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Gravitational field-flow fractionation for the characterisation of active dry wine yeast ☆

R. Sanz[a], L. Puignou[a,*], P. Reschiglian[b], M.T. Galceran[a]

[a]*Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1–11, E-08028 Barcelona, Spain*
[b]*Dipartimento di Chimica "G. Ciamician", Università di Bologna, Via Selmi 2, I-40126 Bologna, Italy*

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Abstract

Gravitational field-flow fractionation (GrFFF) is applied to the fractionation of active dry wine yeast. An experimental approach to the analysis of the effects that field variation by changing mobile phase composition and flow-rate have on the fractionation process of standard particles (polystyrene) was first developed to further obtain effective fractionation of wine yeast by GrFFF. Scanning electron microscopy and Coulter counter particle size measurements were used to monitor the fractionation extent and capabilities of GrFFF to describe the distribution of yeast cells populations.

Keywords: Gravitational field-flow fractionation; Field-flow fractionation; Wine; Yeast; *Saccharomyces cerevisiae*; Polystyrene

1. Introduction

Over recent years the use of active dry wine yeasts as starter cultures for wine production has gained increasing acceptance [1]. The ready commercial availability of strains of *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces fermentati* has prompted their widespread use. Yeasts are ancient cultivated eukaryotes that reproduce by budding or fission [2]. A single yeast cell is usually spherical to ellipsoidal in shape but may be cylindrical, ogival, pyramidal, or apiculate; yeast cell size varies from one species to another [3]. The genus *Saccharomyces* is widely used in the baking, beer and wine industries, and for the production of biomass. It has been demonstrated that fermentation yield and productivity of yeast are related to the overall cell growth processes. For instance, *S. cerevisiae* ferments rapidly and may produce up to 18–20% of ethanol [4]. However, most of the strains of this yeast type cannot be readily identified by classical biochemical methods [5].

Field-flow fractionation (FFF) constitute a set of separation techniques that are able to characterise supramolecular species in a size range spanning many orders of magnitude, from macromolecules to micron-sized particles [6]. The FFF instrumentation scheme has been directly derived from classical high-performance liquid chromatography (HPLC). Here though, the column can be just replaced by the

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*Corresponding author. Fax: +34-93-4021-233.
E-mail address: puignou@apolo.qui.ub.es (L. Puignou).

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FFF channel and the detection system is frequently UV–Vis such as in HPLC. Most recently, detectors for absolute molecular mass to size determination like multi-angle laser-light scattering (MALLS) have been on-line coupled [7,8]. Various types of FFF techniques can be distinguished according to the nature of the force field applied to structure separation. Gravitational field-flow fractionation (GrFFF) employs the Earth's gravitational field applied perpendicular to a very thin, empty channel. GrFFF is a very inexpensive technique that has shown suitable for the separation and characterisation of various micrometer-size particles of different origin [9–19].

Because of its intrinsic simplicity and gentle fractionation mechanism, GrFFF and, most recently, the subset dielectrophoresis GrFFF (DEP/GrFFF) has shown to be specifically suitable for the separation and further characterisation of living samples like parasites [20,21] and cells [22–31]. In the particular case of yeast fractionation, centrifugal sedimentation field-flow fractionation (SdFFF), flow field-flow fractionation (FlFFF) and its subset DEP/FlFFF have been already applied [32–34]. However, the big advantage of GrFFF when compared to FFF techniques and other methods for cell fractionation lies in its very low cost and easy implementation in a standard HPLC system, as well as in its simplicity and softness, which reduce the risk of sample degradation. Most recently, moreover, a method to obtain quantitative particle size distribution (PSD) analysis of micronsize dispersion by GrFFF was developed [9,35,36]. Such a method shows promising to implement in GrFFF characterisation capabilities based on size distribution analysis of dispersed micron-size samples like cells.

The aim of this study was to determine the applicability of GrFFF in the fractionation of cell samples of great importance in the agricultural, food and biotechnology industries like yeast. Wine-making yeast has been chosen here as sample model. Given the low density of yeast cells (ca. 1.1 g ml^{-1}) [37], the possibility of field programming in GrFFF by means of flow-rate and density variation of the carrier liquid was explored [38,39]. Fractionation performance was first tested with standard polystyrene (PS) particles, the density of which is similar (1.05 g ml^{-1}) to that of wine-making yeast. Several types of *S. cerevisiae* were, then, studied by obtaining fractograms characterised by different profiles. If differences in GrFFF profiles corresponded to difference in size distribution of the sample, this was determined by scanning electron microscopy (SEM) and Coulter counter measurements.

