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Flor yeast metabolism in a model system similar to cellar ageing of the French "Vin Jaune": Evolution of some by-products, nitrogen compounds and polysaccharides

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Summary

French flor-sherry wine "Vin Jaune" (Jura region) made from Savagnin grapes is aged under a film of *Saccharomyces cerevisiae* flor yeasts for 6 years and three months. During this ageing, velum develops and falls corresponding to yeast multiplication and autolysis. In a model system using glass receptacles ("Fioles de Roux") containing sterilised Savagnin wine inoculated with a pure culture of *S. cerevisiae* flor yeast, the evolution of velum was studied for 15 weeks. At the same time, nitrogen compounds (amino acids, peptides and proteins) and neutral polysaccharides were quantified in relation to glycerol, volatile acidity and acetaldehyde. Compounds such as glycerol, acetic acid and amino acids including proline are consumed by the flor yeasts whereas other compounds such as peptides and neutral polysaccharides are released. At the same time, yeasts produce other compounds, namely acetaldehyde.

K e y w o r d s : Vin Jaune; nitrogen compounds; neutral polysaccharides; ageing yeast; *Saccharomyces cerevisiae* flor yeasts.

Résumé

Les vins jaunes, élaborés à partir du cépage Savagnin, sont vieillis sous voile de levures, *Saccharomyces cerevisiae* pendant 6 ans et 3 mois. Le vieillissement se caractérise par le développement et la chute successive du voile où les levures se trouvent soit en phase de croissance soit en phase d'autolyse. Dans un système modèle utilisant des "Fioles de Roux" contenant du vin issu du cépage Savagnin et ensemencé à l'aide d'une levure à voile de type *S. cerevisiae*, l'évolution du voile a été étudiée pendant 15 semaines. Les composés azotés (acides aminés, peptides et protéines) et les polysaccharides neutres ont été quantifiés en relation avec l'évolution du glycérol, de l'acidité volatile et de l'éthanal. Des composés tels que le glycérol, l'acide acétique et les acides aminés y compris la proline sont consommés par les levures à voile alors que d'autres comme les peptides et les polysaccharides sont libérés. Pendant cette même période, de l'éthanal est produit par les levures à voile.

Introduction

Similar to the "Fino Sherry" in the Jerez region (Spain), the production of French flor-sherry wine "Vin Jaune" in the Jura region involves the development of film-forming yeasts (flor yeasts) on the surface of the wine, which is essential for flavor development (BOTELLA *et al.* 1990; MARTINEZ *et al.* 1995; IBEAS *et al.* 1996, 1997). The French flor-sherry wine "Vin Jaune" is produced using cv. Savagnin grapes; after the alcoholic and the malolactic fermentation are completed, it matures in oak barrels (228 l) for 6 years and three months. During this period, the wine undergoes no racking. During velum development and fall, flor yeast multiplication and autolysis occur as a consequence of ageing. Film formation and oxidative metabolism might reflect adaptative mechanisms which allow cells to survive under such conditions. During biological ageing, a respiratory metabolism occurs in the yeast with ethanol as the fundamental source of energy. Ethanol is oxidised first to acetaldehyde and then to acetic acid (WEBB *et al.* 1976; BOTELLA *et al.* 1990). The metabolic activity of the flor yeast also results in a drastic reduction in glycerol and acetic acid concentrations (BRAVO 1984). Amino acids are utilized for yeast growth and as a consequence alcohols such as isobutanol or isoamylalcohol are produced (MARTINEZ DE LA OSSA *et al.* 1987; BOTELLA *et al.* 1990). Due to ethanol oxidation, there is a large increase in the concentration of acetaldehyde which is mainly responsible for the typical flavor of fino sherry (MARTINEZ *et al.* 1997) and which is used to determine the overall flavor development during biological ageing (MARTINEZ DE LA OSSA *et al.* 1987). Acetaldehyde, glycerol and volatile acidity are used to determine cellar ageing. Until now, the evolution of nitrogen compounds and polysaccharides has not been studied in detail.

The aim of this investigation was to study the evolution of nitrogen compounds and neutral polysaccharides during the successive velum development and fall in relation to the glycerol, acetaldehyde and volatile acidity evolution in a model system. By comparing the evolution of

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compounds during ageing of both uninoculated wine and wine inoculated with a flor yeast strain, we were able to elucidate the metabolic activity of the yeast.

