Release of Lipids during Yeast Autolysis in a Model Wine System

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The release of lipids during the aging of sparkling wines in contact with yeast can influence wine sensory attributes and, especially, foam characteristics. Model systems allow study of the autolysis process in a reasonable period of time compared to natural conditions, at which it can last several months. In this paper, the release of the different classes of lipids during the autolysis of three commercial yeast strains in a model wine medium has been monitored. Due to the absence of accurate quantitative methods, an HPLC method for separating and quantifying the different neutral and polar yeast lipid classes was developed. Lipids were eluted through a YMC PVA-Sil column with a complex solvent mixture. Detection was carried out with a light-scattering detector. The yeasts were suspended in the model wine buffer and incubated at 30 °C for up to 12 days. A release of triacylglycerols, 1,3-diacylglycerols, 2-monoacylglycerols, free fatty acids, sterol esters, and sterols was observed over the first 2 days, a period that corresponded to the maximum loss of yeast viability. A decrease in most of these lipids was observed from day 2, possibly indicative of the release of yeast hydrolytic enzymes due to the breakdown of the cell wall. Phospholipids were not detected in any of the autolysates. The mean lipid content in the autolysates as a percentage of the total lipid content in the yeasts was 8.6% for sterol esters, 3.8% for sterols, 2% for triacylglycerols, and <2% for 1,3-diacylglycerols and free fatty acids.

Keywords: *Wine; lipid classes; yeast autolysis; Saccharomyces cerevisiae; light-scattering detection*

INTRODUCTION

One of the most important processes taking place during the aging of sparkling wines in contact with yeast is yeast autolysis, which releases substances that exert a major impact on the sensory characteristics of the wine. Few studies have dealt with the release of lipids, notwithstanding the influence that lipids may have on the characteristics of the foam, one of the most salient sensory attributes of quality sparkling wines. Certain authors have reported relationships between the total contents of certain fatty acids (Pueyo et al., 1995) and other lipids (Dussaud et al., 1994) and the foaming characteristics of sparkling wines and the base wines. Certain microbial lipopeptides have been found to be good foaming agents in model systems (Razafindralambo et al., 1998). Lipids may also affect wine flavor in that the fatty acids released may give rise to volatile components with low sensory thresholds such as esters, ketones, and aldehydes (Charpentier and Feuillat, 1993).

The main reason for the dearth of quantitative data on the lipids released during yeast autolysis is the absence of methods for quantifying the different families of lipids present in yeast autolysates. Some of the components of the lipid fraction in sparkling wines, namely, free and total fatty acids and free and esterified sterols, have been quantified using gas chromatography (GC) (Ferrari et al., 1987). Silva et al. (1990) employed nuclear magnetic resonance (NMR) to analyze the phospholipids in wines aged in contact with yeast. In a study of a second fermentation in a white wine, Troton et al. (1989) separated the families of lipids by thin-layer chromatography (TLC) and then analyzed the corresponding fatty acids using GC. Hernawan and Fleet (1995) used TLC to determine the contents of the different classes of lipids released in the autolysis of three strains of wine-making yeasts in an aqueous medium (phosphate buffer, pH 4.5). Because accurate quantitative analysis is not possible when TLC is used, the results were expressed as the percentage decrease with respect to the initial lipid content of the yeasts.

High-performance liquid chromatography (HPLC) employed in combination with newly developed light-scattering detectors represents a major advance in one-step analysis of the different lipid families. This methodology has substantially improved the sensitivity and reproducibility of analysis of the lipids over the levels afforded by TLC while at the same time making possible accurate quantification of the lipids. The first separations were performed using normal-phase columns (Christie, 1985; Christie and Morrison, 1988; Lutzke and Braughler, 1990; Redden and Huang, 1991), but because of the poor reproducibility achieved using those columns, they have been widely replaced by columns packed with chemically bonded silica columns, for instance, aminopropyl-, diol-, or cyanopropyl-bonded stationary phases (Christie and Urwin, 1995).

