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ORIGINAL ARTICLE

# The application of flow cytometry in microbiological monitoring during winemaking: two case studies

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**Abstract** In this work, we exploit a general flow cytometry technique involved in the differentiation of live and dead yeast cells for two applications in winemaking. The discrimination of yeast populations is achieved using two fluorescent dyes that measure the metabolic activity and membrane integrity of the yeast. This analytical approach is first applied for quality control of active dry yeast. Results are discussed in comparison with the Codex Oenologique International (International Oenological Codex) of the International Organisation of Vine and Wine (OIV), demonstrating that analysis using flow cytometry is a valuable alternative, given the ease of execution and the high quality of results obtained in terms of reproducibility, repeatability, and confidence interval. In the second case, we apply flow cytometry as a technique for monitoring the production of sparkling wines using the "Champenoise" method, and describe the evolution of yeast through the production process. In this case, results are directly compared with those obtained with the two methods (plate counts and direct microscopic count) listed in the OIV standards, in order to ensure a thorough understanding of the improvements related to the use of flow cytometry.

**Keywords** Flow cytometry · Single-cell analysis · Active dry yeast · Alcoholic fermentation · Sparkling wine

## Introduction

Flow cytometry (FCM) is an effective alternative to traditional methods of microbiological analysis based on plate or microscopic counts of microorganisms. In wine science and in the wine industry, the plate count method is recognised as the reference standard in microbiological analysis (Codex Oenologique International 2014a, b), despite the length of time required to carry out analysis and the poor recovery of cells present in the samples, especially in the case of a "viable but nonculturable" state (Oliver 2005; Salma et al. 2013). Microbiological analysis using plate counts also requires specific environmental conditions and trained personnel, two features often absent in wineries. These factors discourage the use of plate counts for monitoring oenological fermentation in a winery setting, relegating the technique to specialised applications carried out in the laboratory. Microscopic counts, on the other hand, which ensure results in a few minutes and provide information regarding microflora viability (Fiala et al. 1999), are better suited for use in controlling the evolution of alcoholic fermentation (Ibarra-Junquera et al. 2010). Unfortunately, results obtained with this technique are heavily influenced by the analyst performing the procedure, thereby resulting in low reproducibility and effectively limiting its application.

FCM is a process for measuring cells that are suspended in a liquid. This technology allows the simultaneous measure and analysis of multiple characteristics of single cells as they pass through a beam of light (Fiala et al. 1999). The technique is widely used today in certain areas of the biotechnological industry, given its speed, reproducibility, adaptability to different contexts, and reasonable equipment costs. Some examples of FCM applications in oenology have already been published (Fiala et al. 1999; Boyd et al. 2003; Gerbaux and Berger 2009; Portell et al. 2011; Bouix and Ghorbal 2013). In this work, we describe two case studies in which FCM represents an improvement compared to microbiological monitoring approaches in use today.

First, we describe a method for the analysis of active dry yeast (ADY) employed in wine fermentation. The use of an appropriate ADY is a key component of winemaking (Reed and Chen 1978; Fleet 2007), as it influences not only the

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evolution of alcoholic fermentation, but also the sensorial properties of the wines produced and the succession of steps such as malolactic fermentation that are involved the winemaking process (Mazzei et al. 2013). The need for a rapid and efficient method of analysis is evident in the large number of ADYs available on the market today, in most cases devoid of rigorous scientific information concerning their microbiological features. The number of viable cells present in a batch of ADYs is one of the principal features that must be assayed in order to ensure the effectiveness of yeast inoculation in grape must, along with information on the physiological state and degree of stress to which yeasts are subjected during dehydration (Franca et al. 2007). In this work, validation of FCM analysis is based on determinations performed on more than 80 ADY samples, which are compared to results obtained using the standard method established by the International Organization of Vine and Wine (OIV) (Codex Oenologique International 2014a).

The second "case study" concerns the microbiological monitoring of sparkling wine production using FCM. This procedure represents one of the most complex processes in winemaking, given the difficult conditions present when secondary alcoholic fermentation takes place. The effervescence that characterises sparkling wines is generally obtained by inducing secondary alcoholic fermentation in the closed bottles in which wine will be sold (Pozo-Bayon et al. 2009; Torresi et al. 2011). Secondary fermentation thus occurs in an environment unfavourable for microbial development, as it is characterised by a number of stress factors (Carrascosa et al. 2011; Penacho et al. 2012). In this situation, stuck fermentation is frequently observed; consequently, the process must be carefully monitored to ensure homogeneous results in any batch of wine. Flow cytometry may therefore provide a satisfactory solution, combining good reproducibility and plate count accuracy with rapid execution, while offering the depth of information typical of microscopic counts. We utilized FCM to monitor the complete production process for a high-quality sparkling wine, Trento DOC, made in northern Italy in the hills around the city of Trento. We began our observations with the cultivation of the *pied de cuve*, terminating our research once secondary alcoholic fermentation had concluded. Each stage was followed using the three analytical methods described above, providing the tools necessary to understand the specific characteristics of each technique and the benefits associated with the implementation of FCM in the winemaking process.

