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# Determination of Nutrient Supplementation by Means of ATR-FTIR Spectroscopy during Wine Fermentation

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**Abstract:** Nitrogen is a limiting factor for the development of wine alcoholic fermentation. The addition of nutrients and different nitrogen sources is a usual practice for many winemakers. Currently, there is a market trend toward wine that is additive-free and there are also restrictions on the amount of ammonium fermentation agents that can be added to the wine. In this work, the changes produced on the alcoholic fermentation by the addition of different nitrogen sources were evaluated by the use of ATR-FTIR. The results showed the feasibility of this technique to observe differences in the growth yeast capacity depending on the type of the nutrients added. A high influence on the development of the alcoholic fermentation was observed, especially at its exponential and the stationary phases. Moreover, the changes observed in the recorded spectra were related to the proteins and lipid esters composition of the yeast cell wall. This technique should be a useful tool to evaluate nitrogen deficiencies during winemaking although further studies should be done in order to evaluate more influential factors.

**Keywords:** fermentation; infrared spectroscopy; grape juice; chemometrics; yeast

## 1. Introduction

The authenticity and traceability of beverages is a current target in avoiding fraud [1]. The evaluation of the authenticity of wine involves checking several claims, such as the declaration of origin, vintage, and production method [2]. Wine quality is affected by numerous factors, from grape harvest to the fermentation process and ageing. Among these, low temperature winemaking, novel (additive-free) techniques, additions, or even new isolated yeast strains may clearly influence its quality [3–7]. Wines originate as a result of winemaking practices including, among others, the addition and supplementation of some enological products, such as grape juice nutrients. These additions do not usually appear on the labeling and may be misrepresented. Detecting the additions performed during winemaking, at that moment, even if it is legal supplementation, is important for fraud detection and consumer protection. The wine yeast nutrition is essential to ensure their right growth during the wine alcoholic fermentation [8]. The grape juice is the main source of nutrients and its quantitative and qualitative composition strongly influences the fermentation kinetics. Nitrogen is one of the most important nutrients and it is critical to the growth of *S. cerevisiae* [8–11]. The grape juice nitrogen composition influences the final wine quality since it has an important effect on the wine aroma and the health-related metabolic by-products [12–14]. Yeast available nitrogen (YAN) content is limited in the wine alcoholic fermentation process and therefore the addition of nitrogen sources is a usual practice in the winemaking process.

The nitrogen addition allows yeasts growth, the activation of the fermentation process, and the formation of the end-products. Otherwise, the limited nitrogen content directly restricts the metabolic activity of yeasts and may induce sluggish fermentation or even a fermentation stop [15]. Moreover, the undesirable production of by-products, such as $H_2S$, produced by a deficient nutrition environment is considered a great winemaking concern [16,17]. Several studies have shown that *S. cerevisiae* requires minimum levels of YAN (ranged from 120 to 180 mg/L) to obtain the optimum fermentation kinetics [18,19]. Nitrogen addition is recommended to enhance the characteristics of wines [20] and to ensure the end of the fermentation process [9]. The supplementation time is also important, and it seems to be most effective when nitrogen source is added during the acclimation phase of the yeasts, at the beginning of the alcoholic fermentation [9,19]. At this stage, the nitrogen addition has a direct effect on the fermentation kinetics, through the protein synthesis reactivation [21]. On the other hand, a late addition may prevent the sugar transportation and its assimilation by the yeast cells [22]. The timing and also the nature of nitrogen are important factors on the fermentation kinetics, being the organic source the most adequate in some cases [23]. However, the addition inorganic nitrogen sources, mainly the ammonium chloride and the diammonium phosphate salts, can reduce the slow down fermentation risks and the formation of undesirable sulphur compounds [8,13] and improve the aromatic profile of wines [20]. Therefore, the amount, timing, and type of nitrogen supplement can substantially modulate wine volatiles composition [13].

There is currently a market trend toward wine that is additive-free and also there are restrictions on the amount of the ammonium fermentation agents that can be added to the wine [24]. In this way, infrared spectroscopy has been widely used in winemaking as a rapid technique to determine some quality control parameters [25]. In this way, this study was to evaluate the feasibility on the use of ATR-FTIR to determine the wine nitrogen supplementation during alcoholic fermentation.

