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Evaluation of Brewer's Spent Yeast To Produce Flavor Enhancer Nucleotides: Influence of Serial Repitching

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ABSTRACT: The present work evaluates the influence of serial yeast repitching on nucleotide composition of brewer's spent yeast extracts produced without addition of exogenous enzymes. Two procedures for disrupting cell walls were compared, and the conditions for low-cost and efficient RNA hydrolysis were selected. A HILIC methodology was validated for the quantification of nucleotides and nucleosides in yeast extracts. Thirty-seven samples of brewer's spent yeast (*Saccharomyces pastorianus*) organized according to the number of serial repitchings were analyzed. Nucleotides accounted for 71.1−88.2% of the RNA products; 2′AMP was the most abundant (ranging between 0.08 and 2.89 g/100 g dry yeast). 5′GMP content ranged between 0.082 and 0.907 g/100 g dry yeast. The sum of 5′GMP, 5′IMP, and 5′AMP represented between 25 and 32% of total nucleotides. This works highlights for the first time that although serial repitching influences the content of monophosphate nucleotides and nucleosides, the profiles of these RNA hydrolysis products are not affected.

KEYWORDS: *brewer's spent yeast, flavor enhancers, HILIC, mechanic yeast disruption, nucleotides, nucleosides, serial repitching*

INTRODUCTION

Ribonucleotide derivatives and nucleosides are known to have various physiological effects. Benefits of nucleotides related to enhanced repairing of gastrointestinal tract damage, impact on fatty acid metabolism, and improvement in immune response are described.[1] Attention has also been paid to the influence of dietary nucleotides in infant nutrition[2,3] and the role of nucleotides as signaling molecules between cells.[4] Furthermore, 5′-monophosphate nucleotides are responsible for umami taste. Among all 5′nucleotides, guanosine 5′-monophosphate (5′GMP) is an active flavor enhancer and adenosine 5′-monophosphate (5′AMP) is a precursor of the well-known flavor enhancer inosine 5′-monophosphate (5′IMP).[5] These compounds have little flavor or aroma themselves but can enhance the flavor and mouthfeel of other compounds. The flavor-enhancing activity of 5′GMP and 5′IMP is more than 100 times greater than that of monosodium glutamate (MSG), a widely used flavor enhancer.[6−8]

Yeasts, namely, *Saccharomyces*, 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.[6,9] Moreover, it is a GRAS (generally regarded as safe) microorganism and has good nutritional characteristics.[10] Yeast extracts containing 5′GMP can be prepared by hydrolysis of RNA from baker's yeast cells by heating and using 5′-phosphodiesterase. Alternatively, brewer's spent yeast, the second major byproduct from the brewing industry, is an interesting raw material for the production of flavor-enhancing 5′-nucleotides, due to its low cost.[9,11] In the brewing process, serial repitching of *Saccharomyces* biomass is usual; thus, yeast is reused four to six times before its disposal.[10,12] Therefore, it can be of interest to understand the influence of yeast repitching for flavor enhancer production; however, to the best of our knowledge, studies related to the influence of yeast generation on monophosphate nucleotide and nucleoside composition of brewer's spent yeast have not been reported.

The separation of intracellular yeast compounds for use in food applications requires efficient means of disrupting cell walls and separating the useful products. Several methodologies for yeast breakdown have been reported, namely, physical, chemical, and enzymatic methods.[9,13−17] However, among the methods for disrupting yeast cell walls, autolysis is the only one easily applied at industrial scale.[18] In this process cell components are solubilized by activation of the degradative enzymes present within the cells.

Yeast autolysis can be induced by exposing the cells to elevated temperatures (40−60 °C), salts, or organic solvents.[7] Through an autolysis process, the yeast's own enzymes break down the RNA into mononucleotides, polynucleotides, and nucleosides. Yeast autolysis is strongly influenced by temperature and pH. Following autolysis, enzymatic treatments have been developed to further increase the final yield of 5′-nucleotides, thus increasing flavor enhancement properties of the yeast autolysates.[18] Applications of RNases and 5′-phosphodiesterases are recommended procedures.[13] RNA extracted from yeast cells can be degraded into four 5-nucleotides: 5′GMP, uridine 5′-monophosphate (5′UMP), cytidine 5′-monophosphate (5′CMP), and 5′AMP, but only 5′GMP possesses favorable properties. Additionally, 5′AMP can be converted into 5′IMP by AMP-deaminase.[9,13] Despite a high production yield, this procedure is not attractive to manufacturers owing to the relatively high capital investment cost. More recently, mechanical disruption, using glass beads,