Material and Methods

Biological ageing in a model system: "Fioles de Roux" glass receptacles (400 ml) containing 250 ml of centrifuged Savagnin wine with 13.2 % (v/v) ethanol were inoculated with the *S. cerevisiae* strain P3 (isolated by the Laboratoire Départemental d'Analyses Agricoles du Jura from French "Vin Jaune" velum). The specific surface (surface/volume ratio) in these receptacles was 0.8 cm^{-1}. A non-inoculated control was also included. The control was analysed at the onset and after 15 weeks of ageing. Two glass receptacles were analysed after 0, 5, 8, 10 and 15 weeks of biological ageing (inoculated wines) and were then discarded.

Biomass and viability determination: Each time, two glass receptacles were agitated until the yeast cells were homogeneously resuspended. 3 ml of the samples were filtered using preweighed filters (0.45 µm, Millipore) and dried for 24 h at 105 °C. These filters were then weighed again to determine the yeast biomass. Viability was determined according to FINK and KUHLES (1993) using methylene blue staining.

In the following analyses, the wine was centrifuged (10,000 *g*, 20 °C, 30 min) and the supernatant filtered through a 0.22 µm Fluoropore (Millipore) filter.

The volatile acidity was determined according to the continued flux method (DUBERNET 1976). Acetaldehyde and glycerol were determined using an enzymatic method (Boehringer Mannheim, Mannheim, Germany). Neutral polysaccharides, isolated by microprecipitation in the presence of 80 % alcohol were hydrolysed in a concentrated sulphuric acid medium and the released sugar monomers determined according to DUBOIS *et al.* (1956). Total nitrogen was determined according to the Kjeldhal method (KJELDHAL 1970) with a Kjeltec digestor and a Kjeltec distillation unit.

Preparation of protein and peptide fractions: To separate compounds with a high molecular weight (proteins) from compounds with a low molecular weight (peptides and amino acids), wine was ultrafiltered in a cell (V= 400 ml, d= 90 mm) at room temperature using a cellulose ester membrane retaining substances with a molecular weight ≥ 5000 Daltons (Millipore). Consequently, the retentate contained proteins and filtrate peptides and amino acids. In order to obtain the peptide fraction (500-5000 Daltons), the filtrate was ultrafiltered again in a cell at 6 °C using a cellulose ester membrane retaining substances with a molecular weight ≥ 500 Daltons (Millipore). Protein concentration in the retentate was determined using the Lowry method (LOWRY *et al.* 1951).

Analysis and quantification of the amino acid: Analysis of the free amino acid fraction was performed directly on a sample of wine after previous filtration.

40 µl of an internal amino acid standard (2.5 $mmol \cdot l^{-1}$ α-amino-butyric acid (AabaSI)) and 760 µl of MilliQ-water were added to 200 µl of wine. 70 µl of borate buffer and 20 µl of AQC reagent (6-aminoquinolyl-N-hydroxy-succinimidyl carbamate) were added to 10 µl of the above solution, mixed for 12 s and placed at 55 °C for 10 min (COHEN and MICHAUD 1993). For liquid chromatography, two pumps (Waters™ 626) and a system controller (Waters™ 717 plus) were combined with a scanning fluorescence detector (Waters™ 474). Separation was carried out using a Nova-Pack™ C18 column at 37 °C (3.9 x 150 mm) filled with silica spheres (pore size: 6.0 nm; particle size: 4 µm) with fluorimetry detection at 395 nm. The precolumn had the same characteristics and was 2 cm long. Elution was realised with eluant A (sodium acetate buffer, pH 5.8), eluant B (acetonitrile) and eluant C (water) at a flow rate of 1 ml min^{-1}. The gradient was t 0: 100 % A, t 35 min: 86 % A and 14 % B, t 40 min: 86 % A, 14 % B, t 51 min: 0 % A, 60 % B, 40% C, t 54 min: 100 % A. Amino acids were identified and quantified by comparing the retention time and peak area of the amino acid fraction with the retention time and peak area of the amino acid standard, chromatographed as a mixture of amino acids at a known concentration. The standard solution contained leucine (LEU), valine (VAL), phenylalanine (PHE), aspartic acid (ASP), glutamic acid (GLU), lysine (LYS), isoleucine (ILE), methionine (MET), tyrosine (TYR), glycine (GLY), histidine (HIS), alanine (ALA), arginine (ARG), serine (SER), threonine (THR, proline (PRO) (2.5 $mmol \cdot l^{-1}$) and cysteine (CYS) (1.25 $mmol \cdot l^{-1}$). An internal standard consisting of α-amino-butyric acid (AabaSI) was added, along with asparagine (ASN), glutamine (GLN), ornithine (ORN) and γ-amino-butyric acid (GABA) (2.5 $mmol \cdot l^{-1}$).