## 2. Materials and Methods

### *2.1. Nutrient Additions and Enological Analysis*

This study was performed by "Grenache Blanc" white grapes provided by Gandesa winery from Tarragona (Gandesa, Spain). The obtained must was sulphited (40 mg/L of sulphur dioxide), clarified (10 mg/L of commercial enzymes), and settled during 24 h to remove the solid fraction. Then, three batches of cleaned must (150 mL) were inoculated by the E491 *S. cerevisiae* strain (Zymaflore X5®, Laffort, Bordeaux, France). The recommendation provided by the supplier to reach an initial concentration in must of $10^6$ cfu/mL was considered (200 mg/L of inoculum). Afterwards, 100 mg/L of ammonium salts with thiamine was added as inorganic nitrogen source (20 mg/L of YAN, Thiazote, Laffort, Bordeaux, France) and 300 mg/L of amino acids, vitamins and minerals (21 mg/L of YAN, NutriStart OrganiQ, Laffort) was added as organic nitrogen source. Nutrients were added during the yeast rehydration process, following the supplier instructions. Fermentations were performed in triplicate at 21 °C using a thermostatic water bath, and the wine density and the glucose plus fructose content were daily monitored. The basic oenological analysis (pH, acidity, Brix, turbidity, and alcohol content) were performed according to the methods established by the Organisation Internationale de la Vigne et du Vin [26]. Acetic acid and yeast assimilable nitrogen (YAN) was calculated according to the enzymatic methodologies developed by BioSistems S.A.

### *2.2. Infrared Spectroscopy Measurements*

Infrared analyses were performed at 0 h (initial point), 18 h (early exponential phase), 42 h (exponential phase), and 90 h (stationary phase). Samples (1.5 mL) were taken by the use of sterilized laboratory material and centrifuged (15,900× *g* for five minutes at room temperature). Then, the supernatant was carefully removed, and the obtained pellets were washed with a standard sodium chloride solution. Spectral data was acquired with a zinc selenide crystal accessory in reflectance mode from 1.5 µL of sample. Six spectra per each sample were collected in the mid-infrared region (from

4000 to 800 $cm^{-1}$ with 2 $cm^{-1}$ of resolution) by 32 average scans. Spectra were recorded by the use of a Thermo Nicolet 380 FTIR spectrometer with an ATR ultra-high-performance and the OMNIC™, as control software.

### 2.3. Statistical and Chemometric Analyses

SAS® software (SAS® System for Windows™, 8.02, 1999; SAS Institute, Cary, NC, USA) was used and a Tukey test was performed at $p < 0.05$. Spectra were exported to the Pirouette® multivariate analysis software (version 4.0, InfoMetrix, Inc., Washington, DC, USA). The spectral data were mean-centered, transformed to their second derivative using a 15-point Savitzky-Golay polynomial filter, and vector-length normalized. Mahalanobis distance and sample residuals were used to determine outliers. SIMCA was used as predictive model based on the PCA construction models for each class. SIMCA class models were interpreted according to the misclassifications, the class projections, the discriminating power and the interclass distances.

## 3. Results and Discussion

### 3.1. Enological Parameters and Fermentation Process

Table 1 shows the enological parameters of the grape juice used to produce the corresponding wines. The YAN content of the initial must was enough to the strain used (*S. cerevisiae* E491) to grow and complete the alcoholic fermentation on the control samples. All sugars were consumed without any nutritional problems [9,19]. The total acidity (5.5 g/L) and pH (3.1) also presented normal values for "Grenache Blanc" musts [27].

**Table 1.** Enological parameters of the "Grenache Blanc" grape juice.

| Initial Must | Mean ± SD |
|---|---|
| Brix degree (°Bx) | 20.3 ± 0.2 |
| Alcoholic potential strength (% vol.) | 11.8 ± 0.2 |
| YAN (mg/L) | 170 ± 6 |
| pH | 3.1 ± 0.1 |
| Total acidity (g/L) | 5.5 ± 0.1 |
| Turbidity (NTU *) | 18 ± 2 |
| Press yield (%) | 54 ± 0.7 |
| Clarification yield (%) | 81 ± 0.8 |

Different upper-case letter superscripts differ significantly at $p < 0.05$. * Nephelometric Turbidity Units.

The percentage of alcohol produced during the fermentation process showed significant differences (Table 2). Control wines obtained lower alcohol concentration than wines with inorganic and organic nitrogen, in agreement with other authors who reported that an increase of nitrogen favors the formation of the end-products [28,29]. Fermentations were monitored by the daily measurement of residual sugar content (Table 3). The sugar content of samples at the early stage of the exponential phase (18 h) showed significant differences. The samples with inorganic nitrogen showed the highest sugar content (164.7 g/L) at this time. Moreover, lower significant sugar content was also observed at exponential (42 h) and at stationary phase (90 h) in control wines. According to other authors, the nitrogen addition at the beginning of the alcoholic fermentation was quickly assimilated by yeast and had clearly effects on the fermentation kinetics, in this case, the fermentation activity and the formation of the end-products was increased [21,28,29].

