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Improved performance of gravitational field-flow fractionation for screening wine-making yeast varieties

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Abstract

Performance of gravitational field-flow fractionation (GFFF) is improved here with respect to the ability to fractionate and distinguish different varieties of wine-making yeast from *Saccharomyces cerevisiae*. A new GFFF channel with non-polar walls has been employed to enhance fractionation selectivity and reproducibility. Since GFFF retention depends from first principles on particle size, Coulter counter measurements were performed in order to compare size distribution profiles with GFFF profiles. From such a comparison, GFFF was shown to be able to reveal differences in yeast cells other than size. This could make use of GFFF for screening different varieties of wine-making yeast towards future quality assessment procedures based on a possible correlation between yeast cell morphology indexes and quality indexes.

Keywords: Field-flow fractionation; Gravitational field-flow fractionation; Wine; *Saccharomyces cerevisiae*; Size distribution analysis

1. Introduction

Active dry wine yeast is currently employed as a starter for commercial wine production. However, there is still a need for methods for screening and characterising different yeast varieties to correlate yeast quality indexes with quality of the final product. In analytical chemistry, quality assessment procedures are often assisted by analytical separation methods. These methods can either give ''fingerprints'' of the analytes and/or reduce sample complexity for further characterisation through uncorrelated, and more specific techniques. Field-flow fractionation (FFF) is a family of separation methods already applied to separate yeast cells (*Saccharomyces cerevisiae*) [1–4]. In these first works, FFF fractograms were found able to point out cell features related to the growth state of the cells. In FFF, separation occurs by differential retention of the analytes in a liquid stream flowing through a thin, empty channel. Separation is structured across the channel thickness, perpendicularly to the liquid stream, by an applied force field or gradient. Various types of FFF techniques such as sedimentation FFF, flow FFF, thermal FFF, and electrical FFF can be sorted according to the nature of the applied force field [5].

Gravitational FFF (GFFF) is a subset of sedimentation FFF that employs perpendicular applica-

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tion of Earth's gravitational field to the channel. This technique can be simply implemented in a standard high-performance liquid chromatography (HPLC) setup. It is soft, low-cost, and its application niche involves micron-size particles of any origin. More specifically, it has been shown particularly successful for separation and further characterisation of cells and microorganisms [6–14]. In two previous papers [3,4], GFFF has been shown for the first time able to fractionate few types of commercial, dry wine-making yeast. However, the GFFF system therein employed was not able to find significant differences in the fractograms from different yeast types. In this work, a new GFFF channel specifically designed to increase compatibility with yeast cells shows able to improve separation performance and quantitative reliability. Because of the higher retention levels achieved with this channel, fractionation selectivity has been increased to a sufficiently high level to find out differences in the fractograms of different yeast varieties. Since retention in GFFF is not only dependent on size but also on other sample indexes and their relevant distributions within the sample (the so-called ''multipolydispersity matrix'' [15]), an uncorrelated, well-established technique for sizing as the electro-sensing zone technique (commonly known as Coulter counter) was employed to compare fractograms to size distributions. The clearly, in some cases drastically different response given by GFFF and Coulter counter indicates the possibility to screen different varieties of wine-making yeast by means of GFFF-sensible morphology indexes different than size. These findings could open up future GFFF-based procedures in the framework of yeast quality assessment.

2. Experimental

2.1. Methods and equipment

The GFFF system here employed was designed differently than in the previous works on yeast [3,4]. The depletion wall was here made of polycarbonate (PC) and the accumulation wall of polyvinylchloride (PVC). The plastic channel was clamped together by means of a properly designed aluminium frame. The use of the aluminium frame, instead of Plexiglas blocks commonly used for glassy GFFF channels (as reported in Refs. [3,4]), showed some technical advantages when used with plastic walls. With the aluminium frame the torque applied to the bolts, when the channel is tightened up, allows for its more homogeneous distribution around the channel contour. The dimensions of the ribbon-like channel were 0.0151 cm thick, 2 cm wide and 30 cm long.

