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Release of Nucleotides and Nucleosides during Yeast Autolysis: Kinetics and Potential Impact on Flavor

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Nucleotides, particularly 5′-nucleotides, are important flavoring agents found in many foods and beverages. Their precise effect on the flavor of wines aged on lees has not been examined previously. In this study nucleotides and nucleosides released by yeast during autolysis in a model wine system and in Champagne wines were identified and quantified, and their impact on wine flavor was determined. Ribonucleotides only were detected in yeast autolysate and in Champagne wines. In wines ribonucleotides were quantified by tandem mass spectrometry coupled to HPLC. The maximum concentration of total nucleotides was very low with a maximum of ∼3 mg/L in wine aged on yeasts for 9 years. In young wines the most important nucleotide was 5′-UMP, but after 2 years of aging its concentration decreased and the concentration of 5′-GMP slowly increased. The threshold values of the most representative nucleotides in Champagne wines were higher than the concentrations found in the same wines. However, it is known that there is synergism between the different nucleotides and also in the presence of glutamic acid. This phenomenon could explain the difference observed in descriptive profiles of wines spiked with nucleotides.

KEYWORDS: Yeast autolysis; nucleotides; nucleosides; wine flavor

INTRODUCTION

Nucleotides, particularly 5′-nucleotides such as 5′-GMP and 5′-IMP, are important flavoring agents found in many foods and beverages. These compounds have little or no flavor or aroma themselves but can enhance the flavor and mouthfeel of other compounds. They may be added to foods as a component of yeast extract produced by the autolysis of brewer's yeast. Yeasts such as *Saccharomyces cerevisiae* are the preferred source of nucleic acids for production of 5′-nucleotides due to their high nucleic acid content (∼8−11% RNA by dry weight) (*1*).

Zhao and Fleet (*2*, *3*) investigated the influence of temperature and pH on the degradation of RNA and DNA during yeast autolysis. They showed that the most favorable temperature and pH, in terms of rate of degradation, were 40 °C and pH 7 for DNA and 50 °C and pH 7 for RNA. In such conditions during autolysis, cell DNA was 40−50% degraded, releasing mainly 3′- and 5′-deoxyribonucleotides, and RNA was 90% degraded, releasing primarily 3′- but also 5′-ribonucleosides, with smaller amounts of 2′-ribonucleotides (tentatively identified), ribonucleosides, and bases.

The precise effect of nucleotides on wine flavor has not been examined. They can arise from yeasts in wines matured on yeast lees such as sparkling wines produced according to the "méthode champenoise" process (secondary fermentation in bottle and aging on lees). It is conceivable that flavor nucleotides may participate by enhancing the toasty, mushroom flavors considered as an element of the "methode champenoise" sparkling wine autolysis character (*4*).

Leroy et al. (*5*) showed a loss of nucleic acids (measured by the orcinol method) in yeast cells and an increase in the concentration of nucleic acid materials in the wine, which occurred mainly in the first 10−15 months of aging on lees. Thus, at the end of the 15 months of storage, the nucleic acid amount of the yeast cells decreased by 16% of the original value and the yeast dry weight by ∼8%. This slow release or nucleic acid degradation is due to the unfavorable conditions for the autolysis process of champagne making (low pH of wine and low storage temperature).

Courtis et al. (*4*) studied the total nucleic acids content of a sparkling wine matured on lees for a period of 5 months at 21 °C. A rapid release of nucleic acids was observed with the total nucleic acid content of the wine rising from 130 to 340 μg/mL within 80 days. After this period, there was relatively little

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release of nucleic acids into the wine (a further 4% in 50 days), but the formation of flavor nucleotides during this process was not studied.

To adapt yeast to low pH of wine and low storage temperature, the traditional method of starter preparation used in Champagne consists of growing yeasts in a wine preferably identical in composition to the "cuvée wine" supplemented with sugar and diammonium phosphate.

Thus, this study was undertaken with the objective of identifying and quantifying nucleotides and nucleosides, released by yeasts grown in wine during autolysis, in a model wine system and in Champagne wine after different times of aging on lees and studying their influence on wine flavor by sensory analysis.

MATERIALS AND METHODS

Chemicals and Chromatographic Standards. The nucleic acids degradation products, nucleotides and nucleosides, were obtained from Sigma (St. Louis, MO) except the mixture 2′-GMP/3′-GMP (38:59), which was purchased from Fluka BioChemica (Buchs, Switzerland). Potassium sulfate, magnesium sulfate, malic acid, sodium hydroxide, Rectapur potassium dihydrogenphosphate (KH_2PO_4), and orthophosphoric acid (84%, w/w) were acquired from Prolabo (Paris, France). Analytical reagent grade dipotassium hydrogenphosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$) was obtained from Merck (Darmstadt, Germany). Surfin ethanol (96.2%, v/v) was bought from Charbonneaux-Brabant (Reims, France), and the ion-pair reagent, tetrabutylammonium dihydrogenphosphate (TBA), was acquired from Sigma. Acetic acid and HPLC grade methanol were purchased from Carlo Erba reagents (Rodano, Italy); HPLC grade water was purified using a Milli-Q system (Millipore Corp.).

Yeast Strains and Adaptation to Wine. A yeast strain MC001, species *Saccharomyces cerevisiae*, selected by Moët & Chandon Cie (Epernay, France) and conditioned in active dry yeast by Bioprox (Château-Renaud, France), was used.

Traditional starter culture preparation in wine was as follows: The procedure of adaptation to wine was made according to Moët & Chandon protocol (*6*). The dried yeast (2 g/L) was rehydrated in sugared water (100 g/L) at 35 °C; the growth medium was then supplemented with ammonium phosphate (300 mg/L) and the temperature progressively reduced to 20 °C. After 24 h, the medium was diluted 5 times with wine to adapt the yeast to wine conditions. The yeasts were then propagated during ~4 days in wine to which 60 g/L sugar and 0.5 g/L ammonium phosphate had been added, at a temperature of 16 °C. When the yeast population reached 5×10^7 yeast cells/mL, they were chilled at a rate of 2 °C per day to a temperature of 2 °C. At the end of the propagation, the medium contained 13.5% (v/v) ethanol and 20 g/L sugar.

