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Rapid Method for Proline Determination in Grape Juice and Wine

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ABSTRACT: Proline is typically the most abundant amino acid present in grape juice and wine. The amount present is influenced by viticultural and winemaking factors and can be of diagnostic importance. A method for rapid routine quantitation of proline would therefore be of benefit for wine researchers and the industry in general. Colorimetric determination utilizing isatin as a derivatizing agent has previously been applied to plant extracts, biological fluids, and protein hydrolysates. In the current study, this method has been successfully adapted to grape juice and wine and proved to be sensitive to milligram per liter amounts of proline. At sugar concentrations above 60 g/L, interference from the isatin−proline reaction was observed, such that proline concentrations were considerably underestimated in grape juice and dessert wine. However, the method was robust for the analysis of fermentation samples and table wines. Results were within ±10% agreement with data generated from typical HPLC-based analyses. The isatin method is therefore considered suitable for the routine analysis required to support research into the utilization or release of proline by yeast during fermentation.

KEYWORDS: *grape juice, HPLC, isatin, proline, wine*

INTRODUCTION

The availability of assimilable nitrogen during fermentation of grape juice by yeast can be critical to yeast biomass formation, fermentation rate and completion, and formation of aroma active compounds.[1−5] For this reason, knowledge of the quantitative and qualitative nitrogen content of juice can be helpful in predicting and modulating fermentation outcomes. However, the amino acids and ammonium which constitute the bulk of juice assimilable nitrogen are a chemically disparate group, and their quantitation can be challenging, especially within the time and resource constraints of a typical commercial winery. The ideal approach of deriving total yeast assimilable nitrogen (YAN) content from an accurate determination of the content of individual amino acids and ammonium, usually by high-performance liquid chromatography (HPLC),[6,7] is rare in a winery setting, but more common in research laboratories. More rapid assays have been extremely useful to winemakers, but such methods typically give an approximation of total assimilable nitrogen, the magnitude of which is dependent on the precise nature and amounts of amino acids present and which may or may not include proline.[8,9] The interest in proline is multifold and stems from the conflicting notions that proline either (i) is not utilized during winemaking or (ii) is, or can be, utilized. Furthermore, proline is considered a physiological indicator of stress in many plants, including grapevines, and is believed to accumulate in response to water deficit, salinity, temperature, and nutrient deficiency.[10,11]

While proline is often the predominant amino acid in grape juice,[5] it is of little nutritional importance to yeast under strictly anaerobic conditions because of the dependence on molecular oxygen for its catabolism.[12,13] The widely held view is in fact that proline content changes little during winemaking.[13,14] For this reason a specific determination of proline might allow a subtraction of any contribution made by this amino acid to rapid determinations of assimilable nitrogen content, thereby increasing the precision of such determinations. Alternatively, there is growing evidence that proline content is not in fact static during fermentation and that both increases and decreases occur. Catabolism of arginine[15] or novel winemaking practices[16] can lead to increases, whereas significant removal of proline, whether involving catabolism or direct incorporation, has also been reported.[14,17,18] In addition, several groups have described novel yeast strains engineered for greater utilization of proline or reduced proline formation from arginine under enological conditions as a means of improving fermentation reliability of low-nitrogen media.[17−20]

To facilitate further research into the importance of proline under such conditions, a simple method for proline determination, which is economical in terms of reagents and instrument costs, is required. Additionally, the appropriate method also needs to overcome limitations associated with ninhydrin-based assays; i.e., interference from other amino acids (reviewed by Elliott and Gardner[21]), or sugar concentrations of up to 100 g/L,[22] as may occur even in the late stages of fermentation. HPLC-based analysis methods have been employed for the detection of amino acids, including proline, derived from various sources, e.g., plants, food, and wine.[23,24] Flow injection analysis (FIA) methodology and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) have also been reported for proline determination in wine.[25,26] However, these methods are complicated and expensive compared with a candidate protocol that utilizes isatin (2,3-indolinedione) as a derivatizing agent. The isatin method was developed for the determination of proline in biological fluids and protein hydrolysates[21,27] and has since been adapted to a microtiter

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format.[28] An evaluation of the isatin-based, colorimetric method for specific determination of proline in grape juice, fermentation samples, and table wines is reported herein.

