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Grau de Química

Treball Final de Grau

Method validation of enzymatic ammonia method and colorimetric free amino nitrogen method applied to wine industry.
Validació del mètode enzimàtic de nitrogen amoniacal i del mètode colorimètric de nitrogen amínic lliure en la indústria vinícola.

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Science is the progressive approximation of man to the real world.
Max Planck

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REPORT

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1. SUMMARY

The branch of enology is one of the most complex areas in the alcoholic beverage field. Nowadays there is still much information to discover about microbiological processes which occur on must fermentation. Year after year, enology industry is developing new methods to control and optimize fermentation in order to obtain very specific sensorial characteristics. Since the 90's, wine makers have focused on strain yeast to achieve the required characteristics. But right now experts seem to aim that nutrient addition has also a relevant role. Huge varieties of nutrients are entering the wine market and consequently, are taking importance in wineries.

The necessity to analyse assimilable nitrogen is irrefutable and new methods are being implemented. In this work, it was studied the reliability of two methods for nitrogen analysis: ammonia nitrogen by enzymatic method and free amino nitrogen by NOPA method. Both methods are spectrophotometric methods and the sum of both results gives to analyst the yeast assimilable nitrogen.

Neither of these methods is present in O.I.V. (International organization of vine and wine) compendium of international methods for wine and must analysis. However, nowadays there are a lot of wineries which wager for them. Principal benefits of these methods are the analysis speed, the easy automatization and the diminution of human mistake.

This project guarantees the main analytic parameters needed to implant these methods as reliable methods. Linearity, limits of detection, exactitude and robustness were studied to validate them. Methods were verified for different kind of samples; water, must, finished wine, fermenting wine and also the nutrients addition effects over previous samples.

Keywords: NOPA, ammonia enzymatic method, yeast assimilable nitrogen, FAN, wine, method validation, nutrients, TDI

2. RESUM

La branca de l'enologia és una de les àrees més complexes de les begudes alcohòliques. Avui dia encara hi ha moltes incògnites sobre els processos microbiològics que es produeixen en la fermentació del most. Any rere any, la indústria està desenvolupant nous mètodes per controlar i optimitzar les fermentacions amb la finalitat d'obtenir unes característiques sensorials molt específiques. Des dels anys 90, els enòlegs s'han centrat en el tipus de llevat per aconseguir les característiques desitjades. Però actualment els experts apunten a que l'addició de nutrients també pot tenir un paper rellevant. Una gran varietat de nutrients estan entrant en el mercat del vi i, en conseqüència, estan prenent importància en els cellers.

La necessitat d'analitzar el nitrogen assimilable és irrefutable i nous mètodes estan sent aplicats. En aquest treball es va estudiar la fiabilitat de dos mètodes d'anàlisis de nitrogen; Nitrogen amoniacal per el mètode enzimàtic i nitrogen amino lliure pel mètode colorimètric NOPA. Tots dos mètodes són mètodes espectrofotomètrics i la suma dels seus resultats proporciona a l'analitzador el nitrogen total assimilable.

Cap d'aquests mètodes està present en el recopilatori de la O.I.V. (Organització Internacional de la Vinya i el Vi) sobre mètodes internacionals d'anàlisis de vins i de mosts. No obstant això, avui dia hi ha cada cop més cellers que aposten per ells. Els principals beneficis d'aquests mètodes són la velocitat d'anàlisi, la fàcil automatització i la disminució de l'error humà.

Aquest projecte garanteix els principals paràmetres analítics necessaris per implantar aquests mètodes com a mètodes fiables. Es va estudiar la linealitat, els límits de detecció, la exactitud i la robustesa per a validar-los. Es van verificar els mètodes per a diferents tipus de mostres: aigua, most, vi acabat, vi en fermentació i també els efectes de l'addició de nutrients sobre les mostres anteriors.

Paraules clau: NOPA, mètode enzimàtic amoniacal, nitrogen fàcilment assimilable, vi validació de mètodes, nutrients, TDI.

3. INTRODUCTION

Wine has an important historic role in Catalonia. It was introduced by the Greeks in Empordà and expanded throughout Catalonia during the Roman Empire. In the eighteenth century, winemaking was promoted on the coast and pre-littoral areas for export to the south of France and America. With the arrival of the insect phylloxera to France, in 1865, producers oriented their exports to the south of France to satisfy the great French demand. Later on, phylloxera reached Catalunya destroying the entire Catalan vineyard in 1900. The subsequent replanting brought a change in the productive areas and Penedès became the largest production zone. Since 1911, the "Mancomunitat de Catalunya" promoted the creation of wine cooperatives, coinciding with the "modernism movement" that led to the construction of large cooperative wineries called "wine cathedrals" due to their architectural significance.

During First World War there was an exceptional demand, however it was slow down by Spanish civil war and Second World War. In 1960 the cultivation was renewed and there were introduced new varieties of grape and new winery methods, emphasizing the Penedès area as a pioneer in technology. Nowadays many localities owe their wealth to wine production, making viniculture vital in Catalonia.[1] [2]

The art of winery has always been related to climate and terrain properties; nevertheless, nowadays wine can be produced anywhere thanks to technology and globalization. Importing the grapes when necessary, the production no longer depends only on the environment. On the other hand, exhaustive control of the fermentation parameters allows winemakers to achieve the desired characteristics for their products in a very reproducible way.

It is worth noting that, around 1980, the advances in biotechnology allowed specific strains of yeasts to be commercialized, giving rise to a technological race in the wine-making sector. Yeasts have been the technological focus over the last decades; commercial providers of yeasts offer winemakers the possibility of producing wine with very specific properties dependent on the selected strain. As a matter of fact, it seems that yeast nutrition also has a decisive role in the final properties of a wine. Therefore, it is not only the strain but also the fermentation medium that must be considered to elaborate a specific sensory profile and wine composition.[3]

3.1. ALCOHOLIC FERMENTATION PROCESS

The first step of winemaking begins in the vineyard, which gives an intrinsic composition to the grape, and consequently to the corresponding wine. There are many factors that cannot be modified once the fruit has been harvested, so it is important to pay attention to this step in order to enhance the wine properties.

The grape must fermentation is done usually by inoculated yeast, which needs nutrients to grow. Nutrients are also needed to build more yeast cells, as well as to produce enzymes and other proteins, which have a relevant role in metabolite transport.

Saccharomyces cerevisiae is the main yeast on industrial fermentations. This yeast ferment sugars, converting hexoses (6-carbon sugars) to two pyruvate molecules (3-carbons atoms); the energy of this bond breaking is captured in the form of ATP. This biochemical process is called glycolysis and produces two ATP molecules.

The next route after glycolysis depends on oxygen availability. In aerobic conditions Krebs cycle is done, producing ATP and NADH molecules. The produced NADH is oxidized in the respiratory chain by oxidative phosphorylation in the mitochondrion. This oxidative process creates three ATP molecules for each NADH. Adding up all the routes, on aerobic conditions, each initial hexose can produce 36 ATP molecules.

On the other hand, on anaerobic conditions, the two NADH molecules created during glycolysis cannot be oxidized by the respiratory chain without oxygen. In order to deoxidize NADH to NAD^+, the enzymes pyruvate decarboxylase and alcohol dehydrogenase converts pyruvate into ethanol and carbon dioxide. As a consequence, each hexose produces 2 ATP molecules.[4,5]

Despite the oxygen availability is the main factor to decide which metabolic route is done, it has been seen that some yeast species such as *S. Cerevisiae* use fermentation even in the presence of oxygen, provided the glucose concentration is sufficiently high. The use of fermentation in presence of oxygen and at high glucose concentrations is known as Crabtree effect.[6] Nowadays, Crabtree effect is not used in wineries.

3.1.1. Nitrogen involved on fermentation

Nitrogen availability is important for wine making: it regulates the formation of yeast biomass and, in turn, the fermentation rate and the time to complete it. Slow or stuck fermentation is often related to a deficient nitrogen concentration. The nitrogen status may also affect the production of many volatile compounds that contribute to wine flavour, alcohols with more than two carbon atoms synthesis, fatty acids and their ethyl ester or acetate ester derivatives. The levels of nitrogen sources have also effect on the activities of metabolic routes involved in the production of glycerol and organic acids.