2. Experimental

The GrFFF system employed was built up as described elsewhere [10,40]: two mirror-polished glass plates were clamped together over a polytheraftalate sheet from which the channel volume had been removed. The cut space was rectangular with tapered ends. Channel dimensions of the resulting ribbon-like channel were 0.0133 cm thick, 2 cm wide and 30 cm long.

The channel void volume V_0 was experimentally determined by eluting an unretained spectroscopic standard Na_2CrO_4 (Aldrich, WI, USA) at 373 nm incident wavelength and was found to be 0.732 ml. The channel just replaced the column of a standard HPLC system. The samples were injected by means of an injector Model 7125 (Rheodyne, Cotati, CA, USA) with a loop of 20 μl. The switching valve was an automatic Rheodyne 7010. The carrier flow was generated by a Model 2150 HPLC pump (LKB, Bromma, Sweden) and the channel outlet was connected to a Model System Gold 168 UV-diode array detection (DAD) system (Beckman, Fullerton, CA, USA) operating from 260 to 360 nm. All the fractograms were obtained at 330 nm.

PS standard particles of different diameters (nominal 2.764±0.136 μm, 6.096±0.401 μm) (Polysciences, Warrington, PA, USA), dispersed at 0.1% (w/v) in the carrier liquid, were used for the optimisation of the elution conditions. Various types of wine-making yeast were investigated. Samples were active dry wine yeast from *S. cerevisiae* (Fermol bouquet, Cryoaromae, Fermol rouge, Killer, Awri 350) (Escola d'Enologia de Tarragona, University of Rovira i Virgili, Tarragona, Spain), and they were dispersed at 0.1% (w/v) in the carrier liquid and kept at 4–5°C before the analysis to prevent cell growth of yeast samples. Both samples, standards and yeasts, were sonicated for 2 min in the carrier liquid and left to equilibrate for 1 min before injection into the GrFFF channel. The amount in-

jected was always 10 μl, the injection time was 45 s with an injection flow of 0.2 ml min^{-1} and the stop flow time was 6 min. The carrier liquid was Milli-Q water (Millipore, Bedford, MA, USA) with 0.1% (w/v) of NaN_3 (Fluka, Buchs, Switzerland), commonly used in FFF practice as bactericide and to control the ionic strength. Gradient-grade quality methanol (MeOH) (Merck, Darmstadt, Germany) was used at different concentrations to modify density of the carrier. For surfactant-added carrier liquids, the non-ionic surfactant Triton X-100 (Merck) or the anionic surfactant sodium dodecyl sulfate (SDS) (Fluka) was used. Flow-rates ranged from 0.1 to 0.3 ml min^{-1}. All the experiments were performed at room temperature.

Viscosity values were obtained through a Cannon–Fenske viscosimeter Model 25 (Afora, Barcelona, Spain). Coulter counter size measurements were performed through a Multisizer II (Coulter Corp., Hialeah, FL, USA) set for 256-channel analysis. Aperture size was 70 μm (measure rank: from 2 to 60% of nominal aperture size), samples were counted from 2.0 to 7.0 μm and instrumental calibration was performed with Calibration Standard PS Latex, 18.5 μm (Coulter Electronics, Luton, UK). Sample populations in the carrier liquid were diluted 1:1000 with commercial phosphate saline buffer (Isoton II, Coulter Corp.) previously filtered on a 0.2-μm pore filter. The analytical volume was 500 μl. Fractions of each sample were collected after six consecutive runs under identical experimental conditions at the same time interval. The total analysis time was 4 h at controlled room temperature. The fractionated samples were diluted 1:20 with the buffer, analytical volume was 2000 μl. All measurements were replicated three times.

Scanning electron micrographs were obtained with a scanning electron microscope Model S-2300 (Hitachi, Tokyo, Japan) at 15 kV; samples were dry–frozen with liquid N_2 and sputter-coated with ca. 500 nm of Au.