The GFFF channel just replaced the column in a HPLC system HP Model 1050 (Hewlett-Packard, Palo Alto, CA, USA). It was equipped with a quaternary pump, an injector Model 7125 Rheodyne (Cotati, CA, USA), and a variable-wavelength UV–Vis detector. Channel void volume V_0 was measured as 0.831 ml, determined by eluting Na_2CrO_4 (Aldrich, Milwaukee, WI, USA) at 373 nm. The injected amount was always 20 μl, and the injection time 45 s at an injection flow-rate of 0.2 ml min^{-1}. For sample relaxation, stop-flow time was always 6 min. The carrier liquid was 0.5% Triton X-100 in MeOH–water (20:80, v/v), which was found most suitable for GFFF of wine-making yeast [3,4]. All the fractograms were obtained at 330 nm at room temperature with a flow-rate of 0.2 ml min^{-1}.

Coulter counter size measurements were performed using a Multisizer II (Coulter, Hialeah, FL, USA) set for 256-channel analysis. Aperture size was 70 μm (measure rank: from 2 to 60% of the nominal aperture size). Instrumental calibration was performed with Calibration Standard PS Latex, 18.5 μm (Coulter Electronics, Luton, UK). Yeast cells were dispersed before the measurements in the carrier liquid and diluted 1:1000 with the conducting fluid Isoton II solution (Coulter). Because the analytical response from the Coulter counter is proportional to yeast cell volume, results can be expressed in terms of equivalent numbers of cells of a given diameter as though they were spherical in shape. For each yeast type, at least three replicates were considered ($n=3$).

2.2. Wine-making yeast samples

Samples were eight different types of active dry wine-making yeast from *S. cerevisiae*: Fermol bouquet, Cryoaromae, Fermol rouge, Killer D-47, Navarra 33, L1033, Awri 350, and Bourgoblanc. They were supplied by Dr. J. Guasch (Group of Química Analítica Enològica i dels Aliments, Uni-

versity Rovira i Virgili, Tarragona, Spain). All the samples were dispersed using sonication (1 min) at 0.05% (w/v) in the carrier liquid, kept at 4 °C before the analysis and sonicated for 15 s more before injection into the GFFF channel. All injections were made at room temperature. In all cases the sample recoveries, measured as the yeast cell fraction eluted after collecting the whole fractogram, ranged from 70 to 75%, which were calculated from Coulter counter measurements before and after FFF analysis.

3. Results and discussion

In general, cell–surface interactions are known to easily occur if both cells and surface have polar nature [16–18]. Particle–wall interactions in FFF can generally induce poor reproducibility in the fractograms and irreversible adhesion of the sample to the channel walls (the so-called potential barrier effect [19]). With glassy GFFF such interactions were already observed with inorganic particles [20,21] and cells [6]. Because of reduced cell–wall interactions and higher bio-compatibility, non-polar walls were already proposed for FFF of red blood cells (RBCs) [12,22]. Specifically, in GFFF it was first used siliconized glass [6] and, in further works, also PC [23]. In the first paper on GFFF of yeast with a bare glass channel, good run-to-run and day-to-day precision for retention ratio ($R=t_0/t_R$, where t_0 is the void time and t_R the retention time) and peak width values was indeed observed for four different types of yeast (Table 2 of Ref. [3]). However, the found variability (RSD data in Table 2 of Ref. [3]) indicated that differences in R values for the four different types of yeast were not statistically significant. In other words, the different yeast samples could not be distinguished by retention ratio values. Otherwise, all the R values obtained in that first study were relatively high ($0.19 \div 0.22$).

