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Application of differential pH technique to the determination of urea in Italian wines

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Summary

A method for the quantification of urea in wine, based on measuring the change in pH when urease is added to the sample, is presented and compared to the conventional dual enzyme (urease/glutamate dehydrogenase) approach. The method is linear in the range 0-30 mg·l^{-1} in red, white and "raisin" wines, and the detection limit (0.3 mg·l^{-1}) is lower than for the usual enzymatic method. The differential pH technique presented here gives accurate quantification of urea in wine, being unaffected by the presence of ammonium. The amounts of urea in 195 still and sparkling commercially available wines with designation of geographic origin from the most renowned Italian grape growing areas were quantified. 17.4 % of samples were over the 3 mg·l^{-1} level suggested by the International Organisation of Vine and Wine for urease treatment to limit the potential risk for ethyl carbamate formation during wine ageing. Yeast strains EC1118 and SP665 can minimise urea content in wine.

K e y w o r d s : differential pH technique, urea, wine, yeast strains.

Introduction

The main reason for accurately analysing urea content in wine is that it is deemed to be the main source of ethyl carbamate (EC) (Ferreira Monteiro *et al.* 1989), otherwise known as urethane, a known animal carcinogen (Nettleship *et al*. 1943) for which Canadian legislation was the first internationally to establish a legal limit (30 µg·l^{-1}) in 1985. Three years later, the US Food and Drug Administration accepted an agreement to reduce EC levels in wine and, from the 1995 vintage on, it was established that no more than 1 % of table wine production must have > 25 µg·l^{-1} and no more than 1 % of dessert wine must have > 90 µg·l^{-1} urethane (Butzke and Bisson 1997).

Urea produced during the metabolism of arginine in *Saccharomyces cerevisiae* by arginase is believed to be the most important precursor of EC in wine (Ferreira Monteiro *et al.* 1989; Ferreira Monteiro and Bisson 1991). However, arginine can be degraded through the arginine deiminase pathway yielding carbamyl phosphate as a secondary product, and both urea and carbamyl phosphate can react with ethanol to form ethyl carbamate (Ough *et al.* 1988; Arena *et al*. 1999). Urea can also be produced as a result of the degradation of purines, producing allantoin and allantoic acid, but their levels in grapes are low (Baumann and Zimmerli 1986; Ough *et al.* 1988; Ferreira Monteiro *et al.* 1989; Ferreira Monteiro and Bisson 1992). The use of nitrogen fertilisation in vineyards contributes towards urea accumulation in wines, as it increases the content of nitrogen nutrients in the relevant juices (Ough *et al*. 1989; Spayd *et al*. 1994). However, Ough *et al.* (1991) stated that if a great excess of amino acids is present, the metabolism of arginine by yeasts is reduced, with a lower production of urea. Ough and colleagues (1988) observed that the same amount of urea produced less EC in red than in white wine, as a consequence of possible reactions or bonds of urea with phenols. High temperatures of aging and storage, and high concentrations of ethanol, urea and citrulline enhance EC formation (Ough *et al.* 1988; Stevens and Ough 1993; Kodama *et al*. 1994; Hasnip *et al.* 2004), while the influence of pH is controversial (Ough *et al.* 1988; Stevens and Ough 1993) and light has no effect (Tegmo-Larsson and Spitter 1990). Consequently, storage temperatures of over 24 °C and urea concentration of over 5 mg·l^{-1} should be avoided (Stevens and Ough 1993).

In the light of the aforementioned relationships between urea and EC, a quick and accurate method for quantifying urea is welcome. Because of the peculiarities of different matrices, a variety of analytical techniques has been developed, *i.e.* indirect methods involving enzymatic degradation of urea into ammonia prior to detection, and direct methods (Francis *et al.* 2002; Francis 2006). One of the most popular, conventional and easiest methods for detecting urea/ammonia is the enzymatic Boehringer Mannheim UV-method (1987), but in wine this suffers from low sensitivity (Fujinawa *et al.* 1990), the natural amounts of ammonium, sodium and potassium in wines causing possible interference (Francis *et al.* 2002). Nevertheless, to date a method for detecting urea in wine is missing in the "Compendium of International Methods of Analysis of Wines and Musts" of the International Organisation of Vine and Wine, and in the Official Methods of Analysis of AOAC International.