Model systems have been used to study complex processes generally of long duration, such as the aging of sparkling wines in contact with yeast. The objective of this study was to monitor the release of the different classes of lipids during yeast autolysis in a model wine medium. To that end, an HPLC method was developed for separating and quantifying the neutral lipids (triacylglycerols, diacylglycerols, monoacylglycerols, free

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Table 1. Gradient for the Elution of Lipids

time (min)	% solvent A	% solvent B	% solvent C	flow rate (mL/min)
0	100	0	0	0.4
5	80	20	0	0.4
15	48	47	5	0.6
20	48	42	10	0.6
32	40	25	35	1.0
32.1	30	70	0	1.0
35	100	0	0	1.0
37.9	100	0	0	1.0
38	100	0	0	0.4

and esterified sterols, and free fatty acids) and the polar lipids (phospholipids) that may be present in autolysates of strains of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Yeast Strains and Autolysis. Three different commercial active dry yeasts were used, *S. cerevisiae* EC1118 supplied by Lallemand (Madrid, Spain) and *S. cerevisiae* BC and PM supplied by Agrovin S.A. (Madrid, Spain). The yeasts were rehydrated following the manufacturer's instructions. After rehydration, yeast cells were harvested by centrifugation at 7000*g* for 10 min at room temperature and washed three times with 0.9% NaCl. The washed yeasts were suspended (10 g of yeast dry weight/L) in the model wine buffer (Feuillat, 1987). The wine buffer contained ethanol (10%, v/v), tartaric acid (4 g/L), malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate (0.025 g/L). pH was adjusted to 3.0 with sodium hydroxide. Autolysis was conducted by incubating the cell suspensions at 30 °C for up to 12 days with shaking (G25 shaker, New Brunswick Scientific Co., Inc., Edison, NJ) at 100 rpm. During autolysis, samples of the suspensions were removed for analyses of dry weight, cell viability, and lipid composition.

Dry Weight of Yeast. Samples (10 mL) of the autolysates were vacuum-filtered through a preweighed 0.45 μm membrane filter. The residue of cells was dried at 120 °C for 24 h. The weight of the cells was determined by subtracting the weight of the filter.

Cell Viability. Viability was determined by plate counting on malt extract agar (ADSA micro, Spain). Plates were incubated at 30 °C for 3 days. These analyses also provided a check on culture purity.

Extraction of the Lipids from the Yeast. Washed yeasts (0.5 g of dry weight) were suspended in 20 mL of methanol and broken by vortex stirring with 40 g of glass beads (2 mm diameter), 8 × 60 s, with ice cooling. Chloroform (40 mL) was added and mixed with a magnetic stirrer for 2 h at room temperature. The suspension was then centrifuged (5 min, 400*g*), and the residue was re-extracted with 60 mL of chloroform/methanol (2:1, v/v) for 2 h. This was repeated twice. The combined organic solutions were dried with anhydrous sodium sulfate, filtered, and concentrated to dryness in a rotatory evaporator at <35 °C. The residue was dried with a nitrogen stream and was kept under nitrogen atmosphere until it was dissolved in 0.4 mL of chloroform containing 500 mg/L of 2,6-di-*tert*-butyl-4-methylphenol (BHT), for HPLC analysis. Samples were prepared and analyzed in duplicate.

Extraction of the Lipids from the Autolysates. The autolyzed suspension (200 mL) was centrifuged (2700*g*, 5 min). The supernatant was filtered through a 0.45 μm membrane filter and was concentrated 10-fold in a rotary evaporator at <35 °C. Lipids were extracted with 20 mL of chloroform/methanol (2:1, v/v), four times. The organic fractions were prepared for HPLC analysis as described above.

