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Validation and comparison of analytical methods used to evaluate the nitrogen status of grape juice

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Abstract

Three methods used to evaluate nitrogen grape juice status were studied and compared in this work. HPLC was used as reference in the determination of amino acids. Some validation parameters such as quantification limits, precision and accuracy levels were determined with FAN (free amino nitrogen) and formaldehyde methods. Afterwards, these methods were compared with NOPA (*o*-phthaldialdehyde/*N*-acetyl-L-cysteine assay) and with HPLC. Both formaldehyde and FAN methods showed high level of precision and low analytical limits. After validation, the methods were used to determine assimilable nitrogen in 28 grape juices from 10 different grape varieties grown in the Douro Region. The comparison between methods indicates that the formaldehyde procedure yields higher values ($p < 0.05$) than HPLC, FAN and NOPA methods. Thus, ammonium analysis must be run separately and NH_4^+ nitrogen values should be added to the figures obtained by HPLC, FAN and NOPA, in this case no significant differences were observed between the four methods.

Keywords: Grape juice; Assimilable nitrogen; HPLC; FAN; Formaldehyde; NOPA

1. Introduction

Insufficient assimilable nitrogen (free amino acid plus ammonium) in grape musts is known to be the major cause of stuck and sluggish fermentations (Boulton, Singleton, Bisson, & Kunkee, 1996; Henschke & Jiranek, 1993; Kunkee, 1991) and sulphide production (Giudici & Kunkee, 1994; Henschke & Jiranek, 1991; Henschke & Jiranek, 1993). The total assimilable nitrogen content of must can affect the aroma composition of the wine (Rapp & Versini, 1991) and ethyl carbamate production (Ough, Crowell, & Mooney, 1988). It is therefore advantageous to assay grape musts to enable determination of assimilable nitrogen if and whenever necessary. This will allow for its prediction and correction prior to fermentation. The existence of rapid, accurate and precise methods of assimilable nitrogen determination would indeed constitute a valuable tool for winemakers. Several analytical methods are usually used. However, their analytical quality is insufficiently known. The FAN (free amino nitrogen) method described in 1972 by EBC (the European Brewing Commission) deals with the α-amino acids. The formaldehyde procedure provides a useful approximate index of the nutritional status of the juice. A more recent method using *o*-phthaldialdehyde (NOPA) (Dukes & Butzke, 1998) is able to detect free α-amino acid nitrogen by derivatization from primary amino groups with *o*-phthaldialdehyde (OPA). Due to the specificity of this reaction to primary amino acids, the amino acid proline cannot form a derivative and is thus not taken into account in this method. With the NOPA procedure the ammonium ion, the second major source of assimilable nitrogen, is recovered at about 3.5%. Shively and Henick-Kling (2001) claimed that the formaldehyde and NOPA methods yielded similar results. Gump, Zoecklein, Fugelsang, and Whinton (2002) on the other hand, reported an increase of 28.5% in nitrogen values using the formaldehyde reaction in comparison to those obtained with the NOPA procedure.

The purpose of this work is to evaluate some of the validation parameters of FAN and formaldehyde methods,

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and comparing these with HPLC (specific assay) and NOPA procedures. In addition, the advantages of the methods investigated in the present study and discrepancies found between them will be discussed.

2. Materials and methods

The grapes used in this study were picked up from 8-year-old vines grown in the Douro Region, in the north-western of Portugal. Samples (600 berries from each grape variety) with a similar Brix content of about 20 Brix were collected. The juices obtained from these grape varieties were analysed either separately or in mixtures.

2.1. Sample preparation

The grapes were manually crushed, centrifuged at 11,200*g* at 10 °C using a Damon/IEC Division IECB-20A centrifuge. The samples were stored at −20 °C and were allowed to thaw to 20 °C before being analysed.