Even though nitrogen is the primary element of the nutrients, not all nitrogen forms are useful for yeast. Yeast Assimilable Nitrogen (YAN) is described as the nitrogen that can be used by yeast. It is compounded mainly by ammonium ions, primary or alpha amino acids and small peptides. It is worth noting that proline, a dominant secondary amino acid in many grape varieties, cannot be assimilated under anaerobic conditions. Therefore, the principal focus of nitrogen will be the ammonia (NH_4^+) and free amino nitrogen (FAN), excluding proline. YAN can be approximated as the sum of FAN and ammonia, because the peptides are in very low concentration.[7]

YAN concentration has a direct effect on fermentation processes. In general terms, a grape juice containing less than 150 mg/L YAN should be supplemented at least to 150-200 mg/L, in order to avoid a slow or stuck fermentation. Low YAN concentration also increases the risk of H_2S producing[8] as well as “higher” alcohol production, which is characterized by fuel-like odours that have a negative impact on wine aroma, mainly because they mask the fruity characters. High YAN concentrations enhance fatty acids ethyl esters and acetate esters production. These metabolites can have interesting implications for wine flavour, because they are generally responsible for fruity aromas, but a high concentration can also give unwanted sensorial characteristics and may be carcinogenic.[3,9] There is not a specific concentration of YAN to optimize fermentation; it depends also, for instance, on yeast’s strain, temperature, aeration or presence of grape solids. It is needed to know each winery characteristics to calculate the nutrient supplements required.

Although all nitrogen compounds that are classified as YAN are assimilated by yeast they do not all support growth equally. Glutamine, glutamate, asparagine and ammonium sources are preferred, since they allow a high specific growth rate. Otherwise, proline, allantoin and urea

are non-preferred nitrogen sources. Yeast uses a regulation mechanism called nitrogen catabolite repression (NCR) to enhance the nutrition through the preferred nitrogen sources. Additional regulatory mechanisms involving the plasma membrane SPS sensor also regulate amino acids transportation.[10]

In grape juice there is a complex mixture of ammonium and amino acids. The substrates can be classified into four groups based on the time and the percentage of removal during fermentations. The first group is consumed almost totally during the first hours of fermentation; the second group is removed gradually throughout the process; the third group is only used after the depletion of the compounds of the first group. Finally, proline, which is not assimilated under anaerobic conditions, constitutes the fourth group.[10]

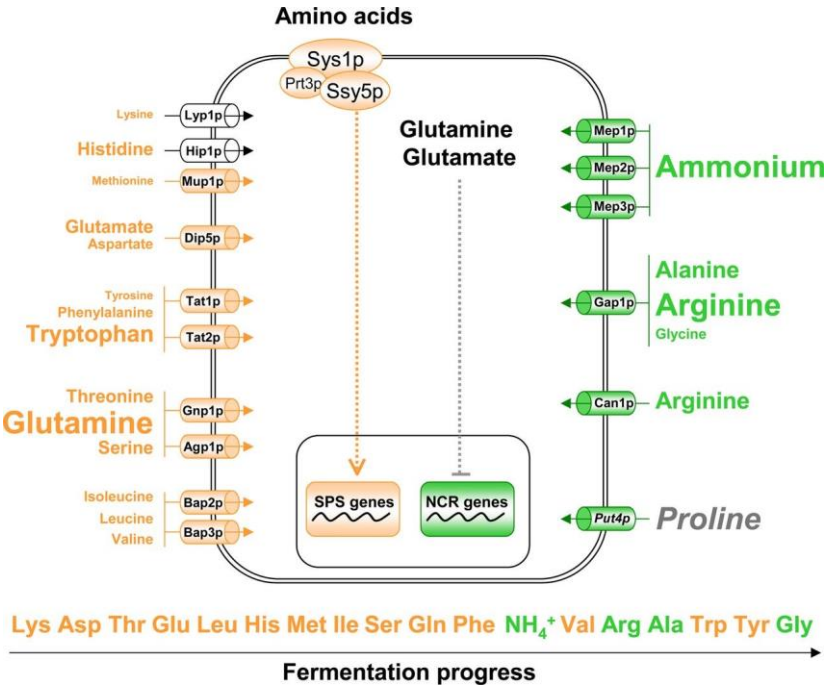

Figure 1. Sequental use of nitrogen compunds by *S.cerevisiae*.
(*Image extracted from ref.10*)

3.1.2. Supplementing nutrients

Grape must YAN composition has generally ammonium ions and amino acids. The major sources of amino acids are proline and arginine (30 to 65% of amino acids content). They are located mostly in grape's skin, so grapes' processing practices are crucial.

Therefore, the importance of controlling YAN levels is clear. There are several kinds of nutritional supplements, but they can be separated in two groups: inorganic and organic supplements. The most common inorganic nutrient is DAP (diammonium phosphate) which has a 21% of nitrogen. DAP's main disadvantages are wine acidification and phosphate concentration. On the other hand, the organic supplements use to be mixtures of amino acids. They provide a more regular fermentation and better organoleptic results. None of these nutrients have a relevant effect on the final ethanol concentration. It has been proven that nitrogen addition at one-third of the fermentation process is the best option.[11] At this time all grape nutrients have been consumed by yeast for the multiplication phase and to build biomass. It is not recommended to add nutrients at the beginning as it leads to a very high yeast population, a sudden increase in fermentation speed, accompanied by an exothermic reaction and high nitrogen depletion.[11]

3.2. ANALYSIS METHODS OF NITROGEN

There are many sources of nitrogen but only YAN is important to wine fermentation. On the next sections, the main methods of YAN determination will be explained. OIV suggest only three analysis methods for measuring nitrogen in wines and musts: two for analyses of total nitrogen (OIV-MA-AS323-02A:R2009 and OIV-MA-AS323_02B:R2009) and one for ammonia nitrogen (OIV-MA-AS322-01:R2009).[12] The three methods proposed are manual and require a significant effort in both time and human resources. Moreover, no method is proposed by OIV for FAN analysis.

3.2.1. Ammonia determination

There are different methods suggested for ammonia determination, but the fastest one is the enzymatic analysis. Furthermore, it can be easily automatized and it reduces human mistakes.

OIV method is based on the separation of ammonia ions by a chromatographic column and followed by the titration with hydrochloric acid. Another method relies on the use of a specific electrode which has a similar principle than pH-meters but measuring ammonia ions instead.

This project will focus now on the enzymatic method, which consist in reducing NADH to NAD^+ in the presence of glutamate dehydrogenase. Reagents are 2-oxoglutarate (1) and ammonia (2) to form L-glutamic acid (3) in acid medium.

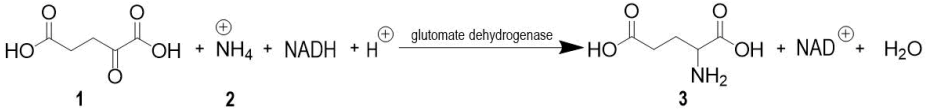

Figure 2. Ammonia enzymatic reaction.

The amount of NAD^+ that is formed in this reaction is stoichiometric with the amount of ammonia. The consumption of NADH is measured by the decrease of absorbance at 340 nm. The time of reaction is 2 minutes at 37 ºC, or 5 minutes at 25 ºC.

Enzymatic reactions are very specific and often have problems with the presence of macromolecules which can mask the active centre of the enzyme. On wine samples, polyphenols are the higher macromolecules which could mask the enzymatic reaction. This problem is resolved by adding PVP to the wine samples in order to mask polyphenols and avoid the obstruction of the active centre

3.2.2. FAN determination

There are two methods described, but only one is actually useful to regular wine analysis. The chromatographic methods (HPLC and GC) are expensive and slow. It is normally used by researchers, but not for a quality control. Therefore, this project will focus on the NOPA method.

NOPA method is a colorimetric method and it is the most used for NOPA determination. It has the advantage of not quantifying proline amino acid, which is the only alpha-amino nitrogen that yeast cannot assimilate in standard fermentation conditions because it needs oxygen to be hydrolysed. The result of this method provides the FAN value to the analyst.

The reaction is done by o-phthalaldehyde (4), n-acetyl-l-cysteine (5) and the primary amino acid (6); it forms an isoindole product (7), which has a maximum absorbance at 340 nm.