## Materials and methods

### ADY samples and microorganisms

The OIV characterization (Codex Oenologique International 2014a) of ADY describes it as a dehydrated form of yeast isolated from a vineyard or winery environment (commonly belonging to the *Saccharomyces cerevisiae* species), which is selected, purified, and reproduced for use as an alcoholic fermentation starter in winemaking. In this work, we collected 87 ADY samples, purchased on the Italian market in 2012. The *Saccharomyces cerevisiae* ATCC 9763 strain was employed as a reference strain to validate the FCM method. Sparkling wine was produced using two strains of *Saccharomyces cerevisiae* belonging to the Edmund Mach Foundation collection (FEM 111 and FEM 222).

### Plate counts and microscopic counts

ADY reactivation was performed using the OIV method (Codex Oenologique International 2014a). Samples were diluted 1:10 with a 1 % sucrose solution (Sigma-Aldrich, St. Louis, MO, USA) and mixed using a Stomacher blender (Seward Ltd., Worthington, UK) for 2 min. The samples were then incubated for 15 min in a water bath at 25 °C. This procedure was repeated twice. The ADYs were again homogenised using a Stomacher blender (2 min), and further analysed within 10 min. In the case of sparkling wine, samples were subjected to direct analysis. For analysis of ADY, we performed nine decimal dilutions in phosphate buffered saline (PBS) solution before plate spreading; in the case of sparkling wine, the dilutions ranged between the third and sixth decimal according to the production stage.

Plate and microscopic yeast counts were performed according to OIV standards (Codex Oenologique International 2014a, b) using Wallerstein Laboratory (WL) nutrient agar medium (Oxoid Ltd., Basingstoke, UK). Petri plates were incubated for 3–5 days at 25 °C. Colonies were identified as yeast through microscopic observation. Cellular concentration (*C*) was expressed as colony-forming units (CFUs) per gram (or mL) of sample, according to the equation

$$C = \frac{N1+N2}{1.1\times V1\times D}$$

where N1 and N2 are the number of colonies counted on two plates derived from two consecutive decimal dilutions, D1 is the dilution rate of N1, and V is the volume of the sample on the spread plate [OIV - Recueil international des méthodes d'analyses (International Compendium of Analytical Methods) 2014b].

Direct counting of yeast cells using a microscope was performed by placing a drop of the yeast cell suspension with the appropriate dilution (typically 3-decimal dilution for ADY, 1-decimal dilution for wine) on the surface of a Bürker chamber, after staining with a solution of methylene blue. After incubation at room temperature for 10 min, we counted the yeast cells present inside 20 quadrants of the Bürker chamber, separating viable and dead cells on the basis of their colour (unstained cells live, blue cells dead). Cellular concentration was expressed as cells per gram (or mL) of sample, according to the equation

$T = C \times 0.25 \times 10^6 \times dilution\ factor$

where $C$ is the average number of cells counted in 20 squares and $T$ is the population in the sample [OIV - Recueil international des méthodes d'analyses (International Compendium of Analytical Methods) 2014b].

## Flow cytometry analysis

ADYs were reactivated as described in the previous paragraph, then diluted to reach a concentration of $10^5$ cells/mL using a PBS buffer. For wine analysis, 1 mL of sample containing approximately $10^5$ of cells, obtained by appropriate dilution in the PBS buffer, was filtered through a 30-μm filter (CellTrics®: Sysmex Partec GmbH, Görlitz, Germany). After dilution, samples were incubated for 10 min at 30 °C in the presence of 10 μL of fluorescein diacetate 5 mg/mL solution in acetone (both purchased from Sigma-Aldrich, St. Louis, MO, USA) (Ross et al. 1989). Diluted samples were mixed and added to 10 μL propidium iodide 2 mg/mL solution in water (Sigma-Aldrich, USA). The double-stained samples were homogenised and subjected to FCM analysis within 10 min.

FCM analysis was performed using a CyFlow Cube 8 cytometer (Sysmex Partec GmbH, Germany) equipped with a solid-state blue laser, emitting at 488 nm (main parameter in Table 1). Through the use of four band-pass filters, we considered the following signals: a combined forward-angle light scatter (FSC); a side-angle light scatter (SSC); and two fluorescence signals, the first with a 530-nm band-pass filter to collect green fluorescence (FL1 channel) and the second with a 630-nm long-pass filter to collect red fluorescence (FL2 channel). FCM analysis was performed using logarithmic gains and specific detector settings, adjusted using a sample of unstained *Saccharomyces cerevisiae* ATTC 9763 to eliminate background and cellular autofluorescence. Data were collected and analysed using FCS Express 4 software (De Novo Software, Glendale, CA). The yeast cell population was identified and gated in an FSC/SSC dot plot; live and dead cell differentiation was performed using an FL1/FL2 dot plot, adjusted for the appropriate compensation between the two signals, considering the subpopulation of yeast gated in the FSC/SSC dot plot.