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was shown to be a promising procedure to maintain RNase viability at low economical costs.[17]

Nucleotides and nucleosidases are separated by chromatographic methodologies, such as reversed-phase high-performance liquid chromatography (RP-HPLC),[3,5] ion-exchange liquid chromatography,[19] and detection with diode array detection coupled to dual electrospray atmospheric pressure chemical ionization time-of-flight mass spectrometry.[20] Other techniques include enzymatic assay, capillary electrophoresis, capillary electrophoresis−inductively coupled plasma mass spectrometry, capillary electrochromatography, and ion-pair reversed-phase chromatography.[2] More recently, hydrophilic interaction liquid chromatography (HILIC) has found increased use for the separation of highly polar molecules, namely, nucleotides that are often unretained under RP-HPLC.[21−24]

The present work was undertaken to obtain 5′-nucleotide-rich yeast extracts from brewer's spent yeast without addition of exogenous enzymes and evaluate the influence of serial yeast repitching on extract composition concerning monophosphate nucleotides and nucleosides. For this purpose two procedures for disrupting cell walls that can be used at industrial scale were tested, and the optimum conditions for low-cost and efficient RNA hydrolysis were selected. A HILIC methodology coupled to diode array detection was validated for quantification of adenosine, uridine, xanthosine, cytidine, guanosine, 5′GMP, 5′IMP, 5′AMP, 5′CMP, 5′UMP, xanthosine 5′-monophosphate (5′XMP), adenosine 2′-monophosphate (2′AMP), adenosine 3′-monophosphate (3′AMP), guanosine 2′-monophosphate (2′GMP), and guanosine 3′-monophosphate (3′GMP) in yeast extracts.

METHODS

Reagents. Disodium salts of 2′GMP, 5′AMP, 5′CMP, 5′GMP, 5′UMP, 5′XMP, 2′ AMP, 3′AMP, 2′ GMP, and 3′GMP and adenosine, cytidine, xanthosine, guanosine, and uridine, with 95−100% purity, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bradford reagent and bovine serum albumin (BSA) were also from Sigma. Perchloric acid, potassium hydroxide, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Ammonium acetate, ammonium phosphate, and glacial acetic acid (gradient grade) were supplied by Panreac (Barcelona, Spain). Acetonitrile was acquired from Fluka (Buchs, Swetland). Ultrapure water was obtained from a Seral-Seralpur Pro 90 CN (<0.055 μS) water purification system from Belgolabo (Overijse, Belgium).

Standards. Stock standard solutions of individual nucleotides and nucleosides were prepared by weighing 10 mg of each compound into a volumetric flask (10 mL). Standards were dissolved in deionized water, except for 5′GMP, guanosine, 2′GMP, and 3′GMP, which required a few drops of 10% KOH to promote dissolution. The stock standard solutions were stored at 4 °C in a dark environment for up to 2 weeks. The standard solution mix of nucleotides and nucleosides was prepared daily by dilution with ultrapure water.

Equipment. The chromatographic analyses were carried out in an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with a low-pressure quaternary pump (Jasco, PU-1580 intelligent HPLC pump), a degasification unit (Jasco, DG-1580-54 4-line degasser), a type 7981 Jones Chromatography column heater (Jones Chromatography, Hesperia, CA, USA), a type 7725i Rheodyne injector (Rheodyne, Rohnert Park, CA, USA), and a diode array detector (Jasco, MD 910). Chromatographic separation was achieved with a TSK-GEL Amide-80 (Tosoh Bioscience, Japan) chromatographic column 5 mm (250 × 4.6 mm) from Waters (USA), at room temperature; the loop volume selected was 20 μL. Data acquisition was accomplished using Borwin Controller software, version 1.50 (JMBS Developments, Le Fontanil, France). Eluents were degassed using an ultrasonic bath (Fungilab, Barcelona, Spain).

