

Development of a reference material for copper and iron in wine

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A B S T R A C T

Reference materials are essential tools to achieve comparability and traceability between measurements. However, reference materials in food matrices are scarce. In this article, the development of a reference material for copper and iron in wine is described. To assign the reference values, methods for measurement of copper and iron in wine by flame atomic absorption (FAA) were previously validated. Special attention was paid to sample treatment, since the wine matrix could affect the accuracy of measurements. For copper, the sample treatment was microwave digestion, while simple dilution of sample was enough to avoid matrix effects in iron measurements. The assigned reference values \pm expanded uncertainties were 2.986 ± 0.102 mg/L for iron and 0.154 ± 0.004 mg/L for copper.

Keywords:

Wine

Reference material

Metals

Flame atomic absorption

1. Introduction

Comparability and traceability between measurements have become increasingly important issues at the national and international levels. The use of reference materials is one important step to achieve comparability and traceability in the international trade. However, reference materials in food matrices to fulfill this need are scarce. In Chile, one of the most important industries of export products is the wine industry. In wine, some reference materials have been prepared and used (Frias et al., 2003; Díaz et al., 2003), but still some new specific reference materials are needed. During the process of production and certification of the final product, many chemical analyses are done. There is practically no reference material in this matrix, and laboratories at wine industries and external certification laboratories provide a big amount of analytical results without the aid of adequate reference materials.

Due to the importance of this issue, the Chemical Metrology Center (CMQ) has focused part of its activities on the development and production of reference materials in Wine. Taking into account that the concentrations of certain metals are not only a requisite for exported wines (Commission Regulation (EC) No. 466/2001 of 8 March 2001), but they are also related to an eventual toxicity of the product, it was considered that the

development of a reference material with a reference value for metals (copper and iron) was relevant.

There are different methods for the measurement of metals in wine (Galani-Nikolakaki et al., 2002; Sauvage et al., 2002; Sastre et al., 2002; Marisa et al., 2003; Benítez et al., 2002; Azenha and Vasconcelos, 2000); however, the production of a reference material requires a method with the highest accuracy leading to a measurement with the lowest uncertainty possible. This implies the study of different methods for the determination of total concentration of these metals.

In this paper, we report on the development of a new reference material for copper and iron in wine, using different methods for sample treatment with a final measurement by FAA spectrometry. The work presented in this article corresponds to a pilot study for wine reference material developed in CMC. After results were obtained, an analog reference material was prepared in our Center under the same conditions described in this article, with some modifications in bottles for storage, and used as unknown sample for a CCQM Pilot Intercomparison Study (CCQM P-12.1, lead, iron, copper and cadmium in wine, coordinated by UK's LGC).

2. Experimental

2.1. Reagents

The following reagents were used in the experiment: aqueous reference material for copper and iron, from NIST (SRM 3114 and SRM 3126a, respectively), aqueous reference Material for copper and iron, from High Purity Standards; ethanol (absolute) for

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analysis, from Merck; high-purity acids (nitric and hydrochloric), from Merck; hydrogen peroxide, from Merck.

2.2. Equipment

Atomic absorption measurements were done using a FAA instrument Analyst 100, from Perkin-Elmer. Digestions were performed with a high performance microwave digestion unit, MLS 1200 Mega, from Milestone, equipped with an EM-45/A Exhaust Module, and a Mega 240 Control Module.

2.3. Evaluation of ethanol influence in measurements

To test the possible interference of ethanol in the determination of copper and iron, two sets of standards were prepared from NIST SRMs. One of the sets consisted of five standards in aqueous solution (2% nitric acid). The other set consisted of five standards in aqueous/ethanol solution (10% ethanol, 2% nitric acid). In addition, two more sets of standards were prepared for iron, with 2% and 1% ethanol (2% nitric acid). For copper, standards were in the range of 0.05–0.5 mg/L. For iron, standards were in the range of 0.1–0.35 mg/L approximately. The difference in the response was evaluated by means of covariance analysis of the different sets of calibration standards. Moreover, a control solution was prepared from a reference material (High Purity Standards), both aqueous and 10% ethanol.