MATERIALS AND METHODS

Chemicals. Amino acids, including proline, were purchased from Sigma-Aldrich (St. Louis, MO). The derivatizing agents isatin and fluorenylmethyloxycarbonyl chloride (FMOC) were obtained from Sigma-Aldrich and Fluka Chemie (Buchs, Switzerland), respectively. Solvents were HPLC-grade from Merck (Darmstadt, Germany) or Scharlau (Barcelona, Spain), and water (18.2 MΩ/cm resistivity) was distilled and deionized (ELGA LabWater's PURELAB Maxima system). Chemically defined grape juice medium (CDGJM) was prepared according to Henschke and Jiranek.[1] All other reagents were of analytical grade.

Colorimetric Determination of Proline Following Derivatization with Isatin. Proline was quantified according to a modified version of the colorimetric method reported by Elliott and Gardner.[21] Samples (40 μL) were placed in a 13 mL disposable glass tube and diluted with citrate buffer (0.5 M, pH 4.1, 40 μL). To this mixture was added a 0.075% (w/v) solution of isatin in acetone (250 μL) and ethanol (500 μL). The tubes were evaporated to dryness by heating in a boiling water bath for 5 min. The blue proline−isatin residue obtained was dissolved in aqueous acetone (3 mL, 2:1, v/v) and the absorbance of the resulting solution measured at 595 nm using a 1.5 mL quartz cuvette and a spectrophotometer (Thermo Spectronic, Cambridge, England).

HPLC Determination of Proline Following Derivatization with FMOC. Proline was quantified according to the HPLC method reported by Bütikofer and co-workers.[29] Analyses were performed on an Agilent (Palo Alto, CA) 1100 series HPLC instrument, equipped with a vacuum degasser, a quaternary pump, a thermostated column oven, and an Agilent 1200 series fluorescence detector (FLD). Separation was achieved with an ODS (C18) HyperClone column (250 × 4.0 mm, 5 μm, Phenomenex, Lane Cove, Australia) at an operating temperature of 42 °C. A binary elution gradient was utilized (Table 1) with a flow rate of 1 mL/min. Mobile phase A consisted of 30 mM sodium acetate trihydrate, 0.1 M titriplex III, and 0.25% tetrahydrofuran in water (pH 7.2); mobile phase B comprised 20% 100 mM sodium acetate trihydrate and 0.1 M titriplex III in water (pH 7.2) and 80% acetonitrile.[29] Precolumn derivatization was carried out before injection: an aliquot of diluted sample (25 μL) was added to borate buffer (0.4 M, pH 10.6, 250 μL), and then FMOC (2.5 g/L in acetonitrile, 25 μL) reagent was added and the reaction mixture allowed to stand for 2 min, as described by Herbert and co-workers.[30] The injection volume was 10 μL. The excitation/emission wavelengths of the fluorescence detector were set at 266 and 313 nm.

Table 1. Elution Gradient for Proline Determination by HPLC

time (min)	eluent A portion (%)	eluent B portion (%)
0	100	0
13	70	30
20	60	40
25	0	100

Method Validation. The precisions of the isatin and HPLC methods were validated by a series of standard addition experiments. For the isatin method, calibration standards were prepared (in triplicate) in water at concentrations of 0, 50, 100, 150, and 200 mg/L. The assay was also validated in CDGJM containing 500 mg/L proline and 200 g/L sugar, i.e., as a model substrate. The impact of different grape juice components on proline determination was investigated by quantifying proline in CDGJM (in duplicate), with the salt, vitamin, sugar, and mineral components individually excluded.

