ELSEVIER

Journal of Chromatography A, 907 (2001) 155–164

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Purification method for the isolation of monophosphate nucleotides from Champagne wine and their identification by mass spectrometry

Jérôme Aussenac[a], David Chassagne[a,*], Catherine Claparols[b], Monique Charpentier[c], Bruno Duteurtre[c], Michel Feuillat[a], Claudine Charpentier[a]

[a]*Université de Bourgogne, Institut Universitaire de la Vigne et du Vin, Campus Universitaire, B.P. 27877, 21078 Dijon Cedex, France*
[b]*Service de Spectrométrie de Masse, FR 1744, IPR 8241, Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse Cedex 04, France*
[c]*Moët & Chandon, Laboratoire de Recherche, 6 Rue Croix de Bussy, 51200 Epernay, France*

Received 20 July 2000; received in revised form 4 October 2000; accepted 4 October 2000

Abstract

Monophosphate nucleotides are difficult to identify in Champagne wine because they are present in small concentrations in a complex mixture. A method for the isolation, separation and identification of reference compounds, which achieved on average 79% recovery (except for cytidine derivatives), was developed and applied to wine. Some monophosphate nucleotides were then isolated from a Champagne wine aged on lees for 8 years, by ultrafiltration followed by a semi-preparative HPLC step using a strong anion-exchange column. The fraction obtained was subjected to HPLC in a reversed-phase column to remove the salt previously introduced, before identification of compounds by HPLC coupled to a mass spectrometer. For the first time in wine, 5′-IMP, 5′-AMP, 5′-CMP, 5′-GMP, 5′-UMP and the 3′- and/or 2′-isomers of the four latter compounds were identified by comparing their HPLC and electrospray ionization mass spectrometry data with those of reference nucleotides.

Keywords: Wine; Food analysis; Nucleotides

1. Introduction

In the food industry, monophosphate nucleotides, particularly 5′-nucleotides such as 5′-guanosine monophosphate (5′-GMP) and 5′-inosine monophosphate (5′-IMP) [1] have commercial applications as flavoring ingredients and can be produced from enzymatic RNA degradation during yeast autolysis [2–4]. RNA represents more than 95% of the total content of nucleic acids within yeast cells [5] and is degraded more rapidly than DNA during autolysis [6]. One aspect of the traditional production of Champagne is the long aging on lees during which yeast autolysis occurs [7], releasing into the wine intracellular yeast constituents such as the degradation products of nucleic acids [8]. Formation of such nucleotides in Champagne wine can affect its sensory qualities [9], hence our interest in their identification. Some monophosphate nucleotides have already been detected and quantified by re-

*Corresponding author. Tel.: +33-3-8039-6392; fax: +33-3-8039-6265.

E-mail address: dchassa@u-bourgogne.fr (D. Chassagne).

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01019-0

versed-phase ion-pairing high-performance liquid chromatography (HPLC) with UV detection, in yeast extract [10], yeast autolysates [11] and beer [12]. On the other hand, unlike other nitrogenous compounds, nucleotides have received little attention in wine because their detection in this beverage is quite difficult. Wine monophosphate nucleotides are in an extremely complex mixture, together with organic acids, polysaccharides, phenolic compounds, proteins, peptides, amino acids, etc., which are present in higher concentrations and can therefore interfere with their measurement. Up to now, except for preliminary investigations [13,14] performed with spectrophotometer observations, no study has led to the unequivocal identification of monophosphate nucleotides in Champagne wine. Generally, nucleic acids have been measured by methods based on HPLC with spectrometric detection. There are some difficulties using these methods due to low sensitivity and selectivity; indeed, complex mixtures contain many different types of products that interfere with the chromatographic detection. Although HPLC methods with electrochemical [15,16], fluorometric [17–20] and chemiluminescent nitrogen-specific detection [21] for the determination of some nucleotides have been reported, the method of choice for the identification of polar compounds such as nucleotides is mass spectrometry (MS), due to the development of soft ionization techniques such as electrospray ionization (ESI) [22].

The aim of our work was to develop a purification procedure for monophosphate nucleotides from Champagne wine to allow their identification by HPLC coupled to ESI-MS.

2. Experimental

2.1. Chemicals and chromatographic standards

The monophosphate nucleotides studied and their abbreviations were as follows: adenosine 5′-monophosphate (5′-AMP), adenosine 2′-monophosphate (2′-AMP), adenosine 3′-monophosphate (3′-AMP), cytidine 5′-monophosphate (5′-CMP), cytidine 2′-monophosphate (2′-CMP), cytidine 3′-monophosphate (3′-CMP), guanosine 5′-monophosphate (5′-GMP), guanosine 2′-monophosphate (2′-GMP), guanosine 3′-monophosphate (3′-GMP), inosine 5′-monophosphate (5′-IMP), inosine 3′-monophosphate (3′-IMP), uridine 5′-monophosphate (5′-UMP), uridine 2′-monophosphate (2′-UMP), uridine 3′-monophosphate (3′-UMP), xanthosine 5′-monophosphate (5′-XMP), thymine 5′-monophosphate (5′-dTMP), thymine 3′-monophosphate (3′-dTMP), 2′-deoxyadenosine 5′-monophosphate (5′-dAMP), 2′-deoxycytidine 5′-monophosphate (5′-dCMP), 2′-deoxyguanosine 5′-monophosphate (5′-dGMP), 2′-deoxyuridine 5′-monophosphate (5′-dUMP). All of them were obtained from Sigma (St. Louis, MO, USA) except the mixture 2′-GMP–3′-GMP (38:59) which was purchased from Fluka (Buchs, Switzerland). A stock standard solution of 1000 mg/l of this compound mixture was prepared in Milli-Q quality water (Millipore, Bedford, MA, USA) and stored at −20°C. Working standard solutions were made daily depending on their concentration by diluting the stock standard solution.