Peptides were hydrolysed to amino acids by adding 0.1 ml internal standard (AabaSi) and 0.4 ml 6 M hydrochloric acid to 0.5 ml of the sample. This mixture was sealed under vacuum in vials and incubated for 24 h at 110 °C. After acid hydrolysis, 0.4 ml of the hydrolysate was removed and dried under a stream of nitrogen. The residue was then resuspended in 1 ml MilliQ-water and filtered through a 0.22 µm Fluoropore (Millipore) filter. Since acid hydrolysis of the samples caused destruction of tryptophan, the results do not include values of tryptophan. To identify and quantify the amino acids which constitute peptides, the above mentioned method of amino acid analysis was used.

Results and Discussion

Evolution of velum, biomass and viability during biological ageing: Glass receptacles filled sterily with Savagnin wine and inoculated with the velum P3 flor yeast strain were incubated at room temperature without any physical disturbance. After only two weeks, islands of flor yeasts aggregated on the wine surface and a ruffle of yeast cells developed on the walls of the glass receptacles. The first velum developed during the following weeks and finally, after 6 weeks, dropped to the bottom of the receptacles. As the first velum dropped,

a second one was formed. Due to the presence of the first velum on the bottom of the glass receptacles, it was difficult to visually distinguish between the dropping of the second velum and the formation of the third velum.

The biomass and the viability of the flor yeast were determined during the 15 weeks of biological ageing (Fig. 1). At the onset of biological ageing, 25 % of the flor

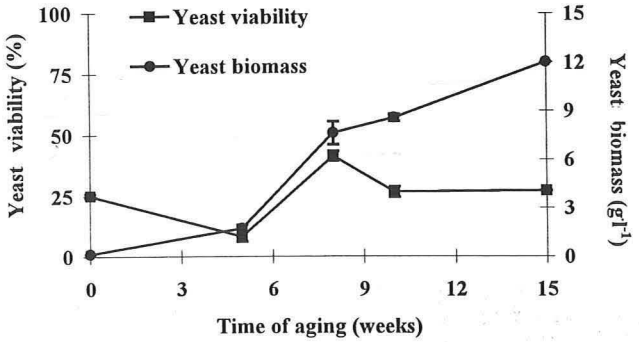

Fig. 1: Evolution of yeast biomass ($g \cdot l^{-1}$) and viability of cells (%) during biological ageing.

yeasts were viable with a biomass of 0.134 $g \cdot l^{-1}$. After 5 weeks of biological ageing, the biomass increased to 2 $g \cdot l^{-1}$ of which only 8 % of the cells were viable. Between the 5th and the 8th week, a significant increase in flor yeast biomass (8 $g \cdot l^{-1}$) was observed which was correlated with a significant increase in the viability of the yeast cells (41.8 %). This increase in biomass corresponded to the second velum development, during which the yeast biomass increased 4-fold, even if less nitrogen and carbon (glycerol) were available. The decrease in viability during the first 5 weeks can possibly be explained by the low pH (3.0) and the high alcohol level (13 %) that are unfavourable for yeast growth. The significant increase in viability during the second development probably indicates the adaptation of the flor yeast to these unfavourable growth conditions. Concerning the uninoculated wine, no velum and no yeast proliferation could be observed.

Evolution of volatile acidity, acetaldehyde and glycerol during biological ageing: Acetaldehyde and volatile acidity are important indicators of the potential quality of the product during biological ageing. They determine wine health, and one of the oenological rules is to have the lowest possible volatile acidity. A significant increase of volatile acidity (0.92 $g \cdot l^{-1}$ sulfuric acid) occurred after the first 5 weeks of biological ageing, thereafter the volatile acidity decreased to minimal values of 0.17 $g \cdot l^{-1}$ sulfuric acid (Fig. 2). This corresponds to previous reports on French flor-sherry (Arbault *et al.* 1977) and fino sherries (Martinez de la Ossa *et al.* 1987). The decrease of volatile acidity is probably due to the assimilation of acetic acid by the flor yeasts and its transformation into acetyl-CoA which is utilized in various metabolic pathways such as the tricarboxylic acid, the glyoxylate and the fatty acid pathway or in the formation of ethanol via acetaldehyde (Arbault *et al.* 1977). No significant difference in volatile acidity was observed for the uninoculated wine between the onset (0.31 $g \cdot l^{-1}$ sulfuric acid) and after 15 weeks (0.34 $g \cdot l^{-1}$ sulfuric acid) of ageing.