HPLC Analysis. *Chromatographic Apparatus and Conditions.* The HPLC system consisted of three Beckman (Fullerton, CA) M116 pumps, a Waters (Milford, MA) M717Plus automatic injector, and an ACS model 750/14 mass detector (Applied Chromatography Systems, Macclesfield, U.K.). The settings of the mass detector were as follows: attenuation range, 16; photomultiplier sensibility, 5; time constant, 5 s; evaporator set, 40; internal air pressure, 1.96 bar. Equipment control and obtention and processing of data were carried out using the Beckman Gold System program. Lipids were separated according to the method proposed by Christie and Urwin (1995) as modified by Christie (personal communication, 1997). A 100 × 3 mm column packed with 5 μm YMC PVA-Sil (YMC Europe GmbH, Schermbeck, Germany) was used. The column was housed in a column oven maintained at 25 °C. Three different solvents were prepared: solvent A, isohexane/methyl *tert*-butyl ether (98:2); solvent B, 2-propanol/butanone (50:50); solvent C, propanol/acetonitrile/methanol/water/*N*-ethylmorpholine/acetic acid (56:21:14:9:0.42:0.144). The elution gradient is described in Table 1. Lipid class identification and quantification were done by using the method of the external standard. Quantitation of peak areas was carried out with a deconvolution program (Peakfit V3.11B; Jandel Scientific, GmbH, Erkrath, Germany).

Calibration. The standards used for the quantitation of the different lipid classes were tristearoylglycerol for triacylglycerols; 1,3-dipalmitoylglycerol for 1,3-diacylglycerols; 1,2-dipalmitoylglycerol for 1,2-diacylglycerols; 1-monostearoylglycerol for 1-monoacylglycerols; 2-monopalmitoylglycerol for 2-monoacylglycerols; stearic acid for free fatty acids; cholesteryl palmitoleato for sterols esters, and cholesterol for sterols. All of these standards were purchased from Sigma (St. Louis,

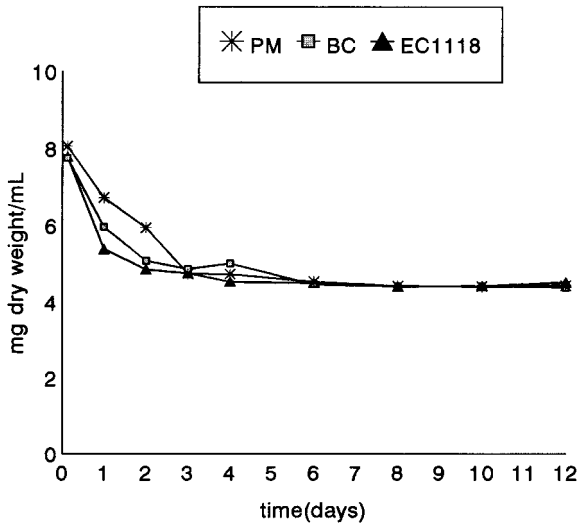

Figure 1. Evolution of dry matter (mg/mL) during the autolysis experiment.

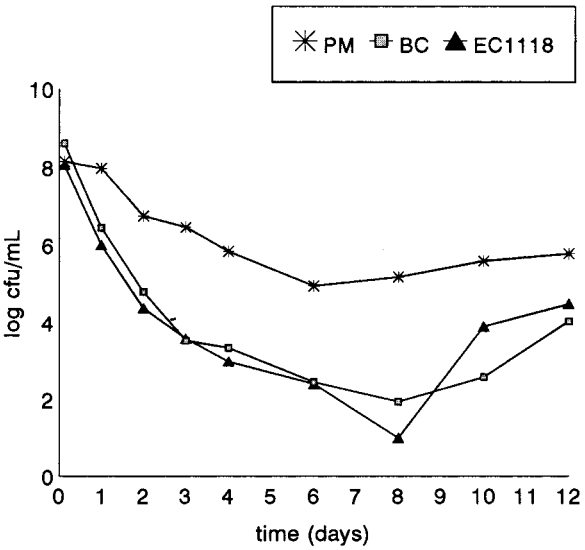

Figure 2. Evolution of yeast population (log cfu/mL) during the autolysis experiment.