For the HPLC method, calibration standards were prepared (in duplicate) in water at 0, 1, 2, 3, 4, and 5 mg/L concentrations. Potential interference from other amino acids was investigated by analyzing a CDGJM containing a mix of 19 amino acids and a commercial white wine sample. The methods were further validated by performing a series of repeatability, reproducibility, and recovery tests. To evaluate instrument repeatability, proline standards of low and high concentration (i.e., 50 and 200 mg/L) were repeatedly measured (n = 5). Reproducibility of sample preparation was evaluated by preparing five replicates of a CDGJM fermentation sample and a white wine sample. The accuracy of each method was determined by means of recovery tests, performed by measuring the proline concentration of the CDGJM fermentation sample and the white wine sample (n = 5), before and after they were spiked with known concentrations (i.e., 30 and 120 mg/L) of proline. Recovery was determined by comparing the observed and expected proline concentrations.

Juice and Wine Samples. Grape juice samples (Table 3) were prepared from the fruit of several different grape varieties harvested in 2010 or 2011 and frozen at −20 °C until required for analysis. Berries were defrosted and then crushed manually, and the resulting juice was clarified by centrifugation (5000g, 5 min) and filtered through a 0.22 μm filter (Millipore, Cork, Ireland). The sugar content was determined enzymatically (Boehringer-Mannheim/R-Biopharm, Darmstadt, Germany), adapted for a 96-well plate. Wines (Table 4) were sourced commercially and included a range of grape varieties and vintages.

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) using Genstat (10th ed., VSN International Ltd., Herts, U.K.). Mean comparisons were performed by least significant difference (LSD) multiple-comparison tests at $P < 0.05$.

RESULTS AND DISCUSSION

In preliminary work, the protocol of Elliott and Gardner[21] was followed. However, when samples containing high concen-

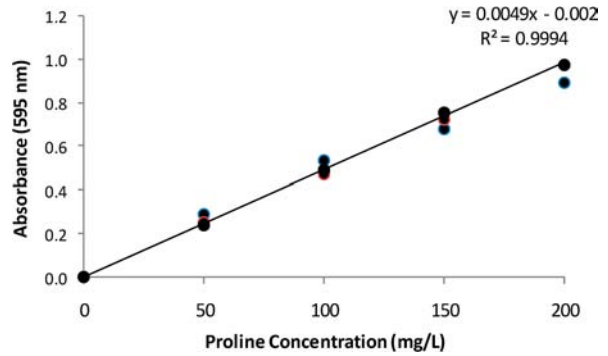

Figure 1. Calibration function for proline in water determined using the isatin colorimetric assay.

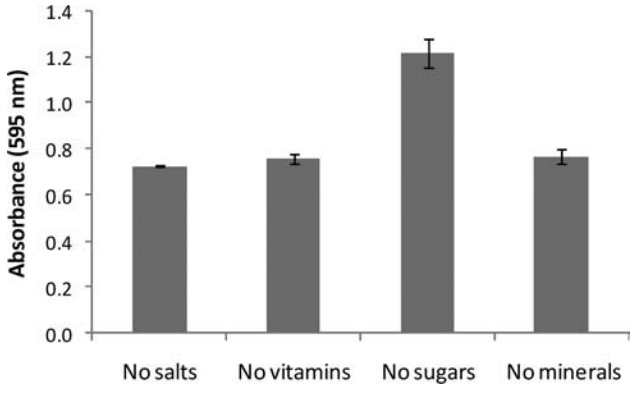

Figure 2. Absorbance of proline (300 mg/L) in CDGJM with the exclusion of salts, vitamins, sugars, and minerals. Data are means (n = 2). Error bars show 2 standard errors of the mean.