Acetaldehyde is considered to be the most important compound contributing to the organoleptic characteristics of fino (John *et al.* 1977; Martinez de la Ossa *et al.* 1987) and French flor-sherries (Arbault *et al.* 1977). During the biological ageing of sherries, acetaldehyde is formed at concentrations ranging from 300 to 400 $mg \cdot l^{-1}$, although values of around 700-800 $mg \cdot l^{-1}$ have been also reported (Arbault *et al.* 1977). After reaching a maximum of 873.5 $mg \cdot l^{-1}$ acetaldehyde after the first 5 weeks of biological ageing, the acetaldehyde concentration decreased to 123 $mg \cdot l^{-1}$ (Fig. 2). This decrease is due to the fact that acetaldehyde is an intermediate in the metabolism of ethanol by flor yeasts and is the precursor for various other compounds, such as 2,3-butanediol, acetic acid and acetoin, that is found in sherry wines (Reed *et al.* 1991). While the formation of acetoin could be the result of an acetonic con-

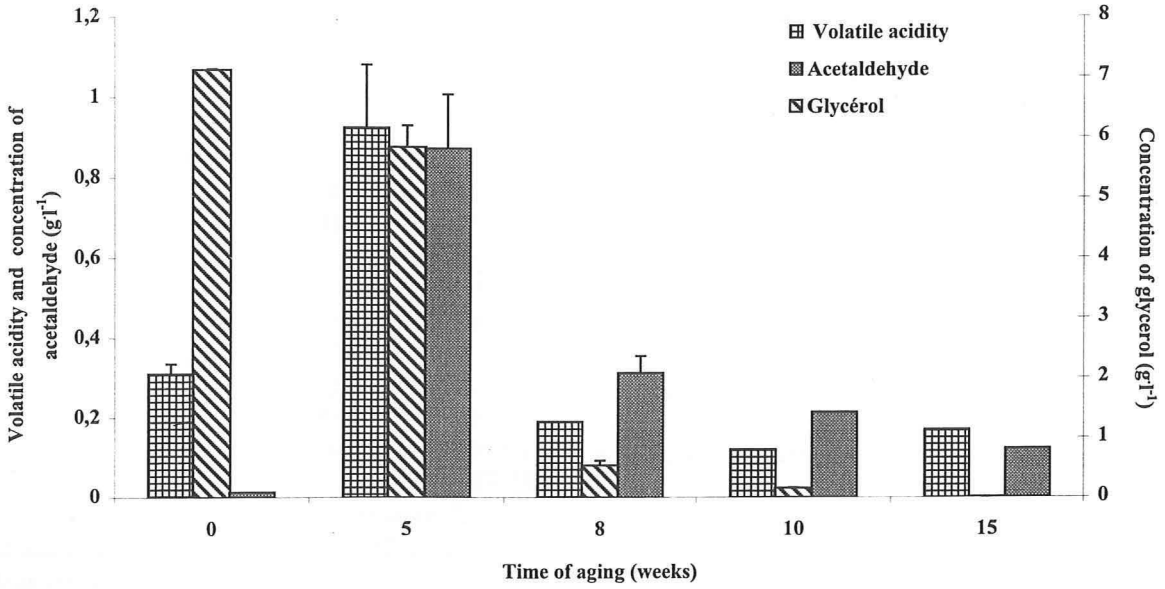

Fig. 2: Evolution of volatile acidity ($g \cdot l^{-1}$ sulfuric acid), acetaldehyde content ($mg \cdot l^{-1}$) and glycerol content ($g \cdot l^{-1}$) during biological ageing.

densation of two molecules of acetaldehyde by flor yeasts (MARTINEZ *et al.* 1995), the formation of 2,3-butanediol may have its origin in a reduction of an acetoin molecule (REED *et al.* 1991). By condensation with various alcohols, for example glycerol, acetaldehyde can also be the precursor of acetals such as *cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxan and *cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxalan which were isolated from French flor sherry wine (ETIÉVANT 1979). By condensation with ketonic acid as α-ketobutyric acid, acetaldehyde can also be the precursor of lactones such as sotolon which were also isolated from French flor-sherry wine (PHAM *et al.* 1995). The formation of these compounds possibly explains the simultaneous decrease of the acetaldehyde and glycerol content. For the uninoculated wine, no significant difference was observed between the onset (13 $mg \cdot l^{-1}$) and after 15 weeks of ageing (26 $mg \cdot l^{-1}$).