Differential pH-metry (DpH) has been applied in the medical field since the early 1980s (Luzzana *et al*. 1983; Ceriotti *et al*. 1984; Luzzana *et al.* 1984) to measure several compounds, including urea (Ripamonti *et al.* 1984). More recently it has been used in food analysis, *i.e.* in milk

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to quantify urea (Luzzana and Giardino 1999) and in must and wine to measure sugars (Cecchini and Morassut 1995; Larcher 1999; Moio *et al.* 2001) and acetic (Di Paolo *et al.* 2006), lactic and malic acids (Palleschi *et al.* 1994).

In this paper a method using differential pH-metry to measure the urea content in wine was established. Secondly, the method was applied in order to verify the content of urea in 195 still and sparkling Italian wines with denomination of origin and to check variability due to yeast strains used to ferment real juices on a semi-industrial scale.

Materials and Methods

Instruments and reagents: A differential pH-meter (mod. CL-10 Plus; Eurochem Diffchamb, Ardea, Rome, Italy) was used. This instrument quantifies urea by measuring the pH variation between 2 electrodes caused by urea hydrolysis in a buffered medium into ammonia following the addition of urease. The buffer (pH 7.1; stable for two weeks at 2-8°C after reconstitution), urea standard (30 mg·l^{-1}) and urease solution were from Eurochem Diffchamb (WCP688). For calibration, a blank sample must be prepared with 10 ml of wine, corrected to pH 6.9 ± 0.1, amended with 300 µl urease solution, incubated at 37 °C for 30 min and then heated to 80-90 °C for 5 min. The analyses of blank and urea standard (400 µl) set the "offset" and "slope" values respectively, and must be checked every 10 samples. The samples (400 µl) adjusted to pH 6.9 ± 0.1 with NaOH, after degassing in the case of sparkling wines, are analysed within 24 hours to avoid losses of urea.

Evaluation of the method: Increasing amounts of urea (NIST SRM 912a) in the 0-30 mg·l^{-1} range were added to blank samples of different matrices (white, red, and raisin wines) to check linearity, accuracy and precision (RSD % of 10 replicates). The detection limit (DL) was defined as 3 standard deviations measured in 10 repeated tests of the blank sample.

The proposed DpH method was compared with the conventional dual enzyme approach (urease/glutamate dehydrogenase; Boehringer Mannheim/Biopharm, Darmstadt, Germany) by measuring the urea content in natural and properly spiked wines in different technological categories.

Materials: An extensive sample of commercially available wines (N = 195) of different compositions and types (*e.g.* dry, sweet, still, sparkling, white, red, young, aged) from almost all the Italian regions were taken directly from wineries and analysed for their urea content using DpH. Only wines with denomination of origin from the most renowned viticultural areas in Italy and the most important varieties from each area (Tab. 1) were included. Wines were classified into 6 technological categories: (a) still red, (b) still white, (c) late harvest and *raisin*, (d) *nouveau* (carbonic maceration), (e) Charmat's method sparkling and *petillant*, and (f) classic-method sparkling. A larger number of wines in the categories "still red" and "classic-method sparkling" were analysed to better represent national production (in the ratio red/white wines) and in order to have a picture of wines usually processed with longer and/or double yeast contact (*i.e.* classic-method sparkling wines).