Yeast Samples. Yeast was harvested from the fermentation vessels (3000 hL) after 11 days of beer fermentation and successively inoculated (repitched) into fresh wort. Thirty-seven samples of 0.5 kg of brewer's spent yeast (*Saccharomyces pastorianus*) were collected; these included 6 samples of yeast biomass repitched twice in the brewing process (coded R2), 10 samples of yeast biomass with four serial repitchings (coded R4), 15 samples of yeast biomass with five serial repitchings in the brewing process (coded R5), and 6 samples of yeast biomass with six serial repitchings in the brewing process (coded R6). All samples were provided as slurry by the brewing industry Unicer, Portugal, and were stored at 4 °C until preparation procedure (1 day maximum).

Debittering of Brewer's Yeast Cells. Brewer's spent yeast was centrifuged at 5000*g* for 15 min at 4 °C to remove beer liquor. The yeast paste was washed three times with phosphate buffer, pH 7 (volume ratio 1:1). After centrifugation at 5000*g* during 15 min at 4 °C, the yeast cell pellet was weighed and stored under refrigerated conditions. Total protein content was estimated by Kjheldal method according to the AOAC,[25] and moisture was evaluated using a Scaltec instrument (Scaltec Instruments GmbH, Heiligenstadt, Germany).

Preparation of Nucleotide-Rich Yeast Extracts. The process involved two phases: yeast disruption and RNA hydrolysis. Yeast biomass (5 g) was suspended in phosphate buffer, pH 7 (volume ratio of 1:2, yeast cell mass/buffer). Two disruption methods were tested: a physical method, applying temperature at 50 °C during 24 h,[9] and a mechanical disruption method, wherein the suspension was mixed with glass beads with a diameter of 0.60 mm (weight ratio of 1:2, glass beads/suspension), cooled to 4 °C in an ice−water bath, and homogenized using a VV3 vortex mixer (VWR International, West Chester, PA, USA) for 1 min to break the cells and release the inner content. This procedure was repeated 10 times, at intervals of 1 min, and the mixture was cooled in an ice−water bath to keep the temperature below 4 °C during the entire process.[26] The mixtures obtained by the two disruption processes were centrifuged at 11000*g* during 30 min at 4 °C, and the supernatant (inner yeast extract) was carefully pipetted out, divided into aliquots, and stored at −20 °C before further analyses. The breakdown of yeast cell wall results in release of protein and nucleic acid materials into the extracellular environment. Evaluation of disruption method yield was performed by quantifying the amount of protein released after disruption using the Bradford method;[27] BSA was applied as standard. For this assay, an aliquot of 100 μL of each sample was mixed with 900 μL of Bradford reagent, and the absorbance was measured at 595 nm (Shimadzu UV-1601 UV−visible spectrometer) after 25 min.

Three RNA hydrolysis procedures were tested as follows: (i) 5 mL of yeast extract added to 3 mL of acetic acid 3% (v/v) and 6 mL of deionized water at room temperature during 15 min, coded extract 1;[28] (ii) 5 mL of the cell extract added to 3 mL of 0.3 M KOH, heated at 37 °C for 24 h with occasional mixing, and final neutralization with 3 mL of 0.5 M $HClO_4$, coded extract 2;[29] (iii) 5 mL of the cell extract added to 3 mL of 0.3 M KOH, heated at 60 °C for 24 h with occasional mixing, and final neutralization with 3 mL of 0.5 M $HClO_4$, coded extract 3.[30] All of the RNA extracts, with the same dilution factor, were centrifuged at 5000*g* and 4 °C to collect the supernatants, which were analyzed for evaluation of RNA content (Herbert method) and RNA purity degree. The RNA concentration was determined by measuring the absorbance at 260 nm using average nucleotide data for calibration: $M \approx 340$ g/mol, $\varepsilon = 10800$ L mol^{-1} cm^{-1}.[29,31] The ratio of the absorbance at 260 nm and the absorbance at 280 nm ($A_{260/280}$) was used to assess the purity of nucleic acids; for pure RNA $A_{260/280}$ is ∼2. Additionally, all of the RNA extracts were filtered through a 0.45 μm Teknokroma syringe filter (TR-200106 PVDF, 25 mm ⌀, PK/100) to remove intracellular proteins and particles before HILIC injection.