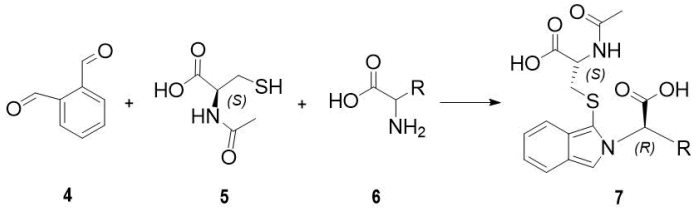

Figure 3. NOPA reaction: Determination of alpha-amino nitrogen.

3.2.3. YAN determination

There are two appropriate technics to determine YAN, Sörense and ninhydrin methods.[4,7] The first one is more frequently used because of its simplicity and availability. It is a titration of the sample with formaldehyde and it is very useful for YAN estimation, even though it has some errors associated. The formaldehyde does not totally react when it has ammonia present in the sample and it also partially reacts with proline. It approximately determines 85% of ammonia, 90-120% of FAN and 17-33% of proline, but it is difficult to know the exact percentage and it also depends on the sample's composition.[10]

Ninhydrin method seems to be more precise, but it also determines proline. This causes trouble when it comes to know the exact FAN concentration. In order to have a more accurate measurement, it is crucial to know proline's concentration and practically the only way of doing it is through a chromatographic determination.

3.3. TECNOLOGÍA DE DIFUSIÓN IBÉRICA, S.L.

Since its inception, in TDI (Tecnología de Difusión Ibérica), it have been pioneers in the Spanish market and our enological analytical philosophy is to adapt the analyser to the needs of each user, reducing analysis cost, and to offer the most comprehensive advice. Being a family business allows them to offer a flexibility that many multinationals do not have, without forgetting the quality, rigour and resources of a large company. TDI offers the widest range of products, which can be from the most sophisticated analyser to a simple titrator, thus being able to advice winemakers at all times on the most suitable instrument for their needs.

It is worth noting that since 2013 a customer has detected some measurement mistakes on a NOPA reagent, which measure alpha-amino nitrogen. Those problems were only visible in

measurements made during nutrient addition and the results seem not to follow any patron, only a dispersion of points with no sense. Before starting to solve the issue, it had to know how and when winemakers use nitrogen kits. The outcome of the request was that all other customers who use kits during fermentation have not found any problems with them. A technical of TDI went to the previous customer's laboratory and verified the anomaly.

Hypothetically, it was speculated that such behaviour might rise as consequence of metabolite interference generated during the fermentation process. On the other hand, not all fermentation processes cause this mistake, so the interference may come from some of the specific supplements added to the grape juice by the particular customer.

In the fermentation process there are so many metabolic routes and in consequence a lot of reactions involved. Each one takes specific nutrients and sugars from the juice to give specific products, which can cause interferences. The proportion of each metabolic route depends not only on substrates' availability, but also on temperature, pressure, yeast strain or oxygen concentration. Therefore, the difficulty to find the interference is remarkable.

At this point, the main objective of the company is to verify ammonia and NOPA methods by a method validation in aqueous, finished wine and fermenting wine mediums. Once these validation errors are detected it will be easier to find the possible interferences or, at least, to better define when to apply each method.

4. OBJECTIVES

- Establish a method validation for ammonia and NOPA methods in aqueous and finished wine medium.
- Study the repeatability and reproducibility on dynamic samples (wine fermenting medium)
- Study the effect of nutrient addition on static and dynamic samples for both methods and for different kinds of nutrients.
- Compare the nutrients behaviour between the customer which has the complaint and other commercial nutrients.
- Find and repair the possible interferences or errors, if they exist.

5. EXPERIMENTAL SECTION

All the experiments have been measured using Miura One® instrument, once in TDI laboratory and the other in "Cooperativa del Sarral" laboratory.

The reagent kits, alpha-amino and ammonium nitrogen, used in the experiments come from the same production batch.

5.1. MIURA ONE® INSTRUMENT

Miura One® is an automatic multiparametric analyzer for chemical analysis by enzymatic, colorimetric and turbidimetric methods for all types of wines and musts. A reagent kit must be bought for each parameter and placed into the instrument. Miura One® also needs systemic and cleaner solutions, which are used for fill up the hydraulic circuits and for the cuvettes washing.

The published technical specifications are:

- Rotary distribution system.
- 120 analysis / hour.
- 20 positions for refrigerated reagents.
- 15 positions for samples with unlimited recharge.
- Pre and post automatic dilution.
- Calibration line from a single standard.
- Validated and widely contrasted analytical techniques.
- Minimum reagent consumption by analysis.
- Automatic washing station for reaction and reading cells.
- Specific program for enology
- Relevant technical datasheet
- 80 incubation and reading cuvettes
- Controlled incubation temperature at 37,0 ºC ± 0,2 ºC
- Programmable incubation and reading times
- Photometric resolution of 0,0001 Abs
- Wavelength range: 340-700 nm

For ammonia and free amino nitrogen methods studied, sample results are given in mg of nitrogen for litre. The sum of both results is the YAN measurement.

5.2. ALPHA-AMINO NITROGEN METHOD – TDI KIT

5.2.1. Reagents

The kit is composed of two reagents to mix with sample; N-acetyl-L-cysteine and o-phthaldialdehyde. These reagents react with free amino acids in the sample to form isoindole derivatives (see Figure 3) which have the maximum absorbance at 340 nm. As the reaction is stoichiometric, it is possible to know primary amino nitrogen concentration by measuring the absorbance of the isoindole solution.

Reagent	Composition	Volume
R1 liquid	o-phthaldialdehyde, stabilizers, preservatives	2x45 mL
R2 liquid	N-acetylcysteine, stabilizers, preservatives.	1x10 mL

Table 1. Reagents of TDI's alpha-amino nitrogen kit.

5.2.2. Samples

This kit is optimized for samples of wine.

In case of turbid samples, these should be filtered or centrifuged.

In case of sample containing CO_2, these must be degasified.

Samples with concentration over the specified linearity limit should be accordingly diluted with distilled water.

5.2.3. Sampling procedure

Firstly, it is necessary to have information about the sample in order to do the previous treatment if it is needed. On the Miura One® instrument, the sample is mixed with the R1 solution in a sample/R1 ratio of 1:100. After 36 seconds Miura reads the absorbance of solution (A1). Then, it adds R2 to the solution in proportion 2:25 (Sample:R2) and waits for 480 seconds before reading absorbance (A2). Absorbance is measured at 340nm.

The Miura One® *carries* on a blank analysis using the same procedure, but replacing the sample for a volume of water. Then, the calculation is performed by subtracting the increase in absorbance from the blank to the increase of the sample.

5.3. AMMONIA NITROGEN METHOD – TDI KIT

5.2.1. Reagents

The kit is composed for two reagents intended to be mixed with sample; enzyme glutamate dehydrogenase (GIDH) and α-chetoglutarate. The enzyme catalyses the condensation of ammonia and α-ketoglutarate pass to L-glutamate with the concomitant oxidation of nicotinamide adenine dinucleotide (NADH). (Reaction shown in Figure 2).

The oxidation of NADH causes a decrease in absorbance at 340 nm, which is proportional to the amount of ammonia in the sample.

Reagent	Composition	Volume
R1 liquid	oGood's buffer, glutamate dehydrogenase, stabilizers, preservatives.	2x45 mL
R2 liquid	NADH, α-chetoglutarate.	1x12 mL

Table 2. Reagents of TDI's ammonia nitrogen kit

5.2.2. Samples

This kit is optimized for samples of wine.

In case of turbid samples, these should be filtered or centrifuged.

In case of sample containing CO_2, these must be degasified.

Samples with concentration over the specified linearity limit should be accordingly diluted with distilled water.

5.2.3. Sampling procedure

The sampling procedure and calculation of ammonia concentration is done just as te sampling procedure of alpha-amino method (see page 16) but by changing the reagent/sample proportion: Sample:R1 at 1:40 and Sample:R2 at 1:4 proportion.

5.4. EXPERIMENTS IN TDI LABORATORY'S (WITHOUT FERMENTATION)

All the experiments done in TDI had aqueous or finished wine medium, so the samples may be stables since there was no fermentation. The aqueous medium was ultrapure water 0.22 µm (Milipore Milli Q®), and the finished wine came from "Cooperativa del Sarral".