3. Results and discussion

3.1. Optimisation of GrFFF elution conditions

In a recent paper, Plocková and Chmelík described the effective possibility of field programming in GrFFF by changing either flow-rate or/and density of the mobile phase [39]. In GrFFF two elution modes are possible; for particles in the micron-size range, the focusing or steric/hyperlayer mode is effective [38]. In this elution mode, the retention ratio, that is the ratio between the channel void volume and the retention volume ($R=V_0/V_r$), can be expressed as:

$$R = s/w \tag{1}$$

where s is the distance of the centre of the eluting sample zone from the channel bottom when the resulting force acting on the analyte equals zero, and w is the channel thickness. Therefore, the value of s and, then, the retention ratio depends on the two counteracting forces acting on the focusing elution mode, which are the gravitational force (F_G) and the hydrodynamic force (F_L):

$$F_G = GV_p(\rho_p - \rho_m) \tag{2}$$

$$F_L = F_{L,I} + F_{L,NW} \tag{3}$$

where G is the gravity acceleration, V_p the particle volume, ρ_p and ρ_m the density, respectively, of the particles and of the mobile phase, and $F_{L,I}$ $F_{L,NW}$ are, respectively, the inertial and the near-wall lift forces contribution [41–43]. The lift force contributions are known to depend also on flow-rate and mobile phase viscosity. As a consequence, retention in focusing GrFFF can be modulated by changes in density, viscosity and velocity of the mobile phase. Chmelík [38] indeed showed that by adding even small amount of methanol (MeOH) to the mobile phase, because of resulting variations in its density and viscosity, retention can be significantly modified in focusing GrFFF [39]. Moreover, these authors also showed that variation of carrier velocity is very effective in modifying GrFFF retention, because of the high dependence of lift forces on fluid velocity. This pioneering work shows promising for the optimisation of the elution conditions in GrFFF of low-density, micronsize particles like cells, which are poorly retained at the density and viscosity values of purely aqueous carrier fluids.

In this work, because of their low density (ρ_p = 1.05 g ml^{-1}), Polybead PS particles are used to

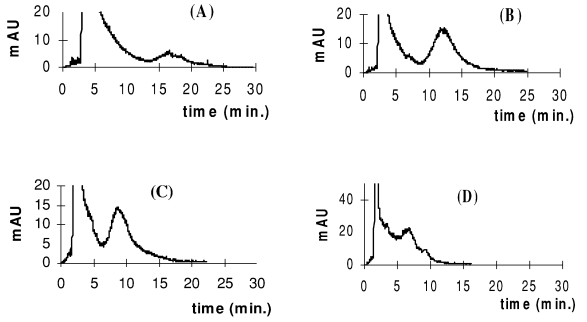

Fig. 1. Fractograms corresponding to 0.1% (w/v) of 3 μm polystyrene in carrier liquid at different carrier velocities: (A) 0.15 ml min^{-1}, (B) 0.2 ml min^{-1}, (C) 0.25 ml min^{-1}, (D) 0.3 ml min^{-1}. Carrier liquid: 0.5% SDS, other conditions as described in the Experimental section.

optimise the elution conditions for the fractionation of yeast. In order to reach a significant level of retention, it was, indeed, necessary to work with flow-rates below 0.5 ml min^{-1}. In fact, because of the low density of the sample, as described in the previous paragraph, at higher flow-rate values lift forces tend to increase to an extent that is able to drastically reduce retention and, thus, the level of fractionation actually reached. Fig. 1 shows as an example, the fractograms obtained for PS 3 μm at four different flow-rates. This study was carried out at the conditions described in the Experimental section. As it can be seen, the optimal flow-rate resulted to be 0.2 ml min^{-1}.

Because of the small differences in density between PS and the mobile phase ($\rho_p - \rho_m = 0.05$), as described by Chmelík [38] and discussed above, in order to increase the resolution between the system peak and the PS peak, in a second step the density and viscosity of the carrier liquid was changed by adding methanol up to 40% (v/v). Furthermore, the presence of a surfactant in the mobile phase it is also known to modulate retention in focusing GrFFF [10,11,14]. In this work, therefore, once determined the best flow-rate value (0.2 ml min^{-1}), the effect of the type and amount of surfactant, as well as the effect of the use of MeOH in the mobile phase had on PS fractionation was explored. Two different surfactants were tested to optimise retention: SDS at 0.5%, as anionic surfactant, and Triton X-100 at either 0.1% or 0.5%, as non-ionic surfactant. The MeOH content was set at 20%. In Table 1 resolution values between the system peak and the polystyrene peak for PS 3 and 6 μm for the two types of surfactant are given. As it can be seen, better resolution was obtained with Triton X-100 at both percentages. At high concentration of Triton X-100 6 μm polystyrene particles showed a high resistance to elute due to the increasing of viscosity, which produced an improvement on resolution. In contrast 3 μm polystyrene particles showed an opposite