The elution mechanism in GFFF for supramicron-sized particles as yeast cells can be either steric or focusing (hyperlayer). In both steric and focusing (hyperlayer) GFFF the elution order is reversed with respect to the normal elution order in Brownian GFFF [24]. However, since transition from steric to hyperlayer elution mechanism is a continuous process that depends on the experimental conditions and sample features, the retention mechanism in reversed elution has been often referred to as steric/hyperlayer. During the elution in steric/hyperlayer GFFF, the Brownian diffusion of supramicron-sized particles has negligible influence in determining the particle position across the channel. Larger particles protrude to faster streamlines of the parabolic flow profile than small particles do, and they are then swept down the channel earlier than smaller particles (reversed elution order). With increasing size and mobile phase flow-rates, because of the action of opposite forces, known as lift forces [25], the particles actually tend to be lifted away from the accumulation wall to migrate at a given position from the surface of the channel wall, at which they are focused at a narrow layer (hyperlayer). The term ''steric/hyperlayer'' may thus generally identify both the elution order and the general elution mechanism under which GFFF of supramicron-sized particles occurs. The relevant relationship between retention ratio (R) and particle diameter (d) was given by Chmelík as $R = 6s/w$ where w is the channel thickness and $s = [(d/2) + \delta]$, where δ is the average distance of particle surface from the accumulation wall during elution, which is $\delta \geq 0$ [24]. Such an average distance is reached during both the relaxation and elution processes undergone of sample particles. It may be, thus, considered as an elevation index that results from the convolution of all real trajectories of sample particles during the overall migration processes along the channel. From the above expression for retention it can be demonstrated that in case of size-based selectivity ($S_d = |(d\log t_R)/(d\log d)|$ lower than unity (as in GFFF), selectivity values should increase with decreasing R [26]. As a consequence, an increase of size-based selectivity can be sought, in principle, by a reduction in R. This is possible by either increasing channel thickness (w) or decreasing the average distance of sample particles from the wall (s). Such a distance s can be reduced by decreasing δ, which could be reduced in GFFF by either reducing mobile phase flow-rates, using non-polar walls [27] or an organic-modified mobile phase (MeOH–water), as first suggested by Plockcová and Chmelík [28] and as further employed in our previous GFFF of yeast [3]. However, attempts to decrease R values of yeast samples by reducing s with the bare glass channel actually

turned out poor fractograms reproducibility. Otherwise, when a silylated glass channel was used [4], after few sample injections a new silylation process was required to obtain a sufficiently good reproducibility for analytical purposes. Following these results, in order to increase fractionation performance and hopefully obtain differences in the fractogram profiles corresponding to different yeast varieties, in the present work yeast fractionation was performed by seeking lower R values through a thicker channel ($w=0.0151$ instead of the $w=0.0133$ used in Ref. [3]) with a plastic, non polar accumulation wall.

The ability of this new GFFF channel to distinguish different types of yeast is first shown in Fig. 1A and B, where fractograms obtained for some of the analysed yeast varieties are compared. Differences within GFFF profiles for different samples are more evident than in Ref. [3]. Incidentally, some yeast types such as L1033 and Killer D-47 even gave a secondary peak at low retention time, while others gave only one peak. It is also evident that retention is considerably increased with respect to the first GFFF work on yeast [3]. In order to eventually establish whether GFFF had been improved to a sufficient level to distinguish different types of wine-making yeast, an extended comparison was performed within fractograms of all samples analysed. A study of sample homogeneity was first carried out for all the yeast types. This was achieved by sampling yeast in four different parts of the package. The relevant, inter-sample variability range for the obtained R values, calculated as RSD, ranged from 1 to 6%. Three replicates were then analysed for each yeast type. In Table 1 peak area and median values of retention ratio are reported. Percent standard deviation values (RSD) of retention ratio ($R\cdot 10^2$) and area values were calculated for each yeast type. Peak asymmetry factor values (only for the main band) are also reported in Table 1 as an additional index for possible differences found in fractogram features. Good reproducibility was observed in all cases, for both R and area values. Standard deviation spanned from 1.7 to 5.9% for R and from 3.6 and 8.5% for area values. This confirms that the so-obtained GFFF of yeast is sufficiently reproducible for either qualitative or quantitative purposes.

Table 1 also reports the median size values obtained by Coulter counter. According to the relationship between R and size (d) above reported, a decrease in R should correspond to a decrease in size. In fact, if retention values in Table 1 are compared to size values obtained by Coulter counter, correlation is poor for the main band while the trend is even opposite for the secondary band. However, it is generally known that in steric/hyperlayer GFFF the value of the average sample elevation from the accumulation wall (s) depends, at constant experimental conditions (i.e., channel thickness, wall compositions, and mobile phase flow-rate as above described), not only on size but also on particle features other than size, like density, shape, rigidity and other morphology and surface indexes [15]. As a general consequence, direct correlation between retention and size must be expected only for spherical, homogeneous particles. For instance, differences in GFFF profiles without corresponding differences in Coulter counter size distributions were, indeed, already observed with RBCs [6,8]. More specifically, GFFF fractograms of RBCs were different when RBCs were fixed, since fixed RBCs do not significantly differ in size from fresh RBCs, but rather in density and rigidity [12]. Large differences in GFFF have been also recently observed between different strains of *Escherichia coli* of similar size, shape and density, due to the presence or not of fimbriae on the bacterial membrane [29].

