Reversed Phase High Performance Liquid Chromatography-Fluorescence detection for the analysis of glutathione and its precursor γ-glutamyl cysteine in wines and model wines supplemented with oenological inactive dry yeast preparations

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Abstract

A Reverse Phase- High Performance Liquid Chromatography-Fluorescence detection (RP-HPLC-FL) methodology involving a pre-column derivatization procedure using 2,3- naphtalenedialdehyde (NAD) in presence of 5 and 0.5 mM of dithiothreitol (DTT) to determine total and reduced glutathione (GSH) and γ-glutamyl-cysteine (γ-glu-cys) in musts and wines has been set up and validated. The proposed method showed good linearity (R^2 > 99 % for reduced and total GSH, and R^2 > 98 % for γ-glu-cys) in synthetic wines, over a wide range of concentration (0-10 mg L^{-1}). The limits of detection (LODs) for reduced GSH in synthetic and real wines were almost the same (0.13 and 0.15 mg L^{-1} respectively) and slightly higher for γ-glu-cys (0.24 mg L^{-1}). The application of the method allowed knowing for the first time, the amount of total and reduced GSH and γ-glu-cys released into synthetic wines by oenological preparations of commercial inactive dry yeast (IDY). In addition, the evolution of these three compounds during the winemaking and shelf-life (0-9 months) of an industrially manufactured rosé wine supplemented with a GSH enriched IDY showed that although GSH is effectively released from IDY, it is rapidly oxidized during alcoholic fermentation, contributing to the higher total GSH content determined in wines supplemented with GSH enriched IDYs compared to control wines.

Key words: RP-HPLC-FL; glutathione; γ-glutamyl-cysteine; inactive dry yeast preparations; wine

1. INTRODUCTION

Currently, the use of winemaking Inactive Dry Yeast preparations (IDY) is gaining interest within the wine industry because of their large amount of potential applications during winemaking. Although they have been mainly used for the improvement of alcoholic and malolactic fermentations, the use of IDY for enhancing wine's sensory characteristics, is one of the most promising and interesting applications [1].

The impact of IDY in wine's sensory properties is due to the ability of yeast components to modify wine chemical composition. As a matter of fact, it has been shown that yeast polysaccharides are able to protect wine colour, because of the interaction of yeast mannoproteins with tannins and anthocyanins, therefore, avoiding or minimising polyphenol aggregation and precipitation [2,3]. In addition, recent research performed in our group have shown that some yeast macromolecules released from IDY may affect the volatility of important wine aroma compounds [4], which could be related to the sensory differences observed in wines supplemented with these preparations compared to control wines [5]. Moreover, the ability of IDY to release nitrogen heterocyclic volatile compounds, likely formed as a consequence of the thermal reactions accounted for in the last steps during their production has been also shown [6].

Besides of the above mentioned effects of IDY on wine aroma, there are currently in the market other types of IDYs, which have been claimed to specifically preserve aroma composition during wine storage. The protective effect of these preparations has been associated to the presence of a relatively large amount of glutathione (GSH). This compound is a yeast intracellular tripeptide (γ-L-glutamyl-L-cysteinylglycine) from non proteic origin of known antioxidant properties, which it is formed from the precursor γ-glutamyl-cysteine (γ-glu-cys) [7]. GSH represents above 1% of the total weight of the yeast, although this concentration depends on the composition of the growth media [8,9].

GSH in musts and wines seems to have an important effect in wine quality, affecting the occurrence of aroma compounds and the prevention of wine oxidation by avoiding must browning and the decreasing of volatile compounds during wine aging [10,11,12,13,14]. Differences in concentrations of GSH in wines seem to be related to the type of wine, and also to different factors during winemaking, such as the pressing conditions [15] and the presence of oxygen [16].

