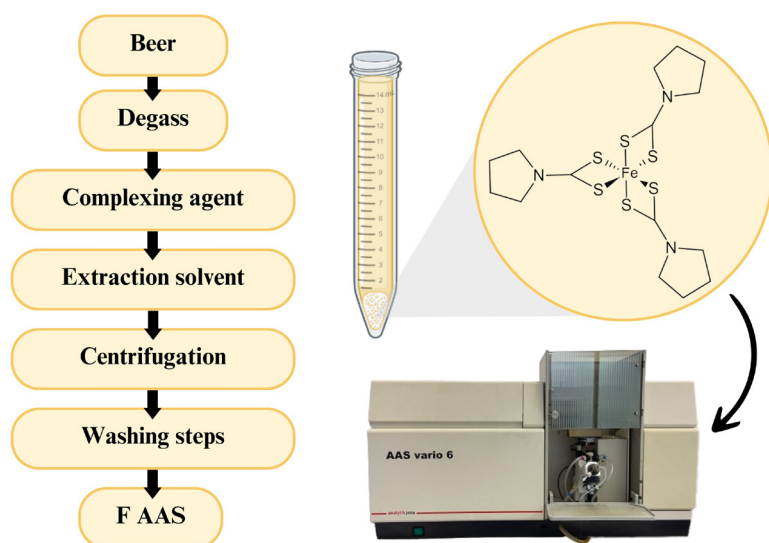


ARTICLE

# A Simple Approach for Fe Determination in Beer by using Dispersive Liquid-Liquid Microextraction and Flame Atomic Absorption Spectrometry

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In this work, a new method for Fe extraction and preconcentration from beer by using dispersive liquid-liquid microextraction (DLLME) and determination by flame atomic absorption spectrometry (F AAS) was developed. Although in DLLME the use of an extraction solvent is mandatory (at least in theory), which is combined with a disperser solvent, in this work only the extraction solvent was used. For Fe extraction by DLLME, the type and volume of extraction solvent, sample volume, type of complexing agent, sample pH, salting out effect, and the number of washing cycles after extraction were evaluated.

Thus, after optimization, 10 mL of beer, 75  $\mu\text{L}$  of 1,2-dichlorobenzene of extraction solvent, 500  $\mu\text{L}$  of 0.5% (w/v) pyrrolidine ammonium dithiocarbamate (APDC) as complexing agent were used. It is important to mention that pH adjustment, salt addition or washing cycles were no required. For Fe determination after DLLME, an external calibration curve was performed ( $R^2 > 0.99$ ) by direct extracts (reference solutions through DLLME) introduction in the conventional nebulization system of F AAS instrument. The accuracy evaluation was performed by results comparison with those obtained after microwave-assisted digestion (MAD) and Fe determination by inductively coupled plasma optical emission spectrometry (ICP-OES). The Fe concentration in the studied samples ranged from 59.7 to 68.2  $\mu\text{g L}^{-1}$  and the limit of quantification for DLLME/F AAS method was 9.0  $\mu\text{g L}^{-1}$ . Finally, the proposed method DLLME/F AAS was applied for Fe determination in several beer samples showing simplicity, low operational costs, suitable accuracy (104 to 119%) and precision (better than 13%), opening the possibility for its use in routine analysis for Fe determination in beer.

**Keywords:** DLLME, iron, beer, F AAS, extraction

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## INTRODUCTION

Currently, beer is one of the most consumed alcoholic beverages worldwide and in Brazil it is the most consumed.<sup>1</sup> The production of beer starts in Mesopotamia, where they cultivated and fed on barley grain.<sup>2</sup> Nowadays, beer is a beverage obtained by the alcoholic fermentation of the brewing wort derived from barley malt in drinking water with the action of yeast and the addition of hops. These raw materials contribute to its complex flavors and to the presence of nutrients, such as proteins, vitamins, organic acids such as ethanol, as well as minerals as Ca, K, Fe, P, Mg and Na.<sup>3</sup>

Iron is the second most abundant metal in the earth's crust, playing primary roles in the physiological and biological environment of animals and plants, as well in the industry and technological environment.<sup>4</sup> The presence of some elements in beer, such as Fe, interferes from the brewing process, where yeast growth can be affected, up to the taste of the final product. The increase of Fe occurrence can be due to the transportation and equipment used in brewing.<sup>5,6</sup>