**Table 1** Setting parameters of the flow cytometry apparatus used for yeast cell count in the active dry yeast and sparkling wine samples

| Parameter | Value |
|---|---|
| FSC - Forward scatter (V) | 130 |
| SSC - Side scatter (V) | 189 |
| First fluorescence channel - FL1 (V) | 302 |
| Second fluorescence channel - FL2 (V) | 290 |
| Threshold (FCS channel) (V) | 0.01029 |
| Compensation (FL1 channel) (%) | 9.3 |
| Flow (μL/s) | 5 |
| Analysed volume (mL) | 0.2 |

## Sparkling wine production: preparation of the pied de cuve and monitoring of wine fermentation

According to the general rules of sparkling wine production (Ribéreau-Gayon et al. 2006), sparkling wine was produced at a winery in the of province of Trento, beginning with a Chardonnay wine from the 2012 vintage (Fig. 1). The wine was inoculated with a yeast culture created in the laboratories of the Edmund Mach Foundation (the pied de cuve), which was prepared using the two *Saccharomyces cerevisiae* strains FEM 111 and FEM 222. Yeast strains were stored at −80 °C and reactivated in nutrient broth (Oxoid Ltd., Basingstoke, UK) with 7 % sucrose at the time of use. Preparation of the pied de cuve began from a yeast growth culture with a concentration of $10^8$ cells/mL, and the volume of the culture was increased by adding a mixture 1:1 of water and wine, supplemented by sucrose (100 g/L) and nitrogen (50 mg/L), as shown in Fig. 1. At each step, the dilution rate did not exceed 5 %, the cell concentration was greater than $10^6$ cells/mL, and the density was greater than 1.005 g/L. Nitrogen supplementation was performed by adding a complex mixture commonly

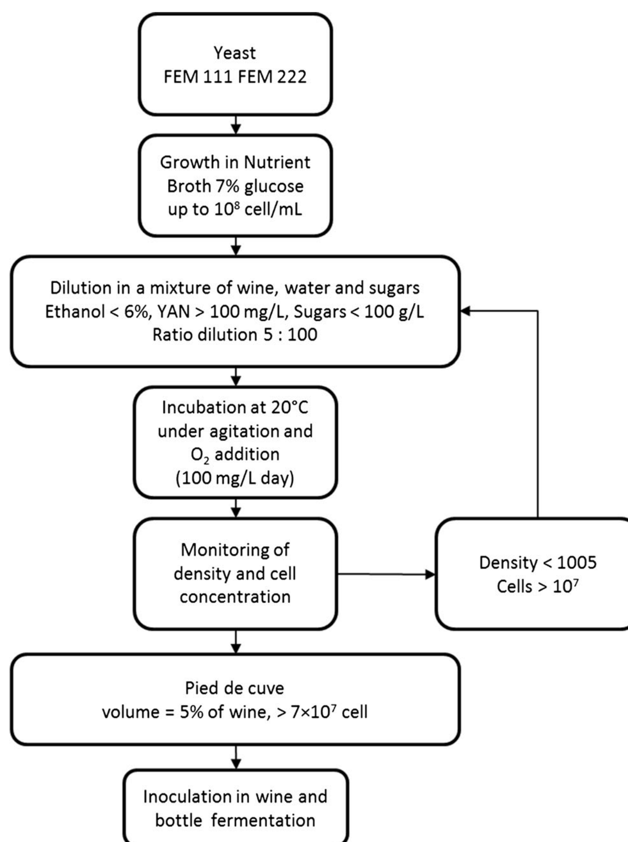


**Fig. 1** Diagram of a sparkling wine production method from pure yeast culture to secondary fermentation in closed bottles

used in oenology (Bioattivante DC; Dal Cin S.p.A., Milan, Italy). The entire procedure, carried out at 20 °C, typically lasts one week. After the addition of sugars (about 20 g/L) and pied de cuve, the wine was bottled and left to ferment at 15 °C in ordinary sparkling wine bottles (0.75-litre). Alcoholic fermentation was followed by regular sampling of wines until stabilization of the pressure at 5 atm occurred inside the bottle.

### Statistics

Statistical analysis was performed using STATISTICA 7 software (StatSoft Inc., Tulsa, OK, USA). Shapiro–Wilk, Huber's, and F-tests were employed for data analysis in each of the experiments, and box plots were used to compare confidence intervals among the methods of analysis.

## Results

### Setting up the FCM apparatus and evaluation of the selectivity of FCM analysis

The physical parameters of the FCM apparatus (FSC and SSC channels) were adjusted using a pure culture of *Saccharomyces cerevisiae* ATCC 9763, and were further utilized in the ADY analysis (Fig. 2). As shown in Fig. 2a, the dot plot of physical parameters (FFS and SSC) clearly shows a population of events recognisable as yeast cells. The dot plot of the FL1 and FL2 channels (Fig. 2b) allows discrimination between the populations of viable and non-viable cells based on features of the two fluorescent dyes involved in the staining of cells. The main parameters for the FCM apparatus settings are provided in Table 1. In order to ascertain the accuracy of FCM in live yeast cell counts, 87 samples of ADYs were analysed using both FCM and plate counts according to OIV standards (Codex Oenologique International 2014a) The results (Fig. 3a) demonstrate that the counts obtained using FCM remained within the confidence interval of the data derived using the culture method. Moreover, by comparing the confidence intervals of the two methods, it is evident that FCM is characterised by higher repeatability and lower confidence intervals, resulting in more accurate measurements (Fig. 3a). In samples with the lowest cell concentrations, the data obtained by FCM tend to be higher than those determined using the culture method (Fig. 3b), the reason for which we will discuss below. In order to assess the accuracy of the method for counting dead yeast cells, the same ADYs were rehydrated and further treated at 80 °C for 10 min in a water bath to kill all vegetative forms (International Fruit Juice Producers 1996). FCM also showed good selectivity in the dead cell count: in heat-treated samples, we did not observe signals in the FL1 channel attributable to live cells, whereas an intense signal in the FL2 channel was associated with the presence of dead cells (data not shown).