At exponential phase, the sugar concentration was 71.6 g/L for control, 56.0 g/L for samples with addition of inorganic nitrogen, and 57.8 g/L for samples with addition of organic nitrogen. At stationary phase, sugar concentrations were 2 g/L for Control and 0 g/L for the samples supplemented with inorganic and organic nitrogen. The nutrient additions at the beginning of alcoholic fermentation is quickly assimilated by yeast and immediately have effects on the fermentation kinetics [21], stimulating

fermentation activity and the formation of the end-products [28,29]. This behavior had been reported by several authors and could explain the significant differences between sugar concentration values detected at exponential and stationary phases.

**Table 2.** Acetic acid and alcohol content of wines.

| Wines | Acetic Acid (g/L) | Alcohol (% vol.) |
|---|---|---|
| Control | 0.42 ± 0.03 $^{a}$ | 12.1 ± 0.1 $^{b}$ |
| Inorganic nitrogen | 0.38 ± 0.02 $^{a}$ | 12.3 ± 0.1 $^{a}$ |
| Organic nitrogen | 0.40 ± 0.02 $^{a}$ | 12.3 ± 0.1 $^{a}$ |

Different upper-case letter superscripts differ significantly at $p < 0.05$.

**Table 3.** Sugar content of must-wine (g/L) at different fermentation times (18, 42, and 90 h).

| Treatment | 18 h | 42 h | 90 h |
|---|---|---|---|
| Control | 162.1 ±1.5 $^{a}$ | 71.6 ± 1.5 $^{c}$ | 1.7 ± 0.5 $^{e}$ |
| Inorganic nitrogen | 164.7 ±1.5 $^{ab}$ | 56.0 ± 1.5 $^{d}$ | 0.0 ± 0.0 $^{f}$ |
| Organic nitrogen | 161.2 ± 0.7 $^{b}$ | 57.8 ± 1.5 $^{d}$ | 0.0 ± 0.0 $^{f}$ |

Different upper-case letter superscripts differ significantly at $p < 0.05$.

### 3.2. Changes during the Alcoholic Fermentation by ATR-FTIR

The representative ATR-FTIR spectra and their second derivative of fresh pellet of *S. cerevisiae* strain E491 fermented with and without the supplementation of inorganic and organic source of nitrogen are shown in Figure 1. The IR bands of highest proportion in the raw spectra were mainly concentrated in two different regions. The first one was located between 900 and 1200 $cm^{-1}$ mainly associated with the polysaccharide absorbing region of *S. cerevisiae* [30] and the second region was located between 1500 and 1750 $cm^{-1}$ mainly formed by the vibrations of proteins and lipid structures [31,32].

The class projections of SIMCA classification model of transformed spectra (1900–800 $cm^{-1}$) of *S. cerevisiae* strain E491 fermented in Grenache blanc must with and without using an extra nitrogen source showed clear differentiation between non-fermented (0 h) and fermented *S. cerevisiae* cells at different fermentation times 18, 42, and 90 h (Figures 2a, 3a and 4a).

Interclass distances (ICD) values further proved these findings since, generally, ICD values above 3.0 are considered significant to discriminate two clusters of samples as a different class [33]. ICD values between non-fermented and fermented *S. cerevisiae* cells without extra nitrogen added, inorganic source and organic source of nitrogen (Table 4) varied from 4.4 to 22.4 showing different pattern of clustering between *S. cerevisiae* cells at early and exponential phase and at stationary phase. Moreover, *S. cerevisiae* cells cluster fermented for 18 h (early exponential phase) without and with nitrogen supplementation showed the highest values of ICD when was compared with the clusters of *S. cerevisiae* cells fermented for 42 h (exponential phase) and 90 h (stationary phase). For instance, in the case of Grenache blanc must supplemented with the organic source of nitrogen (Table 4) the ICD values of *S. cerevisiae* cells fermented for 18 h compared with *S. cerevisiae* cells fermented for 42 h and 90 h were 13.7 and 17.1, respectively. Nonetheless, when *S. cerevisiae* cells fermented for 42 h and 90 h were compared among them, the ICD value was 5.8 and 4.4 for inorganic and organic nitrogen sources, respectively. These results showed that the highest biochemical differences between *S. cerevisiae* cells happened when yeast cells were at early exponential phase in the three matrices tested. Discriminating power of *S. cerevisiae* cells non-fermented and fermented without and with nitrogen supplementation (Figures 2b, 3b and 4b) showed a common band at 986 $cm^{-1}$ linked to $\beta(1\rightarrow6)$ glucans [31]. In the case of discriminating power of *S. cerevisiae* cells non-fermented and fermented with nitrogen supplementation (Figures 3b and 4b), two secondary IR bands at 1026 and 1156 $cm^{-1}$ may be linked to $\beta(1\rightarrow4)$ glucans and C-O, C-OH carbohydrates present in yeast cell wall [31]. According to a previous work made in our laboratory, the compounds mainly responsible of *S. cerevisiae* cells discrimination during the