In Table 1, different area and peak asymmetry values are found within GFFF profiles from injections of the same amount of different yeast samples. It must be recalled that UV–Vis detection response for dispersed samples is known not to be constant with sample size and, consequently, GFFF profiles do not necessarily represent the real mass distributions of the eluted samples [30,31]. It is however known that for supermicron sized particles, extinction efficiency (which is the particle efficiency in removing light by any mechanism and generate a turbidity signal) can be predicted on the basis of a simplified approach [30]. Otherwise, this model shows that particle extinction efficiency in the measured size range of yeast cells can be predicted only if it is exactly known their size, density and refractive index distributions, as well as the detector acceptance angle. Quantitative GFFF analysis of yeast samples stands, thus, beyond the aims of this

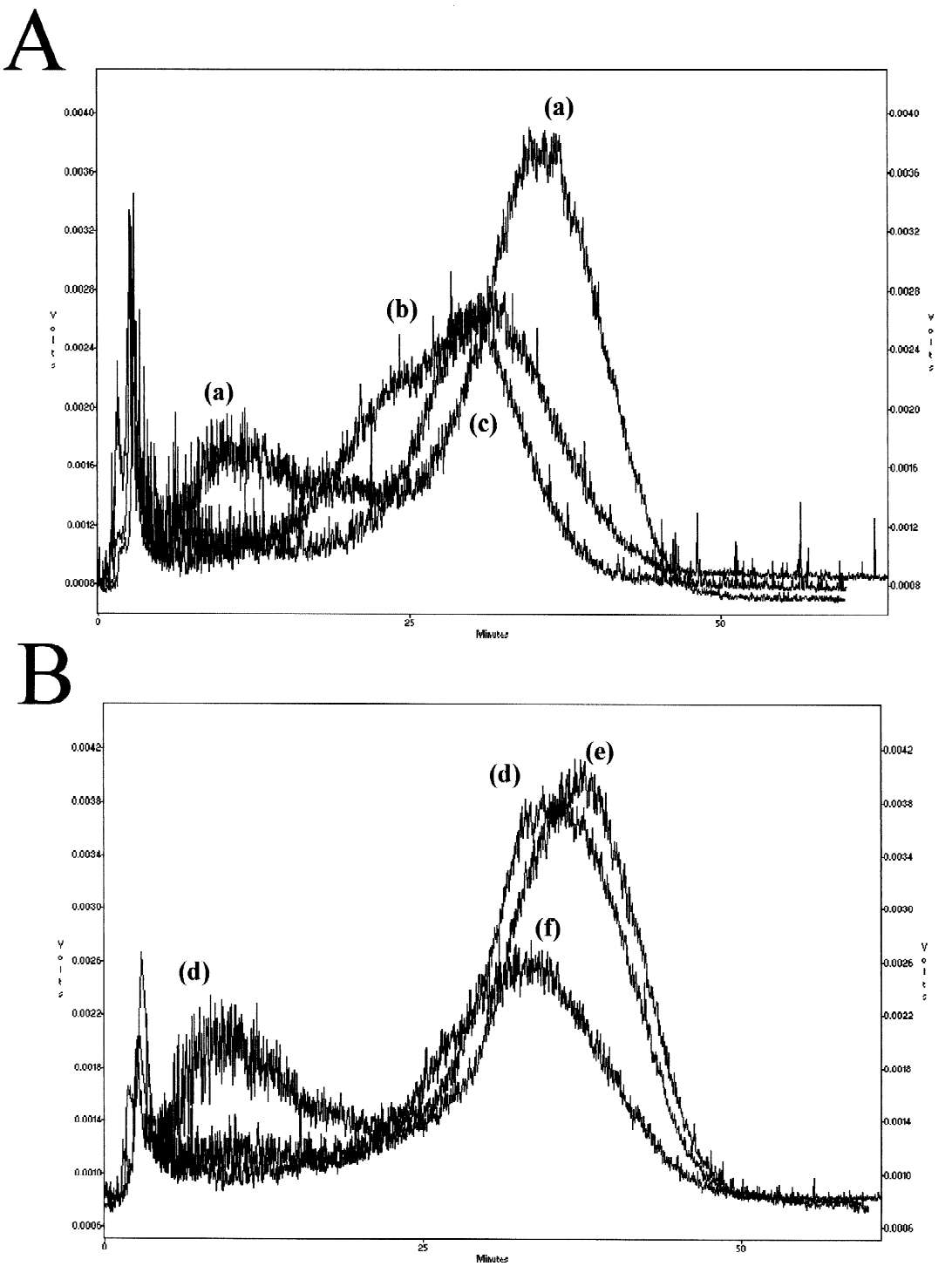