The variability of the final amount of urea left in wine by the yeast strain used in winemaking was checked in white wines produced on a semi-industrial scale at the experimental winery of Istituto Agrario di S. Michele all'Adige. Wines originally produced to check fermentation and aroma production performance of several commercial and pre-commercial *Saccharomyces cerevisiae* strains were used (Nicolini *et al*. 2002) (*28*). They had been processed according to standard procedures from 4 juices (Tab. 2), sulfited (50 mg·l^{-1}), settled (turbidity level < 50 NTU) and enriched with sugar to a high potential alcohol strength (*ca*. 15 % vol.). In the settled juices, assimilable nitrogen (Nicolini *et al*. 2004) and arginine (Mira de Orduña 2001) were measured, while urea was at undetectable levels. Before inoculum, wild yeasts were under 100,000 cfu·ml^{-1}. Commercial strains T 73, CGC62, EM 2, SP665 from La Claire (Verona, Italy) and EC1118 from Lalvin were used (20 g·hl^{-1}) after hydration. To avoid malolactic fermentation and limit yeast lysis, all the wines were kept at 5 °C from the end of alcoholic fermentation to sterile bottling, 3 months later, then the bottles were stored at 10-12 °C.

The data were statistically evaluated and plotted using STATISTICA™ for Windows v. 5.1, 1997 (StatSoft Italia S.r.l., Padova, Italy).

Results and Discussion

The linearity of the data correlation by DpH method is shown in Tab. 3. The regression lines in the range 0-30 mg·l^{-1} have decidedly high and significant ($p < 0.0001$) R^2 values. The slopes of the lines are close to 1 and similar for the 3 wine categories, proving the accuracy of the method and the absence of matrix interference.

The precision of measure, as RSD, and DL were 4.5 % and 0.3 mg·l^{-1} respectively. The same two parameters quantified for the dual enzyme method were similar but slightly higher, being 6.2 % and 0.7 mg·l^{-1}, respectively.

Additions of 10, 25, 50, 75 and 100 mg·l^{-1} ammonium to a blank sample did not affect the quantification of urea using DpH (RSD = 4.3 %).

The correlation of the values observed by comparing the 2 methods on the basis of the content of urea measured in 28 natural wines and wines with urea amendment up to ca. 30 mg·l^{-1} is satisfactory and the slope is close to one. (Fig. 1).

Survey of commercially available wines: The average and median values for urea in the 195 commercially available wines analysed using DpH-metry were 2.13 mg·l^{-1} and 1.74 mg·l^{-1} respectively. Fifty % of the samples had values between 1.07 mg·l^{-1} and 2.51 mg·l^{-1}. Three outlier samples had urea contents above 11 mg·l^{-1} and up to 19.5 mg·l^{-1}. Six samples (3.0 %) were over the 5 mg·l^{-1} level considered as risky for EC, 34 samples (17.4 %) were over the 3 mg·l^{-1} level suggested in 2005 by OIV for urease treatment (Resolution OENO 5/2005),

Table 1

Distribution of samples by region, type of wine, prevailing grape variety and designation of origin (DOC: Registered Designation of Origin; DOCG: Registered and Certified Designation of Origin; IGT: Regional Geographical Indication)