entirely fermentation were the mannoproteins, lipids, and RNA [32]. Moreover, it is important to mention that discrimination power values of 986 $cm^{-1}$ band decreased with the supplementation of inorganic (2990 to 1432 units) and organic nitrogen (2990 to 1646 units) showing higher discrimination when *S. cerevisiae* cells were fermenting without nitrogen supplementation.

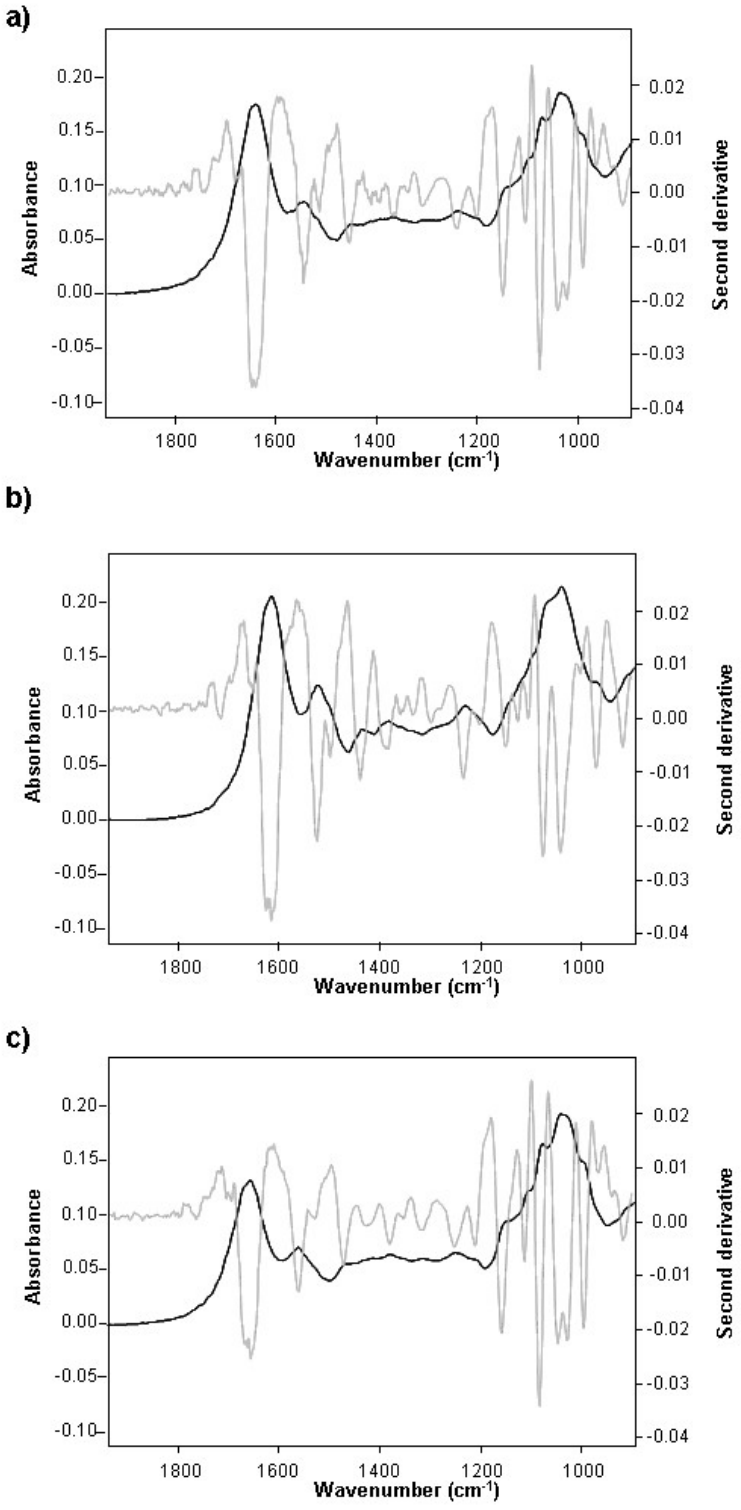


**Figure 1.** Representative ATR-FTIR spectra (in black) and the second derivative (in grey) of *S. cerevisiae* strain ES491 fermented for 18 h without (**a**) and with a source of inorganic (**b**) and organic (**c**) nitrogen. The spectra were taken with a zinc selenide crystal accessory in reflectance mode.