Fig. 1. Fractograms for different yeast types. A: (a) Killer D-47, (b) Fermol bouquet, (c) Fermol rouge. B: (d) L1033, (e) Awri 350, (f) Cryoaromae.

Table 1
Gravitational FFF and Coulter parameters of dry yeast samples

	Coulter data, median size (μm)	Main peak					Secondary peak			
		$A \cdot 10^8$	RSD (%)	$R \cdot 10^2$	RSD (%)	Peak asymmetry factor	$A \cdot 10^7$	RSD (%)	$R \cdot 10^2$	RSD (%)
Cryoaromae	3.82	1.3	3.6	8.9	3.5	0.86	–	–	–	–
Fermol rouge	3.81	1.2	5.8	10.1	4.1	1.15	–	–	–	–
Bourgoblanc	3.68	1.6	5.6	9.1	1.7	1.06	2.5	3.6	24.5	1.3
Fermol bouquet	3.58	0.7	6.3	8.6	5.9	0.50	4.0	3.7	12.5	9.7
Awri 350	3.46	1.7	7.8	7.9	2.5	0.92	–	–	–	–
Killer D-47	3.11	1.9	7.5	8.4	4.1	0.91	2.8	12.6	29.5	2.7
Navarra 33	2.84	2.7	4.7	7.6	2.8	1.31	–	–	–	–
L1033	2.77	1.8	8.5	8.0	2.5	1.16	4.2	1.0	28.0	5.2

R: Retention ratio. *A*: peak area.

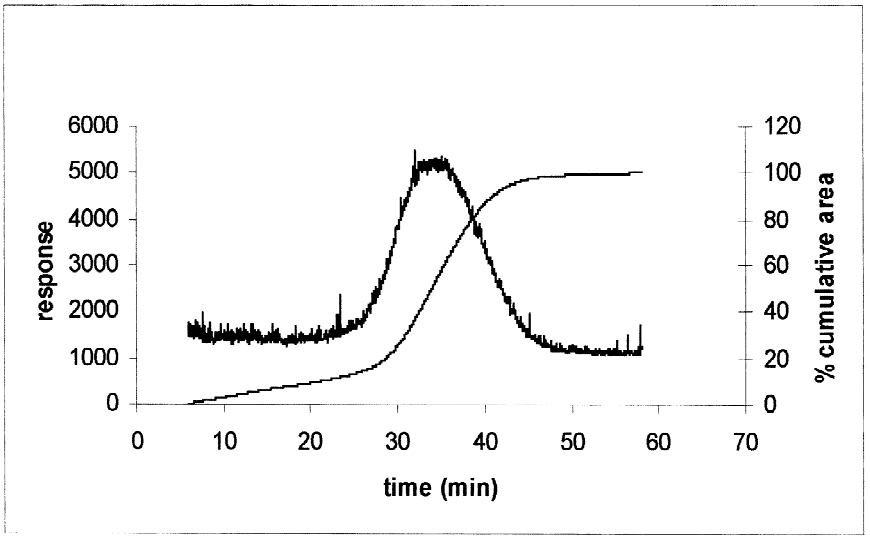
work in which mostly qualitative differences in GFFF profiles were sought to assess the ability of GFFF to screen different yeast types. Quality assessment procedures for wine-making yeast based on quantitative GFFF might likely lie within the aims of further papers.