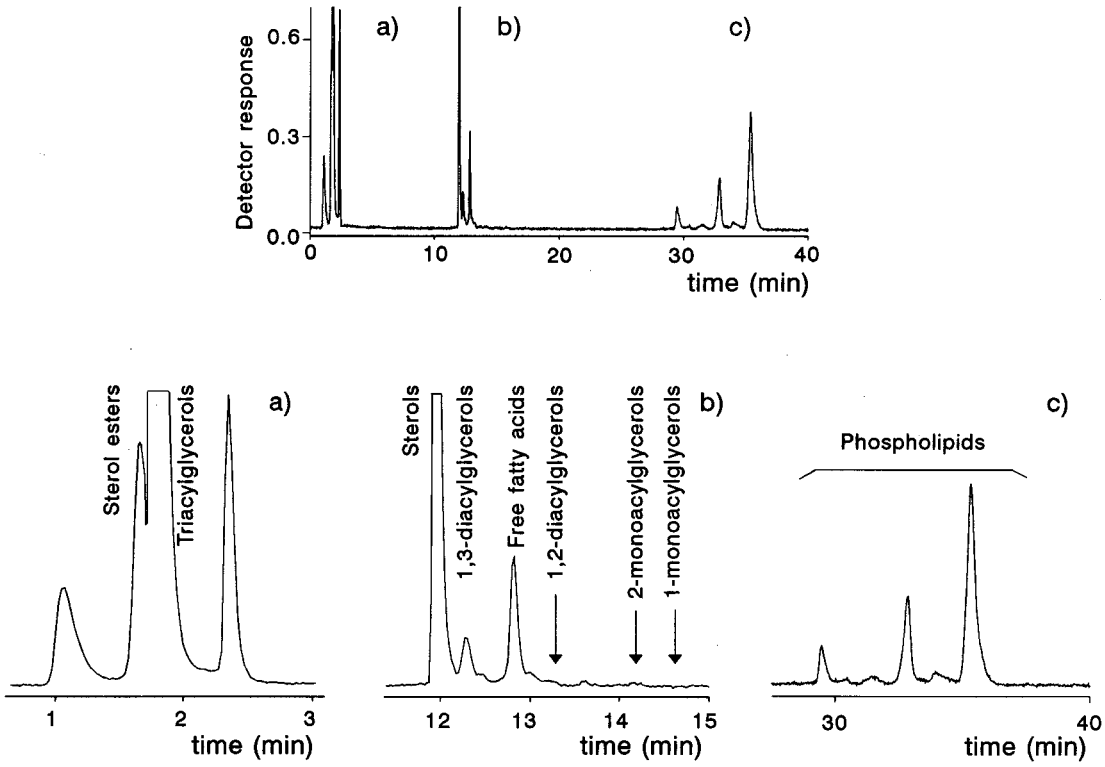

Figure 3. HPLC profile of the lipid fractions from the BC yeast extract. Parts (a), (b), and (c) in the top panel are amplified in the lower panel.

Table 2. Polynomial Regresion ($y = a + bx + cx^2 + dx^3$) for Lipid Area Response versus Concentration[1]

compound	range (μg/L)	a	b	c	d	R^2	s	n	$s/\bar{y}$ (%)
sterol esters	0.26−2.08	−41.691	281.883	−154.387	43.022	0.998	3.87	13	2.77
triacylglycerols	0.5−4.09	7.7156	83.454	ns	ns	0.949	25.80	14	14.7
sterols	0.025−0.20	−4.317	241.399	902.180	ns	0.999	1.09	12	2.86
1,3-diacylglycerols	0.025−0.10	−3.715	187.121	ns	ns	0.997	0.32	12	5.15
free fatty acids	0.06−0.50	−17.672	459.469	−929.500	1582.019	0.997	4.23	14	5.50
1,2-diacylglycerols	0.035−0.14	0.640	ns	1555.860	−6978.910	0.981	0.58	15	8.76
1-monoacylglycerols	0.025−0.10	−8.100	577.6	−11706.7	89881.8	0.998	0.45	14	5.46
2-monoacylglycerols	0.06−0.26	0.985	−39.199	1405.670	ns	0.999	0.52	14	1.62

[1] R^2, coefficient of determination; s, standard deviation of residuals; n, number of data points; $s/\bar{y}$, standard deviation of residual (s) as a percentage of the mean response ($\bar{y}$); ns, nonsignificantly different from zero ($p < 0.05$).

