the copying or artificial production of wine, as well as the production of enocianine in the wineries, article 33 and article 39, respectively [42]. This means that neither wine, nor enocianine can be produced to be added to their own wine, it must be bought from third parties and the cost in Brazil is quite high. Due to this, the producer could be induced to add synthetic dyes to their product, as their cost is far lower than the natural ones. But none of the 12 red wine samples analyzed presented synthetic dyes in their composition, which indicates that this practice has not been adopted by wineries in Brazil. No synthetic dyes were found in the coolers samples as well.

Synthetic dyes were found in all in the entire cocktail and aromatized spirits samples. However in the liquors, six out of eight analyzed samples presented dyes. However, in none of the samples were the levels above the limits allowed by the Brazilian legislation, the limit established is 30 mg/100 mL or 30 mg/100 g.

No synthetic dyes were found in the aperitifs. Only one sample presented a dye, which could not be identified, suspected of being carmine (E-120) (not confirmed), but which was stated on the label by the producer. The confirmation, however, could not be carried out due to the lack of standard. Nevado et al. [18] in their work presented a method for dyes by CE where they determine synthetic dyes together with E-120, demonstrating that a methodology for simultaneous analysis for natural and synthetic colors would be possible. The electropherograms of three analyzed samples are presented in Fig. 2.

4. Conclusions

The use of dyes by the beverage industries has proven to be within the regulations set by the Brazilian legislation, demonstrating a control in the fabrication process.

The capillary electrophoresis method applied for the determination of synthetic dyes in alcoholic drinks here presented has turned out to be very satisfactory in the separation of these additives. Efficiency data can be demonstrated through the analysis of the recovery and repeatability values, and detection and quantification limits values, recommending the application of the methodology to determine the presence of synthetic dyes in beverages. The technique was very simple and efficient to be applied, without the need for previous treatment of the samples, making the analyses easier.

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