Due to the presence of organic compounds in beer composition, a sample preparation step is mandatory for further element determination, mainly when they are present at low concentrations.<sup>7</sup> Conventional acid digestion methods (microwave or convective heating in open or closed systems) can provide the digestion of complete matrices, but requiring long digestion time and high reagent consumption. In addition, the sample dilution during and after digestion procedure is an effect inherent to the process, which worsens the limit of quantification (LOQ) of the method.<sup>8</sup> Alternatively, extraction methods can eliminate some steps of the conventional digestion methods which minimizes some disadvantages of such methods, as the low sample mass required (mainly in closed systems), reduction of dilutions and interferences and allow the determination at low concentrations.<sup>9,10</sup> In this way, dispersive liquid-liquid microextraction (DLLME) is a suitable alternative method, which consists of a ternary system of solvents (liquid sample, extraction solvent and disperser solvent) resulting in two immiscible phases after extraction. The solubility of the analytes in both phases is due to by the relative polarity of the system, with the analytes being generally non-polar and thus showing greater affinity for the organic phase.<sup>11-13</sup>

According to the literature, among the various preconcentration methods applied for liquid samples, DLLME has been widely employed in recent years in view of its efficiency for extraction of elements<sup>14,15</sup> or organic compounds<sup>16</sup>. Some advantages regarding to preconcentration, miniaturization, low reagent consumption, and high selectivity during extraction can be highlighted. On the other hand, the use of chlorinated extraction solvents is still a problem.<sup>17</sup> Although there are several applications of DLLME for liquid samples, no studies regarding to the Fe extraction in beer were found in the literature. It is important to emphasize that there is only one application for element determination (Cu) in beer after DLLME and it was proposed by our research group.<sup>18</sup> Thus, the present study aims to develop a simple, fast, and low-cost method for subsequent Fe determination by F AAS after DLLME.

## MATERIALS AND METHODS

### *Instrumentation*

A flame atomic absorption spectrometry (F AAS) instrument (Analytik Jena GmbH - AAS vario 6, Germany) was used for Fe determination. It was equipped with a deuterium lamp for background correction and a hollow cathode lamp (HCL) with wavelength set at 248.3 nm as radiation source. The following spectrometer conditions were used: HCL current set at 6.5 mA, spectral bandpass of 0.2 nm, and flame composed by a mixture of air and acetylene.

An analytical balance (model BEL, engineer, MARK M214A) with maximum capacity of 220 g and resolution of 0.0001 g was used for weighing. A pHmeter (model 781 pH/Ion Meter, Metrohm, Switzerland) equipped with a glass electrode and a temperature sensor (resolution of 0.01 pH units) was used for the sample pH adjustment. An ultrasonic bath (model Elma/DE-78224, type: TI-H10) was used for sample degassing, useful volume of 8.6 L, frequency of 130 kHz and amplitude up to 100%. A centrifuge (model Q-222T208, Quimis, Brazil), with a maximum capacity of 8 vessels of 15 mL capacity, was used for phases separation after DLLME.

For results comparison, samples were digested in a microwave-assisted digestion system (Multiwave Pro®, Anton Paar, Austria), equipped with a rotor with capacity of 8 high-pressure quartz closed vessels with internal volume of 80 mL, maximum temperature, pressure, and power of 280 °C, 80 bar, and 1400 W, respectively. Iron was determined using an axial-view inductively coupled plasma optical emission spectrometer (Spectro Ciros CCD, Spectro Analytical Instruments, Germany) at 238.204 nm, equipped with a cross-flow nebulizer, a double-pass glass spray chamber, and a quartz torch with quartz injector tube. The plasma, auxiliary, and nebulizer gas flow rates were 12, 1.0 and 1.00 L min<sup>-1</sup>, respectively, and the radio frequency power was set at 1500 W.

### **Reagents and standards**

Water was distilled, deionized, and then purified in a Milli-Q® system (18.2 MΩ cm, Millipore Corp., USA). All the glassware materials were previously decontaminated in a 20% HNO<sub>3</sub> (v/v) solution, for at least 24 h.