2.4. Sample treatment

Two different sample treatments were tested for the determination of copper in wine. These were dealcoholization and microwave digestion. Elimination of alcohol from wine was done by putting 100 mL of wine in open vessels in a thermo-regulated bath at 60 °C approximately. Volume was reduced to approximately 30 mL, and then taken to a final volume of 50 mL. This solution was then diluted to fall in the range of calibration standards. Microwave digestion was performed by taking 10 mL of wine, followed by the addition of 2 mL of hydrogen peroxide and 1 mL of nitric acid (both ultrapure quality). The mixture was allowed to stand for 2 h. Then it was subjected to the next microwave program: 250 W for 5 min; 0 W for 2 min; 350 W for 3 min; 0 W for 5 min; 350 W for 3 min; 0 W for 5 min; 350 W for 4 min; 0 W for 5 min. After microwave digestion, samples were taken to a final volume of 25 mL.

2.5. Recovery studies

To evaluate the efficiency of the extraction and measurement process, recovery studies were carried out. Samples were prepared by the addition of an aqueous standard, to a final concentration (added in wine) of 0.2 mg/L of Cu and 1 mg/L of Fe. Addition was allowed to stand for 12 h before beginning the sample treatment. Samples were treated with the chosen method, based on the previous study of matrix influence. According to this, the sample treatment was microwave digestion for copper and dilution for iron. In the case of copper, a volume of 10 mL of spiked wine sample was subjected to digestion in the conditions previously described. The digested samples were taken to a final volume of 25 mL and measured by FAA. For iron, samples were diluted to the level previously validated, followed by measurement by FAA. Since wine has natively present both cations, the same treatment and measurements were done for non-spiked wine. Evaluation of recovery data was done following the procedure described by Barwick and Ellison (1999), by comparison of the observed concentration with the added concentration, taking into account

the amount of cation present natively in wine. Recovery (R) is defined as the ratio $C_{\text{obs}}/C_{\text{ref}}$ of observed concentration C_{obs} to a reference value C_{ref} for the material tested. The value of R has an associated uncertainty ($u(R)$), which is calculated taking into account systematic and precision factors. R along with $u(R)$ is used to determine whether the recovery is significantly different from 100%.

2.6. Calculation of uncertainty

Type A and Type B uncertainty components were identified and quantified. They were expressed as standard uncertainty and combined according to the procedure described by the ISO GUM, by means of the simplified calculation proposed by Kragten (1994) and Eurachem/Citac Guide (2000). The procedure takes advantage of an approximate numerical method of differentiation, and requires knowledge only of the calculation used to derive the final result (including any necessary correction factors or influences) and of the numerical values of the parameters and their uncertainties. Values of the function considering uncertainty of each parameter are calculated, and combined to get the final uncertainty. In this case, uncertainty due to the recovery of the element in the measurement process was estimated as described by Barwick and Ellison (1999), and added to the final total uncertainty of the measurement.

2.7. Preparation of the reference material of copper and iron

To prepare the reference material, a Cabernet Sauvignon wine was selected. In order to maximize stability of the reference material to prepare, a 3-year-old wine was used, since it is well known that old wines have already gone through processes like oxidation or precipitation. Eight individual bottles of the Cabernet Sauvignon red wine 750 mL each, from the same fermentation barrel, were filtered through cellulose filter 0.45 μm pore size, and mixed to get a final volume of 6 L of wine. This volume was homogenized by circulation for 8 h, and then transferred to 100 mL bottles. The filters were analyzed for copper and iron, indicating that the concentrations of both metals were under the detection limit of the methods.

2.8. Homogeneity tests and assignment of reference value

Homogeneity test and assignment of the reference value for metals in wine were done from data obtained from measurements by FAA of samples digested by microwave in the case of copper, and diluted in the case of iron. Six bottles were selected from the lot of bottles. Selection of bottles was done at random. Four replicates of each bottle were measured by FAA. Data were analyzed by means of one-way ANOVA. Drift in the measurement was discarded by means of regression analysis. The reference value was assigned as the mean of the measurements of the four replicates of each of the six bottles.