### Repeatability of FCM analysis of ADYs and estimation of the measurement uncertainty

Repeatability was estimated as described in the International Organization for Standardization (ISO) 5725–1 (ISO 1994), observing the level of agreement among the results of a repeated test carried out on the same ADY sample. The study was performed considering three ADY concentration levels close to the limit established by the OIV (Codex Oenologique International 2014a) for ADY acceptability ($1\times 10^{10}$ cells/g), and was designed to discriminate and quantify the contribution of the analytical procedure to the total

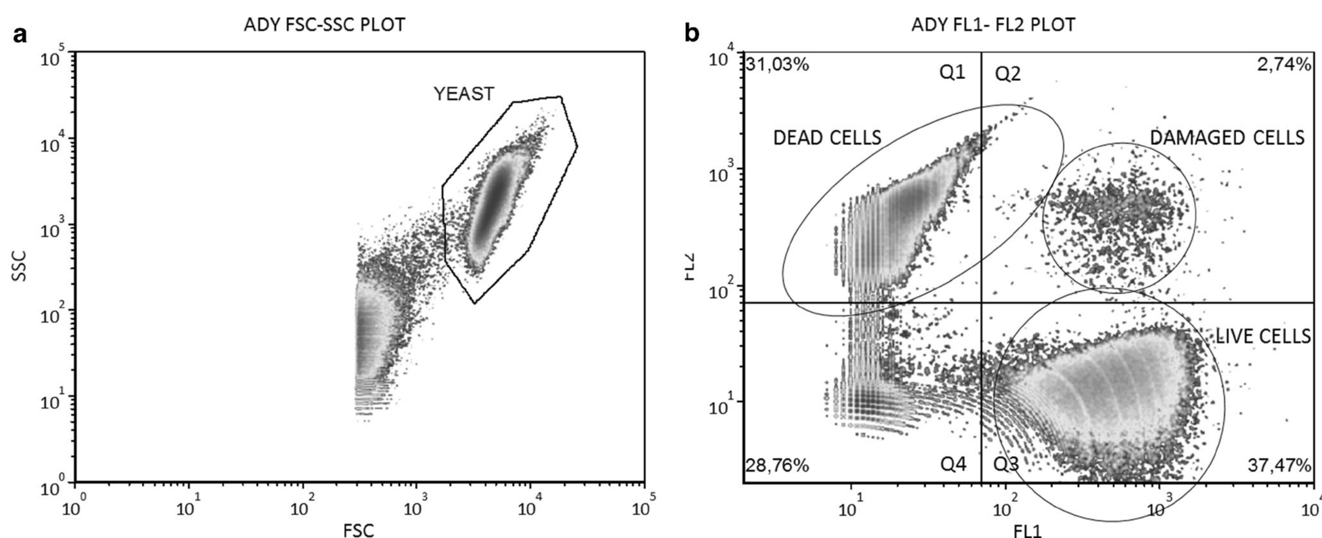


**Fig. 2** Flow cytometry analysis of ADY after rehydration using the OIV method (**a**) Dot plot of physical parameters (FSC forward scatter/SSC side scatter) (**b**) Dot plot of FL1/FL2 signal in discriminating between live and dead cells