MO). An extract containing phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine, which was kindly provided by Dr. González-Rodríguez (García-Guerra et al., 1996), was used as standard for phospholipid identification. A statistical program (Statistica for Windows, release 5.1, Statsoft Inc., Tulsa, OK, 1996) was used for data processing.

RESULTS AND DISCUSSION

Biomass Analysis. Figure 1 graphically represents the dry weight of the yeasts over the 12 days that the experiment lasted. The three strains followed very similar trends. The cells lost ~40% of their weight during the first 3 days. Weight loss slowed between days 3 and 6 (a further 3% loss), and no further changes in dry weight were observed after day 6.

Cell Viability. At the outset of the experiment there were $\sim 10^8$ colony-forming units (cfu). The decrease in the number of viable cells in the early days of the experiment was more pronounced for strains EC1118 and BC than for strain PM (Figure 2). Thus, on the third day of incubation, the number of viable cells was $\sim 10^4$ for strains EC1118 and BC compared with $\sim 10^6$ for strain PM. The number of viable cells increased from day 6 in the case of strain PM and from day 8 in the cases of strains EC1118 and BC, the increase being more marked for these two latter strains. These findings can be interpreted as growth of the most resistant cells using the biomass released by the autolyzed cells as nutrients.

HPLC Separation and Quantitation of Lipids. Figure 3 presents a chromatogram of the total lipid extract from one of the yeast strains used. The peaks identified in the chromatogram included sterol esters, triacylglycerols, sterols, 1,3-diacylglycerols, free fatty acids, and phospholipids. The retention times for lipid components that were not present in the sample, 1,2-diacylglycerols, 2-monoacylglycerols, and 1-monoacylglycerols, are also given. We have previously seen that lipids in the same family, although composed of different fatty acid chains, had the same retention times. Unlike TLC (Troton et al., 1989; Hernawan and Fleet, 1995), HPLC was able to separate 2- and 1-monoacylglycerols. It was not possible to improve the separation of the sterol esters and triacylglycerols because of the similar polarities of both classes of substances.

Light-scattering detector response to lipid concentration is not always linear, and hence polynomial regression analysis of response versus concentration is nec-