HILIC-DAD for Analysis of Nucleoside and Monophosphate Nucleotide Composition of Yeast Extracts. Gradient elution was carried out with a mixture of two eluents.[5,24] Eluent A was 100 mM ammonium acetate buffer (pH 2.5), and eluent B was acetonitrile 100%. The flow rate was 0.5 mL/min, the column was kept at a

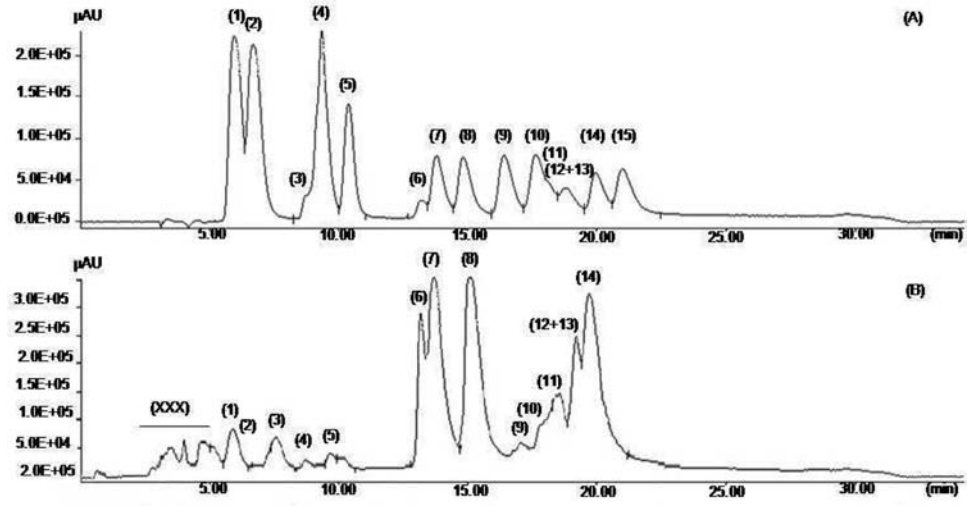

Figure 1. Chromatographic separation by HILIC, UV detection at 260 nm, of a standard mixture of nucleosides and nucleotides (A) and a sample of yeast RNA hydrolyzed extract (B). Peaks: (1) adenosine; (2) uridine; (3) xanthosine; (4) cytidine; (5) guanosine; (6) 2′AMP; (7) 3′AMP; (8) 5′AMP; (9) 5′UMP; (10) 5′IMP; (11) 2′GMP; (12) 5′CMP; (13) 3′GMP; (14) 5′GMP, (15) 5′XMP; (xxx) not identified.

Table 1. Quality Parameters of the HILIC-HPLC-DAD Method (Limits of Detection and Quantification, Working Range, and Linear Regression) for Quantification of Nucleosides and Monophosphate Nucleotides in Yeast Extracts, Precision of Intraday and Interday Assays, and Recovery Percentages

analyte	range (μg mL^{-1})	linear regression	r^2	LOD[a] (μg mL^{-1})	LOQ[b] (μg mL^{-1})	RSD$_R$[c] (%)	RSD$_{IR}$[d] (%)	recovery[e] (%)
adenosine	0.02−125	$Y = 67365X - 13170$	0.9977	0.0063	0.0211	3.72	2.09	72.1 ± 10.1
uridine	0.02−166	$Y = 47761X + 17811$	0.9994	0.0089	0.0217	4.41	6.82	68.4 ± 8.61
Xant + Cyt	0.02−166	$Y = 74195X + 26769$	0.9969	0.0155	0.0213	10.4	14.8	nd
guanosine	2.00−125	$Y = 56418X + 38461$	0.9984	0.4579	1.5263	10.2	12.3	69.2 ± 13.4
2′AMP	0.2−125	$Y = 5159X + 102329$	0.9985	0.0826	0.2752	6.78	8.76	64.1 ± 8.56
3′AMP	0.04−125	$Y = 28988X + 66295$	0.9972	0.0147	0.0490	9.65	10.5	66.1 ± 7.94
5′AMP	0.04−125	$Y = 36215X + 57291$	0.9986	0.0118	0.0392	5.07	6.09	72.1 ± 10.1
5′UMP	0.04−166	$Y = 32049X - 14019$	0.9998	0.0133	0.0343	10.5	13.8	79.1 ± 12.3
5′IMP	0.04−125	$Y = 16837X + 56623$	0.9979	0.0153	0.0443	2.77	4.45	nd
2′GMP	0.20−125	$Y = 6108X - 184508$	0.9992	0.0697	0.2325	11.9	9.07	68.0 ± 9.65
3′GMP	0.05−125	$Y = 18845X + 12740$	0.9998	0.0226	0.0754	6.34	8.90	65.2 ± 10.1
5′GMP	0.04−125	$Y = 32325X + 130052$	0.9994	0.0132	0.0439	5.92	8.21	77.1 ± 10.1
5′CMP	0.04−166	$Y = 23694X + 82464$	0.9996	0.0180	0.0499	4.85	6.76	nd
5′XMP	0.04−166	$Y = 23715X + 12051$	0.9997	0.0167	0.0414	13.8	14.1	84.1 ± 10.8

