CHARACTERIZATION OF YEAST CELL WALL FRACTIONS ACCORDING TO DIFFERENT REDUCED CYSTEINE FORMS

TIRELLI A., FRACASSETTI D., DE NONI I.
Department of Food and Microbiological Science and Tecnology. University of Milan. Via G. Celoria, 2. 20133 Milano

Study presented at Enoforum 2009, 21-23 April 2009, Piacenza, Italy

The thiol groups of reduced cysteine forms (TGRC) can be implicated in numerous important activities in the must and wine. The antioxidant activity of glutathione (GSH) in wine must is well documented; it is capable of interacting with ortho-quinones formed via the reaction of oxidizing enzymes with hydroxycinnamoyl tartaric acids and flavanols (1) as shown in the following reaction diagram (Fig 1).

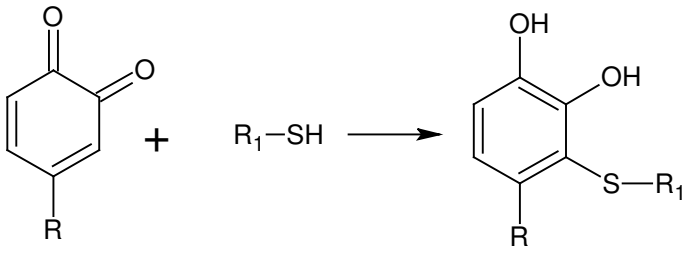

Figure 1. Diagram of 3-S-glutathionyl formation derived from o-quinones

This reaction prevents the accumulation of quinones and the potential formation of brown phenolic polymers which can consequently jeopardize the white wine colour.

Quinone compounds present during fermentation can also bind, following the same reaction mechanism as above, to varietal thiol aroma fractions released via yeast lyase activities on cysteine precursors present in the grapes. In white wines the presence of GSH can also prevent the formation of sotolon (3-hydroxy-4,5-dimethyl-2(5H) furanone), one of the main components responsible for the atypical aging defect, hence conserving varietal thiol aromas longer (2).

The availability of TGRC in the must and wine could similarly prevent the formation of such defects in a significant manner. Enological TGRC consist mainly of free cysteine (Cys), GSH and proteic Cys present in the cell walls of yeast. These last mentioned components have an essential role, even though it is not yet fully understood, during the maturation of white and sparkling wines. Of course also during maturation the glucanase activity of the yeast itself favours the release of mannoproteins (MP) which have a stabilizing action on the saline and colloid equilibriums of the wine. Hence, this further improves the in mouth tactile perception during tasting (3).

However, not much is known about the antioxidant properties of MP, and in general about yeast cell wall derivatives and their TGRC content. The technologies usually used for the production of these enological additives give variable results given the different energetic acid-thermic conditions often used during their extraction. This then makes effective application in the winery difficult. The evaluation of TGRC derivatives from yeast cell walls is further limited by the insolubility of yeast preparations, such as lysed yeast, yeast hulls and lees. Consequently the use of methods based on spectrophotometric determinations of soluble Cys derivatives becomes a complex procedure.

Furthermore, the characterization of individual proteic forms of reduced Cys would require complex fractioning phases which could alter the distribution of the TGRC. The objective of this study was to formulate an analytical methodology capable of evaluating TGRC concentration in yeast preparations and wine, expressed as GSH, free Cys and proteic Cys.
The methodology was consequently applied for the characterization of certain commercial yeast preparations and the lees at the end of fermentation. The thermal damage, due to production

practices, was also evaluated by estimating the intensity of the Maillard reaction using the furosine index.