In this section, the aqueous reference materials used are produced and commercialized by TDI. These standards had been compared with certificate reference materials to pass the quality controls. In addition, it was also used wine standards which actually are not commercialized by TDI. Those standards were prepared following the same steps used in the preparation of the aqueous standards but changing Ultrapure water for finished wine and considering the initial nitrogen concentration on it (see sections 5.4.1 and 5.4.2). Three wine standards were prepared for ammonia nitrogen at 250 mg/L of concentration, and also for alpha-amino nitrogen. Three different wine standards were prepared using red, rose and white wine.

The first experiments carried on at TDI were aimed to obtain the method validation parameters. Experiments were designed following the cited bibliography.[13] Same parameters were determined for ammonia and NOPA methods including:

- Linearity, limits of detection and quantification
- Exactitude (precision and veracity)
- Robustness

5.4.1. Preparation 25 mL of ammonia wine standard

Dissolve the following compounds in specified order in 20 mL of finished wine in a 50 mL flask magnetically fixed at 300-500 rpm.

- 0.0500 g preservative
- 0.0250 mL acidulant
- 0.0050 g preservative
- X mL STD NH_4^+ 5,000 mg/L
- 0.0200 g preservative
- Make up to 25 mL with finished wine

The volume of NH_4^+ standard solution needed depend on experimental ammonia concentration of the finished wine used in the experiment.

5.4.2. Preparation 25 mL of alpha-amino wine standard

Dissolve the following compounds in specified order in 20 mL of finished wine in a 50 mL flask magnetically fixed at 300-500 rpm.

- X g L-isoleucine
- 0.0125 g preservative
- Make up to 25 mL with finished wine

L-isoleucine weight needed depend on experimental alpha-amino concentration of the finished wine used in the experiment.

5.5. EXPERIMENTS IN "COOPERATIVA DEL SARRAL" LABORATORY'S (WITH FERMENTATION)

All the experiments done in "Cooperativa del Sarral" had fermenting wine medium, so samples may not be stable until fermentation was done. The Sarral's Miura One® analyzer was daily used to analyse wine components to control fermentation process. It was also important to have an account that all the fermenting wine sampled contains different kind of nutrient added.

As the methods were validated on the previous section it was not needed to validate again all the parameters. The parameters studied in fermentation samples were the following ones:

- Precision
- Robustness

Figure 4. Façade of Cooperativa del Sarral winery.
(Image extracted from http://www.doconcadebarbera.com).

5.6. NUTRIENT ADDITION

Studying the comportment of ammonia and NOPA methods during the nutrient addition phase is the main objective of this project. The customer complains that nitrogen kits fail when nitrogen is supplied to the fermentation medium. On one hand, the measured nitrogen concentration increase after nutrients addition seems not to fill with expected values. On the other hand, alpha-amino method was no repetitive in short analysis periods.

To carry on our study, the customer provided us with all the nutrients that triggered the unwanted behaviour. One of the nutrients was diammonium phosphate at 99% (w/w) and the other one was a commercial organic nutrient with no information available about its actual composition. The only information about that organic nutrient was that it was similar to the others commercial organic nutrients.

In order to have additional nutrients to extend our study, "Cooperativa del Sarral" provided us an organic nutrient and an inorganic nutrient that they use during the fermentation process. These nutrients came with its own technical data sheet, which is resumed on Table 3.

Nutrient	Dosis (mg/L)	YAN (mg/L)	Ammonia (mg/L)	FAN (mg/L)	Ammonia (%)	FAN (%)	YAN (%)
CooDAP	300	81	81	0	27.00	0.00	27.00
CooORG	300	44	4	40	1.33	13.33	14.67

Table 3. Nutrients used in "Cooperativa del Sarral" and its theoretical nitrogen composition.

Figure 5. Nutrient solutions in an aqueous medium: 0.2% (w/w). Left: Organic nutrient; Right: DAP nutrient

5.6.1. Static samples

Firstly, solubility was studied of each nutrient in ultrapure water. Nutrient providers fix the dosage around 300 mg/L, which is equivalent, approximately, to 0.03 % (w/w). At this concentration, nutrients were expected to be soluble but the experiments that were carried out need the highest concentration as it was possible in order to do the minimal matrix interference.

Concentrations were studied between 0.2% to 2% for each nutrient and the obtained results were compared with those calculated from the technical datasheet. In non-solubility cases, ethanol concentration effect and filtration effect were studied.

On the other hand, repeatability and the stability of nutrients in static samples were studied.

In those experiment, the tested mèdiums were red finished wine and ultrapure water.

5.6.2. Dynamic samples

This section was carried out in “Cooperativa del Sarral” laboratory’s and the samples were fermenting wine from different vats. Nutrient from a stock aqueous solution were added to the fermenting wine samples. Results were compared with the technical datasheet and also with static samples results.

6. METHODS VALIDATION

6.1. LINEARITY AND LIMITS OF DETECTION AND QUANTIFICATION

In order to define the linearity of methods, absorbances of eleven solutions were measured for ammonia and NOPA methods. The solutions were prepared by dilution of the standard stock solution and their concentration ranged from 0 to 500 mg/L. In addition, absorbances of ten samples of ultrapure water (blank solution) were also measured to obtain the limits of these methods, from de blank standard deviation value it was calculated LOD and LOQ. Results from these experiments are shown in Table 4.

Parameter	FAN	Ammonia
Linearity range	0-250 mg/L	0-250 mg/L
Regression curve	Abs = 0.009·[NH_2]+0.0408	Abs = -0.0041·[NH_4^+]-0.0682
r^2	0.9996	0.9999
LOD	5 mg/L	19 mg/L
LOQ	6 mg/L	23 mg/L

Table 4. Linearity parameters and limits for both methods studied in an aqueous medium. (*All results in Appendix 1*)

Both methods offer an excellent linearity up to 250 mg/L, with a good regression factor. The limits obtained differ qualitatively between methods; NOPA method has a low LOD and LOQ while the ammonia method has a higher value.

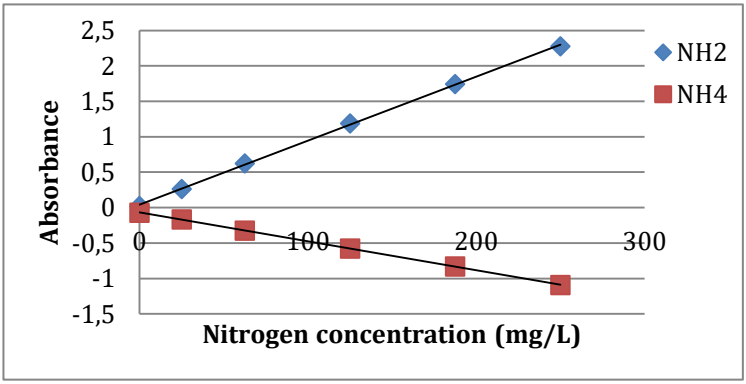

Figure 6. Calibration curve for both methods studied.
Batch number of reagents 2408116421 and 2407116421.

6.2. EXACTITUDE

The exactitude of a method is defined as the combination of veracity and precision. Veracity was checked from water standards and verified by t-student test. Precision was studied in repeatability and reproducibility terms.

6.2.1. Veracity

The first series of experiments were aimed to the measuring of TDI standard (250.0 mg/L) aqueous samples on different dilutions to get more representative values over the entire measuring range. Three level of concentrations (50.0, 125.0 and 250.0 mg/L) were studied. Each of these levels was measured thirty times; veracity and repeatability were calculated from the obtained values.

Method	Average (mg/L)	Std. deviation (mg/L)	RSD (%)	Calculated t	Critic t
FAN	52.3	1.0	2.0	0.435	2.05
	126.8	1.0	1.0	0.316	
	250.9	2.0	1.0	0.084	
Ammonia	47.2	1.1	2.0	0.454	
	124.2	2.1	2.0	0.071	
	247.4	2.4	1.0	0.198	

Table 5. Results and statistic treatment of standard samples of alpha-amino nitrogen. Thirty measures for each concentration's level and method.

As it is shown in Table 5, t-studenCalculated t parameter is lower than t-studenCritic tal parameter so it could be concluded that both methods are veracious over the entire measuring range, in an aqueous medium. Moreover, the statistic parameters of repeatability show a good precision level, perhaps organic nitrogen method seems to be more repetitive than the inorganic one. Both methods are used by winemakers to know approximately the level concentration of YAN on grape must, during fermentation or in finished wines. For such applications it is not needed a high exactitude. These parameters are used to decide the right amount nutrient to add, taking into account that differences below 10 mg/L are negligible. Thus, a standard deviation of 1 or 2 mg/L is considered a good result in this context.