Solutions for external calibration of F AAS instrument were prepared from a monoelement stock solution (Titrisol Merck, 1000 mg L<sup>-1</sup> Fe) as Fe(III), which was diluted for preparing a 10 mg L<sup>-1</sup> in H<sub>2</sub>O, and then the calibration solutions were also prepared in water and ranged from 50 to 250 µg L<sup>-1</sup>. A mixture of gases acetylene (99.8% of purity, White Martins, Brazil) and compressed air, as fuel and oxidant, respectively, was used. High purity extraction solvents, such as carbon tetrachloride (CCl<sub>4</sub>), dichlorobenzene (C<sub>6</sub>H<sub>4</sub>Cl<sub>2</sub>), tetrachloroethylene (C<sub>2</sub>Cl<sub>4</sub>), chloroform (CHCl<sub>3</sub>), and monochlorobenzene (C<sub>6</sub>H<sub>5</sub>Cl) were evaluated. As complexing agents, sodium diethyldithiocarbamate (DDTC) and ammonium pyrrolidinedithiocarbamate (APDC) were evaluated and prepared from the dissolution of the solid reagent in ultrapure water. For the pH adjustment, solutions of 0.1 mol L<sup>-1</sup> NH<sub>4</sub>OH (Merck, Germany) and 0.1 mol L<sup>-1</sup> HCl (Merck) were used.

For the reference method, a mixture of HNO<sub>3</sub> (Merck, 65%, 1.4 kg L<sup>-1</sup>) and HCl (Merck, 37%, 1.39 kg L<sup>-1</sup>) was used, both acids were previously purified in a sub-boiling distillation system (DuoPur, Milestone, Italy).

### **Beer samples**

Three beer samples of different brands were selected and purchased in the local market (Santa Maria, Rio Grande do Sul State, Brazil). The samples were identified as B1 (Pilsen type, 4,8% alcohol content), B2 (Lager type, 5% alcohol content), and B3 (Pilsen type, 5,2% alcohol content). Sample B1 was used for DLLME optimization.

### **Microwave-assisted digestion**

For MAD method, about 5 mL of beer were introduced into the quartz vessels followed by the addition of 2 mL of 14.4 mol L<sup>-1</sup> HNO<sub>3</sub> and 1 mL of 12 mol L<sup>-1</sup> HCl. After, the vessels were closed and fixed in the rotor, and an irradiation program was applied (15 min at 1400 W and 20 min at 0 W for cooling). The digests were diluted up to 15 mL with water and Fe was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

### **Proposed DLLME method**

Previously to the DLLME procedure, samples were degassed in an ultrasonic bath for 1 h at room temperature.

The most important parameter that influences in DLLME, such as type (CCl<sub>4</sub>, C<sub>6</sub>H<sub>4</sub>Cl<sub>2</sub>, C<sub>2</sub>Cl<sub>4</sub>, CHCl<sub>3</sub>, and C<sub>6</sub>H<sub>5</sub>Cl), and volume (25, 50, 75, 100, 125, 150, and 200 µL) of extraction solvent, sample volume (5, 7.5, 10, and 12.5 mL), type (DDTC or APDC) and concentration (0.5, 1.0, 2.0 and 3.0% m/v) of complexing agent, sample pH (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5), and number of washing steps (0, 1, 2, 3, and 4) were evaluated. It should be noted that the disperser solvent was not used in this work, as described in a previous study.<sup>18</sup> It is important to point out that the disperser solvent was not used because when it was injected (mixed with the extraction solvent), a solid material was formed after centrifugation, impairing the aspiration and nebulization by the F AAS system. Surprisingly, when the disperser solvent

was avoided, the cloudy solution was observed and the phases separation provided a clear extract and feasible to be analyzed.

About 5 to 10 mL of beer were transferred to a 15 mL glass tube with conical bottom, followed by adding of 500  $\mu$ L of complexing agent and the fast injection of extraction solvent. After that, the tube was manually stirred by a few seconds. The cloudy solution was centrifuged for 4 min at 3500 rpm. After centrifugation, the supernatant phase was removed and the extract was washed with ultrapure water for eliminating the potential interferences from sample matrix or reagents.<sup>19</sup> Then, the tube was filled with ultrapure water up to about 10 mL, for further Fe determination by F AAS. After the evaluation of all the parameters, a calibration curve was applied by using the optimized conditions of the DLLME, allowing the quantitative analyte determination.