2.9. Stability study

The reference material prepared was stored at 4 °C, and the stability of the material under this condition was evaluated. The stability of the values of copper and iron concentrations was evaluated monthly for a period of four months. The data obtained were analyzed according to the statistical process described by Lawn and Holcombe (2002). The test involves the calculation of a t -value, which is calculated as the difference between the certified value and a value of the material in a subsequent measurement, divided by the standard uncertainty of the

difference. When t -value is higher than 2, it is concluded that there was a significant change with respect to the certified value.

3. Results and discussion

3.1. Evaluation of influence of ethanol in copper and iron measurements

Different methods are in use for the determination of metals in wine. Some of them recommend the direct determination of diluted wine using an aqueous calibration curve. To evaluate if the amount of ethanol present in wine (one of its major components) could interfere in the determination of copper and iron, different calibration curves in the presence and absence of ethanol were prepared.

In the case of the determination of copper, we evaluated the results for an aqueous calibration curve and a 10% ethanol calibration curve. When plotting these two calibration curves, a different slope was obtained for each case. (Equations obtained were $y = 0.140x + 0.002$ and $y = 0.106x + 0.003$ for the aqueous and 10% ethanol calibration curve, respectively). Analyzing these slopes by a covariance analysis gave an F value of 1430 and a critical F value of 6.39. That meant that slopes were significantly different. Additionally, evaluation of a control sample prepared from High Purity Standard showed that bias was present when cross-evaluating control samples in both calibration curves, that is, when quantifying the aqueous control sample in the 10% ethanol calibration curve and vice versa. Therefore, the quantification of copper in wine could give biased results if direct measurement of wine samples was done using aqueous standards. In the case of copper, it was not possible to dilute samples to an extent that ethanol present was not an interference. Copper concentrations are too low to dilute samples and still be in the range of FAA measurements. A sample treatment was necessary to avoid the interference of ethanol in copper measurements.

In the case of iron, four different concentrations of ethanol were tested. Concentrations of iron in wine make possible a higher dilution of the sample to reduce the amount of ethanol, if it was an interference. For that reason, calibration curves were prepared at no ethanol, 1%, 2%, 5% and 10% ethanol. The same statistical analysis as in the case of copper was applied. Analysis of calibration curves of 10% ethanol, 5% ethanol, and aqueous showed that they were significantly different (data not shown). However, when analyzing the 2% ethanol, 1% ethanol and aqueous calibration curves, they were not significantly different. The three calibration curves were overlapped when plotted in the same graphic. (Equations obtained were $y = 0.172x - 0.0004$, $y = 0.171x + 0.0002$ and $y = 0.172 + 0.0005$ for aqueous, 1% ethanol and 2% ethanol calibration curves, respectively.)

F values for the slope, when analyzing calibration curves aqueous and in the presence of ethanol, were 0.16 and 1.08 for 1% ethanol and 2% ethanol, respectively. In both cases, critical value of F was 6.09. Therefore, slopes are not significantly different. In addition, when analyzing an aqueous control sample in these three different calibration curves, there was no significant difference between results for the sample. A one-way ANOVA for the results showed a p -value of 0.594, that is, results were not significantly different. According to this, it was possible to measure iron, just making an adequate dilution that eliminates the ethanol interference. Samples should be measured at a dilution of at least six times (which would reduce the ethanol concentration from 12% to approximately 2%, where there is no significant difference with measurements in aqueous solution). The bigger the dilution, the best, because it reduces even more the matrix from the solution. Concentrations of iron in most wines

allow big dilutions of samples, which makes this a very straightforward method for the measurements of this metal in wine.

3.2. Sample treatment for copper measurements

Two different sample treatments were tested for copper measurements. The first one consisted in an evaporation process with the purpose of eliminating ethanol, since it was an important interference in FAA measurements, as demonstrated in the previous experiment. Six independent samples of wine were prepared as described in sample treatment, and after taking to a final volume their concentration was measured by FAA, using bracketing as the calibration method. NIST SRM 3114 was used as the calibration solution. In parallel, six control solutions (copper in aqueous solution) followed the same procedure, and were measured together with the samples. The results showed a big variation between independent sample measurements, with a percentage variation of 19.9%. However, in control sample, the percentage variation between samples was only of 2.0%. In repeated experiences, similar results were obtained. These results showed that there was a matrix problem when using this sample treatment method. When testing different samples and different dilutions, it was observed that this procedure generated different amounts of precipitate in the sample (depending on the degree of evaporation of the sample, and the type of wine). In some cases, the amount of precipitate present in the sample forced to include a filtration step. All these factors were probably influencing the big variation between samples. These results demonstrated that this method was not suitable for the preparation of a reference material, because it would give a final uncertainty too big for this purpose.

