Validation and Comparison of Formol and FT-IR Methods for Assimilable Nitrogen in Vine Grapes

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In this study, two methods used to evaluate assimilable nitrogen in grape juice were compared, namely: the formol method and the Fourier-transform mid-infrared (FT-MIR) spectroscopy. At the outset the formol method was validated in the laboratory by determining parameters such as precision, accuracy and trueness. Parallel recovery studies were conducted with a model solution and natural grape juice for ammonium and arginine. Average recoveries of ammonium for the formol titration ranged from 77% to 88% and for arginine from 90% to 97%. The repeatability and the intra laboratory reproducibility were ±6.4 and ±31.7 (mg/L of nitrogen) respectively. Formol titration was used as the reference method for the calibration of FT-MIR spectroscopy to determine the assimilable nitrogen in grape juice. Grape juice samples (n=71) for 7 red and 7 white varieties from the Lisbon region in Portugal were analyzed simultaneously by using the formol method and Fourier-transform mid-infrared (FT-MIR) spectroscopy. The results of this study demonstrated that it is possible to explore the applicability of FT-MIR technology to detect the assimilable nitrogen in grape juice for quantification purposes. The correlation coefficient (R^2=0. 993), standard prediction error (SEP; 6.4 mg/L) and the RPD or standard deviation of the data/ standard error of prediction value (Sd/Se; 7.8) proved satisfactory for the calibration of the instrument. In addition, the results obtained by FT-MIR spectroscopy were comparable to those obtained when using the reference method.

INTRODUCTION

The assimilable nitrogen content of the grape must plays an important part in the evolution of the fermentation in winemaking. Insufficient assimilable nitrogen in grape must is one of the major causes of stuck and sluggish alcoholic fermentations (Bell *et al*., 1979; Bely *et al*., 1990a; Boulton *et al*., 1996; Blateyron & Sablayrolles, 2001; Cramer *et al*., 2002), altering the aroma composition of wines (Rapp & Versini, 1991) and moreover their quality (Bell & Henschke, 2005). Therefore, the need for rapid, accurate and precise determination of assimilable nitrogen in winemaking is crucial for winemakers, since it facilitates the correction of the nutritional status of the grape must prior to alcoholic fermentation.

Several analytical methods are commonly used in the wine industry for the determination of assimilable nitrogen (Callejon *et al*., 2008). One of these is the infrared spectroscopy which offers a quicker alternative to conventional methods of analysis (Christy *et al*., 2001). The vibration IR spectrum region varies between 700 nm and 1mm, but in the mid-infrared (MIR) spectroscopy the absorbent region is between 2500 and 2.5 x 10^{-4}nm and it corresponds to a wavelength of 4000 to 400/cm (Smith, 1999). Many enological components such as organic acids, alcohol, volatile acidity, sugars, glycerol, anthocyanins, polysaccharides and aroma precursors may be analyzed by FT-MIR spectroscopy. The largest peaks of the spectrum represent the more abundant compounds of the grape juice or wine, which is why several researchers have studied these vibration areas for the majority of the components. However, the calibration of FT-IR is not always an easy task. Patz *et al.* (2004) referred to the difficulty of calibrating the FT-IR method due to the fact that natural samples do not always provide an even matrix. Different types of must or wines may contain different compounds that can influence the calibration set. Freezing the grapes during storage also proved to have an impact on the calibration accuracy in NIR calibrations (Cozzolino *et al*., 2005). Moreover, Moreira & Santos (2004) concluded that the poor performance in the analytical calibration of the low concentration compounds by FT-IR may be due to spectroscopic interference with the spectra of the most abundant compounds in wine. It is also important to mention that the use of Fourier-Transform infrared spectroscopy requires regular recalibration in order to maintain and control the quality of the results (Patz *et al*.,

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2004). This is difficult to establish for a large part of the wine industry and in addition impacts the cost effectiveness of the method.