In a second serial of experiments, finished wine standards were prepared and analysed to verify the veracity in wine matrix. In this case, it was only analysed the stock solution of 250 mg/L because it was expected to yield the less accurate results. The low accuracy was expected because these standards did not pass any quality control and were no prepared from reference certificate material. Also, in the calculation of these standards, it was used the initial wine concentration from experimental results, which involves more error. The results of those experiments are shown in Table 6.

Method	Kind of Wine	Average (mg/L)	Std. deviation (mg/L)	RSD (%)	Calculated t	Critic t
FAN	Red	245.9	3.5	1.0	0.48	2.05
	Rose	245.3	2.6	1.0	0.73	
	White	247.1	3.6	1.0	0.33	
Ammonia	Red	242.5	2.5	1.0	1.22	
	Rose	248.0	3.0	1.0	0.27	
	White	251.8	1.6	1.0	0.47	

Table 6. Results and statistic treatment of wine standard samples of FAN and ammonia nitrogen. Six measures for each kind of wine and for each method.

Although the veracity of the methods was confirmed in all kinds of wine it was observed a global increase in standard deviation parameter. This increase could be associated to wine matrix but not to the kind of wine. Red wines have more complex matrix than rose wines, and in turn, these have more complex matrix than white wines. More experiments must be perdormed to corroborate this affirmation.

6.2.2. Precision

In order to have more information about the precision about the method, repeatability and the reproducibility were studied deeply. Three wine samples and three aqueous samples were prepared from wine and aqueous standards, respectively, for each method. All these samples were measured along several days after its preparation and each sample was measured six times for day. Table (7) resumes the main statistic results extracted from those experiments (*all results in Appendix 2*).

Medium	Statistic parameter	FAN		Ammonia	
		Within days	All days	Within days	All days
Aqueous	Std.Dev (mg/L)	0.4 – 1.7	0.6 – 1.8	0.0 – 2.6	1.3 -4.0
	RSD (%)	0.4 - 1.2	0.7 – 1.2	0.0 – 3.6	1.5 -2.7
Wine	Std.Dev (mg/L)	1.4 - 8.0	5.0 – 7.2	1.7 – 6.8	3.5 – 5.3
	RSD (%)	1.6 - 11.3	3.2 – 11.6	0.7 – 5.1	2.1 -6.7

Table 7. Standard deviation of six measurements for sample on five different days. Each sample was measured a total of 30 times (counting all days). Three concentration levels were studied (50, 125 and 250 mg N/l).

Results of aqueous samples from Table 7 shows a good repeatability and reproducibility, so the statistic parameters are better than those of veracity experiments (see Table 5). Repeatability was evaluated by calculating statistic parameters of the thirty measurements of each sample. Reproducibility was studied from measurements of five different days for each sample.

Obviously, the higher the concentration the higher the standard deviation and less RSD, but the range was no so high to define an individual standard deviation for each concentration range. In an aqueous medium, both methods are repetitive and reproducible. Additionally, the standard deviation of the methods in an aqueous medium was also extracted from this experiments (see Table 8).

Wine medium affect negatively to both parameters. For the alpha-amino method the increase of the dispersion is higher than ammonia method. Wine medium has a negative effect on repeatability and reproducibility for both methods.

Medium	FAN	Ammonia
Aqueous	1.8 mg N/l	4.0 mg N/l
Red wine	7.2 mg N/l	5.3 mg N/l

Table 8. Standard deviation of alpha-amino nitrogen and ammonia nitrogen methods

Values shown in Table 8 can be treated as the standard deviation of each method. It is expected that these standard deviations are the highest deviations that the methods could have over the entire measuring range. The lower sample concentration is, the lower deviation the method should have.

It can be concluded that alpha-amino method is more reliable in aqueous medium but ammonia method has a better comportment in wine medium.

6.3. ROBUSTNESS

The wine standards shown in Table 6 were used in order to evaluate the robustness of the methods. The first experiment consists in diluting the standards with ultrapure water and studying the matrix dilution impact on the final result. On Table 9 summarizes the dilutions, the expected concentration from experimental results of the standards, and the experimental results of dilutions. Each experimental result comes from six analysis of each sample.

Method	Kind of Wine	Expected concentration (mg/L)	Experimental concentration (mg/L)	Standard deviation (mg/L)	Calculated t	Critic t
FAN	Red	122.9	127.5	3.4	0.546	2.05
		49.2	48.4	0.3	1.252	
	Rose	122.6	124.1	1.6	0.365	
		49.1	50.9	1.2	0.592	
	White	123.6	131.9	2.4	1.418	
		49.4	51.4	0.7	1.202	
Ammonia	Red	121.3	118.2	2.0	0.617	
		48.5	47.2	1.8	0.300	
	Rose	124.0	120.2	2.4	0.652	
		49.6	42.8	2.3	1.192	
	White	125.9	127.3	1.5	0.384	
		50.4	47.8	1.5	0.703	

Table 9. Results and statistic treatment of diluted wine standard samples. Six measures for each dilution.

The results indicate that matrix dilution does not have a significant effect on either method. Second set of experiments were designed to evaluate the impact of colour on matrix. The colour effect was studied by comparing experimental results from discoloured and non-discoloured wine standards. Discolouration was made with active carbon.

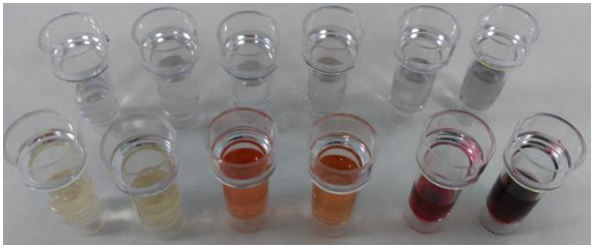

Figure 7. Three kind of wine discoloured samples by active Carbon in front non-discoloured samples. Miura cuvettes used.

Younden and Steiner experiment[13] was designed and the effect of discolouration was compared with $\sqrt{2}\cdot S$ in order to decide if it was a significant effect. The standard deviation used for the comparison was the obtained in Table 8 for wine medium.

Method	kind of wine	Factor	Average (mg/L)	Std. deviation (mg/L)	RSD (%)	Discolour effect	$\sqrt{2}\cdot S$ (mg/L)
FAN	Red	Normal	238.0	4.8	2.0	2.2	10.2
		Discolour	235.8	1.4	0.6		
	Rose	Normal	243.2	2.1	0.9	13.3	
		Discolour	229.9	1.9	0.8		
Ammonia	Red	Normal	236.0	4.2	1.8	5.2	7.5
		Discolour	241.2	3.7	1.5		
	Rose	Normal	243.8	5.1	2.1	5.7	
		Discolour	249.5	1.0	0.4		

Table 10. Results and statistic treatment of diluted wine standard samples. Six measures for each dilution.

Firstly, the effect of discolouration on the NOPA method is notable in rose samples but not in red samples. These preliminary results suggest that discoloured samples reduce the measurement value. This effect can be explained by the adsorption power of active carbon, that adsorbs colourised substance (principally polyphenols) and some amino acids. In the case of red wine, the concentration of polyphenols is very high so the ability of active carbon to adsorb another substance is reduced.

In the case of ammonia method, the tendency was the opposite of that NOPA method. In this case, discoloured samples had more measured values than original samples. This effect was expected as ammonia method is an enzymatic reaction and such reactions are very specific. Macromolecules like polyphenols (responsible of wine colour) present in wine could mask the active centre of enzyme, reducing the efficiency of the reaction. So, when polyphenols are adsorbed by active carbon the reaction efficiency increases.

Although discoloration effect is observed in both methods, this effect does not significantly affect their robustness. In order to have more information, white wine samples were also discoloured.

Method	kind of wine	Factor	Average (mg/L)	Std. deviation (mg/L)	RSD (%)	Discolour effect	$\sqrt{2} \cdot S$ (mg/L)
FAN	White	Normal	240.6	2.0	0.8	11.7	10.2
		Discolour	228.9	2.0	0.9		
Ammonia	White	Normal	247.5	5.1	2.1	8.0	7.5
		Discolour	255.5	5.3	2.1		

Table 11. Results and statistic treatment of diluted wine standard samples. Six measures for each dilution.