HPLC eluents, Rectapur potassium dihydrogenphosphate (KH_2PO_4) and orthophosphoric acid (84%, w/w) were obtained from Prolabo (Paris, France), Analytical-reagent grade dipotassium hydrogenphosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), diammonium hydrogenphosphate $(NH_4)_2HPO_4$, ammonium dihydrogenphosphate $(NH_4)H_2PO_4$ and absolute ethanol (GR grade) were purchased from Merck (Darmstadt, Germany), the ion-pair reagent, tetrabutylammonium dihydrogenphosphate (TBA), triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were from Sigma, potassium chloride (KCl), acetic acid and HPLC-grade methanol were acquired from Carlo Erba reagents (Rodano, Italy) and HPLC-grade water was purified using a Milli-Q system (Millipore).

2.2. Preparation of wine sample

A Champagne wine, which had been aged on lees for 8 years, was first disgorged and degassed under vacuum. A 250-ml volume of this sample was then ultrafiltrated in a cell (volume: 400 ml, diameter: 90 mm) (Millipore) with stirring, at 4°C at a nitrogen pressure of $3 \cdot 10^5$ Pa using a regenerated cellulose low binding membrane with a molecular mass cut-off of 5000 (Millipore). After four washings with 50 ml Milli-Q quality water (Millipore), the ultrafiltrate

of wine was concentrated under reduced pressure at 25°C with an RE-100 Model rotary evaporator (Bibby Sterilin, UK) until 10 ml (25-fold concentration step). The concentrate was then frozen and stored at −20°C until use.

2.3. Semi-preparative ion-exchange chromatography

Semi-preparative HPLC was carried out on a Beckman (Fullerton, CA, USA) Model Biosys 510 protein purification binary solvent delivery liquid chromatography system equipped with a UV detection system. HPLC data acquisition and analysis were controlled by the Gold Nouveau chromatography data system version 1.6 software. Ion-exchange liquid chromatography was performed using an Interchrom (Interchim, Montluçon, France) stainless steel strong anion-exchange (SAX) semi-preparative column (250×10 mm I.D., 10 μm Nucleosil SB silica, 100 Å porosity). The column was preserved under the mixing of 0.3 *M* $(NH_4)_2HPO_4$ and 0.3 *M* $(NH_4)H_2PO_4$ at pH 5 with 10% (v/v) absolute ethanol. Elution was performed with a gradient system consisting of two eluents. Eluent A was an aqueous solution of 0.007 *M* KH_2PO_4 adjusted to pH 4 with orthophosphoric acid. Eluent B was an aqueous solution of 0.25 *M* KH_2PO_4 and 0.25 *M* KCl also adjusted to pH 4. At room temperature, with 4 ml/min flow-rate and 254 nm detection, elution was started isocratically for 15 min with 100% eluent A and 0% eluent B. The ratio of eluent B in the system was increased linearly from 0 to 100% over 45 min, and was maintained at this condition for 20 min. Ion-exchange adsorbents were regenerated by washing with a 2 *M* potassium chloride solution. A 1-ml loop injection was used to introduce the analytes.

2.4. Semi-preparative reversed-phase chromatography

The HPLC apparatus is the same as that described above. A 5 μm particle (porosity: 80 Å), Ultrasphere ODS reversed-phase stainless steel semi-preparative column (250×10 mm I.D.) (Beckman) was used. The chromatographic separation was carried out using a gradient of two eluents. Eluent A was a solution of water–acetic acid (99.85:0.15) and eluent B was 100% methanol. The flow-rate was maintained at 2.5 ml/min throughout the run. Elution was performed at ambient temperature with 254 nm UV detection. Elution began isocratically for 15 min with 100% eluent A. The ratio of eluent B was increased linearly from 0 to 10% over 40 min then from 10% to 40% over 10 min and maintained at this condition for 5 min. Injection volumes were 1 ml.

2.5. Analytical reversed-phase ion-pairing chromatography

Analytical HPLC was performed with a Thermo Separation Products (Thermo-Quest; San Jose, CA, USA) Model P-1000 XR quaternary gradient liquid chromatography system and detection was carried out with a Waters (Milford, MA, USA) Model 991 diode-array detector. Data were recorded using version 6.22 of the detector software. An Interchrom (Interchim) stainless steel C_{18} analytical column (250×4.6 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. The separation was carried out with a gradient system comprised of two eluents as reported by Zhao and Fleet [11]. Eluent A was the addition of 0.005 *M* TBA with the mixing of 0.05 *M* KH_2PO_4 and 0.05 *M* K_2HPO_4 at a final pH 5.45. Eluent B was 100% methanol (HPLC grade). The flow-rate was maintained at 1 ml/min, temperature at 25°C and detection at 254 nm. The elution was started with 100% eluent A. The ratio of eluent B in the system was increased linearly from 0 to 6% over 30 min and maintained at this condition for 5 min. The ratio was then increased linearly from 6% to 9% over 10 min, maintained at this condition for 15 min and increased linearly from 9 to 20% over 20 min. The elution was held at 20% eluent B for 10 min. A loop of 20 μl was used for the injection.