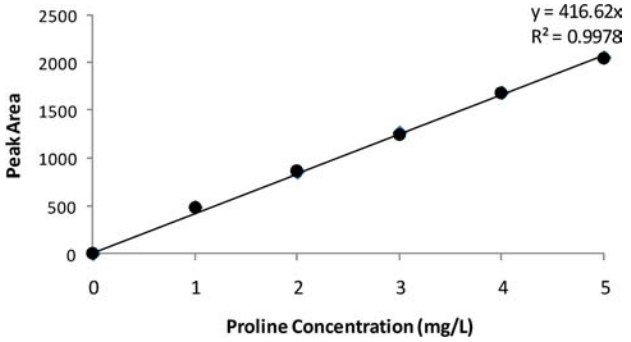

Figure 3. Calibration function for proline in water determined by HPLC following derivatization with FMOC.

trations of sugar were evaporated, heating led to caramelization of the sugars, while alternate evaporation methods not involving heating of the sample produced a large plug of sugar in which proline was not accessible by the isatin. Both outcomes led to inconsistent proline quantitation (data not shown). For this reason, the amounts of sample and citrate buffer were reduced to those volumes reported herein while maintaining the total reaction volume and final citrate buffer concentration. In addition, the heating time was reduced to 5 min. To test the impact of such changes on the protocol, triplicate assays were first carried out using proline standards of known concentration in water. The resulting calibration function showed a high degree of linearity over the working range of 0−200 mg/L, with a correlation coefficient of 0.9994 (Figure 1). From these data, it is clear the assay showed excellent accuracy and reproducibility for aqueous proline standards.

Since the assay is intended for use in the quantitation of proline in fermentation samples and wines, an assay of CDGJM, containing 500 mg/L proline and 200 g/L sugar, was employed as a model substrate. However, the high sugar content within the CDGJM was identified as a source of interference. The major components of grape juice (i.e., salts, vitamins, sugar, and minerals) were individually excluded from the CDGJM to investigate their impact on proline determination. The omission from CDGJM of sugar, as opposed to salts, vitamins, or minerals, produced a significantly higher absorbance reading (A_{595}) (Figure 2), with a concentration effect observed for interference by sugar (data not shown). Thus, at sugar concentrations exceeding approximately 60 g/L, significantly lower absorbance readings were obtained. This was consistent with previous studies[22] and likely indicates a limitation of the method with regard to grape juice analysis. However, for quantification of proline in fermentation samples and wines, the potential for interference is considerably reduced given the lower sugar content.

To further evaluate the performance of the isatin method, comparisons were made with an HPLC method, involving derivatization of proline with FMOC, developed according to methodology reported previously,[29,30] but with modification to optimize sample preparation, injection volumes, and solvent systems. Duplicate assays of aqueous proline standards of