Autolysis Conditions. Yeasts were harvested by centrifugation (Centrikon H401, Kontron-Instruments, Gosheim, Germany) at 7500*g* for 10 min at 4 °C and washed three times at 4 °C with deionized water.

The washed cells were then suspended (27 g/L dry matter) in a model wine buffer (*7*) with minor modification in individual flasks. This wine buffer contained ethanol (11.5%, v/v), malic acid (3.0 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate (0.025 g/L), and the pH was adjusted to 3.5 with sodium hydroxide. Tartaric acid was not added to the medium, as mentioned in the Feuillat patent (*7*), to prevent the salts from precipitating.

Autolysis was conducted by incubating cell suspensions at 30 °C for 8 days with orbital shaking at 200 rpm. Autolysis was performed in duplicate.

Yeast Biomass Determination. After agitation to homogenize the yeast suspension, 5 mL of autolysate was vacuum-filtered through a preweighed 0.45 μm membrane filter. The collected cells were washed with distilled water to remove excess medium. The filters were dried at 105 °C for 24 h and weighed again. The dry matter of biomass was determined from the difference.

Preparation of Autolysate Samples. Every day, a 100 mL sample was collected from each flask in sterile conditions and separated by centrifugation (7500*g* for 10 min at 4 °C) into cell pellet and cell-free (supernatant) autolysate fractions.

On the one hand, a 10 mL sample of each autolysate supernatant was used to determine total nitrogen, and 10 mL of sample was freeze-dried to estimate the dry weight of the supernatant.

On the other hand, 30 mL of each cell-free autolysate fraction was concentrated under reduced pressure at 25 °C with an RE-100 model rotary evaporator (Bibby Sterilin, Staffordshire, U.K.) until reaching a volume of 10 mL (3-fold concentration step) and filtered through a 0.45 μm membrane (Millipore Corp.) with the aim of analyzing nucleic acids degradation products by HPLC.

Champagne Wines. All of the champagne wines were elaborated by Moët & Chandon: 0, blending of Pinot Noir (35%), Pinot Meunier (15%), and Chardonnay (50%) young wines before the bottle fermentation; 0.1, the same wine as 0 after the second fermentation; 2−14, different vintages, aged on lees in the cellar at 12 °C during 2, 3, 7, 8, 9, 11, and 14 years. The base wines are slightly different, but for each of them, the second fermentation was conducted with the same yeast strain and the same type of inoculum preparation.

Preparation of Wine Samples. The sample preparation was carried out following the procedure of Aussenac et al. (*9*) In brief, this procedure included successive ultrafiltration of wine (250 mL) on a membrane (cutoff = 5000 kDa) and a concentration of the filtrate under reduced pressure (25-fold concentration step). The separation of the acidic fraction was performed by an anion-exchange chromatography on a semipreparative column (250 mm length, 10 mm i.d., 5 μm particle size, 100 Å porosity) provided by Interchim (Montluçon, France) and using a gradient system (0.007 and 0.25 M phosphate buffer containing 0.25 M KCl at pH 4). Finally, reversed phase chromatography (Ultrasphere OD column, 250 mm length, 10 mm i.d., 5 μm particle size, 80 Å porosity, purchased from Beckman) was used for desalting and removing the most hydrophobic products.

The average recovery yield was ~80% except for the most hydrophilic nucleotides such as 5′-UMP and dCMP, for which it decreased to 50%.

Analysis of Monophosphate Nucleotides and Nucleosides in Yeast Autolysate by HPLC and UV Detection. Stock solutions (1000 mg/L) of nucleotide and nucleoside mixtures were prepared in Milli-Q quality water (Millipore Corp., Bedford, MA) and stored at −20 °C. Working standard solutions were made daily by diluting stock solutions.

Analytical HPLC was performed with a Thermo Separation Products (Thermo-Quest, San José, CA) model P-1000XR quaternary gradient system, and detection was carried out with a Waters model 991 diode array detector at 254 nm. Data were recorded using version 6.22 of the detector software. An Interchrom (Interchim, Montluçon, France) stainless steel C_{18} analytical column (260 × 4.6 mm i.d., 5 μm Inertsil ODS-2 material, 150 Å porosity) protected by a guard column (15 × 4 mm i.d.) with the same phase (Macherey-Nagel, Düren, Germany) was used for reversed-phase ion-paired HPLC. Elution was carried out according to the procedure of Zhao and Fleet (*2*) as modified by Aussenac et al. (*9*).

The amounts of identified compounds in the different autolysates were determined by comparison of their peak areas with those of the corresponding standard measured in a separate run (external standard calibration). Variation coefficients of the nucleotides and nucleosides quantification were <4%.

HPLC Coupled to Tandem Mass Spectrometry. The mass spectrometer was an API 365 triple-quadrupole from Perkin-Elmer (Perkin-Elmer Sciex, Woodbridge, ON, Canada) fitted with an electrospray ion source. To increase both the sensitivity and the specificity of the detection method of nucleotides, the negative ion mode (*10*) and a parent-ion search mode were selected. The detection of phosphate-containing compounds was monitored by the detection of the PO_3^- fragment at *m*/*z* 79 (M − H fragment). Nucleotides were specifically detected and identified by the determination of their molecular mass M. Such an analysis would eliminate any artifact due to possible product coeluting with nucleotides.