Table 1
Resolution values for 3 and 6 μm polystyrene with different type of surfactant added to carrier liquid

Polystyrene particles (μm)	Resolution		
	SDS, at 0.5%	Triton X-100	
		At 0.1%	At 0.5%
3	1.9	2.9	2.5
6	1.2	1.3	1.9

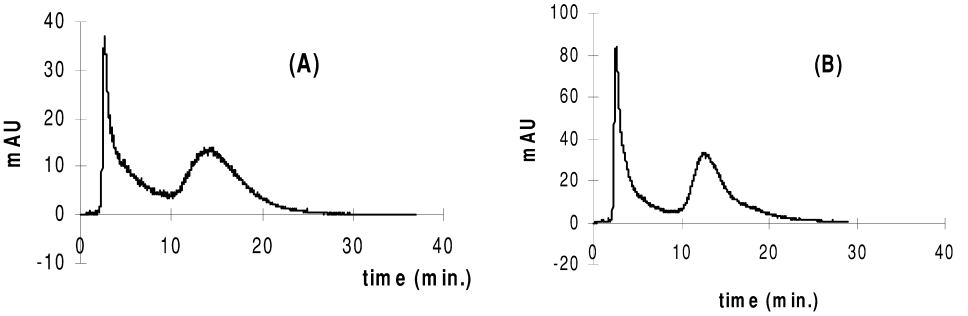

Fig. 2. Fractograms of 3 μm polystyrene dispersed at 0.1% (w/v) in carrier liquid. Carrier liquid: (A) methanol–0.5% SDS (20:80), (B) methanol–0.1% Triton X-100 (20:80). Flow-rate: 0.2 ml min^{-1}.

behaviour probably due to the aggregation of the small particles.

Fig. 2 shows the fractograms obtained with polystyrene 3 μm with the two surfactants. It can also be observed here that Triton X-100 gave better fractogram shapes with respect to those obtained with SDS. Narrow peak width, higher signal, lower analysis time and higher separation efficiency were obtained when the neutral surfactant was used. A proper explanation of this finding would require a full description of the solution properties of the ternary mixture surfactant–MeOH–water with respect to GrFFF elution of PS particles. Such an analysis stands, however, beyond the aims of the present paper. Since Triton X-100 showed better results than SDS, the effects of adding different amounts of MeOH were eventually explored with Triton this latter surfactant. Fig. 3 shows the dependence of resolution and retention ratio values on the density and viscosity variation of the carrier liquid obtained by adding different percent of MeOH. Two different concentrations of surfactant were also employed. It can be observed that at the highest density and lowest viscosity values of the mobile phase, the highest retention ratio values are actually accomplished by a decrease in resolution. Moreover, the carrier liquid with high surfactant concentration (0.5% Triton X-100) provided the best resolution between the system peak and the polystyrene peak. From this study, a carrier liquid solution 0.5% Triton X-100 in MeOH–water (20:80) is here suggested as the optimised mobile phase for the fractionation of low-density samples. Although the effect of MeOH-modified mobile phases on physical features of yeast cells is currently under study. However, it must be noted that the observed elution time values, that are the periods of time during which yeast cells are actually dispersed in the MeOH-modified fluid, were indeed quite short in our experiments.