The LOQ ( $10\sigma$ ) value was estimated for the proposed DLLME method using the calibration curve approach, which was calculated as ten times the standard deviation of ten measurements of a blank, plus the mean of the blanks.<sup>20</sup>

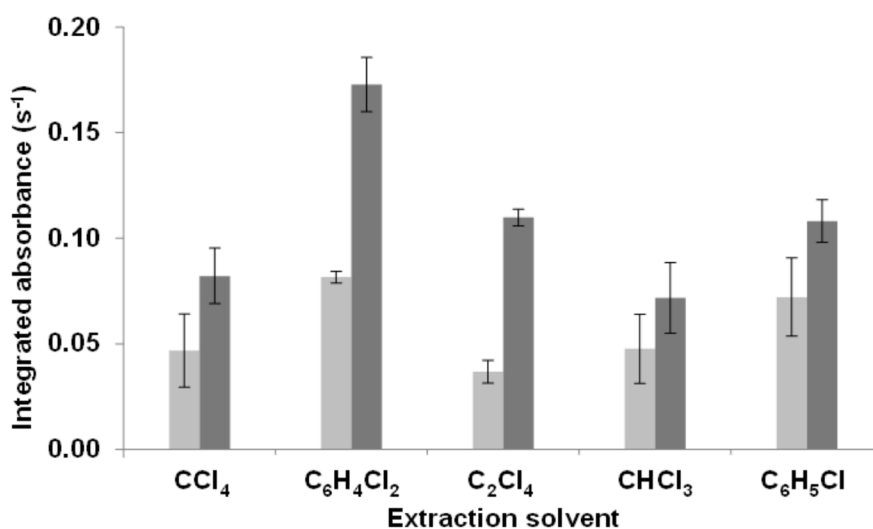
The t-student test (95% confidence level) was used to compare two measurements and ANOVA test (95% confidence level) was used for the comparison of three or more averages. All the statistical evaluations were carried out using the InStat program (GraphPad software).

## RESULTS AND DISCUSSION

In general, DLLME procedures preconize the use of a disperser solvent. However, this type of solvent was not used because of a physical interference, as mentioned before. In addition, it is important to note that during DLLME optimization, all the measurements in the F AAS instrument were performed in integrated absorbance (peak area), considering that a discrete volume of the extract was injected into the nebulization system, generating a transient signal.

### Evaluation of type and volume of extraction solvent

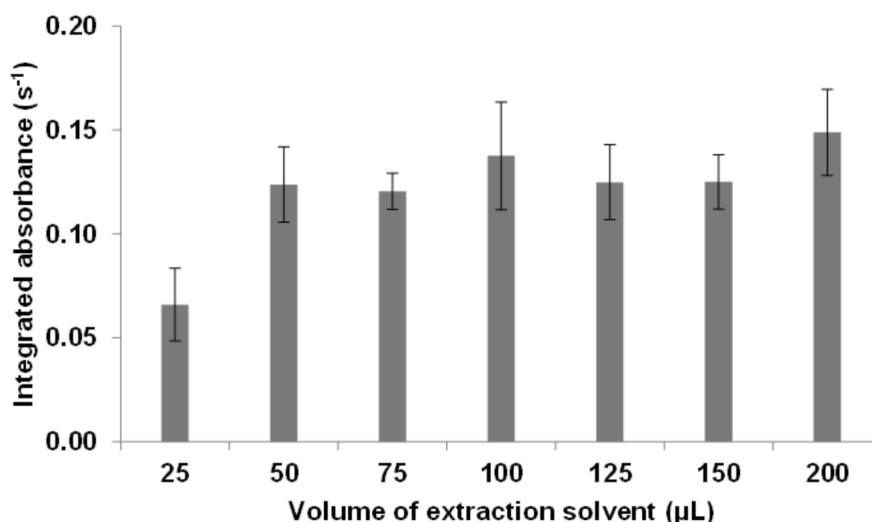
Five extraction solvents ( $\text{CCl}_4$ ,  $\text{C}_6\text{H}_4\text{Cl}_2$ ,  $\text{C}_2\text{Cl}_4$ ,  $\text{CHCl}_3$ , and  $\text{C}_6\text{H}_5\text{Cl}$ ) were evaluated and the extraction was performed by using 5 and 10 mL of beer. Results are shown in Figure 1.



**Figure 1.** Evaluation of the extraction solvents on Fe extraction (light gray bar corresponds to 5 mL and dark gray bar to 10 mL of sample) ( $n=7$ ). Conditions: 500  $\mu$ L of complexing agent (1% DDTC), 100  $\mu$ L of extraction solvent, 4 min of centrifugation at 3500 rpm, and 2 washing steps.