2.6. HPLC–mass spectrometry

HPLC was performed with a Perkin-Elmer (Perkin-Elmer Sciex, Canada) series 200 LC binary solvent delivery liquid chromatography system. An

LC Packings (Amsterdam, The Netherlands) 3 μm particle, 100 Å pore size, PepMap reversed-phase Superba microcolumn (150×1 mm I.D.), protected by a micro-guard column (2×0.8 mm I.D.) with the same phase (LC Packings) was used for the separation of monophosphate nucleotides. The flow-rate generated by the HPLC pumps was 0.6 ml/min and was reduced to 30 μl/min by using a pre-column splitter (LC Packings) located between the pump outlet and the injector. A 5-μl external loop injection was used and the elution was achieved under isocratic conditions for 60 min by aqueous 0.4 *M* HFIP adjusted to pH 7 with TEA.

Electrospray mass spectra were recorded on an API 365 triple quadrupole LC–MS–MS mass spectrometer (Perkin-Elmer Sciex). Both HPLC and MS were controlled by the Mass Chrom 1.1.1 Sciex software allowing simultaneous instrument control, data acquisition and data analysis. The electrospray interface was set to the negative ionization mode and parameters were optimized with standard monophosphate nucleotides to induce partial cleavage (formation of the PO_3^- fragment) while conserving a good intensity of the $[M\text{-}H]^{-1}$ ion. The probe tip was kept at −4.8 kV, the cone voltage was set to −70 V and the source was at ambient temperature. MS data were acquired in scan modes from 78 to 79 u at 0.15 u stepsize and from 300 to 400 u at 0.15 u stepsize. The dwell times were 5 and 2.5 ms, respectively. Nitrogen was used as a drying gas and air as nebulizing gas. Reconstructed mass chromatograms of $[M\text{-}H]^{-1}$ ions from each monophosphate nucleotides were drawn together with that of m/z 79 corresponding to the pyrophosphate anion.

2.7. Quantification of potassium salt

The analysis were performed with a 410 Model flame photometer (Sherwood Scientific, Cambridge, UK) which can directly measure potassium by means of a low-temperature propane gas flame and a source of clean dry pulse-free compressed air at a pressure of 10^5 Pa. Ten, 8, 6, 4, 2 mg/l potassium chloride solutions were prepared by dissolving a stock solution (20 mg/l) with Milli-Q quality water (Millipore) in order to get a calibration graph (average of the three values obtained for the same salt concentration).

2.8. Efficiency of monophosphate nucleotides recovery

The recovery of monophosphate nucleotides after the complete purification procedure was examined in two ways. On the one hand, the HPLC peak areas of a 10 mg/l solution of each standard monophosphate nucleotide, were compared before and after the purification steps by using reversed-phase ion-pairing chromatography. On the other hand, Champagne was spiked with a 2 mg/l solution of each standard monophosphate nucleotides. In this case, recovery was calculated, with the same analytical method, as the peak areas of each monophosphate nucleotide in the spiked sample wine subtracted from the peak areas of the same compounds in the added reference mixture (external standard calibration) at the same concentration. Each test was performed three times and the results (average of the three values) are shown in Table 1.

3. Results and discussion

3.1. Limitation of direct analysis by reversed-phase ion-pairing HPLC

The reversed-phase ion-pairing HPLC was used by Zhao and Fleet [11] for the identification of monophosphate nucleotide isomers from nucleic acid degradation during yeast autolysis in a model system. Direct analysis of a Champagne wine using the method previously cited, showed a complex chromatographic profile with UV detection and no nucleotide was detected unequivocally, even by spiking reference compounds into the sample. The mobile phase used in this chromatographic procedure did not allow the use of a mass spectrometer coupled to a HPLC system because tetrabutylammonium salts as pairing agents, result in poor detection limits. It was necessary therefore to purify monophosphate nucleotides from Champagne wine by using a methodology and reagents compatible with identification by HPLC coupled with ESI-MS.

3.2. Anion-exchange chromatography

The analytical HPLC method described by McK-

Table 1
Efficiency of recovery of reference monophosphate nucleotides mixture in aqueous solution (A) and spiked in Champagne wine (B) after ultrafiltration, anion-exchange and reversed-phase chromatography steps and compounds identified by HPLC–ESI-MS in a Champagne wine aged on lees for 8 years

Nucleotide	Efficiency of recovery		Identification in Champagne wine	Concentration[a]
	A (%)	B (%)		
5′-CMP	81	41	Yes	+
5′-dCMP	90	Not detected	No	
5′-UMP	89	88	Yes	+++
5′-GMP	86	83	Yes	+++
5′-IMP	86	85	Yes	++
5′-dUMP	87	84	No	
2′-CMP	77	Not detected	No	
3′-CMP	85	42	Yes	+
5′-dGMP	84	83	No	
5′-dTMP	84	84	No	
5′-AMP	78	55	Yes	+++
3′-GMP	85	85	Yes for 3′-	++
2′-GMP	87	78	and/or 2′GMP	
3′-UMP	89	85	Yes for 3′-	+
2′-UMP			and/or 2′-UMP	
3′-IMP	89	86	No	
5′-XMP			No	
3′-dTMP	84	80	No	
5′-dAMP	79	63	No	
3′-AMP	87	82	Yes for 3′-	++
2′-AMP	88	75	and/or 2′-AMP	