Due to the increasing importance of determining the occurrence of GSH in musts and wines, different analytical methodologies have been developed for this purpose. HPLC-FL has been previously employed to determine GSH by using precolumn derivatization with *o*-phtaldialdehyde (OPA) [17,18] or 2,3 naphtalenedialdehyde (NDA) [19]. In addition, other methods imply the use of capillary electrophoresis [20] and LC-MS/MS [16]. In most of the cases, GSH has been determined in its reduced form, which seems to be the most active against oxidation. However, total GSH has also been proposed as a good indicator of GSH contained in wines [19], underlining the necessity of sensitive, robust and versatile methods allowing to determine the different forms of GSH present in wines.

On the other hand, although the effect of exogenous addition of GSH to musts and wines before bottling has been already explored [10,14], the impact of using commercial glutathione-enriched IDY preparations (G-IDY) during winemaking on the pool of GSH in wines, has not been study so far.

Therefore, the objectives of this work were to optimise and validate a RP-HPLC-FL method allowing the determination of reduced and total GSH and the precursor γ-glu-cys in wines and synthetic wines, and secondly, the application of the method to determine the ability of commercial IDY and G-IDY preparations to release glutathione into synthetic wines, and to study the stability and evolution of this compound during

the winemaking and shelf-life of an industrially manufactured *rosé* wine from Grenache grapes.

2. MATERIAL AND METHODS

2.1. IDY preparations

Eight IDY preparations were selected for being representative of the current preparations in the oenological market and because they are widely used in winemaking. Four of them: G-IDY-1, G-IDY-4, G-IDY-5 and G-IDY-8 are claimed in reducing the oxidation of wine aroma compounds because of the presence of higher amounts of glutathione. Another four preparations: IDY-2, IDY-3, IDY-6 and IDY-7 were chosen because of their high polysaccharide content, which following manufacturer's information can be used as nutrients and to preserve wine colour. All of them, were supplied by different manufacturers (Agrovin S.A., Lallemand and Oenofrance).

2.2. Synthetic model wines

Model wines were prepared by adding ethanol at 120 mL L^{-1} (VWR, Leuven, Belgium) and 4 g L^{-1} tartaric acid (Panreac, Barcelona, Spain). The pH was adjusted at 3.5 using a 5 M NaOH solution (Panreac). IDY preparations were added to 100 mL of model wines at the same dosage recommended by the manufacturer, 0.3 g L^{-1}, and stirring during 10 minutes. Model wines were kept at 20 ºC during 9 days. Sampling was carried out at 0 days (just 30 minutes after stirring) and 9 days after filtering 1 mL of wine using 0.45 µm Millipore filters (Millipore, Bedford, MA). Samples were kept frozen until the analysis was made.

2.3. Description of the wines

Two different types of monovarietal Grenache rosé wines from the 2008 vintage, a control wine (Cont-W) and a wine manufactured by using a glutathione enriched IDY preparation (G-IDY-W), were industrially manufactured in a cellar from the O.D. Navarra, Spain. To do so, 10,000 L tanks were filled with the same must. G-IDY wine was prepared by adding (20 g HL^{-1}) of G-IDY-1 to the must. A control wine was also made from the same must without IDY addition. To carry out the alcoholic fermentation, the same *Saccharomyces cerevisiae* active dry yeast was inoculated in both types of wines. All the wines were stabilised and clarified in the own cellar. Wines of the same type but from independent fermentation tanks were bottled together and sent to our laboratory. General parameters during winemaking (probable alcohol degree in musts, total acidity, volatile acidity, alcohol degree in wines) were determined according to the official methods of wine analysis. From these determinations, it can be concluded that finished wines had values considered in the normal range for this type of wines (Table 1). After winemaking, wines were kept at 12 ºC during 9 months. Sampling was made in the must, in the wines once alcoholic fermentation was completed, and during the shelf-life of the wines (after 1, 2, 3 and 9 months of aging in the bottle).