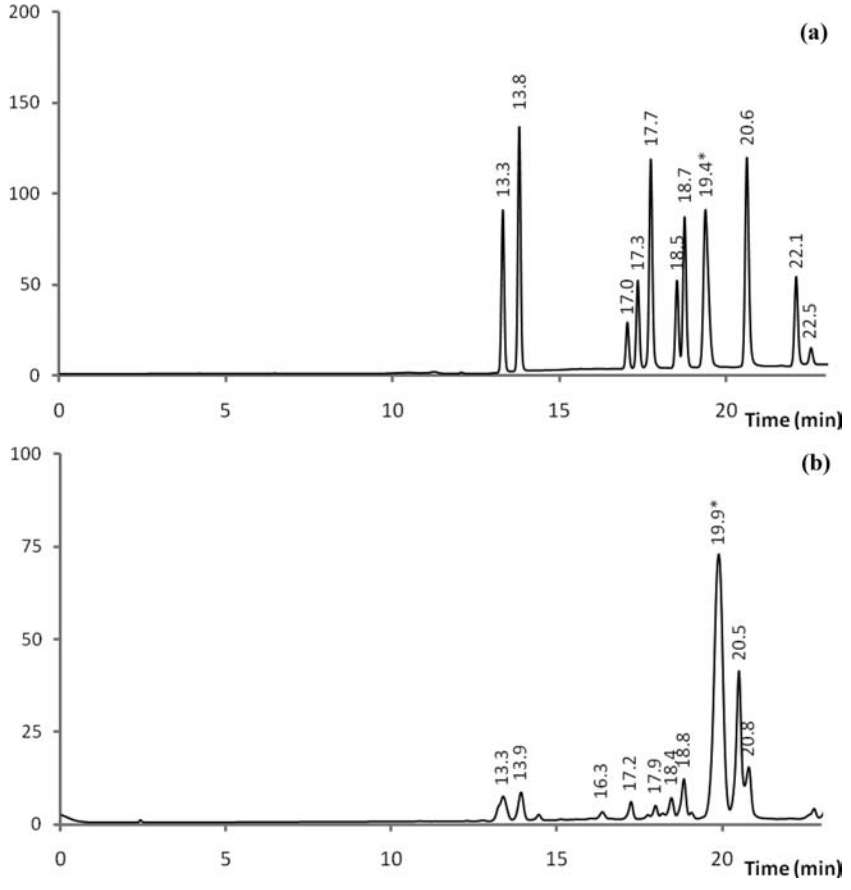

Figure 4. HPLC chromatograms of (a) a CDGJM containing a mix of amino acids and (b) a white wine, following derivatization with FMOC. The proline peak is marked with an asterisk.

Table 2. Method Validation

sample	by isatin method mean (mg/L)	by isatin method CV[a] (%)	by HPLC method mean (mg/L)	by HPLC method CV[a] (%)	n[b]
(a) Instrument Repeatability					
50 mg/L proline standard	53.7	7.8	55.0	4.8	5
200 mg/L proline standard	193.4	3.6	192.0	3.1	5
(b) Reproducibility					
CDGJM fermentation sample	32.7	2.9	38.0	2.0	5
white wine sample	24.6	4.4	28.0	2.0	5
(c) Recovery					
CDGJM fermentation sample					
with 30 mg/L addition (expected)	62.7		68.0		
with 30 mg/L addition (observed)	63.8	5.8	69.6	2.5	5
recovery[c] (%)	101.7		102.4		
with 120 mg/L addition (expected)	152.7		158.0		
with 120 mg/L addition (observed)	160.1	5.0	160.7	1.5	5
recovery[c] (%)	104.8		101.7		
white wine sample					
with 30 mg/L addition (expected)	54.6		58.0		
with 30 mg/L addition (observed)	52.7	5.9	57.5	0.4	5
recovery[c] (%)	96.4		99.1		
with 120 mg/L addition (expected)	144.6		148.0		
with 120 mg/L addition (observed)	154.4	2.8	140.5	1.8	5
recovery[c] (%)	106.8		94.9		

[a]Coefficient of variation. [b]Number of replicates. [c]Observed/expected × 100.

known concentration were performed to construct a calibration function for HPLC analysis. A high degree of linearity was obtained for the working range (1−5 mg/L), with a correlation coefficient of 0.9978 (Figure 3). The HPLC method was subsequently applied to a CDGJM containing a mix of amino acids and a white wine sample to check for interference from other amino acids. The chromatographic conditions employed gave good separation with no interference observed. Proline eluted with a retention time close to 20 min (Figure 4).

Table 4. Concentration of Proline (mg/L) in Wine Determined by Isatin and HPLC Methods

sample	proline concn[a] (mg/L) by isatin method	proline concn[a] (mg/L) by HPLC method	similarity[b] (%)
1999 Semillon	219.7 ± 5.3	243.4 ± 12.5	90.3
2007 Botrytis Riesling[c]	426.5 ± 12.4 a	488.7 ± 14.3 b	87.3
2008 Chardonnay	1002.0 ± 25.4	937.3 ± 15.5	106.9
2009 Cabernet Sauvignon	2707.5 ± 59.0	2551.9 ± 68.9	106.1
2009 Merlot	2223.1 ± 25.3	2082.2 ± 50.2	106.8
2010 Alicante Bouchet	653.1 ± 27.5	604.9 ± 20.4	108.0
2010 Pinot Gris	352.4 ± 17.7	396.8 ± 12.7	88.8
2010 Rosé	1827.9 ± 53.8	1828.9 ± 44.7	99.9
2010 Sauvignon Blanc	408.8 ± 24.9	400.4 ± 9.5	102.1
2010 Semillon	515.6 ± 33.6	560.0 ± 7.7	92.1

[a]Values are means from three experimental replicates (n = 3) ± standard error. Values followed by a different letter within rows are significantly different. [b]Proline concentration determined by isatin/proline concentration determined by HPLC × 100. [c]Containing 168 g/L residual sugar.