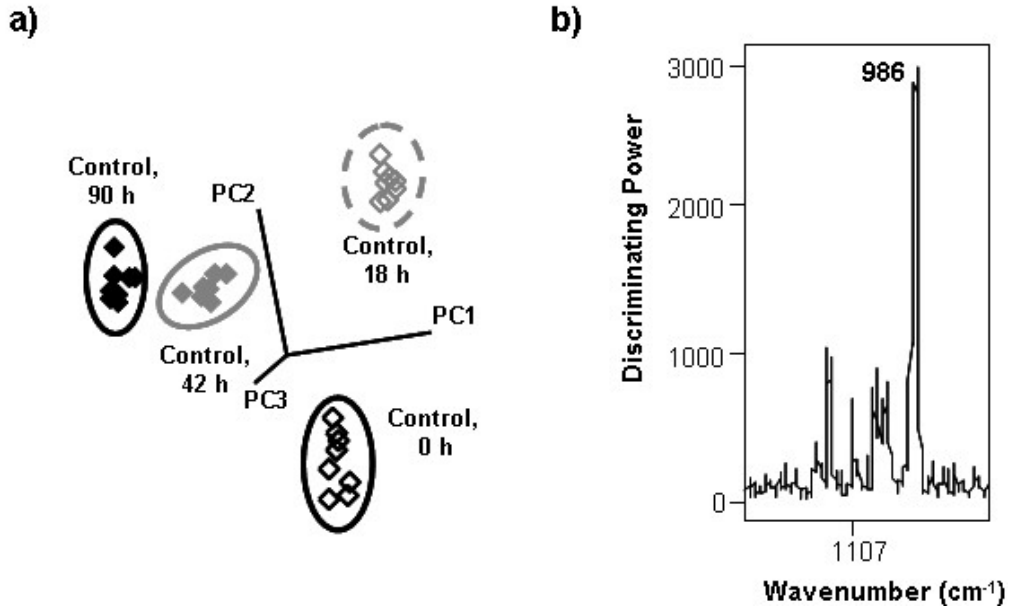


**Figure 2.** Soft independent modeling class analogy (SIMCA) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must without nitrogen supplementation (Control) for 0, 18, 42, and 90 h.

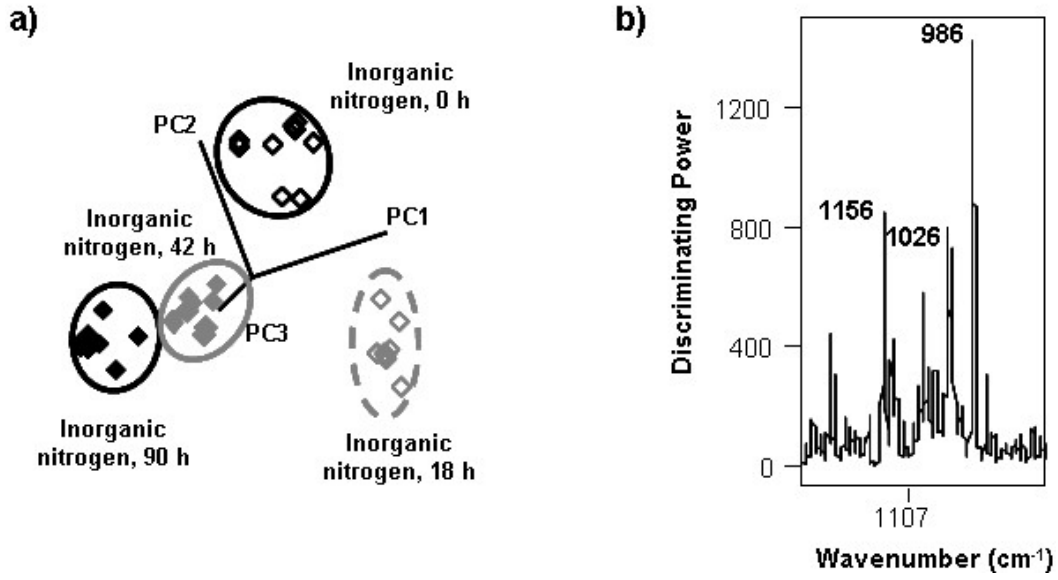


**Figure 3.** Soft independent modeling class analogy (SIMCA) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must with supplementation of inorganic source of nitrogen for 0, 18, 42, and 90 h.