2.4. Determination of γ-glu-cys, reduced and total GSH in synthetic wines and industrial wines supplemented with IDY preparations

In order to determine γ-glu-cys, reduced and total GSH, a first step consisted in developing a protocol by optimizing the conditions described in a previous work [19]. To do so, a reversed-phase HPLC using a liquid chromatograph consisting of a Waters 600 Controller programmable solvent module (Waters, Milford, MA), a WISP 710B autosampler (Waters) and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA) were used. The mobile phase was composed of methanol (Lab-Scan,

145 Sowinskiego, Poland) and phosphate buffer (15:85 v:v). The phosphate buffer was
146 prepared by dissolving $NaH_2PO_4.12\ H_2O$ (10 mM) in highly purified water and
147 afterwards adjusting the pH to 8.5 using 5 M NaOH solution. Finally, the mobile phase
148 was filtered using a vacuum filtration system through 0.45 μm membrane filter. Thirty
149 μL of the filtered sample were placed in a 1 mL vial. For the derivatization of the
150 samples dithiothretiol (DTT) (Sigma-Aldrich) dissolved in borate buffer and 2,3-
151 naphtalenedialdehyde (NDA) (Sigma-Aldrich, Steinheim, Germany) dissolved in
152 ethanol were used. NDA was prepared by dissolving it in ethanol (Panreac) at a final
153 concentration of 5 mg mL^{-1}. DTT solutions were prepared at 5 mM and 0.5 mM in
154 borate buffer to determine total GSH or reduced GSH, respectively. Borate buffer was
155 prepared at 0.2 M H_3BO_4 (Merck, Darmstadt, Germany) adjusting the pH at 9.2. Both
156 solutions were filtered and properly aliquoted in 1 mL vials and kept frozen at -20 ºC.
157 Different amounts of sample and DTT were previously essayed in order to obtain the
158 highest response, which corresponded to a relation sample:DTT:NDA of 2:7:1.
159 Precolumn derivatization was automatically made in the autosampler of the HPLC at a
160 constant temperature of 12 ºC as follows: firstly, 105 μL from the DTT vial were placed
161 in the sample vial; secondly, 15 μL of NDA were also placed in the sample vial; then,
162 two mixtures cycles of the total content of the insert, 150 μL, were carried out. Next,
163 100 μL of the mixture were injected into the HPLC system. Separation was carried out
164 on a Nova Pack C18 (150 mm x 3.9 mm i.d., 60 A, 4 μm) column (Waters) in isocratic
165 mode, with a flow at 1 mL min^{-1} from 0 to 8 minutes, and 1.5 mL min^{-1} from 8 to 20
166 minutes. Detection was performed by fluorescence ($\lambda_{excitation}$= 467 nm, $\lambda_{emission}$= 525
167 nm) and chromatographic data were collected and analysed with an Empower 2-2006
168 system (Waters). The derivatization conditions for the determination of γ-glu-cys were
169 the same previously described for the total glutathione analysis. To do the calibration

curves, solutions of GSH and γ-glu-cys were prepared by dissolving the peptides in water at 1 mg mL^{-1}, and from these solutions, serial dilutions were prepared in a range of concentrations from 1 to 10 mg mL^{-1}, according to those usually found in wines. The analysis of the samples was made in duplicate.

2.5. Chemical composition of industrially manufactured rosé wines

2.5.1. Free amino acids and peptides

Free amino acids and peptides were determined according to the protocols proposed by Doi and co-workers [21]. Free amino acids were determined by the reaction of ninhydrin/Cd with the free amino group (method 5) [21], whereas free amino acids plus peptides were determined by the reaction of the amino group with ninhydrin/Sn (method 1) [21]. Free amino acids, and amino acids plus peptides were determined by measuring the absorbance at 507 and 570 nm, respectively, by using a DU 70 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). To do the calibration curves, leucine was used as standard, and results were expressed as mg N L^{-1}. To obtain the peptide content of the samples, differences between results obtained with Doi's method 1 and method 5 were calculated. Wines were analysed by duplicate.

2.5.2. High Molecular Weight Nitrogen (HMWN) compounds

The concentration of HMWN compounds was determined following the Bradford method [22], based on the reaction of the HMWN compounds with a reagent that contains Coomassie blue (Bio-Rad, Hercules, CA, USA). The absorbance was determined at 595 nm, 15 min after the addition of the reactant in a DU 70 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). To do the calibrations curves, bovine serum albumin (Sigma-Aldrich) was used. Wines were analysed by duplicate, and final results were expressed in mg N L^{-1}.