Isatin and HPLC determinations were then compared by performing a series of repeatability, reproducibility, and recovery tests (Table 2). Instrumental repeatability was evaluated by repeating the analysis of proline standards representing low and high concentrations within the working range of each method. Proline concentrations were found to be highly consistent, with coefficients of variation being 7.8% and 3.6% for the isatin method and 4.8% and 3.1% for the HPLC method. Both methods also demonstrated good reproducibility, with coefficients of variation between 2.9% and 4.4%. Recovery tests were performed with two samples: a sample of fermented CDGJM and a white wine. For the isatin method, recovery of proline ranged from 96.4% to 106.8%, while recovery for the HPLC method ranged from 94.9% to 102.4%. As expected, the HPLC method proved to be the more accurate quantification method, but the isatin method showed acceptable accuracy (i.e., within ±10%). Furthermore, the validation parameters obtained were similar to those reported elsewhere.[29,30]

Proline Determination in Grape Juice and Wine. The proline content of the analyzed grape juices ranged from 194.7 to 3281.9 mg/L by HPLC analysis (Table 3). These

Table 3. Concentration of Proline (mg/L) in Grape Juice Determined by Isatin and HPLC Methods

sample	sugar concn (g/L)	proline concn[a] (mg/L) by isatin method	proline concn[a] (mg/L) by HPLC method	similarity[b] (%)
2010 Chardonnay 1	214.6	1086.4 ± 68.9	1093.2 ± 44.5	99.4
2011 Chardonnay 2	157.9	257.1 ± 10.1 a	380.2 ± 8.4 b	67.6
2011 Chardonnay 3	189.6	3114.3 ± 50.2	3281.9 ± 57.1	94.9
2011 Merlot 1	159.6	481.3 ± 2.9 a	629.0 ± 20.7 b	76.5
2011 Merlot 2	163.0	386.4 ± 5.3 a	596.5 ± 15.8 b	64.8
2011 Sauvignon Blanc 1	152.7	173.5 ± 1.7 a	315.6 ± 3.7 b	55.0
2011 Sauvignon Blanc 2	119.5	60.7 ± 3.9 a	194.7 ± 5.3 b	31.2
2011 Shiraz 1	188.5	300.1 ± 4.1 a	377.3 ± 2.7 b	79.5
2011 Shiraz 2	180.9	257.0 ± 4.4 a	327.4 ± 7.6 b	78.5
2011 Shiraz 3	249.2	645.6 ± 13.3 a	776.5 ± 23.2 b	83.1

[a]Values are means from three experimental replicates (n = 3) ± standard error. Values followed by a different letter within rows are significantly different. [b]Proline concentration determined by isatin/proline concentration determined by HPLC × 100.

observations were consistent with proline concentrations previously reported for grape juice and must.[30−32] With the exception of two Chardonnay samples, the isatin method gave significantly lower proline concentrations than the HPLC method. Similarity ranged from 31.2% to 83.1%. This was partly attributed to interference resulting from the high sugar concentrations, being greater than 119.5 g/L. Consistent results were only obtained for juice samples with extremely high proline levels (e.g., Chardonnay 1 and Chardonnay 3). These samples required the greatest dilution prior to analysis (i.e., to fall within the 0−200 mg/L working range of the assay). Through this process, the sugar content was also diluted, thereby reducing interference. These data further highlight a limitation of the isatin-based colorimetric method for proline determination of grape juice.