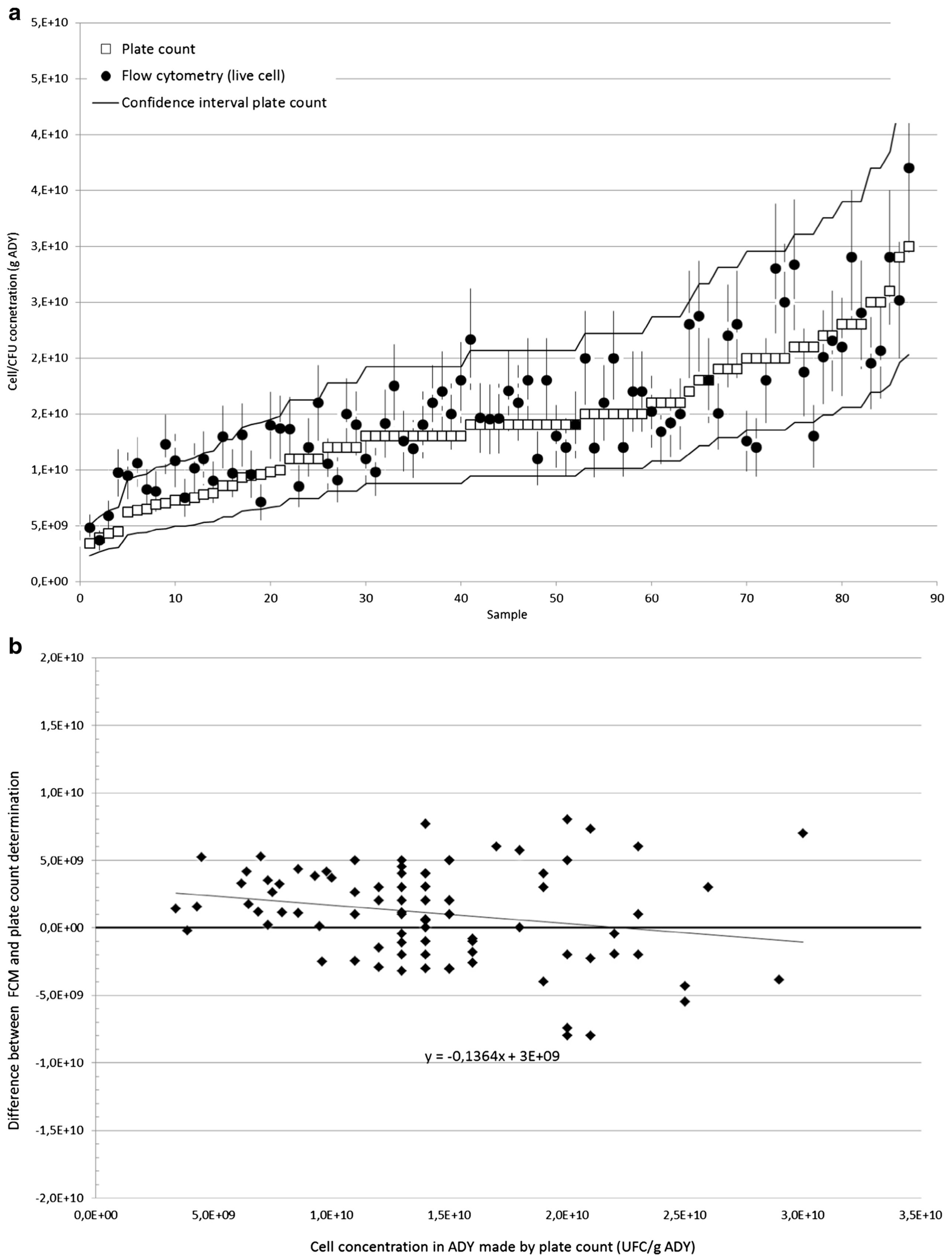


**Fig. 3** Selectivity of flow cytometry analysis in the yeast count (**a**) Comparison between data obtained using flow cytometry (●) and plate count (□) (**b**) Behaviour of absolute differences in determinations performed using the two techniques

variability based on the sample preparation (rehydration, dilution) and instrumental yeast count. Repeatability was determined for both live and dead cell counts. For live cells, a summary of the data from the three measurement sets is given in Table 2. In all cases, the Shapiro–Wilk test ($p$=0.05) showed normal distribution of the data; Huber's test also identified no anomalous results. The F-test ($p$=0.5), showed that the variability of the entire analytical procedure was significantly higher than that of the instrumental phase, at 3.55 (low counts), 6.15 (medium counts), and 12.51 (high counts), compared to 2.82 in the F-test table (11.0, 11.0, 0.05). The evolution in the limit of repeatability ($r$), calculated as the maximum difference between two independent determinations performed under the same conditions ($p$=0.05), is shown in Fig. 4. It is possible to approximate the behaviour of $r$ for the whole range of measurements with the linear equation

$$r = 0.25\ \mathrm{c} + 2.7 \times 10^9$$

where $c$ is the concentration of live cells determined using FCM. A similar procedure was performed to estimate the $r$ limit in the dead cell count (Table 2). In contrast to observations in the previous set of experiments, in the case of the dead cell count, the variability of the entire procedure was not statistically different from that of the instrumental phase (Fig. 4). The behaviour of the repeatability limit ($p$=0.05) can be approximated with the linear equation

$$r = 0.76\ \mathrm{C}$$

where $c$ is the concentration of dead cells determined using FCM.

The uncertainty of the method for differentiating between live and dead yeast cells in ADY using FCM was estimated considering the repeatability ($r$), calculated as described before, and other contributors arising from the variability associated with the use of other instruments involved in the analysis (Table 3). The same approach was followed for both live and dead cells, considering the three yeast concentration levels in the interval as defined above. Data processing describes the evolution of U as a function of live or dead cells (Fig. 5). According to the definition proposed for $r$, the expanded uncertainty of measurement (U) is defined in the interval of measurement as

$$U = 0.20\ c^* + 1.8 \times 10^9 (\mathit{live\ cells})$$

$$U = 0.58\ c^* - 7.0 \times 10^8 (\mathit{dead\ cells})$$

where $c$ is the result of cell counts performed using FCM.