The HPLC device was fitted with a reversed phase chromatographic column (PEPMAT, 150 mm length, 1 mm i.d., particle size = 3 μm,

Table 1. Composition of Base Wine

alcohol % (v/v)	11.4
total acidity (tartaric acid g/L)	6.96
reducing sugars (g/L)	0.77
pH	3.13
total SO_2 (mg/L)	36.0
free SO_2 (mg/L)	14.0
volatile acidity (acetic acid g/L)	0.16

porosity = 100 Å, from LC Packings, Amsterdam, The Netherlands) that was directly connected to the electrospray interface of the mass spectrometer. The liquid flow, adjusted to 30 μL/min by means of a flow splitter located between pumps and the flow injector, was an aqueous solution (0.4 M) of hexafluoroisopropyl alcohol adjusted to pH 7 with trimethylamine according to the method of Apffel et al. (*11*). Coaxial addition of a sheath flow of 2-propanol (10 μL/min) in the HPLC electrospray capillary interface greatly improved the sensitivity.

Before a HPLC-MS-MS experiment was run, the third quadrupole (Q3) was set to the value allowing to detect only ions at *m*/*z* 79. Nitrogen was introduced into the intermediary quadrupole (Q2) to cleave nucleotidic anions by collision. Parent-ion spectra were recorded by scanning the first quadrupole (Q1) while recording the 79^- ions transmitted by Q3. By reconstructing the ion intensity profile during the LC run, only phosphate-containing products would be detected. Specific identification of nucleotides was done by searching, at any elution time, for a response of the *m*/*z* 79 ion that coincides to the transmission of the $(M - H)^-$ ion of a known nucleotide.

The relative retention times and ionic responses of nucleotides were determined by running a standard mixture of known concentration in water.

To improve concentration measurements and to suppress possible matrix effects of coeluted wine components, similar experiments were done on extracts spiked with a known amount of a selected nucleotide. This nucleotide corresponded to the most abundant one present in the extract. Measurement of the relative area variation of the corresponding peak allowed the determination of its initial concentration. Concentrations of the other nucleotides could be calculated by means of the relative ionic response coefficients determined as above.

Determination of Total Nitrogen. Total nitrogen was determined according to the Kjeldahl method (*8*) with a Tecator digestion system and a Kjeltec 1030 autoanalyzer (Tecator AB, Höganäs, Sweden).

Sensory Evaluation. *Sensory analysis* was conducted with a trained panel of 10 subjects (6 women and 4 men) in the Moët & Chandon test room. The mean/average temperature was 22 °C. The lighting was natural. Panel members attended sessions on Monday, Thursday, and Friday between 11 and 12 a.m.

5′-IMP, 5′-GMP, and 5′-UMP (Sigma) were added to base wine (vintage 1999) 2 h before tasting, and the samples were kept at 8 °C until serving. The base wine characteristics are shown in **Table 1**. Samples (50 mL) were presented at 8−9 °C, in tulip-type transparent

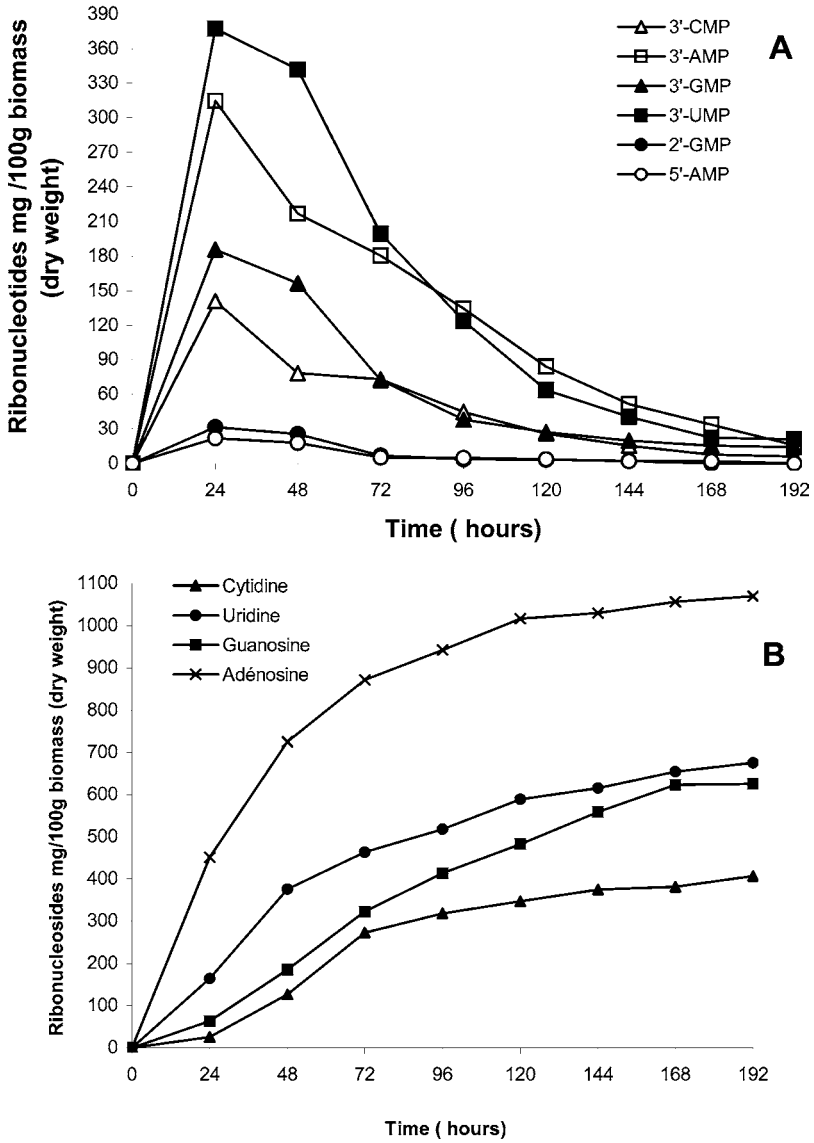

Figure 1. Change in the concentration of ribonucleotides (**A**) and ribonucleosides (**B**) released in supernatant during yeast autolysis in model wine.