The set of experiments can be treated the blank assays because white samples had initial color the polyphenols concentration is expected to be lower. Tendency observed in Table 10 was seen again in this experiment. It will be necessary to study if active carbon adsorb amino acids. If this hypothesis is refused, another option could be the colourimetric interference of the residual active carbon present in samples after filtration.

It was also confirmed that active carbon has a positive effect in ammonia method, because it removes substances present in wines that mask the enzymatic reaction.

In summary, it can be concluded that both methods are not robust. There are different responses in water medium than in wine medium. On the other hand, when methods are used only for wine samples, the sample dilution had not effect on the results over the entire measuring range. Additionally, discolouration is not useful for the alpha-amino method because it reduce FAN measurement, otherwise for ammonia method could have a positive effect but it must be deeply studied.

Figure 8. Miura One®'s interior. Rotary sampling arm, reagents' and samples' positions, rotary cuvettes system (red circle).

7. PERFORMANCE DURING FERMENTATION PROCESS

In this section, it was studied the repeatability and reproducibility from dynamic samples, where fermentation ongoing. As the samples were obtained from fermenting vats, it was impossible to control the concentration levels of each sample.

7.1. REPEATABILITY AND REPRODUCIBLY

Two experiments were designed in order to analyse repeatability and reproducibility during the fermentation process. The first one consists in the analysis of two wines with a similar nitrogen concentration.

Results are shown in Table 12.

Method	Sample	Average (mg/L)	Standard deviation (mg/L)	RSD (%)
FAN	99	74.7	0.7	1.0
	104	74.9	1.0	1.0
Ammonia	99	32.4	0.8	2.6
	104	35.6	0.7	2.0

Table 12. Results and statistic treatment of measures of fermenting wine samples with alpha amino nitrogen and ammonia methods. Ten measures for sample.

As it is shown in the results of Table 12, both vats exhibited similar behaviour for both methods. The standard deviations were lower than what could be expected from method validation (see wine samples of Table 8). It was concluded that the methods still being repetitive.

In the second experiment, results of five aliquots from the same wine vat sample were compared, analysing three times for each aliquot. The experiment was done in two different days. Since fermentation is ongoing, samples are not stable. This fact complicates the reproducibility's study. In order to obtain some information about it, the standard deviations obtained were compared. Results summarized in Table 13 exhibit a good concordance between standard deviations.

Method	Day (dd/mm/yy)	Sample	Average (mg/L)	Standard deviation (mg/L)	RSD (%)
FAN	25/10/16	71	39.40	0.46	1.16
		77	234.20	0.62	0.27
	27/10/16	71	46.67	0.74	1.59
		77	11.53	0.45	3.90
Ammonia	25/10/16	71	21.1	0.80	3.9
		77	58.9	1.3	2.2
	27/10/16	71	21.3	1.3	6.1
		77	40.0	0.9	2.3

Table 13. Results and statistic treatment of measures of fermenting wine samples with alpha amino and ammonia nitrogen methods. Fifteen measurements for sample, method and day.
(A*ll results in Appendix 3*)

7.2. ROBUSTNESS

There is suspension solids presence in wines during fermentation process so in this case it was necessary to study the effect of turbidity. Discolouration effect was studied again with red wine despite it was expected to not have any effect. To perform this study a Younden and Steiner experiment was designed with the factors of filtration and discolouration as it is shown in Table 14.

Factor	1	2	3	4	Filtration effect	Discolour effect	√2 S
Filtration	Yes	No	Yes	No			
Discolouration	No	No	Yes	Yes			
[NH_2] (mg/L)	94.6	95.0	92.8	92.6	0.1	2.1	10.2
[NH_4^+] (mg/L)	162.4	159	161	163.6	0.4	1.6	7.5

Table 14. Younden and Steiner experiment design. Results are the average from six measurements of each sample. Standard deviation (S) used from Table 8, in wine medium.

The results proved that filtration effect was not important for the turbidity of the samples analysed. However, it is not clear the role of filtration in more turbid samples. As it was seen, samples did not have effect for red wines. It could be concluded that the method is robust for the parameters studied.

8. NUTRIENTS ADDITION

The main problem that was intended to study in this project was the complaint of one client about TDI nitrogen analysis kits when the nutrient addition is done during the fermentation process. In this section, it was studied the comportment of nutrients of two providers. Inorganic nutrients (CooDAP and CuDAP) were expected to have the same behaviour between providers because it is 99% diammoinum phosphate in both cases. On the other hand, organic nutrient that came from "Cooperativa del Sarral" (CooORG) had a technical data sheet but the other customer's organic nutrient (CuORG) was provided with no information. This fact made difficult to know if experimental results agree with the expected results. In order to have a starting point for this organic nutrient the theoretical FAN concentration was approximated to the "Cooperativa del Sarral".

8.1. STATIC SAMPLES

Before studying nutrient addition it was studied the solubility of nutrients. Provider's datasheet suggests additions of 20-30 g/hL which is approximately 0.02-0.03% (w/w). Thus, a set of aqueous nutrient solutions of different concentration were prepared to determine the solubility. . For non-soluble samples, it was studied the effect of adding ethanol.

The aim of this study was performed in order to prepare stock solutions with a high concentration. High concentration was needed to add the minimal aqueous medium on wine samples during the nutrient addition. For non-soluble solutions, it was also studied the effect of filtration and it was concluded that it had no effect to nitrogen concentration measurement.

As expected for iònic salts, inorganic nutrients were completely soluble in the range of concentrations used in our experiments. However, organic nutrients depend on providers. Organic nutrient used by "Cooperativa del Sarral" (CooORG) were soluble. However, customer's organic nutrient CuORG was not soluble at any studied concentration, and the presence of alcohol reduced the solubility even more.

All the nutrient solutions studied before were filtrated and measured by both nitrogen analysis methods and the results are resumed on Table 15 These results came from the average of measurements of different concentration solutions for each nutrient, except CuORG in alcohol medium which was discard because nitrogen measured was very low in comparison

to other solutions of the same nutrient. It was because organic samples with alcohol medium were not soluble.

Nutrient	Experimental		Theoretical	
	Ammonia (%)	FAN (%)	Ammonia (%)	FAN (%)
CooDAP	14.83	1.14	27.0	0.0
CooORG	0.00	4.26	1.3	13.3
CuDAP	15.22	1.17	27.0	0.0
CuORG	0	3.20	-	-

Table 15 Experimental results in front theoretical results for the nutrients studied.

Both DAP nutrients studied had similar behaviour but it did not agree with the theoretical datasheet. For CuORG there was not knowledge about theoretical concentration; even so, the measured FAN concentration was lower than the concentration of other organic nutrients in the market.

Before studying dynamic samples it was also studied repeatability and reproducibility of nutrients solutions analysis in aqueous and finished red wine medium. In these experiment, only nutrients provided by the customer that complained were used. by the customer who had the complaint were used. This experiment was performed as the 6.2.2 section. Results were summarized in Table 16 (all results in Appendix 4).

Medium	Statistic parameter	FAN Determination of "CuORG"		Ammonia[a] Determination of "CuDAP"	
		Within days	All days	Within days	All days
Aqueous	Std.deviation (mg/L)	0.4 – 2.3	0.8 – 1.8	0.0 – 2.4	1.1 – 2.4
	RSD (%)	0.2 – 2.0	0.7 – 1.5	0.0 – 3.5	0.4 – 3.5
Wine	Std.deviation (mg/L)	2.3 – 8.8[b]	5.8 – 8.8	1.5 – 3.7	2.5 – 6.9
	RSD (%)	1.0 – 9.1[b]	3.8 – 13.4	0.8 – 5.1	0.8 – 3.9

(a) Some values out of linearity interval, for wine medium.
(b) Two values discarded for being out from the tendency.

Table 16. Experimental results *vs* theoretical results for the nutrients studied.

Nutrients presence in the static medium did not affect either repeatability or reproducibility since obtained values in Table 16 are similar than Table 7.

8.2. DYNAMIC SAMPLES

All the nutrients carried on before this section had corroborated that both methods are repetitive and reproducible for the conditions studied. The only thing missing to be studied was the behaviour when nutrients are added to wine during the fermentation process. These experiment were carried on the "Cooperativa del Sarral" over both CooDAP and CuDAP.