	Wines						Wine grape cultivars	
Region	Still red	Still white	Late harvest, raisin	Nouveau (carbonic maceration)	Sparkling (Charmat & petillant) (red & white)	Classic method sparkling	White-fruited	Red-fruited
Friuli	4	3					Sauvignon b., Tokai	Merlot, Cabernet, Refosco
Veneto	5	5			1		Garganega, Pinot bianco, Trebbiano di Soave, Prosecco	Merlot Corvina, Rondinella, Molinara
Trentino Alto Adige	10	4	7	1	3	18	Chardonnay, Pinot grigio, Traminer, Nosiola, Müller-Thurgau	Cabernet, Lagrein, Marzemino, Merlot, Pinot nero, Schiava, Teroldego
Lombardia	3	2		2	3	32	Chardonnay, Pinot bianco,	Pinot nero, Cabernet, Barbera, Nebbiolo, Merlot
Piemonte	3				1			Barbera, Dolcetto, Brachetto
Emilia Romagna	3				7		Sauvignon b.,	Barbera, Bonarda, Pinot nero, Cabernet, Lambrusco
Toscana	14	2	8	1			Malvasia del Chianti, Trebbiano, Vermentino, Vernaccia	Brunello, Cabernet, Canaiolo, Merlot, Sangiovese, Syrah
Umbria	4	1					Trebbiano toscano, Malvasia, Verdello	Sagrantino
Abruzzo	8							Montepulciano
Lazio		1					Trebbiano toscano, Malvasia di Candia	
Campania		1	1				Greco, Biancolella	
Basilicata	5							Aglianico
Puglia	4	2					Verdeca, Bianco di Alessano	Primitivo, Negroamaro
Calabria	1							Gaglioppo
Sicilia	16	6		1			Inzolia, Grillo, Chardonnay	Nero d'Avola, Merlot, Cabernet, Syrah
Sardegna	1	1					Vermentino	Cannonau
Total	81	28	16	5	15	50		
DOC	41	19	15		9	29		
DOCG	16	1			2	21		
IGT	24	8	1	5	4			

Table 2

Composition of the juices

Variety	°Brix	Total acidity ($g \cdot l^{-1}$)	pH	Assimilable nitrogen ($mg \cdot l^{-1}$)	Arginine ($mg \cdot l^{-1}$)
Sauvignon blanc	22.4	5.5	3.43	277	1380
Pinot gris	20.2	5.3	3.46	182	725
Prosecco	17.3	6.0	3.17	110	580
Chardonnay	19.0	7.2	3.23	229	684

Table 3

Parameters of linearity achieved on the basis of the addition of known amounts of urea (NIST SRM 912a) to different types of wines

wine	slope	intercept ($mg \cdot l^{-1}$)	R^2
white	1.0004	-0.0033	0.9921
red	1.0002	0.0029	0.9920
raisin	1.0011	-0.0009	0.9923

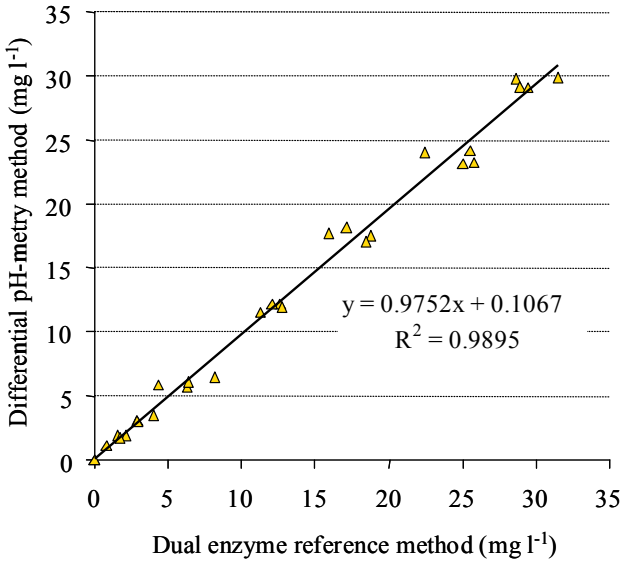

Fig. 1: Regression lines of the values of urea measured in wines using the differential pH technique and the dual enzyme reference approach.

and 147 samples (75.4 %) were over the 1 $mg \cdot l^{-1}$ treatment limit permitted by Reg. 1622/2000 of the European Commission (Fig. 2). Simple statistics relating to the amounts of urea and alcohol in the different types of wines analysed are given in Tab. 4.