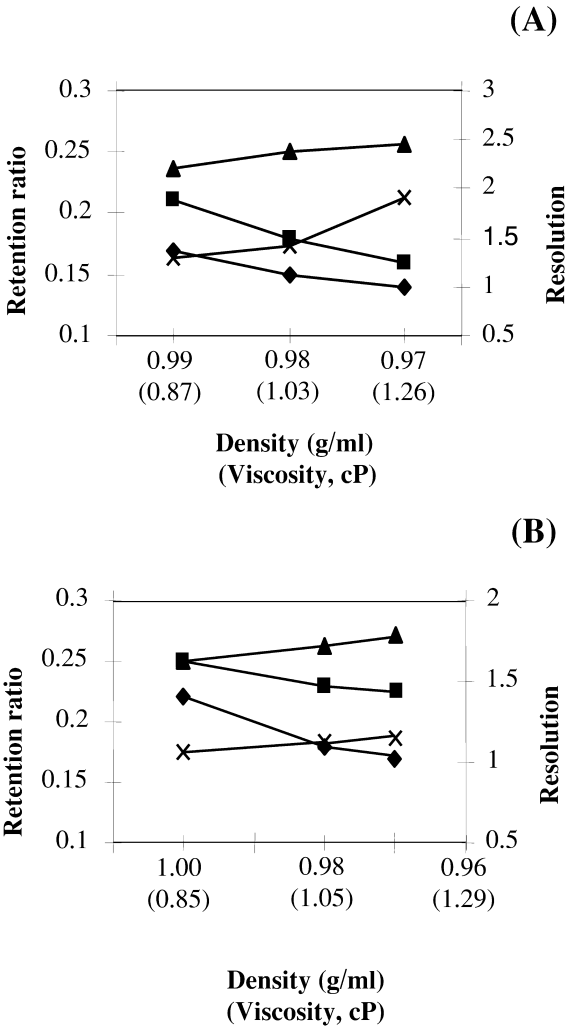

Fig. 3. Dependence of the retention ratio (R) and resolution between the system peak and the polystyrene peak (R_s) on the density and viscosity of carrier liquid: MeOH–Triton X-100, for polystyrene 6 and 3 μm dispersed at 0.1% (w/v) in carrier liquid. (A) using 0.5% Triton X-100, (B) using 0.1% Triton X-100. – ■ – R 6 μm; – ♦ – R 3 μm; – ▲ – R_s 3 μm; – × – R_s 6 μm.

3.2. GrFFF fractionation of yeast

The ternary mixture MeOH–water–Triton X-100 employed above for the GrFFF optimisation with standard PS particles was, thus, used for GrFFF of wine yeast. GrFFF was first used to check the stability of active dry wine yeast dispersions with temperature. When samples of *S. cerevisiae* Fermol bouquet were kept at room temperature fractograms corresponding to replicated injections showed maxims that tended to shift toward lower retention times. This could be an indication of cell growth and reproduction, which change size and shape of cells. Therefore yeast dispersions for GrFFF analysis were always maintained at a low temperature (4–5°C).

The applicability of GrFFF to the characterisation of wine-making yeast was further tested by using

four different types of yeast (Cryoaromae, Fermol rouge, Awri 350, Killer) under the experimental conditions proposed. Retention ratio values and peak widths obtained when injecting three times in 1 day on three different days are given in Table 2. It can be observed that similar retention ratio values were obtained for the four types of yeast. However, different peak width values are shown for different types of sample, suggesting that differences in the ''polydispersity matrix'' (size, density, morphology, rigidity) proper of cells [44,45] might occur within the various types of yeast. This preliminary finding suggests that GrFFF could be promisingly used to discriminate between wine-making yeast types on the basis of physical features of the cells.

The effective capability of GrFFF to characterise wine-making yeast was further investigated on the Fermol bouquet sample. Fig. 4A reports the fractogram of this sample, which shows a broad profile with two maxima that can be ascribed to a multimodal character of the distribution of the Fermol bouquet yeast population. Since retention in focusing GrFFF is known, as above discussed, to depend on size, density, morphology and rigidity of sample particles, the use of uncorrelated techniques for size distribution analysis can establish whether the multimodal character of GrFFF profiles are due to a multimodal dispersion of only size and/or to other particle indexes of the ''polydispersity matrix''. Therefore, to better understand the bimodal character of GrFFF profiles of the Fermol bouquet yeast sample here considered, SEM micrographs were taken on the unfractionated sample. The sample dispersion was filtered through a 0.45 μm pore nylon 66 membrane on a plastic filter holder and washed twice with Milli-Q water, thus obtaining the concentration of yeast cells on the membrane. Furthermore, the histogram of the sample size distribution was obtained under the conditions mentioned in the Experimental section using Coulter counting size measurements by counting a number of 11 618 cells. Results are given in the Fig. 4B. It can be observed a bimodal distribution of the size of sample population, according to what observed for the fractogram of the same sample that can, thus, indicate an actual bimodal character in size. The size distribution by Coulter counter is here given as cell number distribution with the cumulative percentage values of 4.45 μm (10%), 3.66 μm (50%) and 2.89 μm (90%) with a standard deviation of 0.71 μm. The micrograph obtained by SEM also shows cells where two population of different size can be distinguished. However, in order to compare Coulter counter results to the indications given by the GrFFF fractograms, it is important to focus the difference that might exist between a distribution obtained by Coulter counter, which is a number distribution, to a distribution derived from a GrFFF profile, which is proportional to sample mass [9]. Although qualitative indications (e.g., the bimodal character) do not change between the two types of distribution, the relative amount and the values of the statistical moment are known they could be significantly different. However, the derivation of particle size distribution profiles of yeast samples by direct conversion of GrFFF profiles (i.e., the so-called PSAD method in FFF [9,35]) stands beyond the aims of the present paper and it is the object of work in progress.