Care must be therefore taken whenever not only *R* and median size values but also the overall fractogram profiles (and, thus, higher-order distribution moments) are compared to the size distribution moments obtained through techniques whose analytical signals directly give the sample mass or particle numbers as a function of size (as in the case of Coulter counter measurements) [32]. In Figs. 2–3, fractograms and size distribution curves are reported for Navarra 33 and Bourgoblanc, respectively. For better comparison, also the cumulative area and the integrated, size distribution profiles are therein plotted. In Fig. 3 the GFFF profile is bimodal. At low retention time, it is present a second GFFF band that would correspond to a second, distinguished population of yeast cells. In fact, the presence of such a distinguished sub-population is not observed in the relevant particle size distribution. According to the expression for *R* reported above, this sub-population should correspond to particles of higher size. Nevertheless, comparison with the size distribution curve indicates that yeast cells belonging to the sub-population do not significantly differ in size with respect to yeast cells of the main population. They might thus likely differ in other morphology (e.g., shape, flexibility) or surface (e.g., presence of fimbriae) indexes, as well as density, on which GFFF is dependent and selective. These findings should not be considered as a drawback for GFFF-assisted characterisation of yeast samples. In fact, as in the case of GFFF and Coulter counter of RBCs, the present study directly proves that GFFF is sensible to cell features other than size. The use of GFFF can thus yield more information for screening yeast varieties, which can be of practical relevance in the final use of yeast.

4. Conclusion

Several wine-making yeast varieties from *S. cerevisiae* have been effectively fractionated through a specifically designed GFFF channel. High reproducibility of the fractograms (retention ratios and area) has been obtained for all samples. Coulter counter measurements for particle size distribution analysis have been performed for all the yeast types and compared to relevant GFFF fractograms. From such a comparison, fractograms from different yeast types show differences that do not correspond to different cell size. It can be deduced that these differences in the observed fractograms can be ascribed to other morphology or surface indexes of yeast cells. These experimental evidences represent a key feature to make future use of GFFF for quality assessment in yeast-based bio-processes like wine production. This will be possible if some of these cell feature indexes are proved to be related to quality aspects. If a correlation between yeast cell morphology and quality indexes is established, the intrinsic low-cost and easy implementation of GFFF in standard HPLC equipment shall make laboratories

A



B

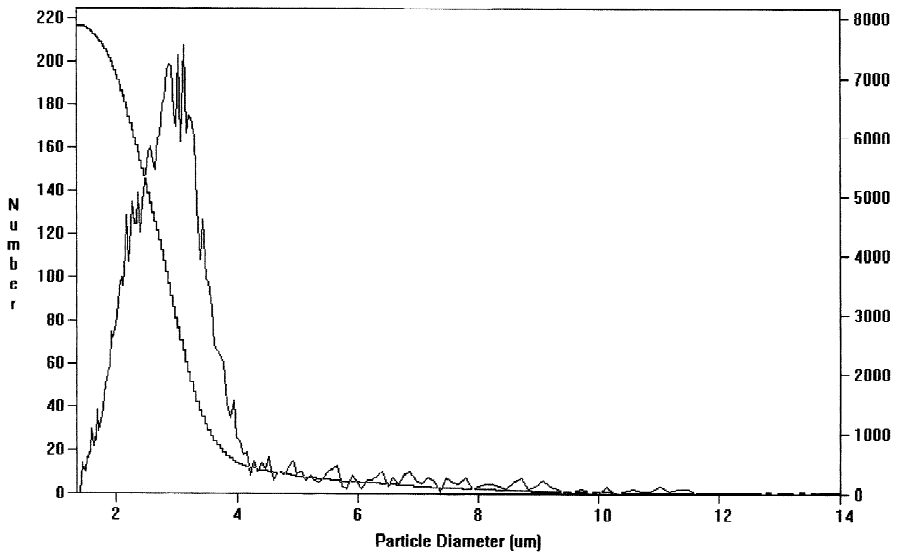
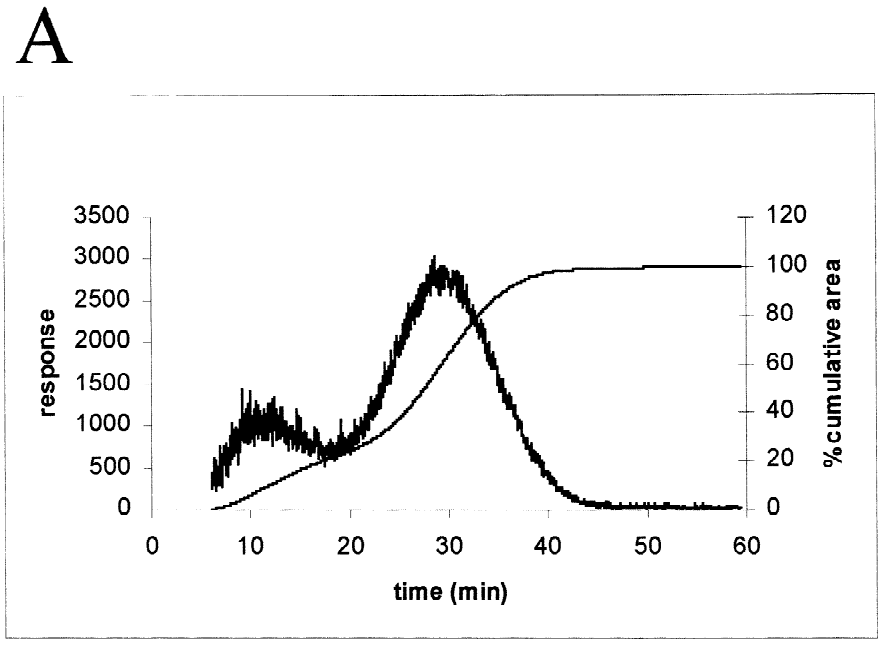