[a]LOD was based on a signal-to-noise ratio of 3:1. [b]LOQ was established as the amount of analyte that produces a signal-to-noise of 10:1. [c]RSD intraday repeatability = SD/mean × 100. [d]RSD intermediate repeatability = SD/mean × 100. [e]Mean recovery ± standard deviation at three concentration levels tested in triplicate. nd, not determined

temperature of 20 °C, and detection was achieved at 260 nm. An injection volume of 20 μL was used. All samples were analyzed in triplicate. The gradient program used was as follows: 0−2 min, 80% of eluent B; 4−6 min, 75% of eluent B; from 75 to 70% of eluent B over 1 min; 9−12 min, 65% of eluent B; 15−25 min, 60% of eluent B; finishing with 80% of eluent B for column re-equilibration during 8 min. Calibration curves were achieved by linear least-squares regression, and quantification of nucleotides and nucleosides was performed by the external standard method. Peaks were identified by comparison of retention time and spectrum correlation with authentic standards, using a similarity index of at least 0.95. Peak purity was also evaluated. The detection limit values (LODs) were based on a signal-to-noise ratio of 3:1, and the quantification limits (LOQs) were established as the amount of analyte that produces a signal-to-noise ratio of 10:1.

The precision of the developed method was evaluated by means of intraday and interday precision. Intraday variability was tested by analyzing the standard mix 10 times consecutively, whereas for the interday variation test, a standard mix was examined in triplicate for nine days; the results were expressed as RSD of peak area. Recovery experiments were carried out using three different concentration levels (100, 200, and 300 mg/L) to assess the accuracy of the method. Each sample was analyzed in triplicate.

Statistical Analysis. All statistical analyses were performed using the software SPSS for Windows, version 20.0 (SPSS, Chicago, IL, USA). Data are presented as the mean ± standard deviation. One-way analysis of variance (one-way ANOVA) at the 5% significance level, with Duncan's post hoc test, was carried out to ascertain significant differences between yeast biomass repitching groups.

RESULTS AND DISCUSSION

Yeast Disruption and RNA Hydrolysis. The efficiency of yeast breakdown and release of intracellular content after heating at 50 °C during 24 h and after glass bead disruption during 10 min was evaluated by measuring the soluble protein content in yeast extracts. Extracts obtained by autolysis at 50 °C during 24 h contained <18% of protein referred to w/w dry yeast cell, whereas those obtained by glass bead disruption contained between 43.5 and 47% of protein referred to w/w

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Table 2. Nucleoside and Nucleotide Composition of Brewing Yeast after Serial Repitchings Expressed as Grams per 100 g Dry Yeast