To test the second sample treatment method, six independent samples were again prepared. These samples were subjected to the procedure described in experimental section. After digestion, samples were measured by bracketing with standards prepared from NIST SRM 3114. Variation between samples in repeated experiments was approximately 2–3%. In consequence, this was a good method to treat wine samples for copper measurements, as it would eliminate the matrix, and give low between-sample variation, which would give rise to adequate final uncertainty levels. To further test the method accuracy, recovery studies were done.

3.3. Recovery studies

Samples for recovery studies were prepared as described in Experimental. Special attention was put on leaving a long period of equilibration of the sample after the addition of the metal to the wine sample. When performing the recovery tests described by Barwick and Ellison (1999), recovery of iron in samples treated by simple dilution was 0.958, with an uncertainty for recovery of 0.038. In the case of copper, recovery was 0.997, with an associated uncertainty of 0.026. In both cases, variation between samples was adequate for purposes (between 2% to 3%). These results validated both methods for determination of copper and iron in wine, with the objective of preparation of a metal reference material.

3.4. Homogeneity tests and assignment of reference value

To assign the reference value to the reference material prepared, six bottles were selected at random from the entire batch. Those six bottles were treated as described, both for copper and iron, that is, microwave digestion and dilution, respectively.

Four replicates for each bottle were prepared and measured in the method conditions defined previously, by bracketing. Along with wine samples, control samples were taken to assess the presence of bias in measurements. In order to evaluate this, a statistical test described by NIST was applied (CAC Quality Assurance Task Group, 1992). To test the homogeneity, a one-way ANOVA was applied for measurements in copper and iron. Results showed that the reference material was homogeneous for copper and iron (Figs. 1 and 2; Table 1).

Results of the analysis of one-way ANOVA clearly demonstrate that there were no significant differences between bottle averages,

that is, the reference material was homogeneous in copper and iron concentration. In addition, bias test with control samples showed that there was no detectable bias. No drift was detected in measurements when applying a regression analysis to data obtained in sequential order.

The reference value was calculated as the mean of measurements of the four replicates of those six bottles selected (24 independent measurements in total), for both iron and copper. Reference value for copper was 0.154 mg/L; for iron, reference value was 2.986 mg/L.

3.5. Uncertainty of copper and iron concentration in the reference material

All type A and type B components of uncertainty were identified and quantified, according to the rules described in ISO/IEC GUM (1993). Calculations for copper measurements in the reference material gave a final expanded uncertainty of 0.004 mg/L (with a coverage factor of 2), which correspond to a relative uncertainty of 2.3%. In the case of iron, expanded uncertainty was 0.102 mg/L (coverage factor $k = 2$), corresponding to 1.7% as relative.

3.6. Stability of the reference material

The time stability of copper and iron concentration in the reference material was evaluated by measuring monthly the concentration of both metals. Measurements were done by replicating conditions used in the assignment of the reference value. Controls were also measured along with samples, to assess the existence of bias in measurements by FAA. No bias was detected in measurements. After all data were collected, they were analyzed with the test of stability proposed by Lawn and Holcombe (2002). This analysis calculates a t -value, based on the value obtained in measurements, the value of certification (initial value for assessment of stability) and the corresponding associated uncertainties. When the t -value is bigger than 2 (in absolute), it means that the reference material lost its stability.

When analyzing with this test the concentrations of copper and iron, results showed that the reference material was stable for a period of approximately 4 months, after which concentrations of both metals varied longer than it was adequate for the satisfactory use of the reference material. Figs. 3 and 4 show the plot of the t -value versus time.

In the case of copper, no tendency was observed in the t -value. However, this value exceeds 2 after the third month of evaluation of stability.