To further check the fractionation extent obtained by GrFFF of this sample, three fractions of the yeast peak were collected from the GrFFF outlet at different time intervals (fraction 1, 2 and 3 from the

Table 2
Retention ratio (R) and peak width (w) variability

Type of wine-making yeast	Run-to-run				Day-to-day			
	R	RSD (%)	w*	RSD (%)	R	RSD (%)	w*	RSD (%)
Cryoaromae	0.22	2.1	17.0	0.4	0.22	3.8	16.4	3.8
Fermol rouge	0.20	3.7	15.1	1.1	0.19	4.2	15.1	4.9
Killer	0.21	1.2	19.5	1.8	0.21	3.8	18.9	5.1
Awri 350	0.20	0.7	19.4	2.9	0.21	4.0	19.2	6.7

Run-to-run ($n=3$) and day-to-day precision ($n=9$, three measurements per day on 3 days) for four different commercial wine-making yeasts. *Peak width measured in minutes at the baseline.

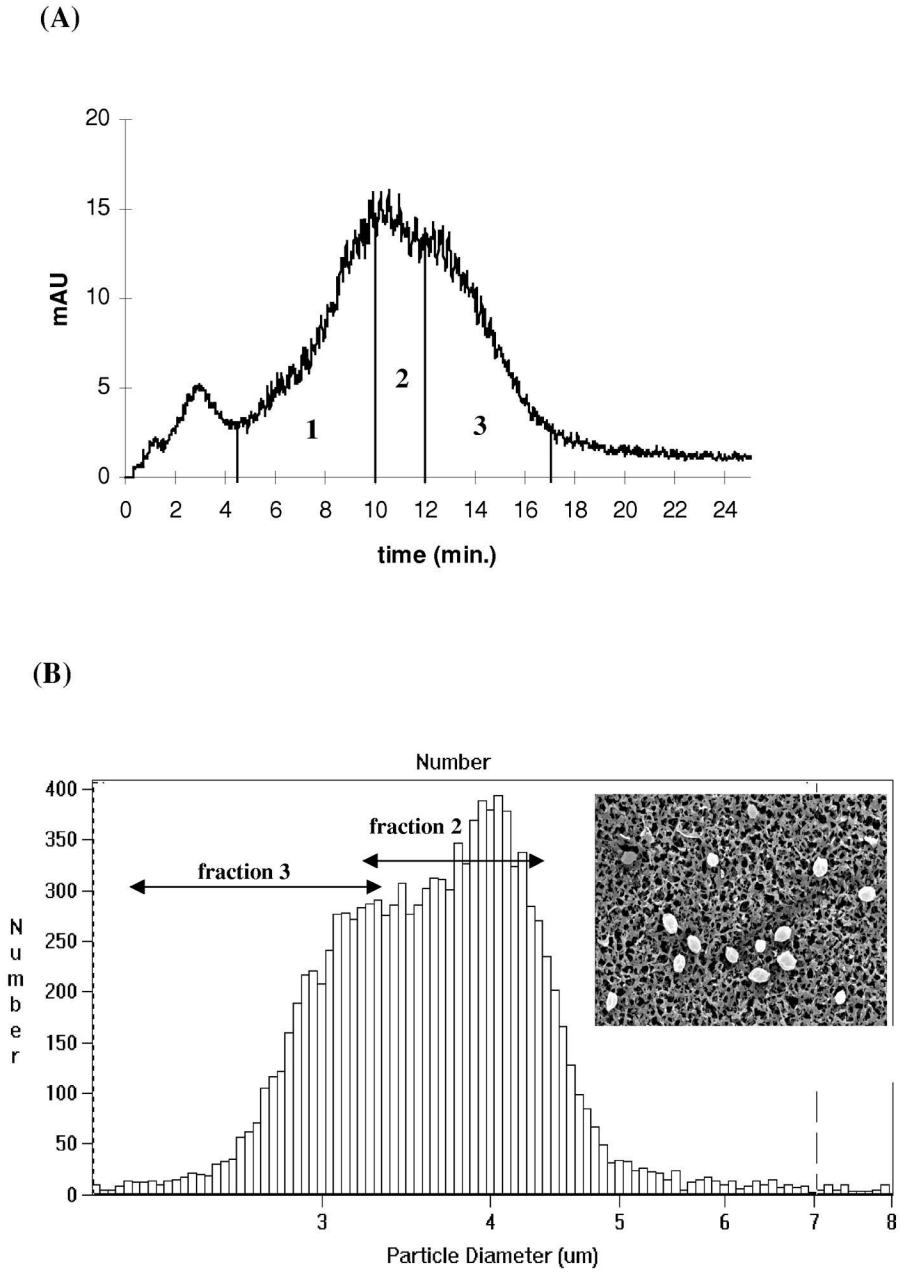

Fig. 4. (A) Fractogram of the yeast sample Fermol bouquet showing the three fractions collected. Fraction (1) from 4.5 to 10 min. Fraction (2) from 10 to 12 min. Fraction (3) from 12 to 17 min. (B) Size distribution and scanning electron micrograph of the Fermol bouquet population, obtained under the conditions described in the Experimental section. Total cells counted from 2 to 7 μm: 11 618. The fractions of (a) are tentatively indicated on the Coulter's size distribution pattern.

fractogram in Fig. 4A) and analysed by the Coulter counter technique. The histograms corresponding to each fractionated sample confirmed a rather homogeneous size population of the yeast cells. In fraction 2 the mean size of the population was 3.49 μm with a standard deviation of 0.95 μm. In the fraction 3 the

mean size of the population was 2.51 μm with a standard deviation of 1.56 μm. These results qualitatively confirms Coulter's findings on the total (unfractionated) sample population, where two different cell size distributions were observed which were tentatively identified as fraction 2 and fraction 3 (see Fig. 4B). GrFFF results and Coulter counter size measurements eventually provide good potentiality for the fractionation and characterisation of wine-making yeast. The methodology developed was applied to several yeast samples from *S. cerevisiae* showing similar retention but