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Comparison of different methods for the determination of assimilable nitrogen in grape musts

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ABSTRACT

Different available methods for the determination of assimilable nitrogen in grape musts were studied. This study was carried out on 10 musts which were fermented at laboratory scale. We first validated the measurement of the actual assimilated nitrogen by yeasts according to the Kjeldahl method as a reference method. Then, the values of assimilable nitrogen obtained by Fourier Transformed Infra Red Spectroscopy (FTIR), formoltitration, [orthophtaldehyde (NOPA) + ammoniacal] nitrogen and [amino acids (automatic analyzer using ion exchange chromatography) + ammoniacal] nitrogen were compared with actual assimilated nitrogen obtained by the reference method. Results showed that the measurement of [amino acids (automatic analyzer) + ammoniacal] nitrogen is the most reliable way to measure assimilable nitrogen in grape musts. But this methodology remains time consuming and quite expensive. FTIR is also a reliable method for the measurement of assimilable nitrogen, except for atypical musts. Being fast and not sample destructive, it appears as a fitted way of assimilable nitrogen measurement on a routine basis. Formoltitration and to a lesser extent, [NOPA + ammoniacal] nitrogen underestimate assimilable nitrogen in grape musts. This study also differentiates between the nitrogen fractions and therefore allows to a better understanding of nitrogen assimilation during alcoholic fermentation.

1. Introduction

Nitrogen is present in must within several fractions: ammonium, amino acids, peptides, proteins, amidic nitrogen and nucleic acid (purines and pyrimidines). Assimilable nitrogen by yeasts during alcoholic fermentation is mainly composed of amino acids and ammoniacal nitrogen. It also includes some peptides. In enology, assimilable nitrogen plays a key role at two different levels: i) it represents an important nutritional factor for yeasts during alcoholic fermentation due to its function in protein synthesis and yeast growth. Bely, Sablayrolles, and Barre (1990) showed that there is a high correlation between maximum fermentation rate and the assimilable nitrogen content of the must. Below a threshold value of about 140 mg N L^{-1}, yeast growth is limited, and fermentation kinetics remains slow, with a low maximum fermentation rate. ii) assimilable nitrogen is essential for the synthesis of wine quality markers like higher alcohols and esters (Cheynier, Schneider, Salmon, & Fulcrand, 2010).

Nitrogen is the most limiting nutrient for yeast growth during alcoholic fermentation and generally the whole assimilable nitrogen is assimilated by the yeasts during alcoholic fermentation. However, there are some situations where this does not occur: i) in highly clarified musts: for example, Casalta et al. (2010) showed that in a clarified Sauvignon must, only 59% of assimilable nitrogen was consumed, ii) when assimilable nitrogen is added in excess to the must. This situation can lead to a lower efficiency of fermentation and a lower synthesis of higher alcohols and formation of ethyl carbamate, or to microbial instability if the residual concentration of ammonium is too high (Taillandier, Portugal, Fuster, & Strehaiano, 2007).

Thus, a reliable measurement of assimilable nitrogen in grape musts is very important for winemakers. In case of a suspected nitrogen deficiency, nitrogen supplementation of the must can be forecast. Several methods are available for the measurement of nitrogen in must. The Kjeldahl method measures total nitrogen content by mineralization of the organic nitrogen, distillation and titration of the ammonium produced (Kjeldahl, 1883). This widely used method has often been modified in order to decrease the time and volume of mineralization (Campbell, 1986; Guebel, Nudel, & Giulietti, 1991; Mann, 1963). The Kjeldahl method is reliable, but remains time consuming. It is not currently used in enology because it only assess the total nitrogen content of the must whereas assimilable nitrogen represents only a part of it (23–40% according to Feuillat, Charpentier, & Maujean, 1998, 55% according to Blateyron & Sablayrolles, 2001).

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Amino-acids content can be measured using an automatic analyzer. Amino acids are generally derivatized with ninhydrin and separated by chromatography using a cation exchange resin (Benson, 1972). This method is very reliable but needs an expensive equipment and also remains time consuming. Amino-acids content can also be determined according to the NOPA method (Dukes & Butzke, 1998) where only primary amino-acids are allowed to react before their separation with orthophtalaldehyde, excluding therefore the determination of proline and hydroxyproline (Weeks & Henschke, 1999). Crowell, Ough, and Bakalinski (1985) developed a method based on the quantitative reaction of primary α-amino groups with trinitrobenzene sulfonic acid (TNBS). The corresponding trinitrophenyledated amine complex was detected at 420 nm. According to this method, assimilable N remains underestimated due to the fact that one of the most abundant amino acids in must -arginine- contains non primary α-amino groups. Ammonium is generally determined by UV-spectrophotometric determination using an enzymatic method, based on glutamate dehydrogenase activity (Bergmeyer & Beutler, 1985), this technique being fast and very reliable.