To determine the TGRC content in the cell walls of commercial yeasts, 200mg of the dry preparation was suspended in 2mL of 50mM citrate buffer at pH 5. Then 100µL of p-benzoquinone (p-BQ), previously dissolved in methanol at a concentration of 130µM, was added to the suspension (or solution for the MP). After agitating for one minute, 1mL of 200µM 3-mercaptopropanoic acid (3MPA) in 0.3M citrate buffer at pH 3.5 was added. The sample was then centrifuged at 14000 x g/5 min and the supernatant was filtered on a 0.22µM PVDF membrane before following with the chromatographic determination by HPLC. For the mannoprotein samples the centrifugation and filtration phases were substituted by an ultrafiltration centrifugation on a 3kDa membrane followed by 2 washings of the retained fraction with 0.5mL of chloride ethanol (0.1%) (4). These washing were subsequently added, filtered and then dried using a rotary evaporator under vacuum. The dried material was then resuspended in 0.5mL of water. The final aqueous solution was then analyzed via HPLC separately from the ultrafiltrate previously obtained. The determination of the TGRC content in reverse phase HPLC was completed using a phenyl-silica column of 250x 4.6mm, 5 µm, 110 Å (Phenomenex) and using a gradient of H_2O/TFA (0.05% v/v) and acetonitrile/TFA (0.05% v/v) the second mentioned increasing from 5% to 35% in 26 min. The detection was completed using a UV spetrophotometer at 303 nm.

The determination of the furosine level and the protein content was completed following the methodology described by Resini P. *et al.* (5). The acid-thermal hydrolysate obtained for the furosine determination was also used in the HPLC determination of the total Cys according to the method described by Krause *et al.* (6), which involved an initial purification through C18 SPE, then under-vacuum drying and resuspension in sodium carbonate buffer at pH 8.6.

The TGRC determination is based on the reactivity of the p-BQ with thiol groups following this diagram, figure 2.

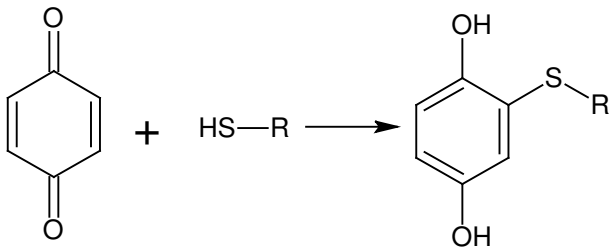

Figure 2. Reaction between p-benzoquinone and thiol compounds.

The reaction with p-BQ leads to the derivatization of accessible TGRC while the excess quinone fraction then successively combines following the addition of excess 3MPA.

The HPLC separation (Figure 3) coupled with mass spectrometry using an ESI interface allowed for the identification and characterization of thiols derived from p-BQ. The 2-S-cysteinyl-p-hydroquinone, the 2-S-glutathionyl-p-hydroquinone and 2-S-(3-mercapto)propionyl-p-hydroquinone are eluted in 6.2 min, 9.0 min and 16.2 min, respectively. Bi- and tri-substituted hydroquinones were also observed but only in trace amounts and therefore they were not considered in the TGRC quantification.

The reduced free Cys and GSH concentration values were calculated directly from the chromatographic peak areas, whereas the reduced proteic Cys concentration present in the yeast preparation fractions accessible to p-BQ was quantified by considering the area given from the derivatization of 3MPA with p-BQ, added in a notable quantity, in the absence of other thiol forms. In order to evaluate the eventual interference exerted by caramelized products on thiol compounds, the determination was also completed on aqueous suspensions of amylopectin and partially caramelized amylopectin (100g/L) using hot acid treatments (120 °C/24h and pH 1 for HCl) containing increasing concentrations (up to around 10mg/L) of Cys and GSH.