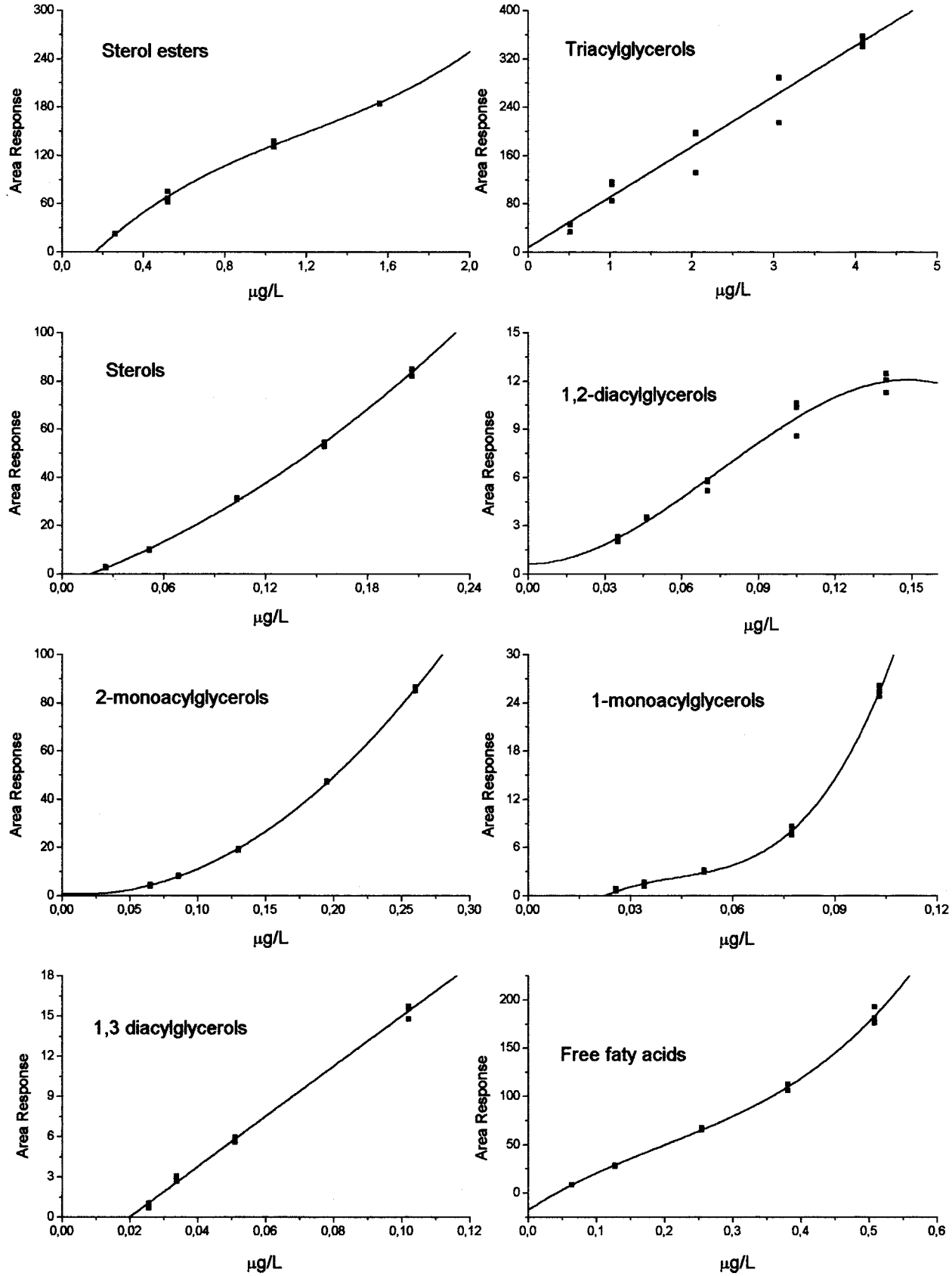

Figure 4. Calibration curves of individual lipids (detector response versus lipid concentration).

essary (Christie, 1985; Redden and Huang, 1991). The calibration curves for the lipid classes detected in the autolysates were fitted to a third-degree polynomial equation, and the significance of each coefficient was tested (Table 2). Equations that contained coefficients that were not statistically different from zero (data not shown) were recalculated with such coefficients omitted. Figure 4 plots the experimental data points and the theoretical curves for each class of lipid considered. The values of the coefficients of determination (R^2) (Table