2.2. Analysis of juice nitrogen components in the juices

The FAN analyses were conducted using the testing method described by the European Brewing Commission. The ninhydrin reacts with the α-amino acids, which are oxidatively descarboxylated with CO_2 formation, NH_3 and an aldehyde. The samples were measured at 570 nm using a Shimadzu UV-2101PC spectrophotometer.

Formaldehyde titration analyses were conducted employing the Aerny procedure (Aerny, 1996). The results obtained in this study were calculated using a general equation: Assimilable nitrogen (mg N/L) = [(vol. NaOH) × (conc. NaOH) × 14 × dilution factor × 1000]/(sample volume). In the titration procedure a potentiometer Crison Basic 20 was used.

NOPA is an *o*-phthaldialdehyde/*N*-acetyl-L-cysteine spectrophotometric assay described by Dukes and Butzke (1998). Amino nitrogen was determined by reading the absorbance of the derivitized samples at 335 nm using a Shimadzu UV-2101PC spectrophotometer. The amount of ammonium was determined through an enzymatic assay (Boehringer Mannheim Reference No. 1112732), in accordance with the manufacture's instructions.

HPLC analysis was conducted using a Gilson HPLC system with Spherisorb S3 ODS C18®, 3 μm, 4.6 × 150 mm, Waters column. The procedure used automated, online derivatization with *o*-phthaldialdehyde (OPA)/2-mercaptethanol (MCE) which reacted with primary amino acids functions, as described by Cooper, Ogden, McIntosh, and Turneel (1984) and Sorensen, Sorensen, Bjergegaard, and Michaelsen (1999). The following amino acids are analysed by this procedure: aspartic acid, glutamic acid, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, valine/methionine, tryptophan, phenylalanine, isoleucine and leucine.

2.3. Analytical limits

The limits were determined in a model grape juice (per litre, D-glucose 100 g, D-fructose 100 g, L-tartaric acid 5 g, L-malic acid 3 g). The pH was adjusted to 3.5 with KOH 0.1 N. In order to assess the detection limits (DL) and quantification limits (QL), 20 samples of model grape juice were immediately analyzed. Detection limits were calculated as follows: Av + 3SD, Av (average) and SD (standard deviation), and quantification limits were: Av + 10SD.

2.4. Spike recovery

The degree of recovery was evaluated in both red and white juice. Several amino acids and ammonium were separately added to the juices in the following concentrations: arginine and proline 250, 500, 750, 1000, 1250, or 1500 mg/L; Ammonium 30, 60, 90, 120, 150, or 180 mg/L; serine and glutamic acid 100, 200 or 300 mg/L. Duplicates of each sample were run using both methods. Samples containing each nitrogen compound were thawed and warmed up to room temperature (±20 °C) prior to analyses.

2.5. Repeatability

In order to assess the repeatability of the methods a mixture of grape juice from 10 grape varieties was used. Ten samples of red and white grape juice were consecutively analyzed using each method. The white grape juice was the first to be tested, followed by the red grape juice. Prior to this analysis, both juices were kept at room temperature (±20 °C).

2.6. Intra-laboratory reproducibility

To enable assessment of intra-laboratory reproducibility of the methods, the red and white grape juices were analysed thrice. The first two analyses occurred in two consecutive days and the third a week later. All three analyses were conducted by the same researcher, in the same laboratory using different reagents and equipments. To test the formaldehyde method, two potentiometers were used (Crison Basic 20 and Crison *micro pH 2002*) whereas for the FAN method, two spectrophotometers, Spectronic 20 Genesys and Shimadzu UV2101PC, were used. Those apparatus were alternatively used in the three different days of the analysis.

2.7. Statistical analysis

Assessment and validation of the quantification limits, repeatability and intra-laboratory reproducibility and calculation of average, range, standard deviation and relative standard deviation were performed using Microsoft Excel 2000 software. For methods comparisons, the Tukey–Kramer test was used by JMP program (JMP 2000). Correla-