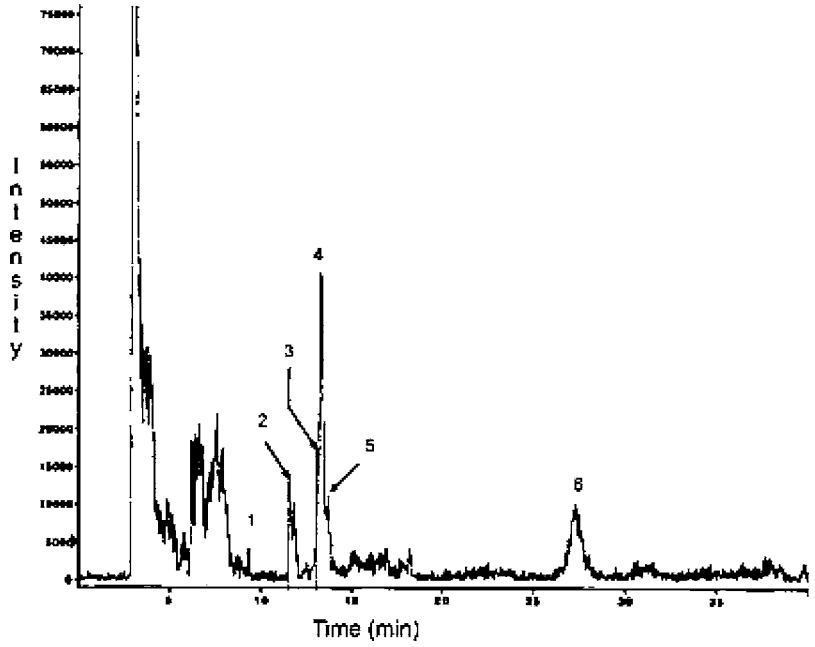

Figure 2. Reconstructed chromatogram of parent ions *m*/*z* 79 (X → 79^-) during a negative ion HPLC-MS-MS run from a 9-year-old Champagne wine extract. The phosphate-containing compounds were monitored by the detection of the PO_3^- fragment at *m*/*z* 79. Nucleotides were identified by recording the full mass spectrum at any time. Peaks: 1, 5′-UMP at *m*/*z* 323; 2, 3′ or 2′-UMP at *m*/*z* 323; 3, 3′- or 2′-GMP at *m*/*z* 362; 4, 3′-CMP at *m*/*z* 322; 5, 5′-AMP at *m*/*z* 346; 6, 3′-AMP at *m*/*z* 346. The unlabeled peaks did not correspond to masses of known nucleotides.

glasses (Norme Afnor NF-V-09-110), total height = 14.5 cm, diameter of top = 4.5 cm.

Thresholds of nucleotides were measured according to ASTM method (E-1432) (*12*) that proceeds to determine individual thresholds and then, in a second step, the group threshold. The concentration range used for the threshold determination was 5,10, 20, 40, 80, and 160 mg/L. Each panelist tasted five or six concentrations, each replicated five times. According to ASTM-E-1432 standard, individual and group thresholds were determined.

Descriptive Analysis. A list of 30 descriptors (color intensity, aromatic intensity, taste intensity, sweetness, acidity, bitterness, ...) was selected for descriptive analysis. Samples were evaluated by each panelist and mark attributed according to the intensity (0, no intensity; 6, very strong intensity).

The scores given by each panelist to each sample were collected on a 7-point ordered scale and treated by TASTEL software (ABT Informatique, Rouvroy sur Marne, France). This software allows the descriptive profile of each wine to be established from the mean mark of each descriptor. The profiles of wines spiked with different nucleotides were compared with the base wine profile, and for each descriptor, significant differences were indicated by the following probability levels: 90, 95, or 99% based on averages comparison by a Student test.

RESULTS AND DISCUSSION

Evolution of Yeast Biomass and Nitrogen Release during Autolysis. During induced autolysis of *S. cerevisiae* MC001, dry matter swiftly decreased by ~40% in the first 2 days and after 6 days reached a plateau close to 45% of the initial dry matter. These results are in good agreement with those published by Leroy et al. (*4*), who observed that lees from a 6-year-old Champagne wine represent only 43% of the initial dry content.

A consequence of the decrease in total dry matter of lees during autolysis is the release of molecules in the extracellular medium (*13*, *14*) and especially nitrogen compounds. After 1 day of autolysis, 4.0 g of nitrogen was liberated by 100 g of yeasts (dry matter).

Evolution of Nucleic Acids Degradation Products during Induced Autolysis of *S. cerevisiae* MC001. After 1 day of autolysis at 30 °C and pH 3 in hydroalcoholic buffer (ethanol 11.5%, v/v), six nucleotides and four nucleosides were identified in the autolysate: 5′-AMP, 3′-AMP, 3′-CMP, 3′-GMP, 3′-UMP, 2′-GMP, cytidine, adenosine, guanosine, and uridine. No deoxyribonucleotides or deoxyribonucleosides were detected in the autolysate. Thus, contrarily to RNA, DNA is not degraded completely during autolysis of *S. cerevisiae* in our conditions.

This absence of deoxyribonucleotides could be explained by a slower degradation of DNA, producing mainly oligonucleotides. This hypothesis is confirmed (i) by the presence of a great majority of endonucleases in *S. cerevisiae* (*15*−*17*), (ii) by the fact that oligonucleotides represent the 20% of degradation products of DNA during autolysis at 40 °C and pH 4 (*2*), and (iii) by enhancement of polynucleotides production by low pH (*2*, *3*).