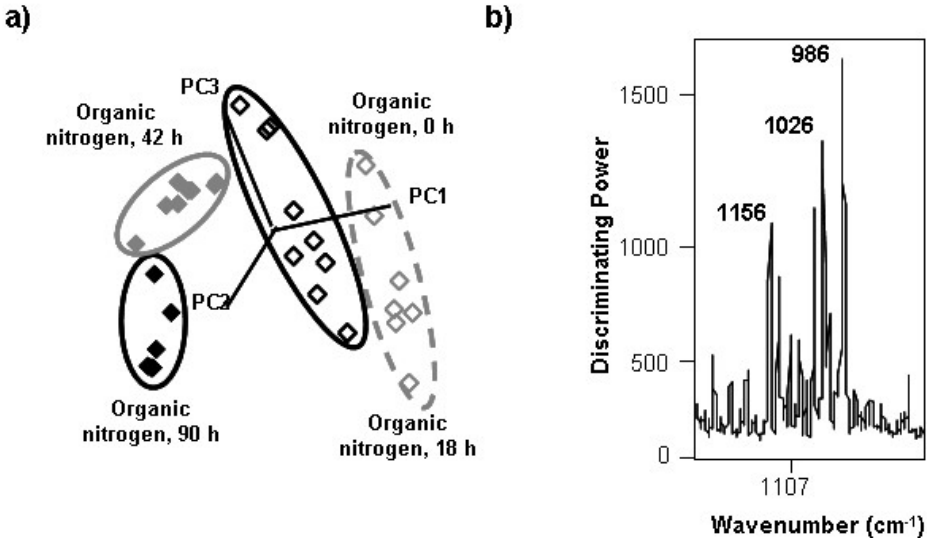


**Figure 4.** Soft independent modeling class analogy (SIMCA) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must with supplementation of organic source of nitrogen for 0, 18, 42, and 90 h.

**Table 4.** Soft independent modeling of class analogy (SIMCA) of interclass distance of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* E491 strain fermented in Grenache must with and without inorganic and organic nitrogen supplementation for 0, 18, 42, and 90 h.

| **Control** | **0 h** | **18 h** | **42 h** | **90 h** |
|---|---|---|---|---|
| 0 h | 0.0 | | | |
| 18 h | 13.3 | 0.0 | | |
| 42 h | 10.3 | 15.6 | 0.0 | |
| 90 h | 16.0 | 22.4 | 6.2 | 0.0 |
| **Inorganic nitrogen** | **0 h** | **18h** | **42h** | **90 h** |
| 0 h | 0.0 | | | |
| 18 h | 7.9 | 0.0 | | |
| 42 h | 7.0 | 13.9 | 0.0 | |
| 90 h | 9.5 | 16.3 | 5.8 | 0.0 |
| **Organic nitrogen** | **0 h** | **18 h** | **42 h** | **90 h** |
| 0 h | 0.0 | | | |
| 18 h | 4.7 | 0.0 | | |
| 42 h | 9.5 | 13.7 | 0.0 | |
| 90 h | 11.7 | 17.1 | 4.4 | 0.0 |

It was also important to study if it was possible to discriminate between *S. cerevisiae* cells fermented without and with nitrogen supplementation. For this purpose, SIMCA models were built up using IR data from non-fermented (0 h) and fermented *S. cerevisiae* cells (18, 42, and 90 h) without and with inorganic and organic nitrogen supplementation (data not shown). The distance between the clusters of non-fermented without and with nitrogen supplementation and fermented samples increased over the time of fermentation excepting for *S. cerevisiae* cells fermented for 42 h (exponential phase) with the addition of nitrogen and *S. cerevisiae* cells fermented for 90 h (stationary phase) with the addition of organic nitrogen (data not shown). In this case, ICD values of non-fermented and fermented *S. cerevisiae* cells without and with the supplementation of nitrogen varied from 1.6 to 19.1 (data not shown). In general, *S. cerevisiae* cells fermented without and with the addition of inorganic source