Aqueous stock solutions were prepared with a concentration of 0.2% (w/w) for each nutrient and for each provider (see experimental values on Table 17, experimental stock solution column). Stock solutions were added to fermenting wine samples on different concentration as it is shown in *Appendix 5*.

All results are summarised in Table 17. Experimental wine additions term is referred to the extrapolation of stock solution concentration. This extrapolation is calculated from results obtained and from the volume added, as in a standard addition.

Nutrient	Experimental stock solution (mg/L)		Experimental wine additions (mg/L)		Theoretical (mg/L)	
	FAN	Ammonia	FAN	Ammonia	FAN	Ammonia
CuDAP	38.7	309.3[a]	27.4	302.7	0.0	540.0
CuORG	74.3	17.3	70.3	15.2	-	-
CooDAP	35.2	317.2[a]	40.9	406.1	0.0	540.0
CooORG	87.67	29.7	79.1	24.1	269.1	26.9

(a) Values out of linearity interval

Table 17. Experimental results *vs* theoretical results for the nutrients studied. Six repetitions for each measurement done.

Results obtained shows that there were no significant differences between the stock solution and the results from wine additions. Wine samples were fermenting and in consequence, there was a nutrient consume. This fact is observed when experimental wine additions are lower than experimental stock solutions.

However, experimental results differed from theoretical values in datasheets.

8.3. NUTRIENT SUMMARY

Nutrient	FAN (%)			Ammonia (%)		
	Theoretical	Aqueous	Wine	Theoretical	Aqueous	Wine
CuDAP	0.00	1.17	2.22	27.00	15.22	24.60
CuORG	-	3.23	3.24	-	0.00	0.70
CooDAP	0.00	1.14	1.88	27.00	14.83	18.33
CooORG	13.33	4.37	3.54	1.33	0.00	1.34

Table 18. Experimental results in front theoretical results for the nutrients studied. Six repetitions for each measurement done.

As it is shown in Table 18 experimental values are lower than theoretical values from datasheet. DAP seems to have more concordance in wine matrix than the aqueous matrix, but it is necessary to study it deeply because the wine matrix used was a diluted matrix.

The behaviour of organic nutrients (CooDAP and CuDAP) was similar, especially in wine medium. But the lack of customer's organic nutrient information makes it difficult to interpret the results.

9. CONCLUSIONS

- Method validation for ammonia and NOPA methods was performed correctly on static samples (aqueous and wine medium) and no error was detected.

- Repeatability and reproducibility on dynamic samples (wine fermenting medium) were verified for ammonia and NOPA methods since results coincided with wine static samples.

- It was studied the effect of nutrient addition on static and dynamic samples for both methods and for different kinds of nutrients. Results obtained corroborate that nutrients addition do not affect to the precision of either method. Veracity should be studied more deeply, but the results obtained seem to indicate that nutritional values of providers' datasheet are greater than the real ones.

- DAP nutrients are composed of diammonium phosphate at 99%, more experiments are needed to know why DAP nutrients has different behaviour than commercial salt used in the laboratory of TDI

- Organic nutrients are principally composed of different amino acids, but providers do not indicate which amino acids are present in their nutrients neither their composition. It is expected that each amino acid has a different sensibility for each method as is described in bibliography.[14, 15] It could not be affirmed that providers are lying in their technical datasheet, but differences are high enough to suspect that they could consider some amino acids as assimilable when they are not. Another option could be that method used has a good sensibility for L-isoleucine, but a bad sensibility for other assimilable amino acids present in nutrients. Both hypotheses will be corroborated or discarded by HPLC or GC experiments in future work.

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11. ACRONYMS

- ATP Adenosine triphosphate
- CooDAP Diammonium phosphate nutrient from “Cooperativa del Sarral”
- CooORG Organic nutrient from “Cooperativa del Sarral”
- CuDAP DAP nutrient from customer with the complaint.
- CuORG Organic nutrient from customer with the complaint.
- DAP Diammonium phosphate
- FAN Free Amino Nitrogen
- GC Gas chromatography
- HPLC High performance liquid chromatography
- NAD^+/NADH Nicotinamide adenine dinucleotide
- NOPA O-phthaldialdehyde/N-acetyl-L-cysteine method
- OIV International Organisation of Vine and Wine
- PVP Polyvinylpyrrolidone
- TDI Tecnología de Difusión Ibérica, S.L.
- YAN Yeast assimilable Nitrogen

APPENDICES

APPENDIX 1: LINEARITY RESULTS

Concentration (mg/L)	FAN Absorbance	Ammonia Absorbance
0.0	0.022	-0.073
25.0	0.261	-0.167
62.5	0.622	-0.324
125.0	1.189	-0.577
187.5	1.743	-0.832
250.0	2.279	-1.091
300.0	2.63	-1.221
350.0	2.951	-1.252
400.0	3.198	-1.251
450.0	3.410	-1.263
500.0	3.545	-1.259

Parameter	NOPA method	Ammonia method
Standard deviation	0.00155	-0.00251
y LOD	0.00510	-0.00827
y LOQ	0.01549	-0.02514
x LOD	5 mg/L	19 mg/L
x LOQ	6 mg/L	23 mg/L

APPENDIX 2: REPEATABILITY AND REPRODUCIBILITY IN STATIC SAMPLES

FAN						
Day (dd/mm/yyyy)	Aqueous			Wine		
	Average (mg/L)	Standard deviation	RSD (%)	Average (mg/L)	Standard deviation	RSD (%)
28/11/2016	50.7	0.4	0.8	43.3	1.9	4.5
30/11/2016	51.0	0.5	1.0	45.9	4.0	8.7
01/12/2016	51.4	0.4	0.8	40.6	4.6	11.3
02/12/2016	51.8	0.5	0.9	37.5	1.4	3.6
05/12/2016	51.2	0.6	1.2	47.4	4.9	10.2
All days	**51.2**	**0.6**	**1.2**	**42.9**	**5.0**	**11.6**
28/11/2016	126.1	0.9	0.7	112.4	5.2	4.6
30/11/2016	126.0	0.6	0.4	109.6	7.3	6.7
01/12/2016	125.9	0.5	0.4	110.1	4.5	4.1
02/12/2016	124.7	1.4	1.1	110.4	6.4	5.8
05/12/2016	126.8	0.7	0.6	118.4	2.7	2.3
All days	**125.9**	**1.0**	**0.8**	**112.2**	**6.0**	**5.4**
28/11/2016	249.3	1.1	0.4	221.4	11.0	5.0
30/11/2016	248.8	1.4	0.6	225.1	8.0	3.6
01/12/2016	250.6	1.0	0.4	220.6	4.9	2.2
02/12/2016	246.9	1.4	0.6	225.2	5.3	2.4
05/12/2016	249.8	1.7	0.7	228.4	3.6	1.6
All days	**249.1**	**1.8**	**0.7**	**224.1**	**7.2**	**3.2**

Ammonia						
Day (dd/mm/yyyy)	**Aqueous**			**Wine**		
	Average (mg/L)	**Std. deviation (mg/L)**	**RSD (%)**	**Average (mg/L)**	**Std. deviation (mg/L)**	**RSD (%)**
28/11/2016	48.5	0.5	1.1	56.2	1.7	3.1
30/11/2016	46.5	0.8	1.8	53.0	1.8	3.4
01/12/2016	47.0	1.7	3.6	57.0	1.8	3.1
02/12/2016	46.0	-	-	50.2	2.6	5.1
05/12/2016	46.0	0.6	1.4	49.8	2.1	4.3
All days	46.8	1.3	2.7	53.2	3.5	6.7
28/11/2016	118.2	2.5	2.1	133.2	5.3	4.0
30/11/2016	115.5	1.0	0.9	126.3	3.4	2.7
01/12/2016	116.3	1.5	1.3	131.7	4.3	3.2
02/12/2016	116.7	1.0	0.9	127.2	2.4	1.9
05/12/2016	115.8	1.3	1.1	124.5	4.5	3.7
All days	116.5	1.7	1.5	128.6	5.1	4.0
28/11/2016	250.2	2.6	1.0	252.3	4.8	1.9
30/11/2016	242.8	2.6	1.1	246.5	3.6	1.5
01/12/2016	246.3	2.3	0.9	254.5	4.2	1.7
02/12/2016	241.2	1.5	0.6	248.0	6.8	2.8
05/12/2016	241.2	0.8	0.3	247.3	1.8	0.7
All days	244.3	4.0	1.6	249.7	5.3	2.1