[a] Relative concentrations were ranked as follow: +, <0.05 mg/l; ++, 0.05–0.1 mg/l; +++, 0.1–0.5 mg/l.

eag and Brown [23] was optimized for this purification step, because it displays a good degree of resolution of 5′-monophosphate nucleotides and separates them from di- and triphosphates, which are eluted later. A mixture of 21 5′-, 3′- and 2′-isomers of reference monophosphate nucleotides, separated by anion-exchange chromatography (Fig. 1a), was used to determine a collection area of these compounds in Champagne wine. Reference compounds were eluted between 25 and 53 min with salt concentrations between 0.10 and 0.33 *M*, and 18 well resolved peaks were obtained. The retention order of the 5′-monophosphate nucleotides, except for 5′-AMP, was similar to that of the method of McKeag and Brown [23].

Champagne ultrafiltered and concentrated 25-fold under reduced pressure, was injected through the same anion-exchange column. The chromatographic profile of this sample (Fig. 1b) shows an initial large signal corresponding to non-polar molecules (e.g., nucleosides, bases), cations and weak anions at pH 4. The pH value of the eluents was appropriate for our application because the majority of positively charged amino acids were eliminated under these conditions. Between 20 and 59 min, nine different peaks were collected together. By comparing wine extract and reference mixture profiles, peak A is in the elution area of 5′- and 2′-CMP; peak C: 5′-, 2′-UMP and 5′-dUMP; peak D: 5′-dTMP; peak E: 5′-IMP; peak F: 3′-CMP; peak G: 2′-GMP, 5′-dGMP and 5′-AMP and peak H: 3′-AMP. The last part between 60 and 80 min probably contains more negatively charged molecules, which are therefore eluted later than monophosphate nucleotides. A good repeatability was achieved with a variation of retention times less than a single minute, in the absence of temperature control.

Before the development of ion-pairing reversed-phase HPLC, anion-exchange chromatography was the method of the choice for the separation and