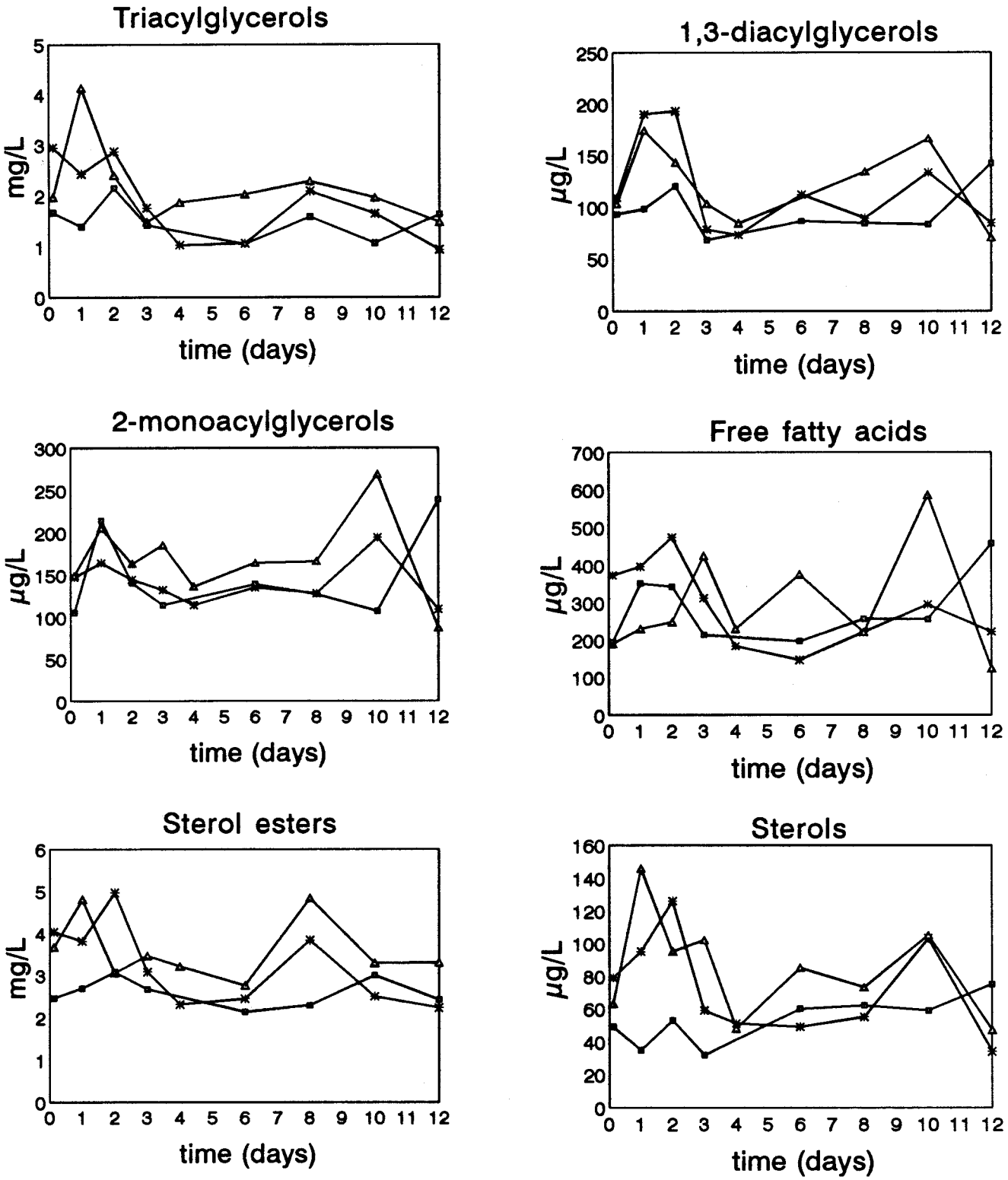

Figure 5. Evolution of the lipid content in the autolysates for the three yeast strains studied: PM (∗), BC (■), and EC1118 (▲).

2) were >0.94 and indicated that the fits were acceptable, with a standard deviation of residuals as a percentage of the mean response ($s/\bar{y}$) <6% for six of the eight classes of lipids quantified. The largest prediction error (14.7%) was for the triacylglycerols, which, as shown in the chromatogram in Figure 3, could not be adequately separated from the sterol esters. Repeatability of the extraction process ($n = 3$) as measured by the relative standard deviation for the peak areas was acceptable, with values for the relative standard deviation of 5% for the sterol esters, 3% for the triacylglycerols, 7% for the sterols, 3% for the 1,3-diacylglycerols, and 6% for the free fatty acids.

Release of Lipids during Yeast Autolysis. Triacylglycerols, 1,3-diacylglycerols, 2-monoacylglycerols, free fatty acids, sterol esters, and sterols were present in the media in which yeast autolysis was taking place. Neither 1-monoacylglycerols nor phospholipids were detected in any of the autolysates, even though they are present as lipid constituents of yeast. The absence of

phospholipids in the autolysates was confirmed by ^{31}P NMR (unpublished data). Other authors (Hernawan and Fleet, 1995) have not detected phospholipids in yeast autolysates. Concentrations of the different classes of lipids that were detected in the autolysates varied considerably (Figure 5). Sterol esters and triacylglycerols were the major lipid fractions present (from 1 to 5 mg/L of autolysate). The values for the free fatty acids, sterols, diacylglycerols, and monoacylglycerols were approximately an order of magnitude lower (between 100 and 600 μg/L). The lipid content of the autolysates of strain BC was lower than that of autolysates of strains EC1118 and PM.