The glycerol concentration decreased from 7.1 $g \cdot l^{-1}$ to 0.01 $g \cdot l^{-1}$ (Fig. 2) during the 15 weeks of biological ageing. Yeasts can use glycerol as a carbon and energy source during respirative growth. Condensation with other compounds, such as acetaldehyde to form acetals, could also explain its total assimilation. For the uninoculated wine, no significant difference was observed between the onset (7.1 $g \cdot l^{-1}$) and 15 weeks of ageing (7.3 $g \cdot l^{-1}$).

Evolution of nitrogen compounds during biological ageing: The evolution of nitrogen compounds is shown in Fig. 3. The total nitrogen content (the sum of amino acids, peptides and proteins) decreased significantly during the first 10 weeks of ageing (- 67 %) whereas a slight increase was observed during the last 5 weeks of ageing (+ 12.7 %). As shown in Fig. 3, only 12 % of the amino acids and 52.2 % of the peptides were consumed during the first 5 weeks of biological ageing. The low amount of amino acids that were assimilated can possibly be explained by the small increase in flor yeast biomass during the first 5 weeks of biological ageing (2 $g \cdot l^{-1}$) and the simultaneous release of amino acids due to autolysis (low yeast viability), or due to peptide hydrolysis (500-5000 Daltons), releasing both oligopeptides and free amino acids. This would then easily compensate for the amino acid assimilation due to the low amount of biomass formed. Between the 5th and the 8th week of ageing, a significant assimilation of amino acids (- 86.9 %) corresponding to a biomass increase of 2-8 $g \cdot l^{-1}$ was observed. During the same period, only 34.7 % of the peptides in the wine were assimilated. Finally, between the 8th and the 15th week, the amino acid content was stable, whereas the peptides increased by 13 % due to protein autolysis.

No significant changes were observed for proteins larger than 5000 Daltons. This is to be expected, since it has been shown that yeast proteases cannot hydrolyse grape proteins (WATERS *et al.* 1992).

Results in the Table show that proline, the most prominent amino acid in the Savagnin wine, increased from 60.4 to 80.3 % during the first 10 weeks of ageing and then decreased to 71.3 %. Flor yeasts have been shown to use proline as a nitrogen source but not as a carbon source in sherry wine (MARTINEZ *et al.* 1995). The metabolism of proline requires both a permease and a proline oxidase which are substrate-inducible and oxygen-dependant (BRANDISS 1979) and occur in the mitochondria (COOPER 1982). During biological ageing, the utilization of proline is not restricted since flor yeasts have exclusively an oxidative metabolism. However, proline is known to be a poor nitrogen substrate assimilated when almost all other

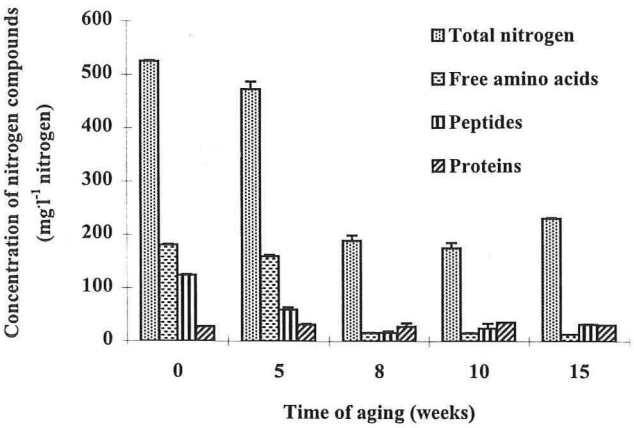

Fig. 3: Evolution of nitrogen content ($mg \cdot l^{-1}$ nitrogen) during biological ageing.

amino acids are totally diminished. Concerning the evolution of amino acid composition during ageing, no significant modifications could be observed (Table). Proline was also the most prominent amino acid in the peptide fraction. While proline represented 60-80 % of the free amino acids, it represented only 12-24 % of the amino acids in the peptide fraction (Table). As for the amino acid composition, the peptide composition did not change. Concerning the control, no significant changes could be observed in nitrogen concentration between the onset and 15 weeks of ageing.