A statistical approach by wine category on the basis of the total data set is difficult, due to the different number, distribution and ageing of the available samples. So, after eliminating the 3 highest values, a subset of samples was created to check possible differences in urea, taking into account only samples of the 3 more numerous wine categories (still red, still white and "classic method" sparkling). Both non-parametric tests - *i.e.* Kruskal-Wallis and Median tests (Vitali 1993) and ANOVA-Tukey's test for samples of unequal number were used, and confirmed that the 3 categories had significantly different ($p < 0.001$) urea content as compared to each other, with amounts of urea being present in white, red and "classic-method" sparkling wines in decreasing order. It may be interesting to note that the 4 richest wines in the overall data set were classic-method sparkling wines produced using 'Pinot' and 'Chardonnay 'grapes. Even though such cultivars generally have a prevalence of arginine (*i.e.* 'Pinot') or presence at high levels in the juice (Nicolini *et al.* 2001), these high values could be due to accidental residues on grape of too late foliar fertilisation with urea.

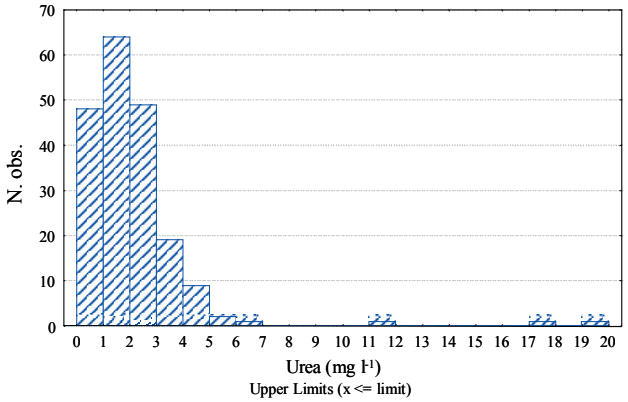

Fig. 2: Histogram of urea contents in the Italian wines in Tab. 1.

In order to minimise the possible effect of different ageing times, and excluding "classic-method" sparkling wines as only the year of degorgement is usually given on the label, 27 red and 26 white wines of 2003 vintage were compared. The reds had significantly lower urea content as compared to the whites (mean ± st.dev.: 1.90 ± 0.65 *vs.* 3.29 ± 1.16 $mg \cdot l^{-1}$, respectively; $p < 0.0001$), in agreement with the ranking discussed by Ough *et al.* (1990) for commercially fermented wines. The same picture was observed by us in wines from 'Pinot noir' and 'Chardonnay' grapes, each variety processed as white and skin-contact "red" wine (data not shown). The usually higher alcoholic strength of the reds reduces urea formation and excretion (An and Ough 1993), thus explaining, at least partially, the lower levels of urea. The same explanation can be taken into account for the low levels observed in the alcohol-rich raisin wines and for the somewhat high levels found in Charmat

Table 4

Amounts of urea and alcohol by wine category (n.a.= not available data)

		Still red	Still white	Late harvest, raisin	Nouveau (carbonic maceration)	Sparkling (Charmat & petillant)	Classic method sparkling
Number of samples		81	28	16	5	15	50
Urea (mg/l)	min	0.3	0.7	< DL	1.4	1.6	0.3
	25° percentile	1.3	2.4	0.7	1.7	2.0	0.6
	Median	1.7	3.4	1.3	2.0	2.8	1.0
	75° percentile	2.1	4.1	2.0	3.0	3.5	1.7
	max	3.6	5.4	4.6	3.2	4.3	19.5
Alcohol (% vol)	min	9.37	11.08	12.65	11.48	6.65	n.a.
	25° percentile	12.74	12.27	13.53	11.88	10.87	n.a.
	Median	13.12	12.65	14.24	12.12	11.89	n.a.
	75° percentile	13.61	12.99	14.70	12.32	12.86	n.a.
	max	15.46	13.95	15.59	13.23	13.14	n.a.

method wines, usually characterised by a relatively low alcoholic degree. Besides, the urea content in raisin wines could be affected by some presence of flor yeasts (Valero *et al.* 1999). The data observed in this sampling of Italian wines do not agree with the ranking given by Waldner and Augustyn (2005) for South African 1997 vintage young wines, with 184 reds and 128 whites averaging 3.64 $mg \cdot l^{-1}$ and 2.11 $mg \cdot l^{-1}$ respectively.