It is well known that the type of extraction solvent in DLLME is important to obtain efficient extraction. According to the results shown in Figure 1, the signal for Fe obtained by F AAS using  $C_6H_4Cl_2$  as extraction solvent was higher in both volumes of sample and the relative standard deviation (RSD) values were below 10%. It should be noted that  $C_6H_4Cl_2$  present high hydrophobicity, as well as the complex formed between Fe and DDTC. Therefore, the interaction between  $C_6H_4Cl_2$  and the complex of  $Fe^{3+}$  and DDTC is more effective compared to the other evaluated solvents.<sup>21</sup> Thus,  $C_6H_4Cl_2$  was selected as extraction solvent for further experiments. Regarding to the sample amount, as expected the higher volume (10 mL) provides higher absorbance values and no increase in the background signal was observed. Thus, 10 mL of beer were chosen for next experiments.

In Figure 2 is shown the effect of the volume of  $C_6H_4Cl_2$  on Fe signals. This evaluation was performed using 10 mL of sample and 500  $\mu$ L of 1% (w/v) DDTC, changing the volume of  $C_6H_4Cl_2$  from 25 to 200  $\mu$ L.

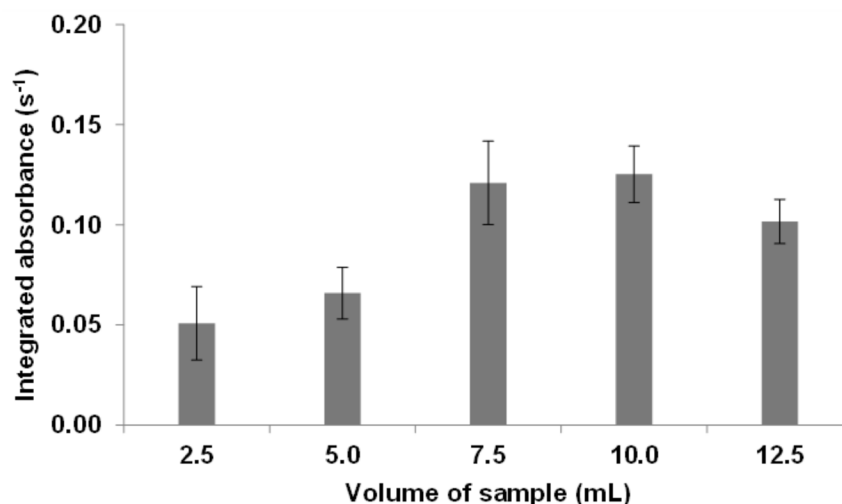


**Figure 2.** Evaluation of the volume of  $C_6H_4Cl_2$  on Fe extraction (n=7). Conditions: 10 mL of sample, 500  $\mu$ L of complexing agent (1% DDTC), 4 min of centrifugation at 3500 rpm, and 2 washing steps.

As can be seen in Figure 2, there was a significant influence on the Fe extraction when using 25  $\mu$ L, showing lowest integrated absorbance signal. Thus, the ANOVA test was applied only for the other volumes. It was possible to verify that there is no significant difference (95% confidence level) between the other volumes (50 to 200  $\mu$ L). Thus, 75  $\mu$ L of  $C_6H_4Cl_2$  was chosen in view of their lowest standard deviation and good integrated absorbance signal.

### **Evaluation of sample volume**

Volumes of sample of 2.5, 5.0, 7.5, 10, and 12.5 mL were evaluated, using 500  $\mu$ L of 1% DDTC, 75  $\mu$ L of  $C_6H_4Cl_2$  as extraction solvent and results are shown in Figure 3.