of nitrogen were clearly differentiated at early (ICD 2.7), exponential (ICD 4.2) and at stationary phase (ICD 3.6). Nonetheless, *S. cerevisiae* cells fermented without and with the addition of organic nitrogen were only differentiated at exponential phase (ICD 2.8). When *S. cerevisiae* cells fermented with the supplementation of inorganic and organic sources of nitrogen were compared, just yeast cells fermented for 42 h (exponential phase) were different (ICD 3.1) and the lowest differences were shown at stationary phase (ICD 1.6). Moreover, *S. cerevisiae* cells cluster fermented for 18 h without and with the supplementation of nitrogen showed the highest values of ICD when was compared with the clusters of *S. cerevisiae* cells fermented for 42 h and 90 h (data not shown). This trend was also detected when SIMCA models of fermented *S. cerevisiae* cells without and with nutrient supplementation were built up separately (Figures 2a, 3a and 4a, and Table 4). Discriminating power (Figure 5) showed a unique IR band at 986 $cm^{-1}$, mainly responsible of the biochemical differences between the compared samples. This band also played an important role on the differentiation of non-fermented and fermented *S. cerevisiae* cells when SIMCA models were built up separately. All the IR bands mainly responsible of the biochemical differences between the samples compared were related with compounds presents in the *S. cerevisiae* cell wall [34–36]. According to these results, supplementing the Grenache blanc must with inorganic and organic sources of nitrogen had an impact on the composition of the yeast cell wall, especially at early exponential phase related to the presence of $\beta(1\rightarrow6)$ glucans.

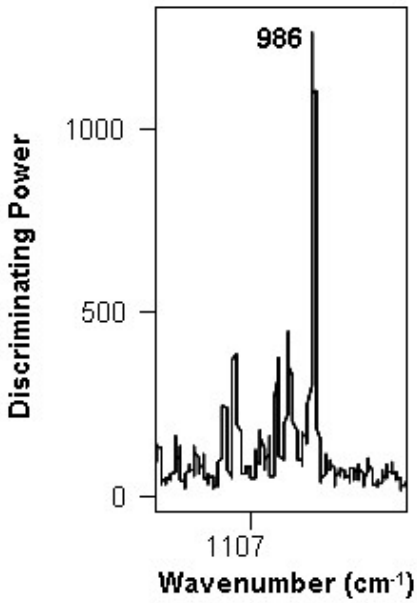


**Figure 5.** Soft independent modeling of class analogy (SIMCA) of discriminating power of *S. cerevisiae* E491 strain fermented in Grenache must for 0, 18, 42, and 90 h without and with inorganic and organic nitrogen supplementation.

In this research it was also interesting to compare among fermented *S. cerevisiae* cells without and with nitrogen supplementation at early exponential (18 h), exponential phase (42 h) and stationary phase (90 h) to study the biochemical differences present in each physiological phase depending on the source of nitrogen used. At early exponential phase, clusters from fermented *S. cerevisiae* cells without (Control) and with inorganic (ICD 4.4) and organic (ICD 3.1) nitrogen supplementation were clearly separated (data not shown). The major discriminating bands observed when 2-classes SIMCA classification models were developed using transformed spectra from *S. cerevisiae* fermented without (Control) and with inorganic (Figure 6a) and organic (Figure 6d) sources of nitrogen were 1538 and 1511 $cm^{-1}$, respectively. The IR bands at 1538 and 1511 $cm^{-1}$ were related to amide II group vibrations of N-H and C-N bounds from peptides [31]. In addition to these major discriminating bands, there was

a small contribution of amide I band resulting from antiparallel pleated sheets and $\beta$-turns of proteins at 1696 $cm^{-1}$ [37] in the discrimination of *S. cerevisiae* fermented without (Control) and with organic (Figure 6d) nitrogen. In the case of *S. cerevisiae* cells at exponential phase (42 h) fermented without (Control) and with inorganic nitrogen (Figure 6b), the biochemical differences were also linked to IR bands (1550 and 1647 $cm^{-1}$) related to different protein structures. Whereas, when SIMCA models were built up using IR data from *S. cerevisiae* cells fermented at exponential phase without (Control) and with organic nitrogen (Figure 6e), the discrimination among clusters was due to one IR band at 1714 $cm^{-1}$ related to C-O stretching of carbonic acid or nucleic acids [37]. Finally, at stationary phase (90 h), *S. cerevisiae* cells fermented without (Control) and with inorganic nitrogen were mainly discriminated by IR bands related to protein structures (1678 and 1541 $cm^{-1}$) and to asymmetric stretching of $PO_2^-$ in RNA and phospholipids and lipid esters (1285 and 1748 $cm^{-1}$) (Figure 6c). In the case of *S. cerevisiae* cells fermented at stationary phase without (Control) and with organic nitrogen (Figure 6f) a unique band at 1714 $cm^{-1}$ linked to C-O stretching of carbonic acid or nucleic acids [37] was mainly responsible of their discrimination.