yeast repitching[a]	sample	adenosine	uridine	Xant + Cyt	guanosine	2′AMP	3′AMP	5′AMP	5′UMP	5′IMP	2′GMP	5′CMP + 3′GMP	5′GMP
R2	1	0.024	0.020	nq[b]	0.143	0.296	0.129	0.171	0.0257	0.026	0.144	0.124	0.167
R2	2	0.086	0.066	0.031	0.324	0.979	0.368	0.349	0.025	0.104	0.521	0.278	0.321
R2	3	0.016	0.025	0.004	0.098	0.595	0.227	0.317	0.044	0.073	0.337	0.184	0.243
R2	4	0.066	0.068	0.011	0.254	0.998	0.305	0.385	0.052	0.078	0.383	0.205	0.305
R2	5	0.019	0.040	nq	0.125	0.619	0.204	0.313	0.041	0.075	0.380	0.185	0.259
R2	6	0.075	0.050	0.021	0.285	0.815	0.311	0.298	0.033	0.066	0.312	0.211	0.258
R4	1	0.145	0.149	0.020	1.327	0.441	0.183	0.211	0.018	0.141	0.250	0.153	0.175
R4	2	0.147	0.146	0.019	1.021	0.403	0.181	0.207	0.019	0.147	0.252	0.153	0.148
R4	3	0.134	0.102	0.038	0.539	1.589	0.858	0.731	0.055	0.135	1.071	0.624	0.855
R4	4	0.040	0.032	nq	0.085	0.344	0.122	0.156	0.019	0.139	0.155	0.111	0.133
R4	5	0.048	0.019	0.024	0.161	0.978	0.271	0.303	0.028	0.131	0.492	0.206	0.263
R4	6	0.073	0.036	0.015	0.312	2.501	0.929	0.829	0.030	0.147	1.283	0.678	0.869
R4	7	0.080	0.043	0.029	0.297	2.157	0.949	0.828	0.047	0.144	1.248	0.637	0.810
R4	8	0.073	0.036	0.015	0.312	2.501	0.929	0.829	0.030	0.147	1.283	0.678	0.869
R4	9	0.075	0.039	0.022	0.297	2.269	0.917	0.809	0.038	0.148	1.235	0.641	0.819
R4	10	0.080	0.043	0.029	0.297	2.157	0.949	0.828	0.047	0.156	1.248	0.637	0.810
R5	1	0.032	0.020	nq	0.246	0.391	0.305	0.339	0.064	0.043	0.129	0.097	0.136
R5	2	0.051	0.017	0.011	0.094	0.500	0.493	0.571	0.103	0.046	0.596	0.478	0.559
R5	3	0.119	0.043	0.018	0.528	0.284	0.167	0.118	0.042	0.029	0.230	0.112	0.123
R5	4	0.024	0.017	nq	0.111	0.248	0.112	0.181	0.020	0.020	0.131	0.099	0.174
R5	5	0.018	0.021	nq	0.149	0.357	0.172	0.208	0.029	0.032	0.154	0.153	0.194
R5	6	0.085	0.032	0.029	0.519	0.604	0.394	0.340	0.089	0.091	0.421	0.241	0.351
R5	7	0.084	0.031	0.040	0.169	0.315	0.123	0.154	0.021	0.021	0.174	0.110	0.131
R5	8	0.089	0.064	0.034	0.284	2.018	0.588	0.555	0.057	0.204	0.968	0.483	0.479
R5	9	0.015	0.024	nq	0.094	0.567	0.216	0.302	0.042	0.069	0.321	0.175	0.231
R5	10	0.019	0.020	0.003	0.073	0.288	0.088	0.111	0.015	0.023	0.111	0.059	0.088
R5	11	0.101	0.029	0.016	0.292	1.337	0.018	0.022	0.012	0.002	0.078	0.019	0.008
R5	12	0.065	0.033	0.015	0.310	2.895	0.997	0.911	0.042	0.153	1.707	0.821	0.907
R5	13	0.059	0.015	0.018	0.129	0.633	0.238	0.231	0.015	0.026	0.306	0.186	0.205
R5	14	0.063	0.011	nq	0.035	0.083	0.058	0.040	nq	0.006	0.052	0.036	0.029
R5	15	0.085	0.018	0.009	0.208	0.958	0.346	0.344	0.027	0.032	0.461	0.281	0.303
R6	1	0.053	0.022	0.009	0.173	0.080	0.088	0.161	0.020	0.025	0.321	0.059	0.119
R6	2	0.296	0.392	0.310	1.271	1.117	0.735	0.791	0.072	0.075	0.427	0.394	0.760
R6	3	0.079	0.057	0.126	0.186	0.505	0.240	0.551	0.031	0.032	0.419	0.203	0.295
R6	4	0.063	0.045	0.287	0.305	0.649	0.317	0.231	0.037	0.045	0.425	0.199	0.198
R6	5	0.120	0.125	0.252	0.798	0.599	0.338	0.395	0.052	0.037	0.423	0.202	0.295
R6	6	0.137	0.227	0.196	0.202	0.558	0.235	0.175	0.026	0.039	0.425	0.172	0.351

[a]R2, biomass repitched twice in the brewing process; R4, biomass with four serial repitchings; R5, biomass with five serial repitchings in brewing process; R6, biomass with six serial repitchings in brewing process. [b]nq, not quantified.

dry yeast cell. The total protein content of spent yeast biomass samples, obtained by using the Kjeldahl method, ranged between 45.8 and 49.4% (w/w dry yeast cell). Thus, >95% of the cell walls were estimated to be ruptured by the glass bead process, which is in agreement with the literature.[32] Glass bead disruption during 10 min was preferred due to its speed and higher efficiency yield.