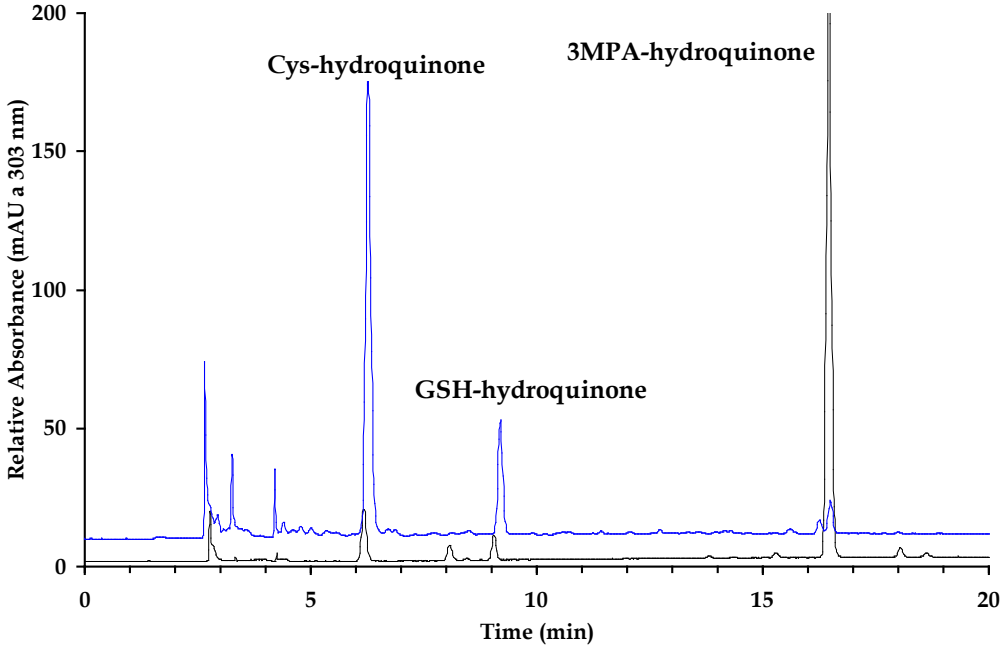

Figure 3. HPLC chromatogram obtained after reaction with p-BQ and 3MPA of (A) a standard solution containing 2mg/L of Cys and 2mg/L of GSH and (B) a commercial yeast hull preparation.

The calibration curves (Figure 4 A-C) do not show notable analytical differences for the two polysaccharides however for both samples a lower precision was noted in the quantification of reduced proteic Cys (Figure 4C) possibly due to error integrated during the quantification of the other thiol forms.