In contrast, one of the most reliable conventional methods for assimilable nitrogen determination is the formol method which is simple and widely used in winemaking. Several authors have compared different methods of analysis and have determined the overall values of formol titration by using other methods such as HPLC, NOPA (an assay based on the derivatization of primary amino groups with an o-phthaldialdehyde/N-acetyI-L-cysteine reagent) and FAN (free amino nitrogen)(Gump *et al.,* 2002; Filipe-Ribeiro & Mendes-Faia, 2007).

This study was initially conducted to assure the quality of the results of the reference method (formol titration) for the determination of assimilable nitrogen content of grape juice.

Subsequently the aim was, to calibrate and validate the FT-IR method for the rapid measurement of assimilable nitrogen concentration; hence enabling simultaneous comparison to the reference method (Formol method).

MATERIALS AND METHODS

Samples

Seventy-one samples of frozen grapes (*Vitis vinifera*, L.) from the Lisbon region in Portugal were used in this study. These include red varieties [Touriga-Nacional, Cabernet-Sauvignon, Castelão, Syrah, Trincadeira (sin. Tinta Amarela), Alicante Bouschet and Aragonez] and white varieties [Alvarinho, Antão Vaz, Maccabeo, Moscatel de Setúbal (sin. Muscat of Alexandria), Arinto, Viosinho, Encruzado] These samples were stored frozen at -20°C and subsequently defrosted to a temperature of 20°C before being analyzed. They were then manually crushed and centrifuged at 6000rpm (G max.= 4300) for 30 min using a VEB MLW MEDIZINTECHNIK LEIPZIG T52.2 (Leipzig, Germany) and immediately analyzed with the reference method (Formol titration) and tested by FT-MIR, to obtain infrared spectra in order to avoid microbial growth and other alterations. The composition of samples covered an approximate concentration range of between 80mg/L and 300mg/L nitrogen.

Reference analysis

The Formol titration procedure described by Gump *et al.* (2002) was used. The reaction of formaldehyde with the free amino groups liberated a proton from the amino group which could be titrated directly with NaOH at pH 8.0. The result obtained in this procedure was calculated according to the general equation: mg nitrogen/L = [(vol 0.05N NaOH) x (concentration 0.05N NaOH) x 14 x (dilution factor) x 1000]/(sample vol). A pH meter by Radiometer Copenhagen, Denmark, Ortec, pHm82 Standard was used in the formol titration analyses. All the reagents used were of analytical grade.

Certain precautions should be taken regarding the implementation of the procedure: It is important that the pH of formaldehyde (37%, w/v) is adjusted to 8.0 before use, because of the instability that it acquires over time. Furthermore, in the last step of the titration, the NaOH should be added gradually by only a few drops at a time while constantly stirring it until pH 8.0 is reached, because of its slow reaction due to the buffering action of the sample.

In order to assure the quality of the results for the determination of assimilable nitrogen in grape juice, a validation of the formol method was implemented determining the analytical limits, the robustness, the spike recovery, the repeatability and the intra laboratory reproducibility according to the standards of the Vine and Wine International Organization O.I.V (OIV, 2011).

Infrared spectroscopy equipment

Fourier-transform infrared analysis (FT-IR) was conducted by using multispec equipment, FT-IR AVATAR 380 NICOLET (Microdom, TAVERNY Cedex – France), coupled to a transmission flow-cell (including a Nicolet EZ Omnic FT-IR software package). The samples were centrifuged prior to spectra acquisition to eliminate suspended solids. Bacchus Acquisition software was used to define measurement parameters. The different absorption zones of amino acid side chains in infrared spectrum is well summarized by Barth (2000) and although the whole mid-infrared spectral range was stored for each sample, the 1480-1800 cm^{-1} area was selected manually for the calculations of the assimilable nitrogen. This zone includes many absorption bands of the major amino acid side chains that are present in grape juice (Goormaghtigh *et al*., 1994a; Rahmelow *et al*., 1998). The absorption in this infrared region is due to vibrations of asymmetric elongation of carboxylic group COO^-, elongation of C=O bond and deformation of NH_2.

Statistical analysis

Validation of the formol method, repeatability, intra-laboratory reproducibility and calculation of average, standard deviation and Anova statistical analysis were performed using Microsoft Excel 2007 software.