For proline analysis in wine, the two methods showed good similarity, i.e., 87.3−108.0% (Table 4). The proline concentration ranged from 243.4 to 2551.9 mg/L by HPLC analysis, compared with 219.7−2707.5 mg/L for the isatin method. With the exception of the 2007 Botrytis Riesling, there was no statistical difference in the proline concentrations measured using the two different methods. The Riesling wine was found to contain 168 g/L residual sugar, typical of a Botrytis-style wine. As such, sugar was again considered to have interfered with the isatin−proline reaction, thereby leading to an underestimation of the proline concentration.

In summary, we have evaluated the application of a colorimetric method using isatin for the determination of proline in grape juice, fermentation samples, and wine. Compared with HPLC-based analyses, the method is more rapid and economical and requires less sophisticated equipment. The method could accurately detect proline at low milligram per milliliter concentrations, but grape sugars were found to interfere with the isatin−proline reaction. As a consequence, proline concentrations were considerably underestimated in undiluted grape juice and dessert wines of high sugar content. Such difficulties were markedly reduced when fermentation samples and wine were analyzed. The isatin method is therefore considered to be suitable for the routine analysis required to support research into the utilization of proline by yeast during fermentation. With consideration of sample sugar content and therefore appropriate dilution, the method may also be useful in monitoring proline in developing berries and resulting grape juices. The findings discussed herein represent the first time the isatin method has been applied to wine analysis.

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Notes

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REFERENCES

(1) Henschke, P. A.; Jiranek, V. Yeasts—metabolism of nitrogen compounds. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwood Academic Publishers: Amsterdam, 1993; pp 77−163.

(2) Alexandre, H.; Charpentier, C. Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol.* **1998**, *20*, 20−27.

(3) Bisson, L. F. Stuck and sluggish fermentations. *Am. J. Enol. Vitic.* **1999**, *50*, 107−119.

(4) Lambrechts, M. G.; Pretorius, I. S. Yeast and its importance to wine aroma—a review. *S. Afr. J. Enol. Vitic.* **2000**, *21*, 97−129.

(5) Bell, S.-J.; Henschke, P. A. Implications of nitrogen nutrition for grapes, fermentation and wine. *Aust. J. Grape Wine Res.* **2005**, *11*, 242−295.

(6) Hernández Orte, P.; Guitart, A.; Cacho, J. Amino acid determination in musts and wines by HPLC after derivatization with phenylisothiocyanate. *Am. J. Enol. Vitic.* **1998**, *48*, 229−235.

(7) Gump, B. H.; Zoecklein, B. W.; Fugelsang, K. C.; Whiton, R. S. Comparison of analytical methods for prediction of prefermentation nutritional status of grape juice. *Am. J. Enol. Vitic.* **2002**, *53*, 325−329.

(8) Ogorodnik, S. T.; Merkureua, Y. S. Determination of amino nitrogen in wine by formol titration. *Sadovod., Vinograd. Vinodel. Mold.* **1971**, *25*, 29−31.

(9) Dukes, B. C.; Butzke, C. E. Rapid determination of primary amino acids in grape juice using an *o*-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. *Am. J. Enol. Vitic.* **1998**, *49*, 125−134.

(10) Bertamini, M.; Zulini, L.; Muthuchelian, K.; Nedunchezhian, N. Effect of water deficit on photosynthetic and other physiological responses in grapevine (*Vitis vinifera* L. cv. Riesling) plants. *Photosynthetica* **2006**, *44*, 151−154.

(11) Verbruggen, N.; Hermans, C. Proline accumulation in plants: a review. *Amino Acids* **2008**, *35*, 753−759.

(12) Duteurtre, B.; Bourgeois, C.; Chollot, B. Study of the assimilation of proline by brewing yeast. *J. Inst. Brew.* **1971**, *77*, 28−35.

(13) Ingledew, W. M.; Magnus, C. A.; Sosulski, F. W. Influence of oxygen on proline utilization during the wine fermentation. *Am. J. Enol. Vitic.* **1987**, *38*, 246−248.