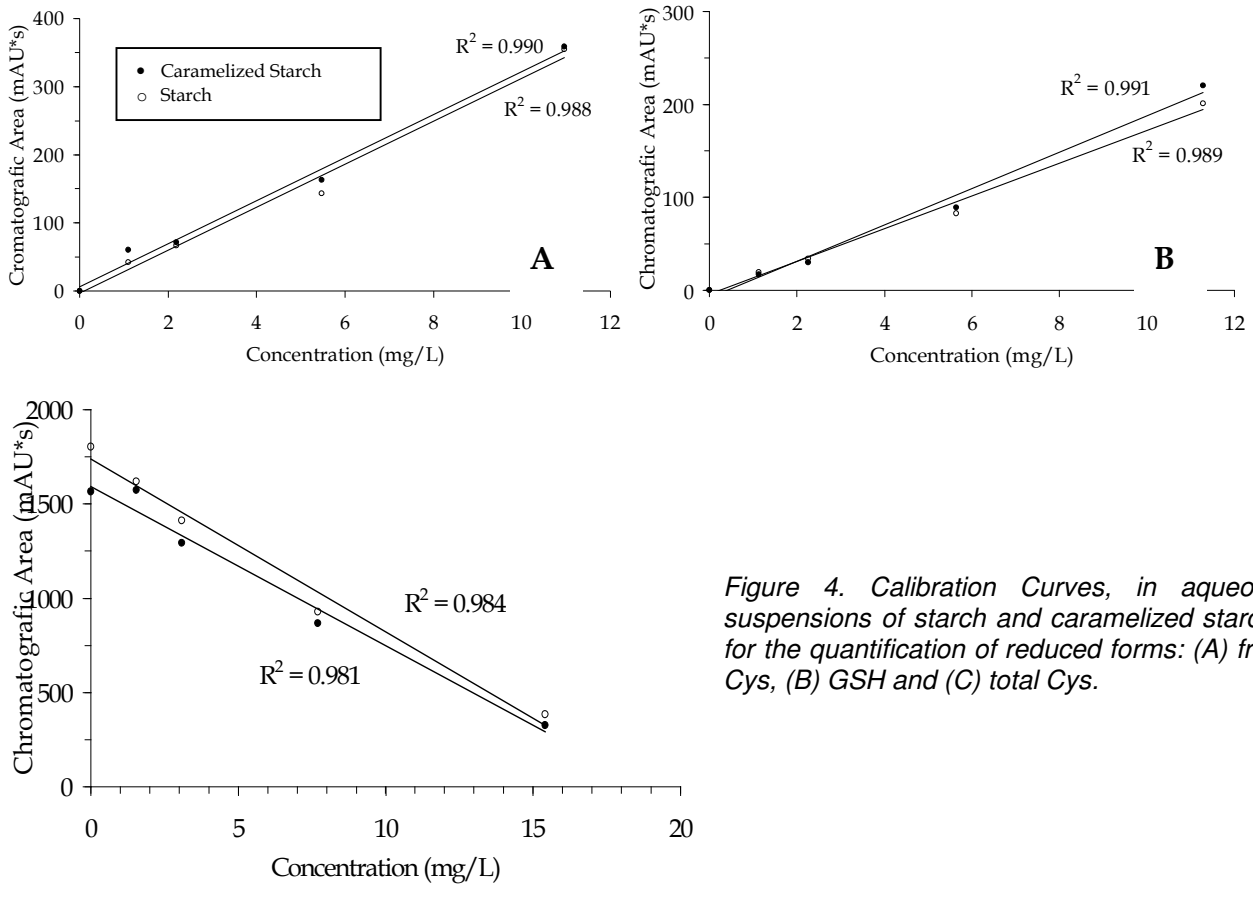

Figure 4. Calibration Curves, in aqueous suspensions of starch and caramelized starch, for the quantification of reduced forms: (A) free Cys, (B) GSH and (C) total Cys.

The methodology when applied to an aqueous solution of lysozyme 30g/L did not show any reaction with p-BQ hence demonstrating the absence of proteic disulfurs interference.

The characterization of the cell wall fractions of commercial yeast preparations overall demonstrated extensive heterogeneity (Table 1).

Table 1. Reduced and total cysteine (Cys) values, glutathione (GSH), protein and furosine found in commercial enological yeast preparations and in winemaking lees.

Yeast Fraction	Protein	Furosine	Reduced free Cys	GSH	Reduced proteic Cys	Total Cys
	(%)	(mg/100 g prod.)	(mmol/100 g product)			
mannoprotein 1	2.86	28	0.00	0.00	0.05	0.55
mannoprotein 2	10.51	254	0.00	0.00	0.05	0.53
mannoprotein 3	9.02	62	0.00	0.00	0.13	2.30
mannoprotein 4	8.77	67	0.00	0.00	1.16	3.03
yeast hulls 1	7.48	17	0.00	0.00	0.11	3.23
yeast hulls 2	8.75	12	0.00	0.00	0.09	4.37
yeast hulls 3	8.87	6	0.00	0.00	0.16	3.26
yeast hulls 4	11.88	12	0.63	0.31	7.31	10.5
lysed yeast 1	9.96	3	0.00	0.18	0.66	6.85
lysed yeast 2	17.94	5	0.00	0.18	0.86	15.6
lysed yeast 3	16.84	38	0.36	4.00	2.28	11.8
lysed yeast 4	14.33	3	0.04	1.26	2.44	8.01
extract 1	21.68	154	0.00	0.00	1.37	37.0
extract 2	23.86	20	0.00	0.00	1.13	25.1
lees at end of AF	7.64	36	0.00	0.00	0.30	in evaluation

The evaluation of the MP, yeast hulls and lysed yeast samples characterized by an absence of free TGRC forms was initially rendered difficult due to the complete sequestering of 3MPA by part of the preparation that prevented the reaction with p-BQ. For these sample the describe methodology was preceded by an initial addition of a notable quantity of Cys in the solution/suspension before the treatment with p-BQ in order to saturate the groups reactive with free TGRC present in the product.

This result shows that some commercial preparation can, not only bring limited antioxidant power to the wine, but can actually lower the content of antioxidant constituents and cause notable losses of thiol aroma fractions. In fact, these preparations could rather have a useful curative application when reduction defects due to thiol mercaptans are present.