APPENDIX 3: REPEATABILITY AND REPRODUCIBILITY IN DYNAMIC SAMPLES

FAN					
Day	**Sample**	**Aliquot**	**Average (mg/L)**	**Standard deviation (mg/L)**	**RSD (%)**
25/10/2016	71	71.1	39.7	1.2	2.9
		71.2	38.7	0.6	1.5
		71.3	39.3	1.5	3.9
		71.4	39.3	1.5	3.9
		71.5	40.0	1.7	4.3
	All aliquots		39.4	1.2	3.2
	77	77.1	233.7	1.5	0.7
		77.2	233.3	3.1	1.3
		77.3	232.7	1.5	0. 7
		77.4	234.7	2.1	0.9
		77.5	236.7	2.1	0.9
	All aliquots		234.2	2.3	1.0
27/10/2016	71	71.1	51.0	1.0	2.0
		71.2	48.3	0.6	1.2
		71.3	45.3	2.5	5.6
		71.4	43.7	1.2	2.6
		71.5	45.0	1.0	2.2
	All aliquots		46.7	3.0	6.4
	77	77.1	12.3	0.6	4.7
		77.2	11.7	0.6	5.0
		77.3	11.7	1.2	9.9
		77.4	11.0	1.0	9.1
		77.5	11.0	0.0	0.0
	All aliquots		12.1	1.6	13.2

Ammonia					
Day	**Sample**	**Aliquot**	**Average (mg/L)**	**Standard deviation (mg/L)**	**RSD (%)**
25/10/2016	71	71.1	20.3	0.6	2.8
		71.2	21.0	1.0	4.8
		71.3	21.7	0.6	2.7
		71.4	22.0	0.0	0.0
		71.5	20.7	0.6	2.8
	All aliquots		21.1	0.8	3.9
	77	77.1	60.7	0.6	1.0
		77.2	58.3	1.5	2.6
		77.3	58.3	0.6	1.0
		77.4	59.3	0.6	1.0
		77.5	58.0	1.0	1.7
	All aliquots		58.9	1.3	2.2
27/10/2016	71	71.1	19.7	0.6	2.9
		71.2	20.3	0.6	2.8
		71.3	22.0	1.0	4.5
		71.4	22.7	0.6	2.5
		71.5	22.0	0.0	0.0
	All aliquots		21.3	1.3	6.1
	77	77.1	41.3	1.0	2.4
		77.2	40.0	0.0	0.0
		77.3	40.3	1.2	2.9
		77.4	40.7	0.6	1.4
		77.5	40.0	1.0	2.5
	All aliquots		40.0	0.9	2.3

APPENDIX 4: REPEATABILITY AND REPRODUCIBILITY IN NUTRIENT STATIC SAMPLES

CuDAP – Ammonia analysis						
Day (dd/mm/yyyy)	Aqueous			Wine		
	Average (mg/L)	Std. deviation (mg/L)	RSD (%)	Average (mg/L)	Std. deviation (mg/L)	RSD (%)
29/11/2016	65.5	0.55	0.84	69.0	3.52	5.10
30/11/2016	66.0	2.28	3.46	66.8	1.47	2.20
02/12/2016	63.0	0.63	1.00	68.5	2.07	3.03
05/12/2016	63.2	1.33	2.10	68.0	2.28	3.35
12/12/2016	66.5	1.22	1.84	70.7	2.50	3.54
All days	**64.8**	**1.9**	**3.0**	**68.6**	**2.6**	**3.8**
29/11/2016	164.8	1.72	1.04	172.7	2.50	1.45
30/11/2016	167.7	1.21	0.72	174.0	3.74	2.15
02/12/2016	163.7	2.25	1.38	174.3	5.01	2.87
05/12/2016	163.3	1.21	0.74	170.7	5.89	3.45
12/12/2016	166.8	2.40	1.44	186.7	1.63	0.87
All days	**165.3**	**2.4**	**1.5**	**175.7**	**6.9**	**3.9**
29/11/2016	290.5[a]	1.05	0.36	298.5[a]	2.81	0.94
30/11/2016	292.0[a]	-	-	297.7[a]	2.50	0.84
02/12/2016	290.0[a]	-	-	298.7[a]	2.34	0.78
05/12/2016	291.3[a]	0.52	0.18	298.5[a]	2.81	0.94
12/12/2016	289.8[a]	1.47	0.51	299.5[a]	2.43	0.81
All days	**290.7[a]**	**1.1**	**0.4**	**298.6[a]**	**2.5**	**0.8**

(a) Value out of linearity interval

CuORG – FAN analysis						
Day (dd/mm/yyyy)	**Aqueous**			**Wine**		
	Average (mg/L)	**Std. deviation (mg/L)**	**RSD (%)**	**Average (mg/L)**	**Std. deviation (mg/L)**	**RSD (%)**
29/11/2016	54.6	0.43	0.79	39.3	8.76	22.29[b]
30/11/2016	54.8	0.47	0.86	42.0	5.40	12.85[b]
02/12/2016	53.7	1.05	1.96	43.1	3.90	9.06
05/12/2016	55.6	0.35	0.63	47.0	3.33	7.09
12/12/2016	55.0	0.37	0.67	47.1	3.43	7.29
All days	**54.7**	**0.8**	**1.5**	**43.7**	**5.8**	**13.4**
29/11/2016	127.2	0.62	0.49	113.6	3.50	3.08
30/11/2016	128.9	0.75	0.58	116.7	3.43	2.94
02/12/2016	127.1	1.03	0.81	116.6	5.96	5.11
05/12/2016	130.6	0.93	0.71	112.7	4.88	4.33
12/12/2016	128.8	0.83	0.65	119.6	12.29[b]	10.27[b]
All days	**128.5**	**1.5**	**1.2**	**115.8**	**6.8**	**5.9**
29/11/2016	244.4	2.32	0.95	235.9	15.81[b]	6.70
30/11/2016	245.6	1.70	0.69	233.1	7.90	3.39
02/12/2016	243.1	1.33	0.55	234.8	7.70	3.28
05/12/2016	246.6	0.46	0.19	237.3	7.88	3.32
12/12/2016	245.1	0.59	0.24	236.9	2.33	0.98
All days	**245.0**	**1.8**	**0.7**	**235.6**	**8.8**	**3.8**

(b) Discarded result

APPENDIX 5: NUTRIENT ADDITION IN DYNAMIC SAMPLES

Sample	Wine volume (mL)	Stock solution volume (mL)	Ammonia (mg/L)			FAN (mg/L)		
			Theoretical from datasheet	Expected from Table 3	Experimental	Theoretical from datasheet	Expected from Table 3	Experimental
Blank	2.0	-	-	-	11.7	-	-	38.0
CooORG 1	2	0.5	14.7	15.3	12.0	84.2	48.0	46.3
CooORG 2	1.5	1.0	17.8	18.8	17.0	130.4	58.0	51.7
CooORG 3	1	1.5	20.8	22.4	21.3	176.7	68.0	64.7
CooORG 4	0.5	2.0	23.9	26.0	26.7	222.9	78.0	73.3
CooDAP 1	2	0.5	117.3	72.8	90.7	30.4	37.4	39.0
CooDAP 2	1.5	1.0	223.0	133.9	170.0	22.8	36.9	38.0
CooDAP 3	1	1.5	328.7	195.0	257.3	15.2	36.3	41.0
CooDAP 4	0.5	2.0	434.3	256.1	313.7[a]	7.6	35.8	39.3
Blank	2.0	-	-	-	7.0	-	-	48.33
CuORG 1	2	0.5	-	9.1	7.0	-	53.5	52.0
CuORG 2	1.5	1.0	-	11.1	10.3	-	58.7	57.3
CuORG 3	1	1.5	-	13.2	14.0	-	63.9	62.0
CuORG 4	0.5	2.0	-	15.3	17.3	-	69.1	67.7
CuDAP 1	2	0.5	126.0	67.5	67.3	38.7	46.4	42.7
CuDAP 2	1.5	1.0	240.3	127.9	117.7	29.0	44.5	41.0
CuDAP 3	1	1.5	354.6	188.4	184.7	19.3	42.5	37.0
CuDAP 4	0.5	2.0	468.9	248.9	253.7	9.7	40.6	33.7

(a) Value out of linearity interval