different elution profiles. Such differences were shown to be due, at least in part, to actual differences in size distribution. Such capability of GrFFF for yeast sizing was proved by SEM micrographs and Coulter counter size measurements on the unfractionated sample and on collected GrFFF fractions of the sample, respectively. However, because of the known dependence of GrFFF retention on the overall ''polydispersity matrix'' (size, density, morphology, rigidity) of cells, the GrFFF elution profiles can be an useful tool to characterise yeast samples with different distributions of also different physical properties of the cells. The potential exploitation of GrFFF for the analytical control of wine-making yeast is thus established.

4. Conclusion

In this work the use of GrFFF for the fractionation of yeast on an analytical scale is shown for the first time. Although further instrumental optimisation and methodological work is still needed to apply GrFFF to routine yeast fractionation, GrFFF is shown here to be capable of working with organic-modified mobile phases in order to increase retention of yeast cells by modulating the actual field through density and viscosity variations of the carrier liquid. The small increase in density difference between the mobile phase and the analyte, together with the relevant increase in viscosity when a relatively small amount of MeOH is added to the mobile phase, is shown here to be able to turn out a significant increase in retention of low-density samples such as yeast cells, thus giving a corresponding increase in the fractionation level. The proposed method could be, thus, proposed in the framework of a characterisation of yeast at low cost and short analysis time, with correlation between the analytical parameters of yeast-made wine and the physical features of yeast cells (size, density, morphology, rigidity) that could be established in this way. Thus, GrFFF is interesting for future applications in the characterisation of active dry wine yeast, the quality of which is necessary to obtain quality assessment in the wine production.

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