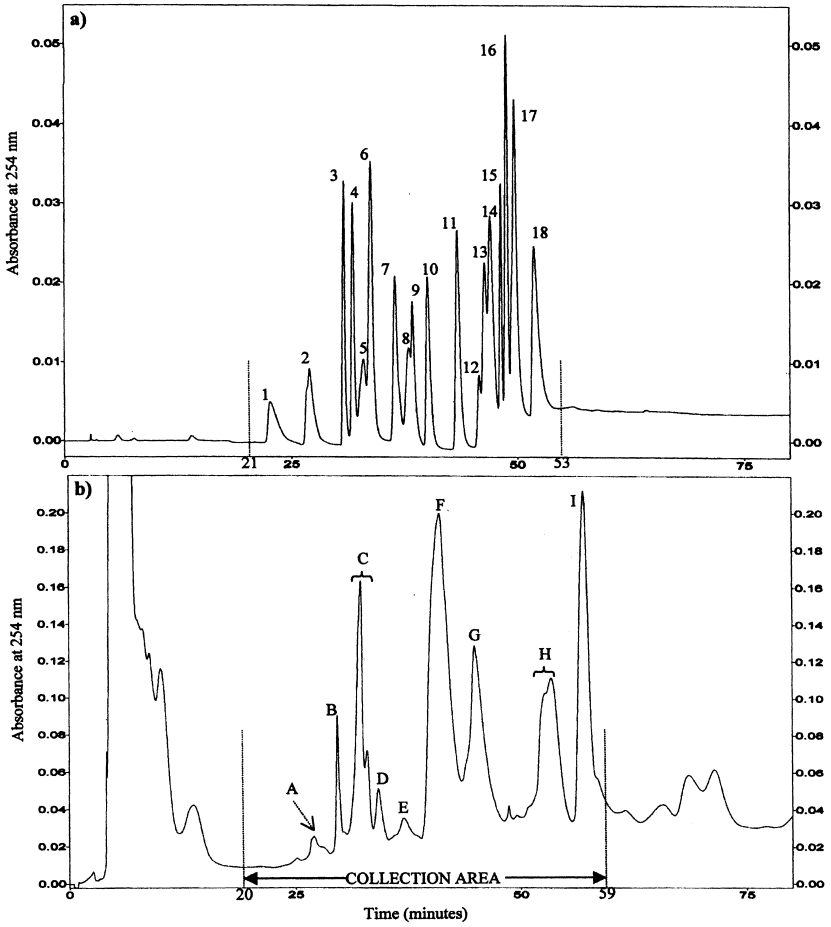

Fig. 1. Semi-preparative anion-exchange HPLC profile at 254 nm of a standard solution of monophosphate nucleotides (10 mg/l each) (a) and of the 25-fold concentrated permeate from a Champagne wine aged on lees for 8 years, ultrafiltered through a membrane with a molecular mass cut-off of 5000 (b). Strong anion-exchange column (SAX). Eluent A, 7 m*M* KH_2PO_4, pH 4. Eluent B, 0.25 *M* KH_2PO_4 and 0.25 *M* KCl, pH 4. Flow-rate, 4 ml/min. HPLC gradient, 0% eluent B for 15 min; the ratio of eluent B increased linearly from 0 to 100% over 45 min and held at this condition for 20 min. Peaks: 1=5′-dCMP; 2=2′-CMP and 5′-CMP; 3=5′-UMP; 4=5′-dUMP; 5=2′-UMP; 6=5′-dTMP; 7=5′-IMP; 8=3′-dTMP; 9=3′-UMP; 10=3′-CMP; 11=5′-GMP and 3′-IMP; 12=2′-GMP; 13=5′-dGMP; 14=5′-AMP; 15=3′-GMP; 16=2′-AMP; 17=5′-dAMP and 5′-XMP; 18=3′-AMP.

quantification of nucleotides [24,25]. This technique is now frequently used as an extraction procedure for nucleotides in complex media using anion-exchange cartridges [20,21,26]. In our study, the HPLC technique was chosen because of its superior selectivity in the purification of nucleotides in wine compared with the solid-phase extraction procedure previously cited, and as reported by Teoule et al. [27], with size-exclusion chromatography used by Somers and Ziemelis [28] and Feuillat and Morfaux [13] for fractionating Riesling and Chardonnay constituents, respectively.

With a view to further analysis, the collected fraction was concentrated under reduced pressure and a 3.2 *M* final salt concentration (estimated by a flame photometer) was produced. In order to identify the monophosphate nucleotides in Champagne wine by MS, the salt must be removed. Indeed, the presence of non-volatile buffer salts in samples is incompatible with MS due to ion suppression and the

formation of cation metal adducts such as ($M+Na^+$) or ($M+K^+$) [29].

3.3. Salt elimination

Preliminary studies have been performed with size-exclusion chromatography as used by O'Mullan et al. [30] and Haff and Smirnov [31], and with cation-exchange chromatography [32]. The first method resulted in only 20% salt removal and the second, although efficient (96.5%), did not allow sufficient latter sample concentration. Therefore, reversed-phase HPLC was used. The separation in a reversed-phase semi-preparative column of 21 reference monophosphate nucleotides produced from the anion-exchange chromatography step is reported in Fig. 2a. Under these conditions, about 99.95% of salts, eluted in the first peak at 5 min, were eliminated.

The Champagne wine fraction collected from the anionic separation step was run through the same reversed-phase chromatography column (Fig. 2b).

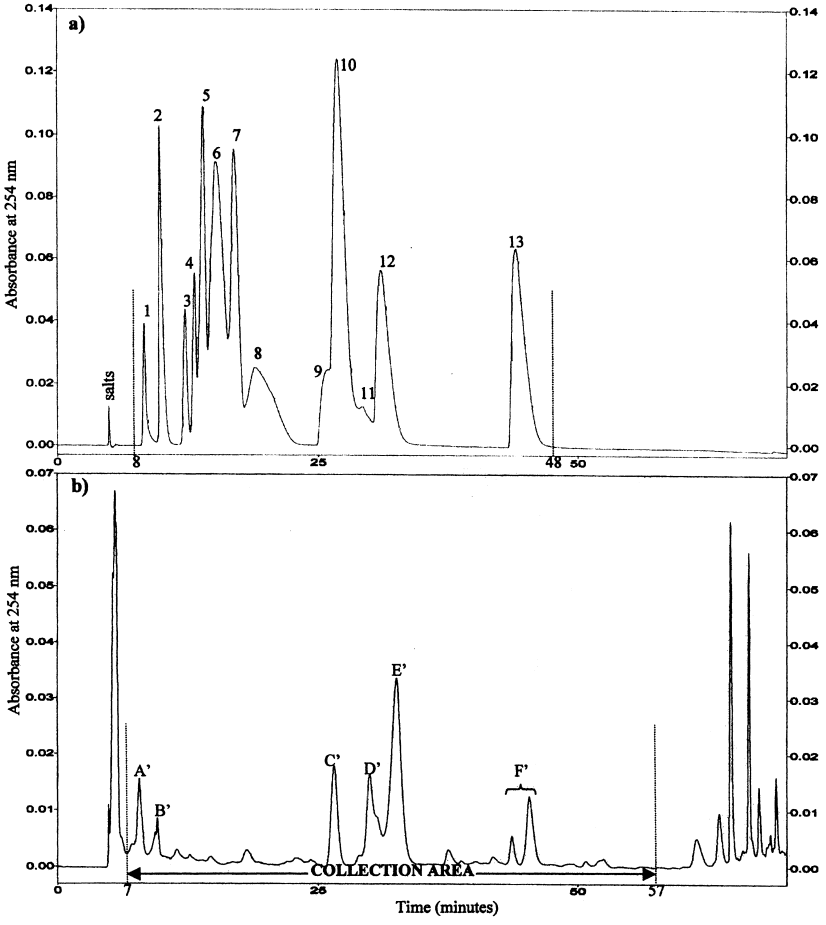

Fig. 2. Semi-preparative reversed-phase HPLC profile at 254 nm of a standard mixture of monophosphate nucleotides (20 mg/l each) (a) and of the anion-exchange HPLC collected fraction (b). C_{18} column. Eluent A, water–acetic acid (99.85:0.15). Eluent B, methanol. Flow-rate, 2.5 ml/min. HPLC gradient, 0% eluent B for 15 min; the ratio of eluent B increased linearly from 0 to 10% over 40 min then from 10 to 40% over 10 min and held at this condition for 5 min. Peaks: 1=5′-CMP; 2=5′-UMP; 3=5′-dCMP; 4=3′-CMP; 5=2′-CMP and 5′-dUMP; 6=5′-GMP and 5′-IMP; 7=5′-AMP and 3′-UMP; 8=5′-XMP and 2′-UMP; 9=3′-dTMP; 10=5′-dGMP, 5′-dTMP and 3′-AMP; 11=3′-IMP; 12=3′-GMP and 5′-dAMP; 13=2′-GMP and 2′-AMP.