(14) Ough, C. S.; Huang, Z.; An, D.; Stevens, D. Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: effects on urea excretion and reabsorbtion. *Am. J. Enol. Vitic.* **1991**, *42*, 26−40.

(15) Brandriss, M. C.; Magasanik, B. Proline: an essential intermediate in arginine degradation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **1980**, *143*, 1403−1410.

(16) Peinado, R. A.; Moreno, J. J.; Maestre, O.; Mauricio, J. C. Use of a novel immobilization yeast system for winemaking. *Biotechnol. Lett.* **2005**, *27*, 1421−1424.

(17) Salmon, J.-M.; Barre, P. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by depression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. *Appl. Environ. Microbiol.* **1998**, *64*, 3831−3837.

(18) Poole, K. Enhancing yeast performance under oenological conditions by enabling proline utilisation. Ph.D. Thesis, The University of Adelaide, Australia, 2002.
(19) Poole, K.; de Barros Lopes, M.; Jiranek, V. Potential for yeast exploitation of proline in grape juice to facilitate fermentation completion. *Aust. N. Z. Grapegrower Winemaker* **2002**, *461a*, 121−123.
(20) Martin, O.; Brandriss, M. C.; Schneider, G.; Bakalinsky, A. T. Improved anaerobic use of arginine by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2003**, *69*, 1623−1628.
(21) Elliot, R. J.; Gardner, D. L. Proline determination with isatin, in the presence of amino acids. *Anal. Biochem.* **1976**, *70*, 268−273.
(22) Mange, C.; Larher, F. High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Anal. Biochem.* **1992**, *200*, 115−118.
(23) Miller, E. J.; Narkates, A. J.; Niemann, M. A. Amino acid analysis of collagen hydrolysates by reverse-phase high-performance liquid chromatography of 9-fluorenylmethyl chloroformate derivatives. *Anal. Biochem.* **1990**, *190*, 92−97.
(24) Hernández-Orte, P.; Ibarz, M.; Cacho, J.; Ferreira, V. Amino acid determination in grape juices and wines by HPLC using a modification of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) method. *Chromatographia* **2003**, *58*, 29−35.
(25) Cataldi, T. R. I.; Nardiello, D. Determination of free proline and monosaccharides in wine samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). *J. Agric. Food Chem.* **2003**, *51*, 3737−3742.
(26) Costin, J. W.; Barnett, N. W.; Lewis, S. W. Determination of proline in wine using flow injection analysis with tris (2, 2′-bipyridyl) ruthenium (II) chemiluminescence detection. *Talanta* **2004**, *64*, 894−898.
(27) Boctor, F. N. An improved method for colorimetric determination of proline with isatin. *Anal. Biochem.* **1971**, *43*, 66−70.
(28) Grainger, D. J.; Aitken, S. A microtitre format assay for proline in human serum or plasma. *Clin. Chim. Acta* **2004**, *343*, 113−118.
(29) Bütikofer, U.; Fuchs, D.; Bosset, J.; Gmür, W. Automated HPLC-amino acid determination of protein hydrolysates by precolumn derivatization with OPA and FMOC and comparison with classical ion exchange chromatography. *Chromatographia* **1991**, *31*, 441−447.
(30) Herbert, P.; Santos, L.; Alves, A. Simultaneous quantification of primary, secondary amino acids, and biogenic amines in musts and wines using OPA/3-MPA/FMOC-Cl fluorescent derivatives. *J. Food Sci.* **2001**, *66*, 1319−1325.
(31) Huang, Z.; Ough, C. S. Amino acid profiles of commercial grape juices and wines. *Am. J. Enol. Vitic.* **1991**, *42*, 261−267.
(32) Spayd, A. E.; Andersen-Bagge, J. Free amino acid composition of grape juice from 12 *Vitis vinifera* cultivars in Washington. *Am. J. Enol. Vitic.* **1996**, *47*, 389−402.