For the calibration, partial least squares (PLS) regression was performed, using Bacchus Quantification software. PLS is one of the most commonly used multivariate calibration methods for the evaluation of mid-infrared spectra (Martens, & Martens, 2001). Using the internal cross-validation, the same samples are used for both the calibration of the model and testing. Sample outliers (n=28) were removed from the calibration data set and a new calibration model was developed using the remaining data points.

Calibration equations were determined by comparing the regression results in terms of correlation coefficient (R^2), standard error of prediction (SEP), as well as the residual predictive deviation (RPD = standard deviation of the data/ standard error of prediction) used to evaluate the predictive ability and performance of the calibration models. In an ideal situation, a RPD value lower than 3 would indicate that the calibration model is unsuitable for quantification, a value between 3 and 5 is suitable for screening and a value bigger than 5 is suitable for quantification (Pink *et al*., 1998; Williams, 1995).

RESULTS AND DISCUSSION

Validation of the reference analysis

In Table 1 the analytical limits, the spike recovery, the repeatability and the intra laboratory reproducibility for the formol method were summarized. These results are in agreement with those obtained by Gump *et al.* (2002) and Filipe-Ribeiro & Mendes-Faia (2007).

The results of the robustness analysis show the ability of this method to remain unaffected by small, deliberate variations of pH. The two sets of experiments with the small difference in pH value (7.5 and 8.5) showed no effect to the quantification results of the sample (data not shown).

Calibration and Validation of Fourier Transform Infrared Spectroscopy

Outliers were pointed out for the spectrum analysis of the samples. A set of 43 samples were used (Fig.1) for both the calibration and testing. The correlation coefficient (R^2) of the calibration curve showed excellent performance (R^2=0. 993), indicating a value close to 1 that is suitable for the calibration model of the experiment, as well as a low standard prediction error (SEP; 5.9 mg/L). The calibration curve also shows a small dispersion of the sample values and a high RPD value (7.8) which is suitable for the quantification purposes (Table 2) with a 13 number factor.

Comparison with the reference method

Compared to the formol method for the analysis of assimilable nitrogen, the FT-IR offers clear advantages in terms of speed and the possibility to have simultaneous analysis of several compounds. The chemical composition of a sample can be obtained within 90 seconds with minimal sample preparation. It is also an environmentally friendly method with relatively low maintenance cost associated with the equipment as well as easy to handle. However, the FT-IR method needs systematic control (calibration must be

TABLE 1
Statistical values for the validation of formol method in the analysis of yeast assimilable nitrogen.

Statistical analysis	Nitrogen (mg/L)	Percentage % ± SD
Analytical limits	7.8 - 324	-
Recovery of ammonium[a]	-	81.54 ± 3.31
Recovery of ammonium[b]	-	82.82 ± 5.06
Recovery of arginine[a]	-	91.27 ± 0.95
Recovery of arginine[b]	-	89.05 ± 7.5
Repeatability	6.4	-
Intra laboratory reproducibility	31.7	-

[a](model solution), [b](grape juice), ±SD (standard deviation).