The residual presence of proteic TGRC in “reactive-thiol” samples demonstrates the lower reactivity of these Cys forms which can therefore exert their reductive action on quinones. Their concentration however is low and cannot guarantee significant oxidation protection for the wine when the permitted quantities are used. This explains the significant additions of Cys and/or GSH by producers noted in certain samples of yeast hulls and lysed yeast (Table 1) where there are up to 4.4mmol of free TGRC per 100g of product as well as high quantities of reduced thiol groups (approx. 2.3mmol/100g) different from those of free Cys and GSH. These yeast preparations could give extended to prolonged protection from oxidation and wine aroma losses. However the abundance of these last mentioned thiol forms, if compared to the quantities of proteic TGRC

found in the other samples, would seem to perhaps not have a proteic Cys origin, even if the lysed yeasts 3 and 4 did not show atypical chromatograms.

The notable response during Maillard reactions, indicated by the furosine level, in the mannoprotein samples seems to confirm the possibility, noted by various winemakers, that many of these enological preparations alter the sensorial impact of the wines by giving abnormal tastes and odours.

The greater amount of proteic Cys found in the lysed yeasts, also characterized by a lower thermal damage, leads to the hypothesis that it could be possible to extract MP fractions that are richer in reduced proteic Cys that could hence improve the shelf life of white wines.

The results for the lees shown in table 1 were taken at the end of the fermentation of an Amarone wine. The sample was initially treated with a French Press to eliminate the cytoplasm contents and then centrifuged and washed with a Tris/EDTA buffer before analytic evaluation. Free TGRC were not found, possibly due to oxidative phenomena which could take place in the must after pressing due to hydroxycinnamic acids. In fact there is a notable quantity of combined TGRC present.

The results obtained demonstrate the need for yeast cell wall fractions for enological use, in particular MP, that have better protective properties for the aromatic fractions and that are richer in endogenous TGRC. This in order to avoid turning to prohibited enrichments with exogenous reduced Cys and GSH.

Futhermore, the analytical approach described could be useful for the evaluation of other enological aspects that involve TGRC within oxidative phenomena in musts and wines.

SUMMARY

The presence of thiol forms from reduced cysteine in enological preparations of yeast cell walls could prevent the development of oxidative phenomena in wines by reducing quinone forms derived from phenolic oxidation. An innovative analytical approach based on the reactivity of thiol groups with p-benzoquinone, was used to characterize mannoproteins, yeast hulls, lysed yeast and yeast extracts and lees, of commercial origin. Many of these enological additives showed a notable linkage activity with free thiol molecules and in general low amounts of either free or proteic TGRC. These products could lower the aromatic patrimony of the wine and could lead to the development of oxidative phenomena, they could be useful however to remove early reductive defects. Some yeast hull and lysed cell samples were shown to contain over 4 mmol/100g of free reduced Cys and GSH as well as only slightly lower quantities of thiols of diverse origins whose association to reduced proteic Cys forms from yeasts needs to be verified. The mannoprotein preparations remained largely damaged by an intense Maillard reaction, measured using furosine index, and had particularly low levels of TGRC. The proposed method could help isolate cell wall fractions and in particular mannoproteins, with better antioxidant properties, as well as evaluate other aspects of the enological activity of TGRC.

REFERENCES

1) M. Salgues, V. Cheynier, Z. Gunata e R. Wylde. Oxidation of Grape Juice 2-S-Glutathionyl Caffeoyl Tartaric Acid by *Botrytis cinerea* Laccase and Characterization of a New Substance: 2,5-di-S-Glutathionyl Caffeoyl Tartaric Acid. J. Food Sci.. 51, 1191 (1986).
2) V. La Vigne e D. Dubordieu. Affinamento sulle fecce e freschezza dei vini bianchi. VigneVini. 31 (7/8), 58 (2004).
3) A. Caridi. Enological functions of parietal yeast mannoproteins. Antonie van Leeuwenhoek. 89, 417 (2006).
4) I. Ummarino, E. Garcia Moruno e R. Di Stefano. Interazione polifenoli - scorze di lievito. Riv. Vitic. Enol. 54 (4), 37 (2001).
5) P. Resmini, L. Pellegrino e G. Battelli. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. Ital. J. Food Sci.. 2, 173 (1990).
6) I. Krause, A. Bockhardt, H. Neckermann, T. Henle e H. Klostermeyer. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. J. Chrom. A. 715, 67 (1995).