Concerning ribonucleotides, 3′-nucleotides are predominant in the degradation products. Their formation is due to the presence of 3′-nucleotides forming exonucleases (*18*−*21*). Despite the existence of 5′-nucleotide-forming exonucleases in yeast cells (*22*−*24*), only 5′-AMP is identified. The presence of 2′-GMP was not expected because yeast cells do not contain 2′-nucleotide-forming exonucleases. The possible pathway for the formation of this isomer is through 2′,3′-cyclic mononucleotide as the intermediate, which could then be converted to either 3′- or 2′-ribonucleotide. RNases producing 2′,3′-cyclic nucleotides have been found in cells of *S. cerevisiae* (*25*). 2′-Nucleotides have been reported in beer (*26*), but it is not known whether the products were from yeast metabolism or from RNA degradation.

Figure 1A presents the evolution of the nucleic acids degradation products during autolysis. During the 8 days of autolysis, the composition of RNA degradation products in autolysate was changed greatly. The highest ribonucleotides content was found the first day. Then, their content decreased by 95% for the following 8 days. This decrease was probably due to their hydrolysis in autolysate, indicating the presence of an extracellular RNase activity. Isomers of 3′-nucleotides were

Table 2. Evolution of Ribonucleotide Content of Champagne Wine during Aging on Yeasts

	concn (mg/L) in Champagne wines during aging on lees								
ribonucleotide	0 years[a]	0.1 year[b]	2 years	3 years	7 years	8 years	9 years	11 years	14 years
5′-UMP	nd[c]	1.25	1.50	0.54	0.23	0.20	0.04	nd	nd
5′-GMP	nd	0.05	0.06	0.08	0.20	0.50	nd	nd	nd
5′-AMP	nd	nd	0.20	0.03	0.04	0.10	0.12	0.04	0.02
5′-IMP	nd	nd	nd	nd	nd	0.08	nd	nd	nd
5′-CMP	nd	nd	nd	nd	nd	0.03	nd	nd	nd
3′- and/or 2′-GMP	nd	nd	nd	0.08	0.10	0.10	1.20	0.60	0.10
3′-AMP	nd	nd	nd	nd	0.05	0.10	0.60	0.50	nd
3′-CMP	nd	nd	nd	nd	nd	0.02	0.40	nd	nd
3′- and/or 2′-UMP	nd	nd	nd	nd	nd	0.02	0.44	0.26	nd

[a] Base wine. [b] End of "prise de mousse". [c] Not detected.

the most released into the autolysate, which is in good agreement with results of Ohta et al. (*27*) and Belem and Lee (*28*), showing that 3′-nucleotides are much released when autolysis is conducted at acid pH.

The presence of ribonucleosides means that some nucleotides were further degraded during autolysis by specific phosphatases (*29*). Other researchers (*2*, *27*, *30*) have also reported the same secondary degradation. The total content of ribonucleosides after 1 day of autolysis was close to that found for ribonucleotides (46% of the total identified nucleic acids degradation products are ribonucleosides against 54% for ribonucleotides) and then swiftly increased for the first 4 days to reach high content. After 8 days of autolysis, ribonucleosides made up 98% of the total degradation products (**Figure 1B**). The increase of ribonucleosides was accompanied by a decrease of ribonucleotides and can be presented as a result of secondary degradation of nucleic acids. The presence of adenosine at 24 h with a high concentration and that of cytidine, guanosine, and uridine after 2 days are probably due to the fast hydrolysis of 5′-nucleotides that are not detected in autolysates. Their concentrations in the autolysate are much higher compared with ribonucleotides. This difference with a ratio of over 3 indicates that ribonucleosides are also formed in the cellular medium and then released in the autolysate.

Evolution of Nucleic Acids Degradation Products during Aging of Champagne. Search for Nucleotides in Champagne Wine. Application of the UV detection method for the detection of nucleotides in yeast autolysate failed to detect them in Champagne wine extracts. Indeed, the UV trace showed a large background from which no peak emerged at the expected retention times for nucleotides. It was likely that nucleotides present in wine, if any, were at concentrations too low to be detected by this method.

In contrast, tandem mass spectrometry coupled to HPLC was successfully applied to detect nucleotides in different wine extracts. The detection limit by this method corresponds to concentrations as low as 0.01 mg/L.

However, nucleotides of the same mass (i.e., positional isomers) are not differentiated by mass spectrometry alone. They could be differentiated if their chromatographic retention times were different. This condition is generally observed in the present solvent system except for 2′- and 3′-GMP or 2′- and 3′-UMP, which have retention times that are too close to be separated by the column (**Figure 2**).

In "base wine" sample, no nucleotide could be detected. This indicates that nucleotides are issued from yeast and not from grapes and they are released in the wine during yeast autolysis. Nucleotides identified in wines were ribonucleotides only. This is in accordance with the results from *S. cerevisiae* autolysis at 30 °C in a model wine.

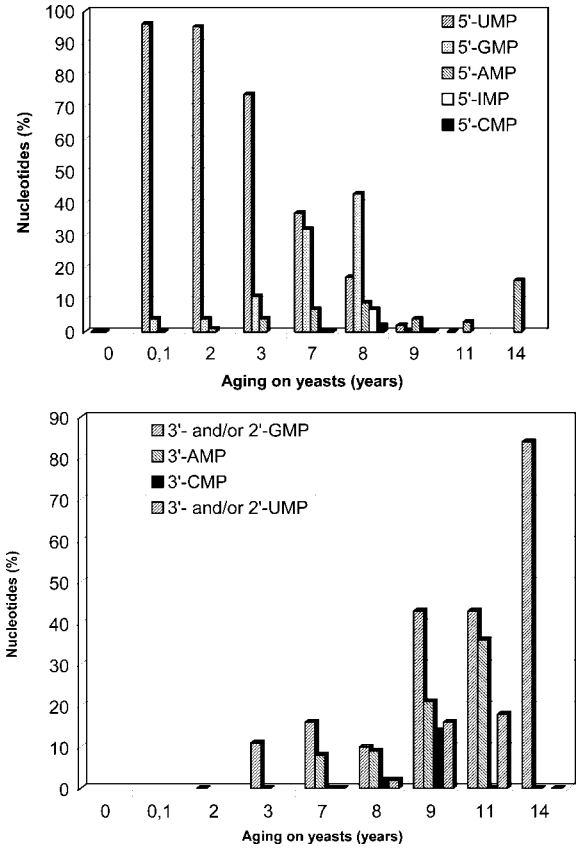

Figure 3. Evolution of 5′- and 3′-nucleotides in Champagne during aging on yeasts (percent variations).