Between 5 and 7 min, signals detected correspond to the elution of salts and hydrophilic molecules. Six different groups of peaks (A′ to F′) were collected together between 7 and 57 min. After 57 min, many signals were obtained, showing the presence in the extract of more hydrophobic compounds than nucleotides. The variation in reversed-phase HPLC retention times was less than 1 min at room temperature with identical profiles obtained throughout several weeks.

Reversed-phase HPLC has been used successfully for desalting oligonucleotides before analysis by MS [31,33] but to our knowledge, this work is the first to show that this procedure is an effective desalting step for monophosphate nucleotides.

3.4. Efficiency of monophosphate nucleotides recovery

The method reported by Zhao and Fleet [11] using reversed-phase ion-pairing HPLC has been optimized to determine the recovery of the monophosphate nucleotides after the complete purification steps. The chromatographic profile of reference monophosphate nucleotides resulted in less co-elution than that reported by Zhao and Fleet [11], particularly for 5′-dTMP and 5′-dGMP compounds. The recovery of monophosphate nucleotides after the ultrafiltration, anion-exchange and reversed-phase chromatography steps is summarized in Table 1. Except for 3′-CMP and 5′-CMP, an average of 79% recovery was observed. Under our purification conditions, 2′-CMP and 5′-dCMP disappeared only in the spiked wine extract. This observation shows that in the wine extract there is competition between all negative molecules to interact with the anionic resins. In the present case, the molecules eluted first (5′-dCMP and 2′-CMP), which have the least affinity with anion-exchange resins, are the most influenced by this competition. This results in weaker retention compared with aqueous solutions and therefore none of these first-eluted molecules were collected under the conditions we used.

Ion-exchange followed by reversed-phase HPLC were found to be complementary and an essential procedure for the purification of champagne monophosphate nucleotides. After these steps, the simplification of chromatographic profiles allowed the use of reversed-phase ion-pairing HPLC to identify and quantify the nucleotides spiked in wine. However, the small concentration of monophosphate nucleotides isolated from Champagne wine can be identified only by MS due to its superior sensitivity.

3.5. Identification and semi-quantification by HPLC coupled to MS

Conditions for electrospraying water solutions of standard monophosphate nucleotides were optimized to induce their partial decomposition, giving the PO_3^- ion (m/z 79) while conserving a high abundance of the $[M\text{-}H]^{-1}$ ion. This was done by adjusting the cone-to-skimmer voltage to an appropriate value.

Preliminary HPLC–ESI-MS runs were performed with an aqueous acetic acid as solvent [32]. This mobile phase induced the formation of an ion at m/z 79, which masked the pyrophosphate anion produced by monophosphate nucleotides fragmentation. This ''interfering'' ion was presumably due to the ^{18}O-isotope of the abundant acetic acid–water cluster. As described by Apffel et al. [34], another solvent was used containing HFIP in water adjusted to pH 7 with TEA. Addition of TEA improves the HPLC separation because it serves as a volatile ion-pairing reagent [35], and reduces adduct formation in electrospray ionization [36]. HFIP is also necessary as a mobile phase additive, because both HPLC separation and electrospray performance using HFIP–TEA solvent system were found to be superior to that obtained with the solvent containing TEA alone [34]. One disadvantage of the HFIP–TEA mobile phase is the background signal generated by the reagents. Although the background spectrum of the mobile phase is dominated by $[HFIP]^{-1}$ and the dimer $[2HFIP]^{-1}$ at m/z 167 and 335, respectively, no ion at m/z 79 was produced by this solvent, indicating nucleotides analysis is possible. Thus, monitoring m/z 79 could be used for the detection of phosphate-containing components in the mixture (Fig. 3), while their superimposition, in the reconstructed mass chromatogram, with nucleotides anions could be used for their identification.

Three monophosphate nucleotides were identified unequivocally in a Champagne wine aged on lees for 8 years: 5′-UMP, 5′-GMP and 5′-IMP, by com-