Fig. 2. Fractogram with the cumulative area for Navarra 33 with relevant size distribution by Coulter counter.



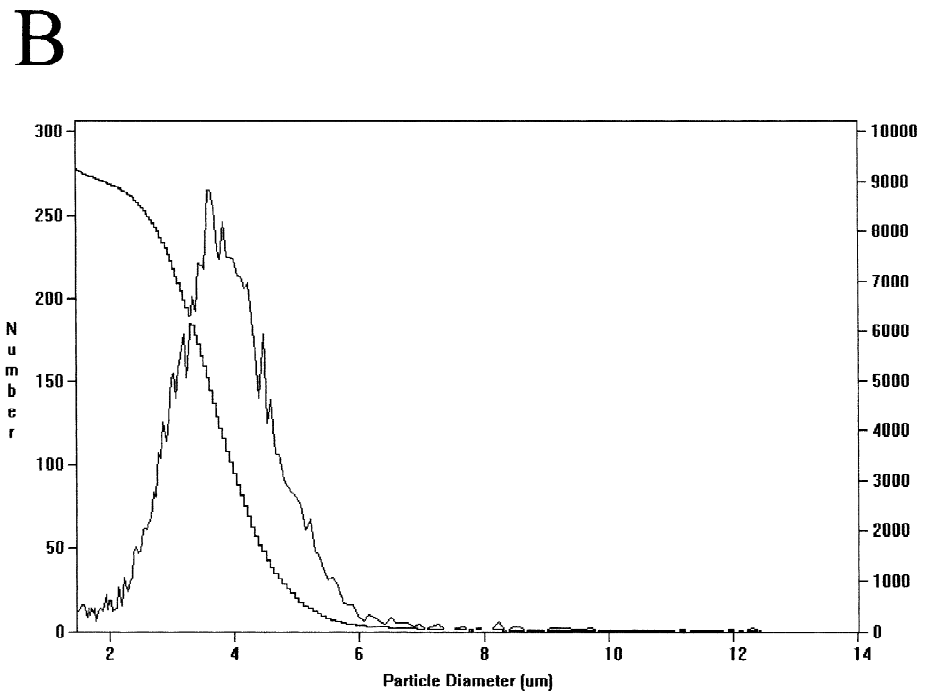


Fig. 3. Fractogram with the cumulative area for Bourgoblanc with relevant size distribution by Coulter counter.

not specialised in the analytical separation of cells to perform sample screening and quality control of yeast-based bio-processes by means of standard HPLC expertise.

The ''soft'' GFFF mechanism, its high bio-compatibility, easy cleaning and sterilisation of plastic GFFF channels increase sample throughput and reduce risks of cross-contamination and cell degradation after the elution. These technical features could make effective use of GFFF also for micro-preparative scale fractionation of yeast cells. Cell pre-sorting should allow for further coupling to other uncorrelated techniques towards the end of quality assessment procedures.

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