The main nucleotides detected in Champagne wine extracts were 5′- and 3′- (or 2′-) UMP, 5′- and 3′- (or 2′-) GMP, 5′- and 3′-AMP, 5′- and 3′-CMP, and 5′-IMP.

Among the ribonucleotides identified in wines, all except 5′-IMP and 5′-UMP are found in the laboratory (as shown in model wine) or in industrial extracts. 5′-IMP and 5′-CMP are identified in wines only after 8 years of aging.

Quantification of Nucleotides in Champagne Wines and Their Evolution during Wine Aging. The HPLC-coupled tandem mass spectrometry method described under Materials and Methods was used for quantification measurements.

It was observed that the ionic response corresponding to each nucleotide is proportional to its concentration in the 0.1−5 mg/L range.

To quantify the amount of each nucleotide in wine extracts, a known amount of a selected nucleotide was added to each extract before HPLC-MS-MS runs. This added nucleotide corresponded to the most abundant one present in the extract.

Table 3. Threshold Values of Ribonucleotides in Base Wine

	individual threshold (mg/L)										
ribo-nucleotide	panelist 1	panelist 2	panelist 3	panelist 4	panelist 5	panelist 6	panelist 7	panelist 8	panelist 9	panelist 10	threshold of the panel (mg/L)
5′-IMP	39.6	nr[a]	29.2	*b*	21.4	33.2	34.2	34.2	19.5	nr	30.0
5′-GMP	*b*	57.7	35.1	79.2	59.1	79.2	46.2	46.2	42.1	*b*	55.3
5′-UMP	nr	68.3	54.8	*b*	69.1	58.5	60.1	60.1	23.4	76.7	55.5

[a] Value not retained. [b] Panelist absent.

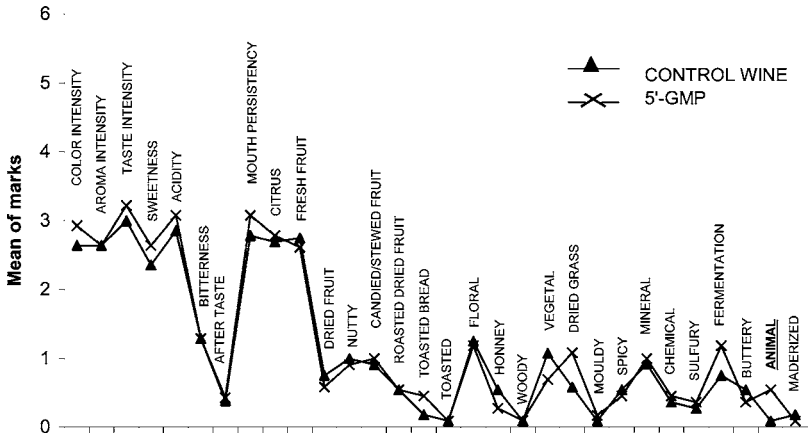

Figure 4. Descriptive profiles of base wine and base wine spiked with 5′-IMP. The underlined attributes are significantly different for base wine and wine spiked with this nucleotide.

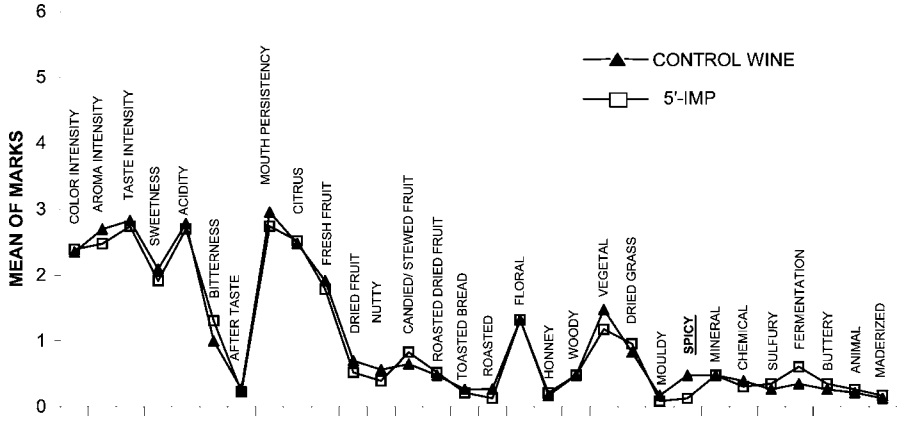

Figure 5. Descriptive profiles of base wine and base wine spiked with 5′-GMP. The underlined attributes are significantly different for base wine and wine spiked with this nucleotide.

The measurement of the area increase of the corresponding HPLC-MS-MS peak led to the determination of the amount of this selected nucleotide originally present in the wine extract. Concentrations of the other nucleotides were calculated from the response coefficients determined from standard mixtures.

The maximum concentration of total nucleotides in wine remained very low. It reached a maximum of ∼3 mg/L in a wine stored in contact with yeasts for 9 years (**Table 2**). Only 5′-nucleotides were identified during the two first years of aging. They were more abundant than the 3′- (or 2′) ones over the first 8 years of aging. After ∼5 weeks (the end of the "prise de mousse" period), only 5′-UMP and to a lesser degree 5′-GMP were detected. The amount of 5′-UMP reached 1.5 mg/L at 2 years of aging. It represents 96% of all nucleotides (**Figure 3**). Its concentration decreased further, but it was still detected in a 14-year-old wine. The concentration of other nucleotides increased slowly to reach a maximum at 8 or 9 years aging. The proportion of 5′-GMP increased to 43% of total nucleotides in an 8-year-old wine.