These variations in the concentrations of triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids were similar to those reported by Troton et al. (1989) during the second fermentation of a champagne wine. Pueyo et al. (1995) reported mean values of 271 μg/L for free fatty acids and 1486 μg/L for total fatty acids in sparkling wines.

The values for the contents of the different classes of lipids in the autolysates followed very similar trends (Figure 5). Lipids were detectable in the autolysis medium from the very first sample (after 5 h). Release of lipids continued over the first 2 days with slight variations according to the class of lipid and the yeast strain concerned. Those same first 2 days were also the period of maximum loss of yeast viability (Figure 2). This initial release of lipids may be attributed to the action of the hydrolytic yeast enzymes on the cell wall components, which thereby increases cell wall porosity and facilitates the release of autolysis products into the surrounding medium (Charpentier and Feuillat, 1993). A decrease in most lipids was observed from day 2, possibly indicative of the breakdown of the cell wall with the concomitant release of hydrolytic enzymes and thus of a more advanced stage of autolysis. A second release of lipid components was observed on days 8−10; this was more pronounced in the cases of sterols, sterol esters, free fatty acids, and monoacylglycerols and less pronounced in the case of triacylglycerols. That period corresponded with a recovery in yeast viability (Figure 2). In a study of changes in the lipid fractions during a second fermentation in a sparkling wine, Troton et al. (1989) observed an increase in triacylglycerols during the yeast division phase, followed by a subsequent decrease in the triacylglycerols during the stationary phase with a concomitant increase in the 1,3-diacylglycerols, monoacylglycerols, and free fatty acids. Phospholipases have been implicated in autolytic reactions in *S. cerevisiae* (Hernawan and Fleet, 1995), although the role of other lipolytic enzymes in autolysis has not received sufficient attention.

To determine the percentage of the total lipid content in the yeasts that was released, the contents of the different lipid families that had been identified in the autolysates were also determined in the initial yeasts (Table 3). In the three strains studied, triacylglycerols (0.836−0.981% dry weight) and sterol esters (0.337−0.411% dry weight) were the major lipids present. Relatively high concentrations of free fatty acids (0.128−0.215% dry weight) were recorded. 1,3-Diacylglycerols were not detected in the initial yeast of the EC1118 strain, whereas 1-monoacylglycerols were not found in the initial yeast of the BC and PM strains. 1,2-Diacylglycerols and 2-monoacylglycerols were not detected in the initial yeast of any of the strains considered. The literature data concerning the composition of the lipid families in yeasts are highly variable. Values reported have been expressed in many different forms, making comparison extremely difficult. The values found in this study can be assumed to fall within the ranges published for *S. cerevisiae* in a review of yeast lipid composition by Rattray (1988) and for baker's yeast by Murakami et al. (1996). The mean lipid contents in the autolysates as a percentage of the total lipid content in the yeasts were 8.6% for sterol esters, 2% for triacylglycerols, 3.8% for sterols, 1.5% for 1,3-diacylglycerols, and 1.7% for free fatty acids.

Table 3. Lipid Composition (Percent Dry Weight) of the Yeast

compound	strain EC1118	strain BC	strain PM
sterol esters	0.358	0.411	0.337
triacylglycerols	0.950	0.981	0.836
sterols	0.015	0.018	0.025
1,3-diacylglycerols	nd[a]	0.079	0.069
free fatty acids	0.128	0.215	0.200
1,2-diacylglycerols	nd	nd	nd
1-monoacylglycerols	0.058	nd	nd
2-monoacylglycerols	nd	nd	nd

[a] nd, not detected.

In summary, by using HPLC and light-scattering detection the one-step quantitative analysis of the different classes of lipids released in the autolysis of yeasts is possible. Three different stages were observed in the lipid trend during the autolysis of three strains of *S. cerevisiae* carried out in this study. In a first stage, lipids, possibly produced by the hydrolytic action of yeast enzymes on its own cell walls, were released to the surrounding medium. In a second stage, lipid concentration in the autolysates decreased, which may be indicative of the release of yeast hydrolytic enzymes to the medium. In a third stage, the number of viable cells increased and a new cycle of lipid release was observed.

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