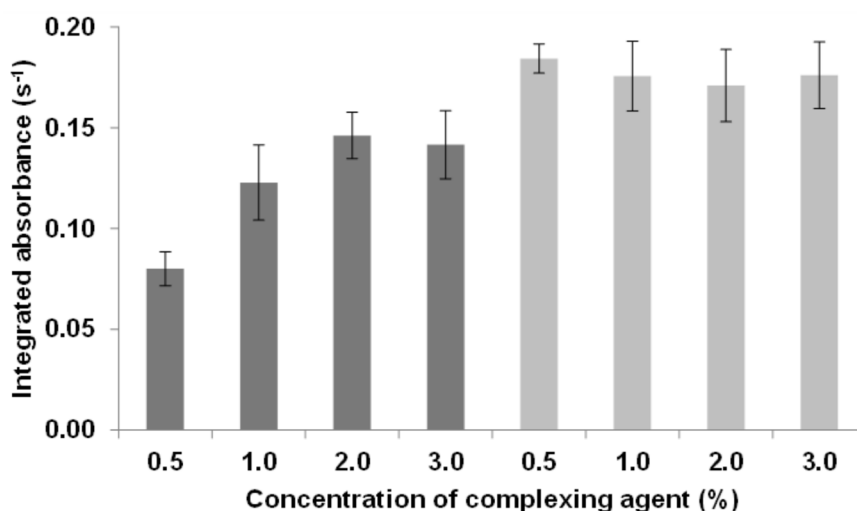


**Figure 3.** Evaluation of sample volume on Fe extraction (n=7). Conditions: 500  $\mu$ L of complexing agent (1% DDTC), 75  $\mu$ L of extraction solvent ( $C_6H_4Cl_2$ ), 4 min of centrifugation at 3500 rpm, and 2 washing steps.

According to Figure 3, the smaller volumes used (2.5 and 5.0 mL) showed the lower integrated absorbance values compared to the higher volumes. When increasing the sample volume, and consequently, the available analyte amounts available to be extracted, the absorbance value increased. However, the decrease of absorbance signal by using 12.5 mL of sample can be explained, probably by the high amount of analyte in this sample volume that cannot be extracted by the low extraction solvent volume decreasing the concentration factor.<sup>22</sup> In addition, the volume of extraction solvent could be not enough to assure the complete Fe extraction. In this way, the volume of 10 mL of sample was chosen for the next experiments.

#### **Evaluation of DDTC and APDC concentration**

In this study, two types of complexing agents were used in order to evaluate the effect of ligand concentration on the analytical response. In Figure 4 are shown the concentrations of DDTC and APDC in the range of 0.5 to 3% (m/v).



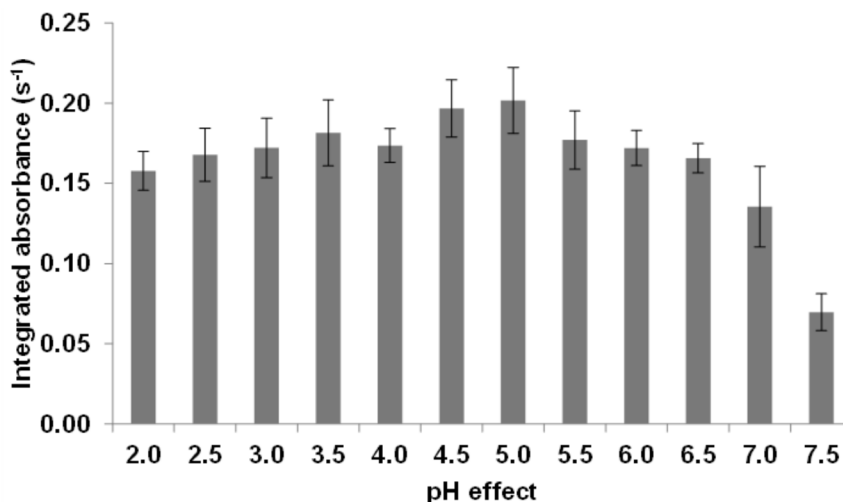
**Figure 4.** Evaluation of the extraction solvents on Fe extraction (dark gray bars corresponds to DDTC and light gray bars to APDC) (n=7). Conditions: 10 mL of sample, 500  $\mu$ L of complexing agents, 75  $\mu$ L of dichlorobenzene ( $C_6H_4Cl_2$ ), 4 min of centrifugation at 3500 rpm, and 2 washing steps.



After comparing the complexing agents, APDC showed higher absorbance values comparing to DDTC, and this low signals can be attributed to the incomplete Fe(III) complexation. Thus, for low amounts of DDTC and consequent low availability of the complexing agent, the reaction is competitive with other ions present in the sample solution.<sup>13</sup> Taking into account that Fe(II) is an unstable species, it was expected that total Fe was determined in the proposed DLLME method, once the conversion of Fe(II) to Fe(III) using APDC for DLLME procedure was described by our research group.<sup>23</sup> According to Figure 4, the complexing agent that provided the higher integrated absorbance signal and lower standard deviation was 0.5% APDC, which was used in further experiments.