For iron, a tendency in the t -value was obtained, which clearly indicated the loss of stability of this metal in time, due to a "loss"

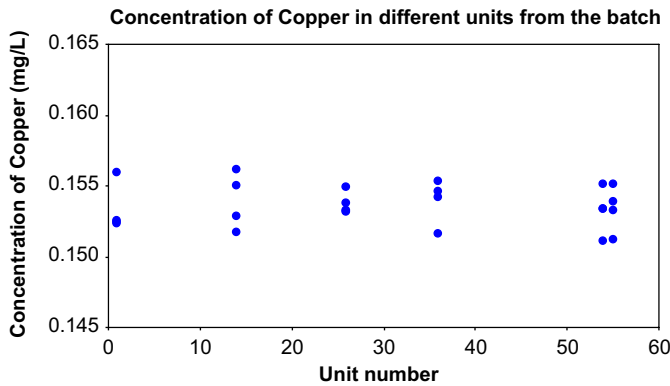


Fig. 1. Concentration of copper in different units from the lot analyzed. Measurements were carried out under reproducibility conditions, and tested for differences among units by means of one-way ANOVA.

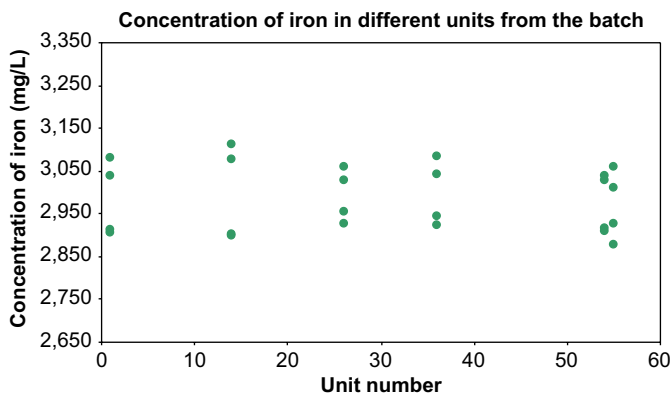


Fig. 2. Iron concentration values obtained for different units from the batch. Measurements were done under reproducibility conditions, and tested for differences among units by means of one-way ANOVA.

Table 1 One-way ANOVA for copper and iron concentration values in wine reference material

Source of variations	Squares sum	Degrees of freedom	Squares mean	F	Probability	Critical value of F
<i>Variance analysis</i>						
Copper						
Between groups	2.10×10^{-6}	5	4.20×10^{-7}	0.160	0.974	2.773
Within groups	0.000047	18	2.62×10^{-6}			
Total	0.000049	23				
Iron						
Between groups	3.19×10^{-3}	5	6.39×10^{-4}	0.091	0.999	2.773
Within groups	0.12653	18	7.03×10^{-3}			
Total	0.129728	23				

F and critical value for F, as well as p-value, demonstrate that there are no significant differences among units from the batch.

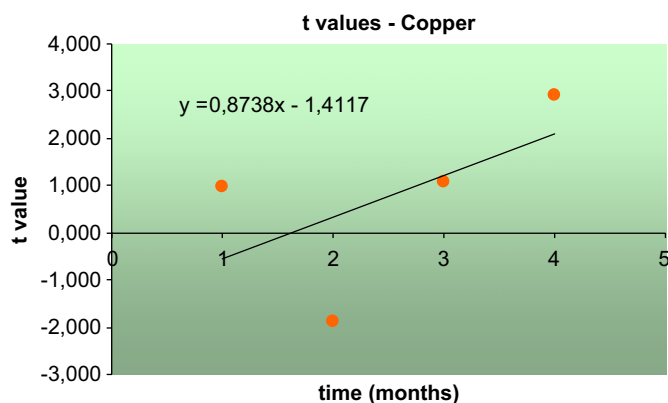


Fig. 3. *t* values for copper in the wine reference material. *t* values were calculated monthly.