The purities of hydrolyzed RNA extracts (coded 1, 2, and 3) were similar ($A_{260/280}$ = 1.8). However, extract 1 presented a lower content of RNA products (5.06%). Extracts 2 and 3 were prepared under similar conditions except temperature, respectively, 37 and 60 °C, but the RNA products of extract 3 (5.93%) were significantly higher than that of extract 2 (5.30%). Thus, this hydrolysis procedure was selected for further studies.

Validation of HILIC-DAD Methodology for Analysis of Yeast Extract Nucleoside and Nucleotide Composition. Different concentrations of buffer in the eluent were examined (10, 25, 50, and 100 mM). Appropriate retention was achieved using 100 mM ammonium acetate. The use of buffer pH in the range of 2.5−7.0 was also investigated, pH 2.5 being the most appropriate. Under the chromatographic conditions optimized, 11 of 15 compounds under study were separated; xanthosine and cytidine (peaks 3 and 4) and 5′CMP and 3′GMP were coeluated (peaks 12 and 13) (Figure 1A).

The analytical performance of the HPLC method for reliable quantification of nucleosides and monophosphate nucleotides in yeast extracts was evaluated. The results obtained are summarized in Table 1. The calibration curve of each compound was established using seven concentration levels

in triplicate. All of the calibration curves presented excellent linear regression. Linearity was maintained over the concentration ranges of 0.02−125 μg/mL for adenosine; 0.02−166 μg/mL for uridine, xanthosine, and cytidine; 2.0−125 μg/mL for guanosine; 0.2−125 μg/mL for 2′AMP and 2′GMP; 0.04−125 μg/mL for 3′AMP, 5′AMP, 5′IMP, and 5′GMP; 0.04−166 μg/mL for 5′UMP, 5′CMP, and 5′XMP; and 0.05−125 μg/mL for 3′GMP. The reliability of the method in terms of LOD and LOQ, precision, and accuracy was also studied. Except for guanosine, LOD and LOQ were less than 0.08 and 0.27 μg/mL, respectively. The precision of the method was good, because the intraday coefficient of variation (CV) ranged between 2.77 and 13.8% and the interday CV ranged between 2.09 and 14.8%. Method performance parameters were in close levels when compared with those published by Ranogajec et al.[5] for analyses of monophosphate nucleotides from mushrooms.

Recovery was evaluated to examine extraction procedure efficiency at three concentration levels. The mean recoveries for each compound are presented in Table 1. These results confirmed that the matrix composition is complex, causing interference effects.[23]

Influence of Serial Yeast Repitching on Nucleosides and Nucleotides Extracts Composition. The RNA content of the yeast extracts varied between 4 and 8% (dry weight) (CV = 34.7%), which is in agreement with yeast RNA content described by other authors.[4,7,32] Biomass groups with two and four reuses showed highest RNA mean content (8.97 and 7.25%), followed by R5 and R6 (5.84 and 4.43%). RNA levels in yeast are closely correlated with specific growth rate.[9] The decrease of total RNA content observed in R5 and R6 can be related to a slower yeast growth after five serial repitchings.

Under the RNA hydrolysis conditions selected previously, five nucleosides and nine nucleotides were identified in yeast extracts: adenosine, uridine, xanthosine, cytidine, guanosine, 2′AMP, 3′AMP, 5′AMP, 5′UMP, 5′IMP, 2′GMP, 5′CMP, 3′GMP, and 5′GMP. Figure 1B shows a typical chromatogram of separation of these compounds in yeast extracts. Peaks 1−5 were identified as nucleosides, and peaks 6−14 were from nucleotides. As expected, yeast extracts contained an array of ribonucleases, nucleotidases, and nucleosidases that contributed to the hydrolysis of RNA. Compositions of RNA degradation products observed in the 37 yeast samples organized by yeast repitching are shown in Table 2. Ribonucleotides accounted for 71.1−88.2% of the RNA products; the predominance of ribonucleotides suggests that nucleases were mainly responsible for the hydrolysis of RNA. The potential flavor enhancer compounds (5′GMP, 5′IMP, and 5′AMP) represented between 25 and 32% of total nucleotides, whereas 2′AMP was the most abundant compound followed by 2′GMP; these two compounds represented approximately 50% of total nucleotides. These results are in agreement with Zhao and Fleet,[7] who found that ribonucleotides represented 65−98% of total RNA hydrolysis products and ribonucleosides were 3−14% of the products obtained under optimized autolysis conditions. According to these authors, at 50 °C and pH 7, RNA hydrolysis released primarily 3′-ribonucleosides (55%) but also 2′-ribonucleotides (27%), 5′-ribonucleosides (18%), and smaller amounts of ribonucleosides and bases.[7]