2.5.3. Analysis of amino acids by RP-HPLC-FL

Amino acids were analysed following the protocol proposed by Moreno-Arribas and collaborators [23] by means of reversed-phase HPLC using the same liquid chromatograph mentioned above. Briefly, samples were submitted to an automatic derivatization with *o*-phtaldialdehyde (OPA) (Sigma-Aldrich) in the presence of 2-mercaptoethanol (Sigma-Aldrich). Separation was carried out on a Nova Pack C18 (150 mm x 3.9 mm i.d., 60 A, 4 μm) column (Waters) and detection was performed by fluorescence ($\lambda_{excitation}$= 340 nm, $\lambda_{emission}$= 425 nm). All the wines were analysed in duplicate.

2.5.4. Statistical Analysis

Data from the analysis of reduced, total GSH and γ-glu-cys released by the eight preparations into model wines were submitted to one-way ANOVA to test the effect of the type of IDY. STATISTICA for Windows (version 7.1) was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

3. RESULTS AND DISCUSSION

3.1. Determination of GSH and γ-glu-cys using RP-HPLC-FL

3.1.1. Optimization of the derivatization procedure

The methodology employed for the determination of γ-glu-cys and GSH was based on that proposed by Marchand and de Revel [19] with several modifications. The most important difference was the use in the present work of dithiothreitol (DTT) instead of ethanethiol employed in the above mentioned work. DTT is a potent reductor agent, that has been shown to increase the fluorescence signal in the determination of GSH in wines, that otherwise, can be reduced due to the influence of quinones and trace metals in wine under basic conditions [20]. In addition, DTT can be used to determine both reduced and total GSH. Using low concentration of DTT allows to determine reduced GSH, but at higher concentration of DTT, oxidized glutathione (GSSG) is converted

220 into GSH [20]. This implies an easier methodology compared to that proposed by
221 Marchand and de Revel [19], which involves the use of the enzyme GSH reductase to
222 determine total GSH, and also a dilution of the wines in PBS (1:20), which might
223 provoke a decrease in the signal.
224 To know the optimal concentration of DTT necessary for the determination of total and
225 reduced GSH, different concentrations of DTT were essayed in wines supplemented
226 with GSH, GSSG, and both of these compounds at a fix concentration of 10 mg L^{-1}.
227 Table 2 shows the areas corresponding to these compounds obtained by adding different
228 concentrations of DTT. The optimal concentration of DTT to determine total GSH was
229 considered as that in which the ratio (wine + GSSG) /(wine + GSH) was similar to 1, so
230 all GSSG will be transformed into GSH by reduction. With a concentration of 5 mM
231 this ratio was 1.01 being, so this concentration of DTT was chosen for total GSH. By
232 decreasing of DTT concentration (from 5 to 0.5 mM), the optimal concentration of DTT
233 for the analysis of reduced GSH were chosen. The optimal concentration corresponds
234 with a DTT concentration that produced a minimum reduction of GSSG and enough to
235 stabilize the reduced GSH during derivatization step. Thus, similar areas of Wine +
236 GSH and Wine + GSH + GSSG (or a ratio near to 1) satisfy the conditions for the
237 analysis of reduced GSH. 0.5 mM of DTT (ratio = 1.11) was chosen to the analysis of
238 reduced GSH, although it is important to notice, that approximately 10 % of GSSG was
239 converted into GSH. In conclusion, DTT at 5 mM and 0.5 mM were used to
240 respectively determine total and reduced GSH in our synthetic and industrial wine
241 samples.
242 *3.1.2. Analytical Quality of the RP-HPLC-FL method*
243 Linearity of the RP-HPLC-FL method was evaluated in both, synthetic and industrially
244 manufactured wines by addition of different concentrations of reduced GSH from 1 to