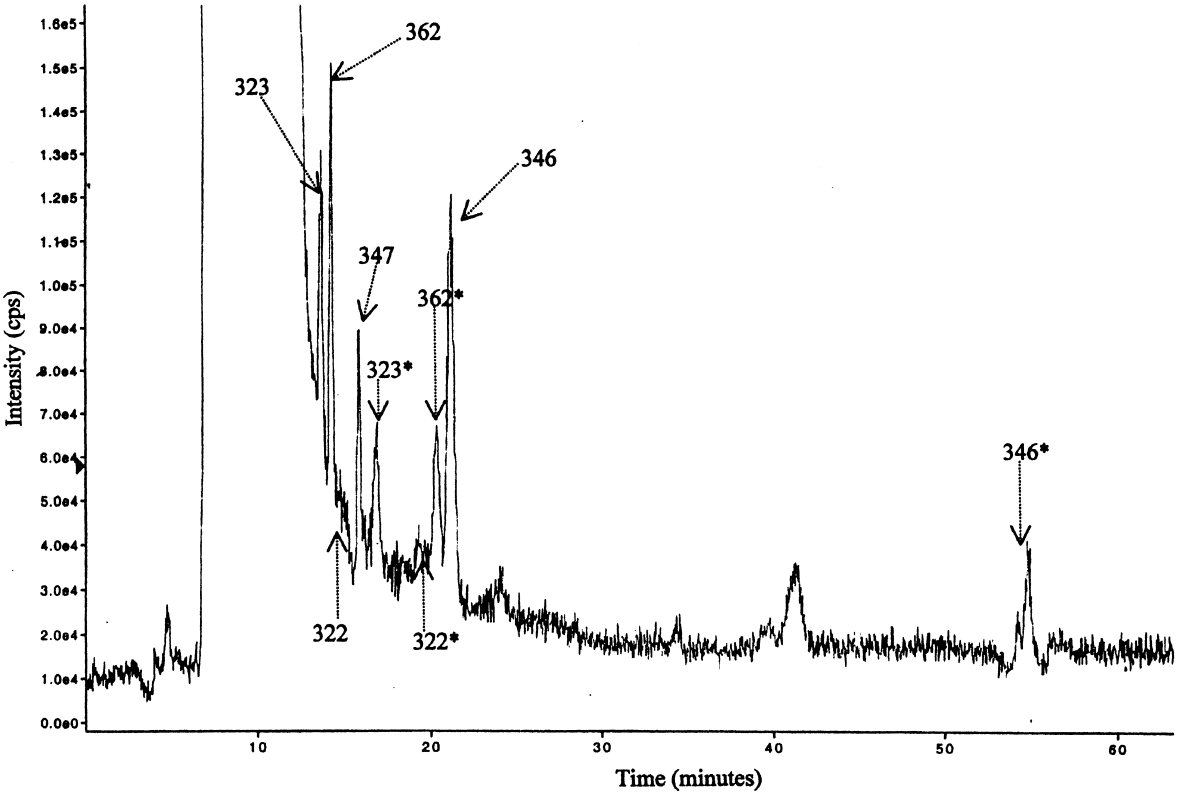

Fig. 3. Scan mode HPLC–ESI-MS analysis of the reversed-phase HPLC collected fraction. Extracted ion chromatogram for m/z 79, PO_3^-. Microcolumn, C_{18}. Eluent, 0.4 *M* HFIP adjusted to pH 7 with TEA. Flow-rate, 30 μl/min. Isocratic elution for 60 min. Peaks: m/z 322, 5′-CMP; m/z 322*, 3′-CMP; m/z 323, 5′-UMP; m/z=323*, 2′- and/or 3′-UMP; m/z 346, 5′-AMP; m/z 346*, 2′- and/or 3′-AMP; m/z 347, 5′-IMP; m/z 362, 5′-GMP; m/z 362*, 2′- and/or 3′-GMP.

parison of mass and HPLC data with those of reference compounds injected individually. With respect to 5′- and 3′-CMP, two peaks in the extracted m/z 322 ion chromatogram correlate with the retention time of reference compounds, but with low intensity, explaining that these peaks are mixed with the background in the extracted m/z 79 ion chromatogram (Fig. 3). For UMP, GMP and AMP, the 3′- and 2′-isomers are co-eluted. Also, in the natural extract, the ions at m/z 323*, 362* and 346* (Fig. 3) corresponding, respectively to the compounds previously cited, cannot attributed to one isomer. 5′-AMP and 5′-dGMP are also eluted as one peak. However, RNA is major (95%) in the total content of nucleic acids within yeast cells [5] and less stable than DNA during autolysis [6]. Consequently, the ion at m/z 346 detected in wine extract, corresponds likely to the parent ion of 5′-AMP.

Comparing HPLC–ESI-MS peaks intensities of the monophosphate nucleotides identified in Champagne wine and those of reference compounds carried out semi-quantitative data (Table 1).

4. Conclusion

A procedure for the identification of wine monophosphate nucleotides was developed for the first time using ultrafiltration, anion-exchange and reversed-phase HPLC columns, and ESI-MS coupled to HPLC.

This methodology can be applied to Champagne

wine which has undergone different aging periods on lees in order to determine what changes occur in the identified compounds, and if some of them can act as ''aging markers'' of this beverage. Among monophosphate ribonucleotides identified, two are well known to have flavor-enhancing properties (5′-GMP and 5′-IMP) in the food matrix [37]. Further investigations such as sensorial analysis, determination of detection threshold are required to evaluate the impact of the nucleotides identified on wine flavor.

Acknowledgements

The authors wish to thank Moët & Chandon for financial support. They gratefully acknowledge Professors J.C. Promé and D. Promé for most valuable guidance in the choice of directions of mass spectrometry research and for fruitful discussion of some results. Our thanks also go to Dr. S. Richelme for their efficient help in the use of the mass spectrometer.