Evolution of neutral polysaccharides during biological ageing: The evolution of neutral polysaccharides is shown in the Table. Polysaccharides were released by yeast autolysis into the wine during a period of 15 weeks of biological ageing. The release of polysaccharides is due to an enzymatic degradation of the cell wall by β-(1-3) endoglucanase (FLEET and PHAFF 1974; HIEN and FLEET 1983). The release of polysaccharides into wine can occur during yeast multiplication (FARKAS *et al.* 1976; LLAUBERES *et al.* 1987; GUILLOUX-BENATIER *et al.* 1993, 1995) and yeast autolysis (CHARPENTIER *et al.* 1986; FEUILLAT *et al.* 1989) as shown in the Table. During the first 5 weeks of ageing, 1.6 $g \cdot l^{-1}$ of yeast biomass was formed, the viability decreased to 8 % and only 65 $mg \cdot l^{-1}$ of neutral polysaccharides were released. This is due to yeast multiplication and yeast autolysis. Between the 5th and the 8th week, a significant increase in flor yeast biomass (+ 5.9 $g \cdot l^{-1}$) was observed which correlated with a significant increase in the viability of the yeast cells (41.8 %) as well as an increase of polysaccharides (+ 283 $mg \cdot l^{-1}$). This release seems to be mainly due to yeast multiplication. Finally, during the last 8 weeks, as the flor yeast biomass increased only by 4.4 $g \cdot l^{-1}$ and the viability

Table

Changes of free amino acids, peptides (500-5000 Daltons) and neural polysaccharides during biological ageing

Time (weeks)	Free amino acids ($mg \cdot l^{-1}$ nitrogen)	Free amino acids (%)	Peptides (500-5000 Da) ($mg \cdot l^{-1}$ nitrogen)	Amino acids constituting peptides (%)	Neutrals polysaccarides ($mg \cdot l^{-1}$)
0	181.0	Pro: 60.4 Lys: 3.7 Ala: 4.8 Glu: 3.2 Leu: 4.2	124.4	Pro: 12.1 Ala: 8.5 Gly: 10.8 Lys: 8.3 Asp+Asn: 9.4 Ser: 7.2 Glu+Gln: 8.9	562
5	157.7	Pro: 61.4 Lys: 3.9 Glu: 5.7 Leu: 2.5 Ala: 5.7	59.5	Pro: 12.9 Ala: 7.9 Gly: 13.2 Ser: 7.8 Asp+Asn: 9.7 Thr: 7.2 Glu+Gln: 9.0 Lys: 7.2	627
8	16.3	Pro: 79.3 Arg: 2.6 Asp: 5.7 Cys: 2.2 Glu: 3.1	16.3	Pro: 19.1 Thr: 8.2 Gly: 18.6 Asp+Asn: 7.5 Glu+Gln: 11.5 Lys: 5.7 Ser: 9.7 Ala: 5.4	910
10	15.7	Pro: 80.3 Cys: 2.4 Asp: 5.8 Arg: 2.3 Val: 3.3	24.5	Pro: 19.5 Asp+Asn: 8.2 Gly: 17.5 Thr: 7.6 Glu+Gln: 11.3 Lys: 6.2 Ser: 8.8 Ala: 5.4	967
15	13.3	Pro: 71.3 Cys: 3.9 Asp: 9.6 Arg: 3.3 Val: 4.5	32.3	Pro: 23.2 Thr: 8.6 Gly: 11.8 Asp+Asn: 7.9 Ser: 10.9 Lys: 5.8 Glu+Gln: 9.4 Ala: 5.5	1353

diminished to 27 %, another strong release of polysaccharides occurred (+ 443 $mg \cdot l^{-1}$). Yeast autolysis rather than yeast multiplication seems to be implicated in this release. In the uninoculated wine, no significant changes were observed between the onset (557.1 $mg \cdot l^{-1}$) and 15 weeks (624.1 $mg \cdot l^{-1}$) of biological ageing.

The study of these different kinetics underlines the relationship between biomass formation and yeast autolysis and contributes to the understanding of the perpetual renewal of velum corresponding to yeast growth and the autolysis of yeasts during biological ageing. The accelerated ageing obtained by the "Fiole de Roux" receptacles permitted better understanding of the evolution of compounds during the biological ageing of the French "Vin Jaune". During the 15 weeks of biological ageing in a model system, volatile acidity, acetaldehyde and nitrogen compounds show opposite evolution depending on the multiplication or autolysis of yeasts. Polysaccharides are released during the entire period of biological ageing. Hence, they may be considered to be more significant biological ageing markers of the French "Vin Jaune" than the nitrogen compounds.

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