The relative proportion of the 3′-isomers increased slowly with aging. 3′- or 2′ UMP, AMP, and GMP remain relatively abundant in an 11-year-old wine (overall = 1.3 mg/L) but quite disappeared after 14 years of aging (0.1 mg/L).

These results are in opposition to those obtained for yeast autolysate in which 3′-isomers are the first detected and, presumably, reflect the relative activities of specific endonucleases and exonucleases.

RNases have been found in a variety of yeast organelles and have a diverse range of properties with regard to their optimal pH and temperature optima, cation requirements for activity, substrate specificity, and specific inhibitors. Aging of Champagne wines is conducted at a lower temperature (12−13 °C) than that of autolysate. The degradation of RNA and 5′-isomers can be expected to be slower than in autolysate. Indeed, Schulz-

Harder (*21*) noted that an increase in RNase activity occurred when *S. cerevisiae* cells were moved to elevated temperature.

Sensory Evaluation. The threshold values of the three most representative nucleotides in Champagne wines (5′-IMP, 5′-GMP, and 5′-UMP) are presented in **Table 3**. They are higher than the nucleotide concentrations found in Champagne wines. However, these values are lower than those cited by Nagodawithana (*1*) when tasted individually in water for 5′-IMP and 5′GMP, respectively, 250 and 150 mg/L. The wine matrix thus appears to reduce the detection threshold of these two nucleotides.

It is known that nucleotides are flavor enhancers of particular interest for food processors because of the synergism that these naturally formed compounds in yeast extract elicit in savory food systems. Several groups have attempted to quantify the synergism among flavor potentiators, by measuring resultant taste thresholds of individual and mixed systems (*31*). These test evaluations have demonstrated dramatic decreases in taste thresholds. 5′-GMP and 5′-IMP are able to enhance flavor when present in concentrations ranging from 0.012% (5′-GMP) to 0.025% (5′-IMP) on a weight per volume basis. Through a synergistic effect (when present in a 1:1 ratio), this can been lowered even further to 0.0063% (*1*).

Moreover, used in combination with 0.8% monosodium glutamate, the resulting combined threshold for 5′-GMP and 5′-IMP was decreased to 0.000031%, representing a dramatic reduction of the taste threshold by a powerful taste synergism. According to Martinez-Rodriguez (*32*) glutamic acid is the most abundant amino acid in the autolysate of yeasts during induced autolysis in a model wine system. Perrot et al. (*33*), in the same yeast autolysate as that used in our study, showed that glutamic acid accounts for ~20% of all amino acids released during *S. cerevisiae* autolysis. An increase of glutamic acid concentration of wine during aging on lees was reported by Feuillat (*34*). The glutamic acid concentration in these studied wines increases linearly from 30 mg/L for wine 2 to 60 mg/L for wine 14. Due to the release of glutamic acid by yeast during aging on lees, the taste thresholds of the nucleotides in Champagne wines are likely to be lower than in base wine.

Quantitative Descriptive Analysis. Thirty sensory attributes were defined, and the intensity of these attributes was expressed by the assignment of marks between 0 (no intensity) and 6 (very high intensity).

Very similar descriptive profiles were found between the control wine ("base wine") and the wine to which 30 mg/L of 5′-UMP was added (**Figure 4**). The two wines appear to be significantly different (significant differentiation with a 90% rate of probability) only in terms of spiciness, which is less pronounced in the spiked wine than in the base wine. There is no significant difference for the other sensory attributes; nevertheless, the bitter aspect is slightly more obvious in the case of the base wine with the addition of 5′-IMP, whereas the acidic taste is weaker.

No differences were observed between the descriptive profiles of the base wine and wine with 55.5 mg/L added UMP.

The wine spiked with 5′-GMP (55.3 mg/L) was significantly different from the base wine by the animal note only (significant differentiation with a 95% rate of probability). Moreover, sweetness, acidity, and persistency on the palate were slightly higher for the wine added with 5′-GMP. Fermentation, toasted bread, and dried grass aroma seemed also higher (**Figure 5**).

These findings suggest a potential influence of nucleotides on flavor of wines. Nevertheless, further research is needed to confirm this hypothesis. Current research is directed toward spiking base wines enriched with glutamic acid with 5′-nucleotides.

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LITERATURE CITED

(1) Nagodawithana, T. Yeast-derived flavors and flavor enhancers and their probable mode of action. *Food Technol.* **1992**, 46, 138−144.

(2) Zhao, J.; Fleet, G. H. Separation of twenty isomers of ribonucleotides and deoxyribonucleotides by reverse-phase ion-pairing high-performance liquid chromatography. *J. Chromatogr. A* **1996**, *732*, 271−275.

(3) Zhao, J.; Fleet, G. H. Degradation of DNA during the autolysis of *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 175−182.

(4) Courtis, K.; Todd, B.; Zhao, J. The potential role of nucleotides in wine flavour. *Aust. Grapegrower Winemaker* **1998**, *409*, 51−53.

(5) Leroy, M. J.; Charpentier, M.; Duteurtre, B.; Feuillat, M. Autolysis during Champagne aging. *Am. J. Enol. Vitic.* **1990**, *41*, 21−28.

(6) Juroszek, J.-M.; Feuillat, M.; Charpentier, C. Effect of the Champagne method of starter preparation on ethanol tolerance of yeast. *Am. J. Enol. Vitic.* **1987**, *38*, 194−198.

(7) Feuillat, M. Autolysats de levures à caractère œnologique et leur procédé de fabrication. Brevet ANVAR 86-16318.