## Monitoring of sparkling wine production using FCM

The evolution of the yeast population during the production of sparkling wine was followed using FCM, beginning with preparation of the active yeast culture (the pied de cuve), through the end of secondary alcoholic fermentation in the closed bottle (Fig. 1). First, we first verified the repeatability of FCM measurements in the yeast cell counts in sparkling wine. For this test, we considered a wine sampled after six days of fermentation in a closed bottle, assuming it as representative of the whole process in terms of the concentration and physiological state of the cells. Figure 6 shows the results, with the mean and confidence interval of 10 determinations performed on the same samples of wine using three different counting methods: FCM, plate count, and microscopic count. The variability of FCM measurements was similar to that of plate counts, with relative standard deviations (RSDs) of 12.5 % (FCM, live cells) and 10.8 % (plate count), whereas the RSD for the microscopic count was 19.4 %. Compared to plate counts, FCM analysis allowed the identification of two other cell populations: dead cells (Fig. 7, Q3 quadrants) and a third population of cells that could be identified as "compromised cells" (Fig. 7, Q2 quadrant). This last subpopulation showed a detectable signal in both the FL1 and FL2 channels, showing itself to be biologically active while at the same time

**Table 2** Flow cytometry (FCM) measurements performed to calculate the $r$ limit. **L** sample with a low yeast concentration, **M** sample with a medium yeast concentration, **H** sample with a high yeast concentration

| | Live cells (× $10^{10}$ cells/g of ADY) | | | | | | Dead cells (× $10^{10}$ cells/g of ADY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FCM count | | | Whole method | | | FCM count | | | Whole method | | |
| | L | M | H | L | M | H | L | M | H | L | M | H |
| Mean | 0.72 | 3.08 | 18.92 | 0.82 | 4.30 | 12.7 | 0.20 | 0.36 | 1.70 | 0.18 | 1.20 | 2.90 |
| Standard deviation (SD) | 0.05 | 0.21 | 0.31 | 0.002 | 0.05 | 1.10 | 0.03 | 0.002 | 0.37 | 0.04 | 0.24 | 0.74 |
| Relative standard deviation (%) | 7.3 | 6.7 | 1.6 | 12 | 12 | 8.7 | 15 | 5.0 | 22 | 24 | 19 | 26 |

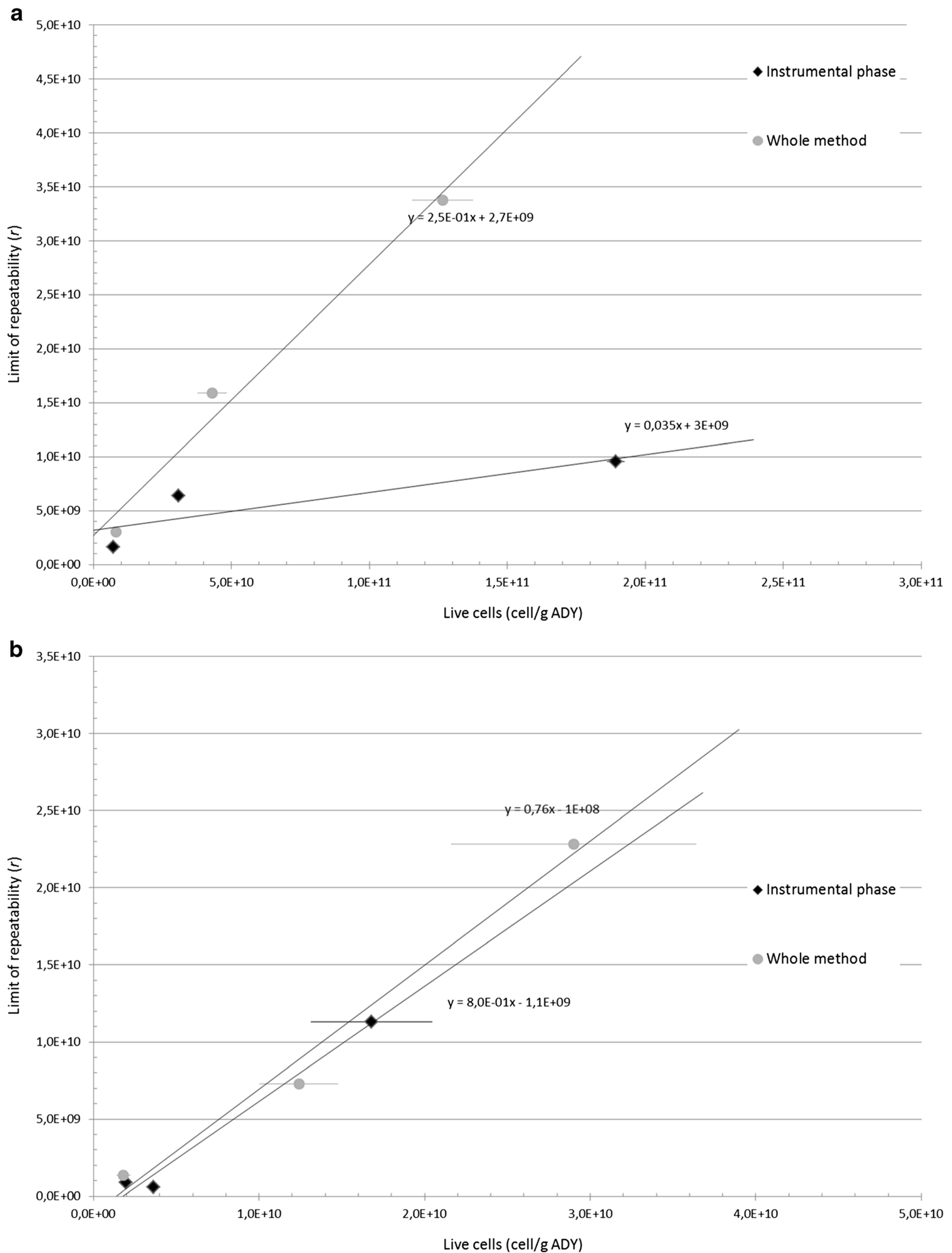


**Fig. 4** Evolution of the limit of repeatability (r) for the instrumental phase (●) and the entire method (□) in the active dry yeast count using flow cytometry (**a**) Live cells (**b**) Dead cells