10 mg L^{-1}. In the whole tested range, the responses were linear when peak area was used for signal evaluation. Determination coefficients (R^2) for reduced and total GSH were higher than 99 % in synthetic wines, while they were slightly minor, 97.4% and 98 % for both compounds respectively in real wines (Table 3). In addition, γ-glu-cys showed adequate R^2 in synthetic wines (98.7 %). The limits of detection (LOD) (concentration for signal / noise =3) and quantification (LOQ) (concentration for signal/noise =10) are also shown in Table 3. The LODs for reduced GSH in synthetic and real wines were almost the same (0.13 and 0.15 mg L^{-1} respectively). In addition, they were very similar to those determined for total GSH (0.18 and 0.13 mg L^{-1} for synthetic and rosé wines respectively). The LODs determined for γ-glu-cys in synthetic wines was slightly higher (0.24 mg L^{-1}) compared to the values determined for GSH. In general, all the calculated limits were low enough to determine reduced, total GSH and γ-glu-cys in wines. The LOQ of reduced GSH was however, lower than that obtained by Du Toit and co-workers [16], but higher than the LOQ reported by other authors [18,20]. In addition, the LOQ for γ-glu-cys (0.43 mg L^{-1}) was very similar than that found by Marchand and de Revel [19]. Therefore, one of the advantages of the methodology developed in this work, is that it allowed to easily determine total GSH with lower quantification limits than that reported in previous works [19].

To evaluate the reproducibility of the method six identical samples of synthetic wines with the G-IDY-1 preparation and rosé wines were analysed in 5 consecutive days. As can be seen, the reproducibility for γ-glu-cys, reduced and total GSH was below 10% which could be considered as good.

3.2. Determination of GSH and γ-glu-cys in synthetic model wines supplemented with commercial IDY preparations

The amount of reduced and total GSH, and their corresponding precursor γ-glu-cys was determined in synthetic wines supplemented with eight commercial IDY preparations widely used during winemaking. Four of them have been recommended by the producers to prevent aroma losses because of their high content in GSH (G-IDY-1, G-IDY-4, G-IDY-5, G-IDY-8) and the other four are mainly used as fermentative nutrients and to prevent the colour losses in wines (IDY-2, IDY-3, IDY-6, IDY-7). Results showed that, from the eight preparations assayed, five of them (G-IDY-1, IDY-3, G-IDY4, G-IDY5 and G-IDY8) were able to release GSH and/or γ-glu-cys into the synthetic wines (Table 4). In general, preparations released very similar amounts of reduced and total GSH. All of them, with the exception for IDY-3, corresponded to preparations specifically recommended to enhance wine aroma in white and rosé wines because of the presence of GSH. In general, these preparations released between 1 mg L^{-1} to 2 mg L^{-1} of reduced GSH in the case of G-IDY-4 and G-IDY-1 respectively, which correspond to the 0.33 and 0.67 % of the total amount of IDY preparations added to the synthetic wines (0.3 g L^{-1}). Papadopoulus and Roussis [10] showed a reduction in the oxidation of some volatile compounds after the addition of GSH (between 2 and 5 mg L^{-1}) into synthetic wines. In the present work, the differences in the manufacturing processes among IDY preparations might be implied in the different ability of IDY to release GSH into the medium. Such differences might comprise the nature of the carbon and nitrogen sources and other nutrients [8,24] in the medium where yeasts grow, or specifically the amount of cysteine, which has been shown to be a limiting factor for GSH biosynthesis [8,25].