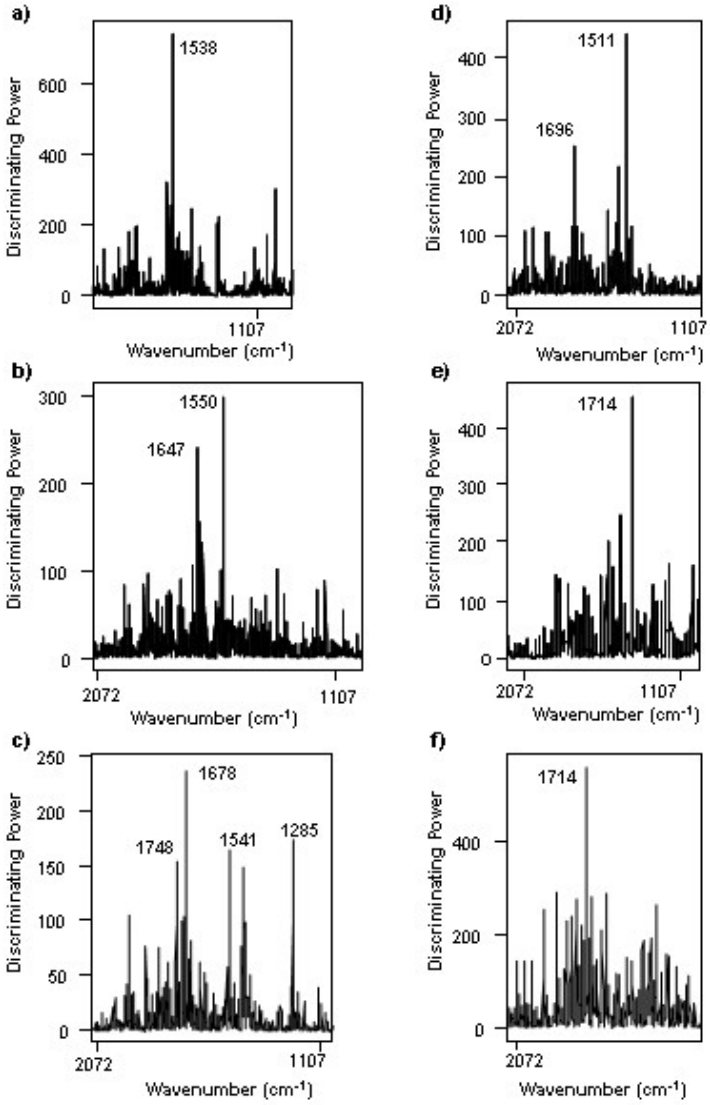


**Figure 6.** Soft independent modeling class analogy (SIMCA) of discriminating power of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must without and with inorganic nitrogen for 18 h (**a**), 42 h (**b**), and 90 h (**c**) and without and with organic nitrogen for 18 h (**d**), 42 h (**e**), and 90 h (**f**).

Summarizing these results, the supplementation of white musts with inorganic and organic nitrogen sources showed biochemical differences, especially when *S. cerevisiae* cells grown at exponential and stationary phase of the alcoholic fermentation. Some authors have described the relation between must nitrogen supplementation and the formation of ethyl esters from medium-chain fatty acids (MCFA) [38] derived from the metabolism of sugars and also amino acids by yeast [39–41]. The synthesis of MCFA ethyl esters is produced by of substrate availability and could be related to the relative increase of fatty acids synthesis due to nitrogen supplementation [38,42]. Other studies showed that the addition of organic nitrogen (mixture of amino acid and ammonium nitrogen) resulted in higher concentrations of MCFA ethyl esters that those samples supplemented with inorganic sources alone (ammonium salts) [13]. In contrast to these results, other authors have suggested that when a high total YAN concentration is naturally present in the grape must, the addition of amino acids can reduce ester concentration due to feedback inhibition suppressing amino acid uptake [43]. As other authors suggested, a relation between total nitrogen and the esters formation was observed [44,45] and the nature of the source of nitrogen used can influence the production of these compounds.

## 4. Conclusions

The combination of ATR-FTIR with chemometrics allowed studying the changes produced during alcoholic fermentation by the supplementation of different nitrogen sources. The feasibility of the use ATR-FTIR spectroscopy as a useful tool to monitor the changes during the white wine alcoholic fermentation produced by the nutrient supplementation has been demonstrated. The results obtained in this work will allow making decision during winemaking process related to the nutrients supplementation of musts. However, further studies should be done in order to evaluate this technique as a rapid tool on the detection of nitrogen deficiencies during fermentation.

**Author Contributions:** M.P.: data curation, writing—original draft preparation. I.A.: investigation, methodology, writing—original draft preparation. S.D.L.-C., R.F.-G.: supervision, writing—review and editing.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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