A similar nucleotide profile expressed as relative percentage of nucleotides was observed for R2, R4, R5, and R6 groups as shown in Figure 2A. Comparison between R2, R4, R5, and R6 shows that the relative percentage of nucleotides is similar in all yeast extracts; thus, serial repitching did not influence the

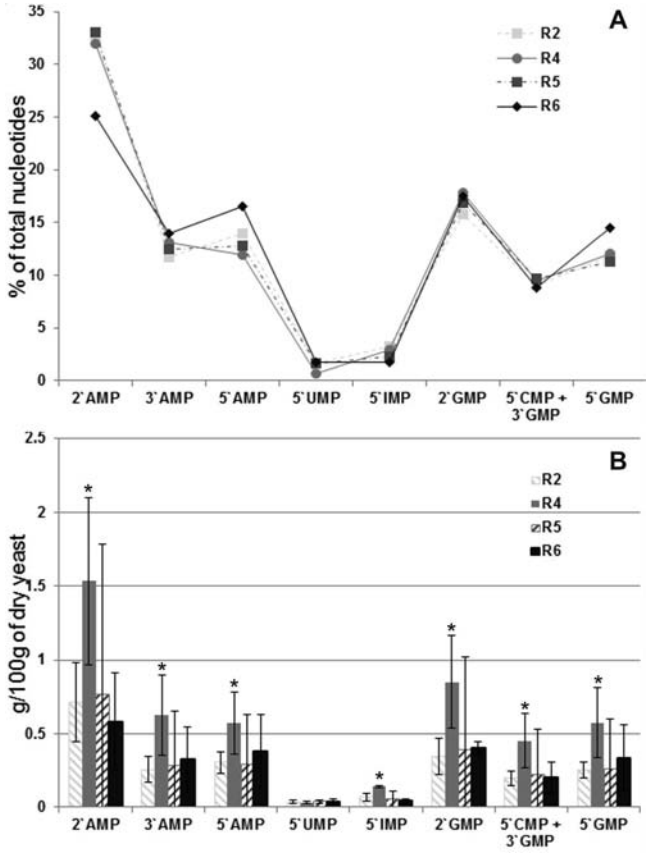

Figure 2. Nucleotide composition of brewer's spent yeast extracts: R2, biomass repitched twice in the brewing process (6 samples); R4, biomass with four serial repitchings (10 samples); R5, biomass with five serial repitchings in brewing process (15 samples); R6, biomass with six serial repitching in brewing process (6 samples); (A) relative percentage of nucleotides; (B) mean nucleotide content. ∗ indicates significant differences (ANOVA and Duncan's post hoc, $p < 0.05$).

profiles of RNA hydrolysis products. Within each group of repitched yeast (R2, R4, R5, and R6) great variability ($p < 0.05$) of yeast extract composition with regard to RNA degradation products was observed for all nucleotides (Figure 2B). However, ANOVA and Duncan's post hoc test indicated significant quantitative differences ($p < 0.05$) between groups with different repitchings. R4 samples presented higher nucleotide content.

The proposed procedure can be scaled up to an industrial process easily and with low investment to produce flavor enhancers. However, the predominance of 3′-ribonucleotides in the degradation products has practical implications because only 5′GMP and 5′IMP have flavor-enhancing properties and 5′AMP is precursor of the well-known flavor enhancer 5′IMP. The use of inhibitors for 3′-nucleotide forming RNases to increase 5′-nucleotides yield is a field to explore.

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ABBREVIATIONS USED

AMP, adenosine monophosphate; UMP, uridine monophosphate; IMP, inosine monophosphate; GMP, guanosine monophosphate; CMP, cytidine monophosphate; XMP, xanthosine monophosphate; MSG, monosodium glutamate; R2, biomass repitched twice in the brewing process; R4, biomass with four serial repitchings; R5, biomass with five serial repitchings; R6, biomass with six serial repitchings

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