From the non-G-IDY preparations, only IDY-3, showed the ability to release reduced GSH into the wines at a concentration of 0.46 mg L^{-1} (corresponding to the 0.15% of the total amount of IDY added to the wine). This amount was significant lower

294 compared to the amounts of GSH released by the G-IDY preparations. This could be
295 due to the naturally occurring GSH present in all the yeast, which in the case of
296 *Saccharomyces cerevisiae* might represent about 0.1 to 1% of the dry cell weight [26]. The
297 absence of GSH released for the rest of IDY might be related to the yeasts strains they
298 belonged and/or to their manufacturing conditions, in which the formation of GSH has
299 not been promoted. In addition, the thermal processing to which these preparations are
300 submitted could influence the final concentration of GSH in the IDY preparation
301 obtained from yeast. In fact, it has been shown that high temperatures can degrade GSH
302 [27]. Even during the drying step that undergo during the manufacturing of these
303 preparation, Maillard reaction can be produced [6] and GSH could also react with
304 reducing sugars [28], thus, disappearing from the final IDY preparation.
305 On the other hand, by comparing the amounts of reduced GSH released into the medium
306 between the first and the ninth day after their addition, it is possible to see that the
307 content of GSH remained quite stable, and only a slight decrease in its concentration
308 was noticed in the synthetic wines supplemented with the preparations G-IDY-1, G-
309 IDY-4 and G-IDY-5 (Table 4). However, the content of total GSH experienced a slight
310 reduction along the essayed time for all the G-IDY preparations.
311 In addition, important differences in the content of γ-glu-cys released by the IDY
312 preparations were also found (Table 4). While this compound was not detected in the
313 wines supplemented with IDY-3, wines supplemented with G-IDY-1 and G-IDY-4
314 showed the highest values of γ-glu-cys (2.62 and 1.60 mg L^{-1}, respectively). The
315 concentration of γ-glu-cys also slightly decreased during the studied time (9 days),
316 although the reasons for this reduction remain unclear. Neither the effect of γ-glu-cys
317 during winemaking has been well established. However, the differences in the release of

γ-glu-cys among preparations seem to be also related to the different conditions employed for their manufacturing.

3.3. Evolution of total GSH, reduced GSH and γ-glu-cys during winemaking and aging in the bottle

The concentration of total GSH, reduced GSH and γ-glu-cys was determined in the must and in the industrially manufactured rosé wines (control and G-IDY wines) immediately after the alcoholic fermentation and along their shelf-life (at 1, 2, 3 and 9 month of aging in the bottle). Figure 1 shows these results. The compound γ-glu-cys was not identified in the must or either in the wines. In fact, this compound has not been previously described in musts, and only has been reported in some white Sauvignon Blanc wines, although at low concentrations (0.6-1.3 mg L^{-1}) [19]. Peptides can be easily consumed by yeast during the alcoholic fermentation which might explain the absence of γ-glu-cys in the wine [29]. However, the content of total GSH greatly increased after alcoholic fermentation in both types of wines (Figure 1). It has been suggested that actively fermenting yeast can produce and release high amounts of reduced GSH during fermentation [30]. However, in other studies a decrease in the total GSH during alcoholic fermentation has been also observed [16]. It seems that depending on the yeast strain used, the evolution of GSH during alcoholic fermentation can be different [20]. In addition, total GSH after alcoholic fermentation was much higher in the wine supplemented with G-IDY-1 than in the control wine, which could be explained by the supplementation of GSH provided by the IDY preparation. Interestingly, the differences in total GSH between the control and G-IDY wine after the alcoholic fermentation were much higher than those expected taking into consideration the amount of total GSH released by the G-IDY-1 preparation, as was previously noticed (Table 4). This could be due, to the additional supplement in nitrogen compounds, and mainly amino acids, provided by the