**Table 3** Synthesis of contributors involved in estimating the value of uncertainty (U) associated with the measure of live and dead cells in samples of active dry yeast. **L** sample with a low yeast concentration, **M** sample with a medium yeast concentration, **H** sample with a high yeast concentration

| Contributor to the relative standard uncertainty | $L_{Live}$ | $M_{Live}$ | $H_{Live}$ | $L_{Dead}$ | $M_{Dead}$ | $H_{Dead}$ |
|---|---|---|---|---|---|---|
| Repeatability (*r*) | 12 % | 12 % | 8,7 % | 24 % | 19 % | 26 % |
| Volume of FCM cuvette | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % |
| Dilution of ADY sample | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % |
| Weight of ADY sample | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % | 2.9 % |
| Volume of sample used in the decimal dilutions | 0.6 % | 0.6 % | 0.6 % | 0.6 % | 0.6 % | 0.6 % |
| Volume of peptone water used in the decimal dilutions | 1.0 % | 1.0 % | 1.0 % | 1.0 % | 1.0 % | 1.0 % |
| Number of dilutions | 4 | 4 | 4 | 4 | 4 | 4 |
| Combined relative standard uncertainty ($u_c$) | 13 % | 13 % | 10 % | 25 % | 20 % | 26 % |
| Degree of freedom | 16 | 16 | 21 | 12 | 12 | 12 |
| Coverage factor (k) | 2.12 | 2.12 | 2.08 | 2.18 | 2.18 | 2.18 |
| Relative expanded uncertainty (U%) | 28 % | 28 % | 21 % | 53 % | 43 % | 57 % |
| Expanded uncertainty (U) | $2.3\times10^{9}$ | $1.2\times10^{10}$ | $2.7\times10^{10}$ | $9.7\times10^{8}$ | $5.4\times10^{9}$ | $1.7\times10^{10}$ |

demonstrating impaired cell membrane permeability. Agreement among the results obtained from the different analytical methods was tested throughout the sparkling wine production process (Fig. 8a). Taking the plate count as the "reference method," we found a linear correlation in comparisons with both FCM and microscopic counts. However, FCM trend data showed the best results in terms of correlation ($R^2$ plate count vs. FCM: 0.9096; $R^2$ plate count vs. microscopic count: 0.66) and in the agreement of results, as indicated by the slopes of the two equations. Despite this, we observed general overestimation by FCM as compared to plate counts. This phenomenon, also occasionally observed in the case of ADY, is dependent upon differences in the nature of the results. We followed the entire process of sparkling wine production using