### pH effect

The pH effect should be considered because it facilitates that the analytes remain in neutral form, allowing the partition of the analyte in the microdroplets of the extraction solvent. In Figure 5 is shown the effect of pH from 2 to 7.5 on Fe complex formation and extraction by DLLME. The pH adjustment was performed by using 0.1 mol L<sup>-1</sup> NH<sub>4</sub>OH or 0.1 mol L<sup>-1</sup> HCl solutions.

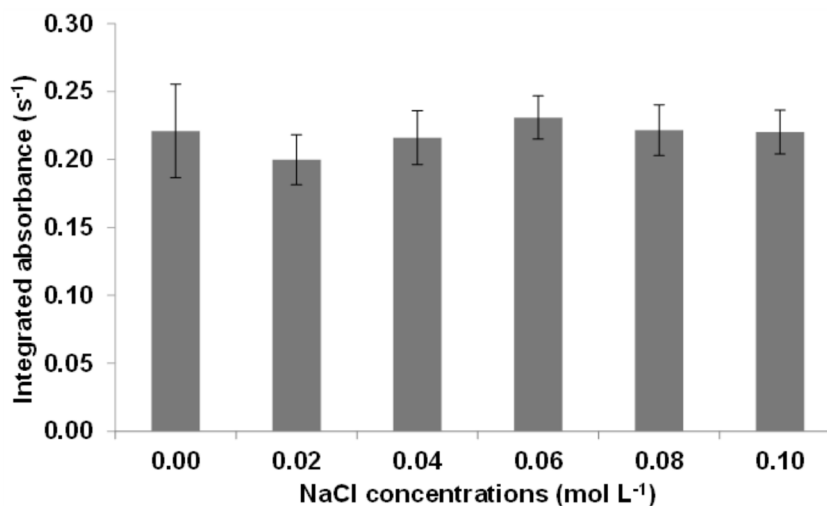


**Figure 5.** Evaluation of samples pH on Fe extraction (n=7). Conditions: 10 mL of sample, 500 µL of complexing agent (0.5% APDC), 75 µL of extraction solvent (C<sub>6</sub>H<sub>4</sub>Cl<sub>2</sub>), 4 min of centrifugation at 3500 rpm, and 2 washing steps.

The metal extraction by DLLME involves the previous formation of complexes with sufficient hydrophobicity for further extraction and phases separation, for obtaining the desired pre-concentration and extraction. It should be noted that the acidity of the sample plays an important role in the formation of the complex. As can be seen in Figure 5, the results show that the signals for Fe were greater in the acid solutions, whereas, in the mildly alkaline solutions (7 and 7.5) the signal decreases significantly. For comparing the pH values from 2 to 6.5, the ANOVA test was applied and showed no significant difference (95% confidence level) between the results. Considering that the samples in this study present pH values between 4 and 4.5, this adjustment was not necessary, minimizing the number of steps in the DLLME procedure.

### Salting out

The effect of salt addition was studied on Fe extraction efficiency by adding NaCl solutions (0.02 to 0.10 mol L<sup>-1</sup>), as well as without the use of salt (0.00 mol L<sup>-1</sup>). According to the nature of the analyte, the salt addition to the sample solution changes the complex solubility and therefore increase the extraction efficiency.<sup>24</sup> The results of salting out effect are shown in Figure 6.