(8) Kjeldahl, P. J. Méthode officielle d'analyse physique et chimique du lait. Arr.8.1.-70. *J. O. Rep. Fr.* **1970**, Jan 25.

(9) Aussenac, J.; Chassagne, D.; Claparols, C.; Charpentier, M.; Duteurtre, B.; Feuillat, M.; Charpentier, C. Purification method for the isolation of monophosphate nucleotides from Champagne wine and their identification by mass spectrometry. *J. Chromatogr. A* **2001**, *907*, 155−164.

(10) Fish, W. W. A method for the quantitation of 5′-mononucleotides in food and food ingredients. *J. Agric. Food Chem.* **1991**, *39*, 1098−1101.

(11) Apffel, A.; Chakel, J. A.; Fisher, S.; Lichtenwalter, K.; Hancock, W. S. New procedure for the use of high-performance liquid chromatography-electrospray ionisation mass spectrometry for the analysis of nucleotides and oligonucleotides. *J. Chromatogr. A* **1997**, *77*, 3−21.

(12) American Society for Testing and Materials Standard. Practice E1432, Defining and calculating sensory threshold from forced-choice data sets of intermediate size; ASTM: Philadelphia, PA, 1990.

(13) Charpentier, C.; Feuillat, M. Yeast autolysis. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwod Academic Publishers: Chur, Switzerland, 1992; pp 225−242.

(14) Fornairon-Bonnefond, C.; Camarasa, C.; Moutounet, M.; Salmon, J.-M. New trends on yeast autolysis and wine aging on lees: a bibliographic review. *J. Int. Sci. Vigne Vin* **2001**, *35*, 57−78.

(15) Pinon, F.; Leney, E. Studies on deoxyribonuclease from *Saccharomyces cerevisiae*. Characterization of two endonuclease activities with a preference for double-stranded DNA. *Nucleic Acids Res.* **1975**, *2*, 1023−1042.

(16) Morolosi, R.; Lusena, C. V. An endonuclease from yeast mitochondrial fractions. *Eur. J. Biochem.* **1980**, *110*, 431−437.

(17) Ezekiel, U. R.; Zassenhaus, H. P. Localization of a cruciform cutting endonuclease in yeast mitochondria. *Mol. Gen. Genet.* **1993**, *240*, 414−418.

(18) Schulz-Harder, B.; Kücherer, C.; Swida, U. A ribonuclease from yeast associated with the 40S ribosomal subunit. *Biochim. Biophys. Acta* **1979**, *565*, 173−182.

(19) Shetty, K. J.; Weaver, R. C.; Kinsella, J. E. A rapid method for the isolation of ribonuclease from yeast (*Saccharomyces carlsbergensis*). *Biochem. J.* **1980**, *189*, 363−366.
(20) Swida, U.; Schulz-Harder, B.; Kücherer. C.; Käufer, N. The occurrence of two ribonucleases depending on growth phase in yeast. *Biochim. Biophys. Acta* **1981**, *652*, 129−138.
(21) Schulz-Harder, B. Heat-shock induction of a ribonuclease in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **1983**, *17*, 23−26.
(22) Stevens, A. Purification and characterization of a *Saccharomyces cerevisiae* exoribonuclease which yields 5′-mononucleotides by a 5′ 3′ mode of hydrolysis. *J. Biol. Chem.* **1979**, *255*, 3080−3085.
(23) Cheriathundam, E.; Chang, L. M. S. Purification and properties of two ribonuclease H enzymes from yeast. *Arch. Biochem. Biophys*. **1982**, *219*, 110−120.
(24) Van Ryk, D. I.; Tigerstrom, R. G. Activity changes of three nucleolytic enzymes during the life cycle of *Saccharomyces cerevisiae*. *Can. J. Microbiol*. **1985**, *31*, 1095−1102.
(25) Stevens, A. Pyridine-specific cleavage by an endoribonuclease of *Saccharomyces cerevisiae*. *J. Bacteriol*. **1985**, *164*, 57−62.
(26) Qureshi, A. A.; Prentice, N.; Burger, W. C. Separation of flavoring compounds by high performance liquid chromatography. *J. Chromatogr*. **1979**, *170*, 343−353.
(27) Ohta, S.; Maul, S.; Sinskey, A. J.; Tannenbaum, S. R. Characterization of a heat-shock process for reduction of the nucleic acid content of *Candida utilis*. *Appl. Microbiol*. **1971**, *22*, 415−421.
(28) Belem, M. A. F.; Lee, B. H. Production of RNA derivatives by *Kluyveromyces fragilis* grown on whey. *Food Sci. Technol. Inst*. **1997**, *3*, 437−444.
(29) Kocková-Kratochvílová, A. Enzymes in yeasts In *Yeast and Yeast-like Organisms*; Kocková-Kratochvílová, A., Ed.; VCH Publishers: New York, 1990; pp 137−152.
(30) Wantabe, S.; Osawa, T.; Yamamoto, S. Studies on enzymatic action for nucleic acid components in microrganisms. II.; Excretion of nucleotides from yeast cells and action of surfactant. *J. Ferment. Technol*. **1968**, *46*, 538−544.
(31) Fuke, S.; Ueda, Y. Interactions between umami and other flavor characteristics. *Trends Food Sci. Technol*. **1996**, *7*, 407−411.
(32) Martinez-Rodriguez, A. J.; Carrascosa, A. V.; Polo, M. C. Release of nitrogen compounds to extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *Int. J. Food Microbiol*. **2001**, *68*, 155−160.
(33) Perrot, L.; Charpentier, M.; Charpentier, C.; Feuillat, M.; Chassagne, D. Yeast adapted to wine: nitrogen compounds released during induced in a model wine. *J. Ind. Microbiol. Biotechnol*. **2002**, *29*, 134−139.
(34) Feuillat, M.; Charpentier, C. Autolysis of yeasts in Champagne. *Am. J. Enol. Vitic*. **1982**, *33*, 6−13.

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