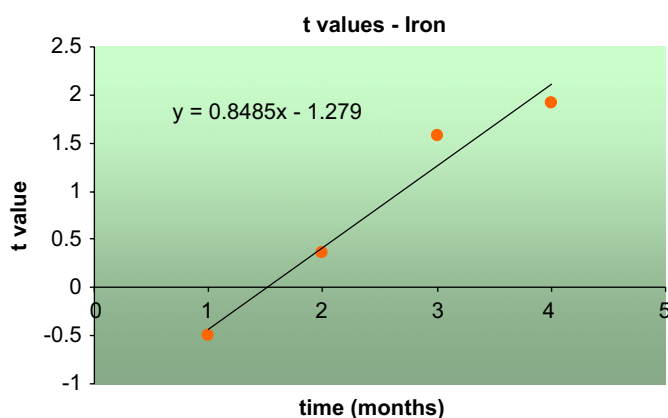


Fig. 4. *t* values for iron in the wine reference material. *t* values were calculated monthly.

of iron in the reference material in time. This “loss” (drop in iron concentration) was probably due to the appearance of a precipitate in the reference material that could possibly drag iron ions adsorbed on it. This precipitate clearly relates with oxidation processes in the wine, when exposed to oxygen. It could be possible to increase the stability of reference materials of this type, by changing storage conditions of the bottles, which is now being conducted in our laboratory.

4. Conclusions

A reference material for copper and iron in wine was prepared. The methods implemented for measurements and assignment of reference value were adequate for the purpose, since they have a good accuracy and uncertainty values, with associated expanded uncertainties of approximately 2% for both methods. The method for preparing the reference material was also adequate, but it can be optimized by changing some conditions of preparation and storage, to reduce the contact of wine with oxygen, increasing therefore the metal concentration stability in the reference material. The latter had a reference value for copper of 0.154 ± 0.004 mg/L. For iron, the assigned reference value was 2.986 ± 0.102 mg/L. The reference material was demonstrated to be homogeneous and stable for 4 months.

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References

- Azenha, M.A.G.O., Vasconcelos, M.T.S.D., 2000. *Food and Chemical Toxicology* 38, 899–912.
- Barwick, V.J., Ellison, S.L.R., 1999. *Analyst* 124, 900–981.
- Benítez, P., Castro, R., Sanchez Pazo, J.A., Barroso, C., 2002. *Food Research International* 35, 785–791.
- CAC Quality Assurance Task Group, 1992. *Use of NIST Standard Reference Materials for Decisions on Performance of Analytical Chemical Methods and Laboratories*, NIST Special Publication 829.
- COMMISSION REGULATION (EC) No. 466/2001 of 8 March 2001. *Setting maximum levels for certain contaminants in foodstuffs*. Official Journal L 77, 16/03/2001, 001–013.
- Díaz, C., Conde, J.E., Estévez, D., Pérez-Olivero, S.J., Pérez-Trujillo, J.P., 2003. *Journal of Agricultural and Food Chemistry* 51, 4303–4307.
- EURACHEM/CITAC Guide, 2000. *Quantifying Uncertainty in Analytical Measurement*, second Ed., EURACHEM/CITAC.
- Frias, S., Conde, J.E., Rodríguez-Bencomo, J.J., García-Montelogo, F., Pérez-Trujillo, J.P., 2003. *Talanta* 59, 335–344.
- Galani-Nikolakaki, S., Kallithrakas-Kontos, N., Katsano, A.A., 2002. *The Science of The Total Environment* 285, 155–163.
- ISO/IEC, 1993. *Guide to the Expression of Uncertainty in Measurement (GUM)*. ISO, Geneva.
- Kragten, J., 1994. *Analyst* 119, 2161–2166.
- Lawn, R., Holcombe, G., 2002. *Stability testing and predicting the shelf-life of reference materials*. LGC/VAM/2002/019.
- Marisa, C., Almeida, R., Vasconcelos, M., Teresa, S.D., 2003. *Journal of Agricultural and Food Chemistry* 51, 3012–3023.
- Sastre, J., Sahuquillo, A., Vidal, M., Rauret, G., 2002. *Analytica Chimica Acta* 462, 59–72.
- Sauvage, L., Frank, D., Stearne, J., Millikan, M.B., 2002. *Analytica Chimica Acta* 458, 223–230.