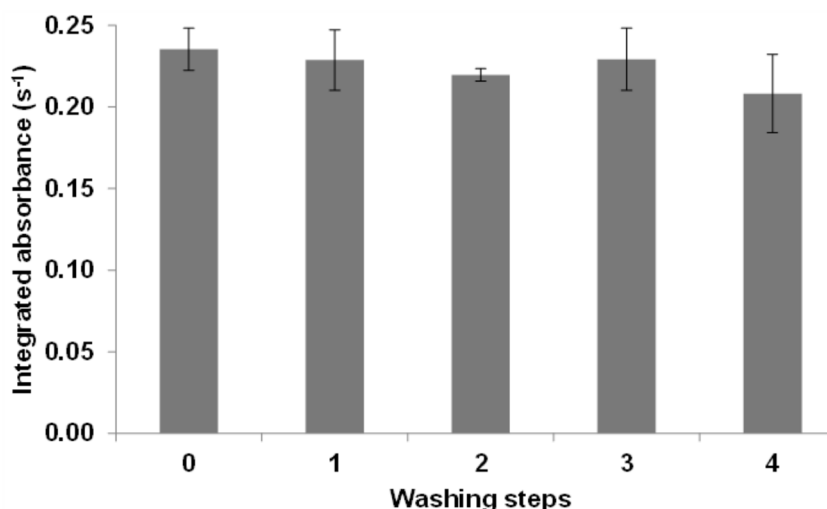


**Figure 6.** Evaluation of salting out effect on Fe extraction (n=7). Conditions: 10 mL of sample, 500  $\mu$ L of complexing agent (0.5% APDC), 75  $\mu$ L of extraction solvent ( $C_6H_4Cl_2$ ), 4 min of centrifugation at 3500 rpm, and 2 washing steps.

According to the results presented in Figure 6, apparently, higher concentrations of salt showed higher integrated absorbance compared to the lower concentration. However, all of them presented no significant difference by applying ANOVA test. Thus, no salt addition was required for subsequent experiments.

### Evaluation of washing steps

To evaluate the number of washing cycles (0, 1, 2, 3, and 4) were performed, taking into account that the remaining matrix solution that can interfere in the determination step by F AAS, because the extract and all the particulate material around the extraction solvent were aspirated.<sup>15</sup> The results are shown in Figure 7.



**Figure 7.** Evaluation of washing steps on Fe extraction (n=7). Conditions: 10 mL of sample, 500  $\mu$ L of complexing agent (0.5% APDC), 75  $\mu$ L of extraction solvent ( $C_6H_4Cl_2$ ), 4 min of centrifugation at 3500 rpm, and 2 washing steps.



The results obtained after the evaluation of washing steps showed no significant difference, therefore, no washing steps were need for further experiments. As a consequence, the elimination of washing steps makes it possible to save time. The long extraction time is one of the disadvantages of conventional extraction methods, visibly noticed since the introduction of microextraction methods.<sup>25</sup>

### **DLLME/F AAS method application and performance**

For the application of the proposed DLLME/F AAS method, 3 samples were analyzed and also applied for obtaining comparative values using MAD/ICP-OES. The results are shown in Table I.

**Table I.** Fe determination by F AAS after DLLME (n=7) and by ICP-OES after MAD (n=3). Results represent the mean and the standard deviation ( $\mu\text{g L}^{-1}$ )

Sample	DLLME/F AAS	MAD/ICP-OES
B1	68.2 $\pm$ 5.6 (119*)	57.2 $\pm$ 5.4
B2	93.7 $\pm$ 12.3 (104*)	89.3 $\pm$ 2.7
B3	59.7 $\pm$ 3.2 (110*)	54.5 $\pm$ 2.1

\*Agreement (%) between DLLME/F AAS and MAD/ICP-OES results.

After applying the DLLME/F AAS method it was possible to obtain suitable accuracy comparing to the reference method, according to AOAC<sup>20</sup> and ICH<sup>26</sup> validation guidelines (criteria acceptable 75 to 125% and 80 to 120%, respectively), with agreement ranging from 104 to 119%. It should be noted that, in other studies, different Fe concentrations in beer were found all below 100  $\mu\text{g L}^{-1}$  independent on the brands or countries. Therefore, the results found in this work are in agreement with those reported in the literature.<sup>27</sup> In addition, the proposed method made it possible to achieve suitable LOQ as 9.0  $\mu\text{g L}^{-1}$ .

### **CONCLUSIONS**

In this work, the DLLME method was developed for extraction and preconcentration of Fe from beer and further determination by F AAS. Some conditions of DLLME method were optimized in order to obtain higher extraction efficiency of Fe in beer samples, showing that the type of extractor solvent and complexing agent were the parameters that presented most influence. The combination of the two approaches (DLLME and F AAS) shows several advantages such as simplicity, relatively low operational costs, reduced time for sample preparation, among others. Although there are several studies highlighting the pH influence on complex formation and analytes extraction, the sample pH does not affect the Fe extraction efficiency. Finally, the proposed method showed suitable accuracy, precision and LOQ values, and the concentrations found in the studied samples was in agree with data found in the literature. In addition, this method can be applied in routine analysis for Fe determination in beer, as well as in samples with similarly matrices.

### **Conflicts of interest**

The authors declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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