The previously described methods, as single methods, measure only a fraction of nitrogen. For the measurement of assimilable nitrogen, e.g. amino acids and ammonium, it is therefore necessary to combine two separate methods. This obviously represents a high time consumption. Only two methods are currently available for the measurement of assimilable nitrogen in grape musts: i) The formol-titration method allows to estimate assimilable nitrogen concentration in must. In this methodology, amine functions of amino-acids are blocked by the addition of formaldehyde, and ammonium salts obtained after NaOH addition are then measured (Aerny, 1996; Sorensen, 1907). Proline and hydroxyproline which are not assimilated by yeasts during fermentation are not detected by this method (Dukes & Butzke, 1998). REACH regulation (2006) aims to improve the protection of human health and environment by encouraging the substitution of toxic products with safer ones. The acute toxicity of formaldehyde represents an important disadvantage for this method. Moreover, measurement of assimilable nitrogen with this method is pH dependent and the result is underestimated when comparing with assimilated nitrogen measured according to Kjeldahl method [difference between total nitrogen in the must and when the fermentation had progressed to 80%] (Blateyron & Sablayrolles, 2001), ii) Fourier Transformed Infra Red Spectrometry (FTIR) is an alternative method for assimilable nitrogen measurement (Dubernet, Traineau, Lerch, Dubernet, & Coulomb, 2000). The interferogram obtained is converted into an infra red spectra using a mathematical operation called Fourier Transform. This automatic technique is fast, reagents saving and not sample consuming.

Only few works focused on the comparison between the different assimilable nitrogen measurement methods. Dubernet, Dubernet, Grasset, and Garcia (2001) compared FTIR results with amino acids measured using the NOPA method and ammonium measured using an enzymatic method. Repeatability and accuracy of the methods were very close. The authors concluded that FTIR is a fast, reliable and not an expensive method, and fits therefore with routine analysis. Bely (1990, 98pp.) showed that the TNBS method underestimates assimilable nitrogen by about 18% when compared with actual assimilated nitrogen. A study by Shively and Henick-Kling (2001) focused on the comparison between NOPA and formaldehyde methods. The obtained results showed that both methods gave similar results. Gump, Zoecklein, Fugelsang, and Whinton (2002) compared formaldehyde, NOPA and HPLC (ammoniacal nitrogen being measured according to the enzymatic assay), and demonstrated that Formol and NOPA methods understate arginine contents. Formol method also understates other amino acids. More recently, Filipe-Ribeiro and Mendes-Faia (2007) reported no significant differences between formaldehyde and NOPA methods, even if a correction is applied for taking into account ammonium ions.

However, to our own knowledge, no exhaustive studies exist on the comparison of the different assimilable nitrogen methods available. In the present work, we first validated a reference method, based on the determination of actual assimilated nitrogen during yeast alcoholic fermentation. Then, we compared the main available assimilable nitrogen measurement methods on different grape musts with the method considered as a reference. Finally, we clarified the idea of assimilable nitrogen by distinguishing between the different nitrogen fractions in the must and during fermentation.

2. Materials and methods

2.1. Musts

Ten musts corresponding to 6 different grape varieties from Languedoc-Roussillon (France) were used, 8 (Chardonnay a and b, Maccabeu a and b, Sauvignon a and b, Viognier a and b) issued from white wine processing and 2 (Merlot and Syrah) from red wine processing (Table 1). All red wines musts were first subject to a flash release process (fast heating by biological vapor at 80 °C for 3 min at atmospheric pressure and quick depression at 50 hPa which causes instant water vaporization). All musts were firstly flash-pasteurized (72 °C, 20 s) and stored at 4 °C until use.

2.2. Culture conditions

2.2.1. Fermentors

Small cylindrical fermentors (1.2 L) fitted with fermentation locks were used. A continuous stirring with a magnetic stirrer (600 rpm) was applied during the fermentation under isothermal conditions (24 °C).

2.2.2. Yeast strain

The commercial enological yeast strain *Saccharomyces cerevisiae* K1 (ICV-INRA, Lallemand, Montreal, Canada) was used. One g of lyophilised yeast was rehydrated in 10 mL of milliQ water supplemented with glucose (50 g L^{-1}). After 30 min at 37 °C, the fermentors were inoculated with $2 \cdot 10^6$ cells mL^{-1} of rehydrated strain.

2.3. Monitoring of fermentation

Fermentation kinetics were followed by automatic measurement of CO_2, every 20 min, from the weight loss of the fermenter. Calculation of the CO_2 production rate was based upon polynomial smoothing (Sablayrolles, Barre, & Grenier, 1987).

2.4. Analytical methods

2.4.1. Sugar content

Must sugar contents were evaluated by refractometry as recommended by OIV (OIV, 2007).

2.4.2. Ammoniacal nitrogen measurement

Ammonium salts were measured by an enzymatic assay (Enzytec, DiasSys Diagnostic Systems GmbH, Holzheim, Germany).

2.4.3. Amino acids measurement

Free amino acids were determined according to Benson, Gordon, and Patterson (1967) by ion exchange chromatography using an