Role of the yeast strain: The results are displayed in Fig. 3. The highest urea content was in wines produced using Sauvignon blanc, while the content in the wines of other varieties was lower and rather similar. The Sauvignon blanc juice had the highest content of assimilable nitrogen, and a remarkable amount of arginine, as reported in the literature (Huang and Ough 1991; Spayd and Andersen-Bagge 1996) (Tab. 2). The 'Chardonnay' juice had the second highest content of assimilable nitrogen. Typically, arginine does not prevail in this variety, particularly in juices from early harvested grapes as in the present case (Millery *et al.* 1986; Nicolini *et al.* 2001). The assimilable nitrogen of 'Pinot gris' and 'Prosecco', varieties where arginine typically prevails (Nicolini *et al.* 2001), was lower. Even though arginine in juice can account for less than 50 % of the urea produced by yeasts (Ferreira Monteiro and Bisson 1991), the overall picture for available nitrogen in the juices contributes towards explaining, in agreement with Ough *et al.* (1990), the relatively low and homogeneous levels of urea in 'Chardonnay', 'Prosecco' and 'Pinot gris' wines as compared to 'Sauvignon'.

Yeast strains EC1118 and SP665 minimised the final urea level in the wine obtained from more potentially risky juice. The performance of EC1118, also known as "Prise de Mousse" and "Premier Cuvée" yeast (Dunn *et al.* 2005), confirms previous findings (Ough *et al.* 1990; An and Ough 1993; Butzke and Bisson 1997). Technologically, a difference of even 6-7 $mg \cdot l^{-1}$ between these 2 strains and the others is of remarkable interest and similar to that recorded by Ough and colleagues (1990) in white wines.

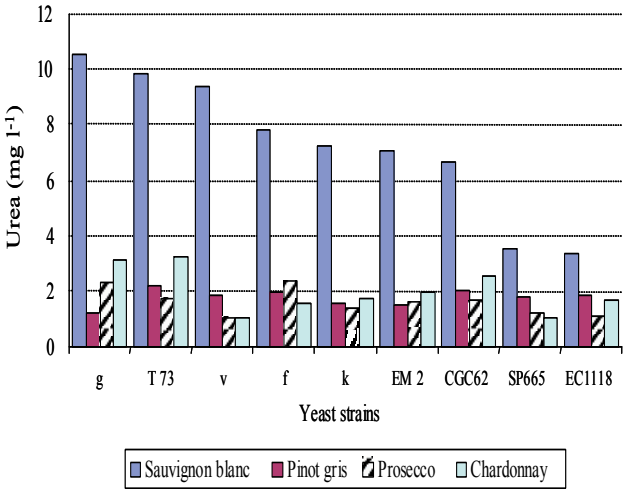

Fig. 3: Urea contents of single variety semi-industrial wines produced using commercial and pre-commercial (k, f, v, g) yeast strains.

Conclusions

This investigation has shown that differential pH-metry can be an alternative approach to the conventional dual enzyme method for measuring urea in wine, having suitable sensitivity, precision, accuracy and robustness with different matrices. Moreover, it is easily automatable.

From a technological point of view, it was highlighted that roughly one sixth of the Italian wines analysed could positively benefit from the urease treatment suggested by OIV, as they exceed urea levels of 3 $mg \cdot l^{-1}$. Furthermore, it was confirmed that the choice of a suitable yeast strain can help to minimise urea production in wine. Significant differences were observed among reds and whites, but it seems that other production factors - e.g. yeast strain, grape variety, juice composition - could affect the final urea content of wine more than the plain skin-contact.

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