343 G-IDY-1 preparation, which have been described to be important contributors for the
344 production of GSH by yeast [30]. To check this hypothesis, the nitrogen composition of
345 the control and G-IDY wines was determined (Table 5). As can be seen, important
346 differences between both types of wines were found. The content of peptides and amino
347 acids was much higher in the G-IDY wine than in the control wine. In the case of amino
348 acids, this effect was mainly due to some amino acids such as glutamic acid, asparagine,
349 glutamine, glycine, arginine, γ-aminobutyric acid, tryptophan and ornithine. The ability
350 of G-IDY-1 preparation to release significant amounts of amino acids into synthetic
351 wines has been already shown [4]. Among all of these amino acids, glycine, arginine and
352 glutamic acid, together with methionine and cysteine, have been described to have a
353 stimulating effect on the production of GSH by *Saccharomyces cerevisiae* [25]. Therefore,
354 during the alcoholic fermentation, the higher nitrogen content in the G-IDY wine might
355 be responsible for the higher formation of reduced GSH.
356 On the other hand, the reduced GSH was the predominant form of glutathione in the
357 must, although the initial concentration was rather low, above 0.5 mg L^{-1}. Other works
358 have also pointed out the low concentration of GSH in musts compared to that found in
359 grapes [18]. This has been explained by the oxidative reaction of GSH with
360 hydroxycinnamates during grape crushing, yielding the "grape reaction product", 2-S-
361 glutathionyl caftaric acid [18]. In addition, other factors during winemaking such as the
362 pressing conditions to obtain the must [15] and/or the must oxygenation might also be
363 involved [16].
364 Surprisingly, after the alcoholic fermentation, none statistical difference was found in
365 the concentration of reduced GSH between the control and G-IDY wine (Figure 1).
366 This seems to indicate that the reduced GSH released by G-IDY-1 preparation might be
367 rapidly oxidized during the alcoholic fermentation. In fact, this effect has been

368 previously observed in the study from Patel and collaborators [15], in which a must added
369 with a high content of GSH (67 mg L^{-1}) decreased considerably its concentration until
370 few milligrams per litre after alcoholic fermentation. In spite of that, it has also been
371 shown that wines from musts supplemented with GSH experienced slighter oxidation
372 symptoms and exhibited better sensory characteristics than control wines (without GSH
373 added to the must) [31]. Finally, the progressive reduction in reduced GSH observed
374 during the shelf-life of the wine (Figure 1b), was higher that observed for the total GSH
375 (Figure 1a) and similar for both types of wines, which is in agreement with the decrease
376 of glutathione during the aging of the wines observed by Lavigne and collaborators [20].
377
378 In summary, the methodology set up in the present work, which involves a precolumn
379 derivatization by using NDA and different amounts of DTT, is a sensitive, robust and
380 versatile method to determine the different forms of GSH and its precursor (γ-glu-cys)
381 present in musts and wines. Its application to oenological IDY preparations has
382 confirmed that all the commercial G-IDY assayed present concentration of GSH (total
383 and reduced) higher than other non G-IDY oenological preparations. However, although
384 GSH is effectively released from IDYs, it is rapidly oxidized during alcoholic
385 fermentation, contributing to the higher total GSH content determined in wines
386 supplemented with G-IDYs compared to control samples. Moreover, nitrogen
387 compounds released by these preparations seem to have an outstanding role on the
388 formation of glutathione *de novo* by yeast during the alcoholic fermentation. In general,
389 it has been also shown that the total pool of glutathione decreases during wine aging.
390 Therefore, these results underline the necessity for a deeper research in order to
391 elucidate the impact of alcoholic fermentation on the formation/degradation of GSH in
392 wines supplemented with IDY.

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FIGURE AND TABLES LEYENDS

Figure 1. Evolution of total (a) and reduced (b) GSH in the control wines (Cont-W) and in the wines produced with G-IDY-1 preparation (G-IDY-W) during the winemaking and aging in the bottle

Table 1. Global composition parameters determined in must, control wine (Cont-W) and wine supplemented with the glutathione enriched IDY preparation (G-IDY-W).

Table 2. Areas obtained by using different concentrations of DTT in the reaction mixture during the derivatization procedure in wines supplemented with reduced (GSH) and oxidized (GSSG) glutathione

Table 3. Analytical performance of the RP-HPLC-FL method for the determination of reduced and total GSH and γ-glu-cys in synthetic and rosé wines

Table 4. Reduced, total GSH and γ-glu-cys released by the commercial IDY preparations into synthetic model wines at 0 (30 minutes) and 9 days after their addition into the wines

Table 5. Nitrogen compounds determined in the control wine (Cont-W) and in the wine produced with the preparation G-IDY-1 (G-IDY-W) after alcoholic fermentation.