References

[1] A. Kuninaka, M. Kibi, K. Sakaguchi, Food Technol. 18 (1964) 29.
[2] M.D. Benaiges, J. López-Santin, C. Solà, Enzyme Microb. Technol. 12 (1992) 86.
[3] B.E.N. Todd, in: C.S. Stockley, A.N. Sas, R.S. Johnstone, T.H. Lee (Eds.), Proceedings of the 9th Australian Wine Industry Technical Conference, Adelaïde, 16–19 July 1995, Winetitles, Adelaïde, 1996, p. 33.
[4] M.A.F. Belem, B.H. Lee, Food Sci. Technol. Int. 3 (1997) 437.
[5] J.C. Mounolou, in: A.H. Rose, J.S. Harrison (Eds.), The Yeasts, the Physiology and Biochemistry of Yeasts, Academic Press, London, 1971, p. 309.
[6] W.E. Trevelyan, J. Sci. Food Agric. 29 (1978) 903.
[7] M. Feuillat, C. Charpentier, Am. J. Enol. Vitic. 33 (1982) 6.
[8] K. Courtis, B. Todd, J. Zhao, Austr. Grapegrower Winemaker 409 (1998) 51.
[9] C. Charpentier, M. Feuillat, in: G.H. Fleet (Ed.), Wine Microbiology and Biotechnology, Harwood Academic Press, Lausanne, 1993, p. 225.
[10] W.W. Fish, J. Agric. Food Chem. 39 (1991) 1098.
[11] J. Zhao, G.H. Fleet, J. Chromatogr. A 732 (1996) 271.
[12] A.A. Qureshi, W.C. Burger, N. Prentice, Inst. Am. Soc. Brew. Chem. 37 (1979) 153.
[13] M. Feuillat, J.N. Morfaux, Conn. Vigne Vin 10 (1976) 33.
[14] M.J. Leroy, M. Charpentier, B. Duteurtre, M. Feuillat, C. Charpentier, Am. J. Enol. Vitic. 41 (1990) 21.
[15] J.B. Kafil, H.Y. Cheng, T.A. Last, Anal. Chem. 58 (1986) 285.
[16] T. Nakahara, A. Shiraishi, M. Hirano, T. Matsumoto, T. Kuroki, Y. Tatebayashi, T. Tsutsumi, K. Nishiyama, H. Ooboshi, K. Nakamura, H. Yao, M. Waki, H. Uchimura, Anal. Biochem. 180 (1989) 38.
[17] M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M.M.E. Merbazani, Z. Tamura, T. Miyazaki, J. Chromatogr. 309 (1984) 63.
[18] H. Fujimori, T. Sasaki, K. Hibi, M. Senda, M. Yoshioka, J. Chromatogr. 515 (1990) 363.
[19] J.O. Svensson, B. Jonzon, J. Chromatogr. 529 (1990) 437.
[20] Y. Ohba, M. Kai, Y. Ohkura, K. Zaitsu, Anal. Sci. 13 (1997) 469.
[21] E.M. Fujinari, J.D. Manes, in: G. Charalambous (Ed.), Food Flavors: Generation, Analysis and Process Influence: Proceedings of the 8th International Flavor Conference, Cos, 6–8 July 1994, Elsevier, Amsterdam, 1995, p. 379.
[22] E.L. Esmans, D. Broes, I. Hoes, F. Lemiere, K. Vanhoutte, J. Chromatogr. A 794 (1998) 109.
[23] M. McKeag, P.R. Brown, J. Chromatogr. 152 (1978) 253.
[24] R.A. Hartwick, P.R. Brown, J. Chromatogr. 112 (1975) 651.
[25] A. Floridi, C.A. Palmerini, C. Fini, J. Chromatogr. 138 (1977) 203.
[26] T.F. Walseth, G. Graff, M.C. Moos, N.D. Goldberg, Anal. Biochem. 107 (1980) 240.
[27] R. Teoule, R. Derbyshire, A. Guy, D. Molko, A. Roget, Nucleic Acids Symp. Ser. 7 (1980) 23.
[28] T.C. Somers, G. Ziemelis, J. Sci. Food Agric. 23 (1972) 441.
[29] S.A. Martin, C.E. Costello, K. Biemann, Anal. Chem. 54 (1982) 2362.
[30] P. O'Mullan, C. Smith, M.J. Byers, Am. Biotechnol. Lab. 16 (1998) 92.
[31] L.A. Haff, I.P. Smirnov, Genome Res. 7 (1997) 378.
[32] E. Witters, L. Roef, R.P. Newton, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, Rapid Commun. Mass Spectrom. 10 (1996) 225.
[33] R.H. Griffey, M.J. Greig, H.J. Gaus, K. Liu, D. Monteith, M. Winniman, L.L. Cummins, J. Mass Spectrom. 32 (1997) 305.
[34] A. Apffel, J.A. Chakel, S. Fischer, K. Lichtenwalter, W.S. Hancock, J. Chromatogr. A 777 (1997) 3.
[35] T. Uesugi, K. Sano, Y. Uesawa, Y. Ikegami, K. Mohri, J. Chromatogr. B 703 (1997) 63.
[36] M.J. Greig, R.H. Griffey, Rapid Commun. Mass Spectrom. 9 (1995) 97.
[37] J.A. Maga, Crit. Rev. Food Sci. Nutr. 18 (1983) 231.