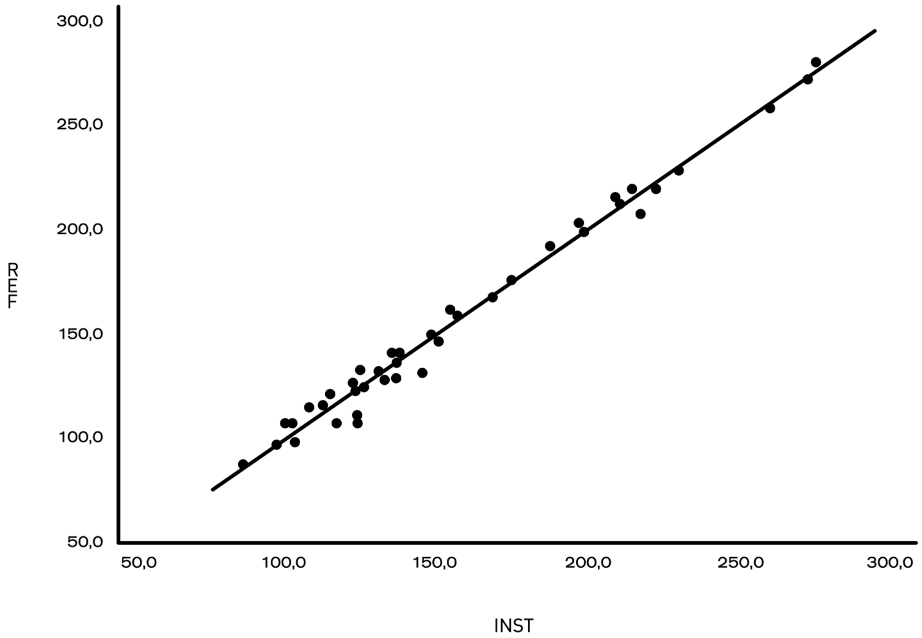

FIGURE 1
Plot between the references values vs. the predicted values for yeast assimilable nitrogen.
REF (references values obtain by formol method in mg/L).
INST(instant values obtain by FT-IR method in mg/L).

TABLE 2
Analysis parameters for yeast assimilable nitrogen predicted by FT-IR

Yeast assimilable nitrogen	YAN
Reference method	Formol method
Samples	43
Range mg/L of YAN	80-300 mg/L
Median	155.4 mg/L
SD	6.4
Frequency region	1480-1800/cm
SEP	5.9 mg/L (3.8 %)
Factor numbers	13
R^2	0.993
RPD (SD/SE)	7.8
	recommend for quantificaton

SD (standard deviation), SEP (standard error of prediction).

done regularly) to assure the quality of the results and has a relatively high initial investment cost. The results of the FT-IR method strongly depend on the quality of the calibration, which may be influenced by unknown adulteration. This procedure also presents several limitations for measuring low concentration parameters.

Nevertheless, several authors have proven the applicability of the method for grape must, while wine parameters like sugars (Urtubia *et al*., 2008), acids and alcohol (Versari *et al*., 2008) which, as shown in this study are efficient in their application for the FT-IR method for the determination of assimilable nitrogen in grape juice for quantification.

The advantages of the formol method have been indicated by several studies (Gump *et al*., 2002; Filipe-Ribeiro & Mendes-Faia, 2007). The procedure shows a high degree of validity and requires only a few reagents and a pH meter. The Formol method offers reliable results for the general overview of the nutritional status of grape juice and has a relatively low initial investment cost and maintenance. The main drawback of this method is the use of hazardous formaldehyde, but this disadvantage can be overcome by taking safety precautions and lowering the quantity of the reagent. This procedure also needs a fair amount of time for the accurate analysis of the samples (maximum 30 samples per day), but it is easy to perform the validation of this method in a laboratory as shown in this study.

Finally, the FT-MIR should be considered as complementary to the formol method rather than as a replacement for determining assimilable nitrogen of grape juice. The calibration and validation of the FT-IR spectrometry strongly depends on the conditions of the analysis, the sampling procedure and on the quality of the reference data.

CONCLUSION

This study proved that the formol method can be an easy, routine method for the determination of assimilable nitrogen in the wine industry and an important tool for winemakers when deciding the applicability of nutrients to musts. The validation of the method is not a complex task to execute in wineries and can provide accurate values for the nutritional status of grape juice. The results obtained in this study were in agreement with the literature already published in this field. The formol method incorporates titration of ammonium (approx. 77% to 88%) and other amino acids such as arginine (approx. 90% to 98%). The validation process showed higher intra laboratory reproducibility (31.7mg/L of nitrogen) than those proposed by previous studies (±20 mg/L).

Using natural samples, it was possible to perform the calibration of the FT-IR spectroscopy in order to analyze the assimilable nitrogen of grape juices. Therefore the comparison to the reference method was efficient. This was due a high correlation coefficient (close to 1) and RPD value (< 7) as well as to an even distribution of the samples and the low SEP value.

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