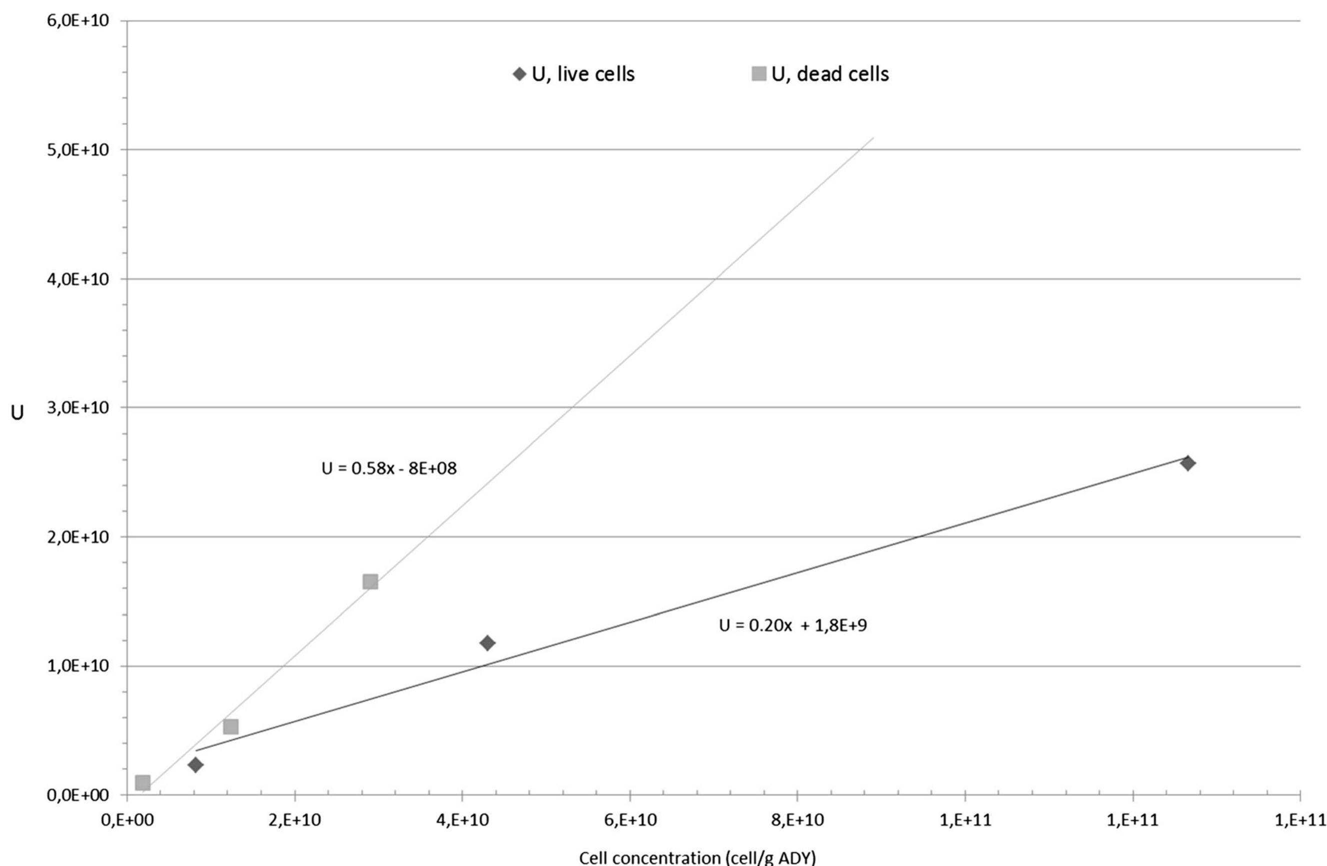


**Fig. 5** Evolution of the value of uncertainty (U) in the active dry yeast cell count using flow cytometry (♦) Live cells (□) Dead cells

Median
25%-75%
Not outlier interval
Outliers
Extremes
Cell concentration (cell/mL)
2,4E+07
2,0E+07
1,6E+07
1,2E+07
8,0E+06
4,0E+06
0,0E-01
Flow cytometry
Plate count
Microscopic count
Flow cytometry (Dead cell)
Microscopic count (Dead cell)

**Fig. 6** Box plot comparing the results and confidence intervals in analysis of yeast during sparkling wine secondary alcoholic fermentation, performed using flow cytometry

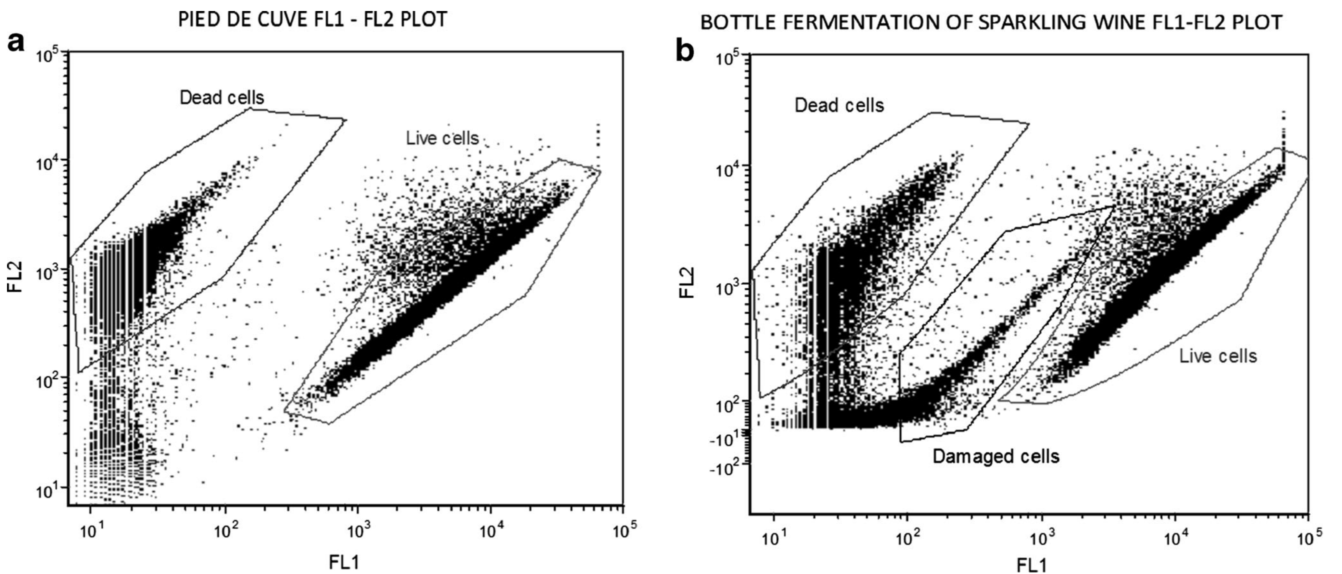


**Fig. 7** Flow cytometry analysis of the yeast population during sparkling wine secondary alcoholic fermentation (**a**) Dot plot of FL1/FL2 parameters of a pied de cuve (**b**) Dot plot of FL1/FL2 signals of bottle-fermenting wine; the appearance of a third population of events corresponding to the damaged cells is evident

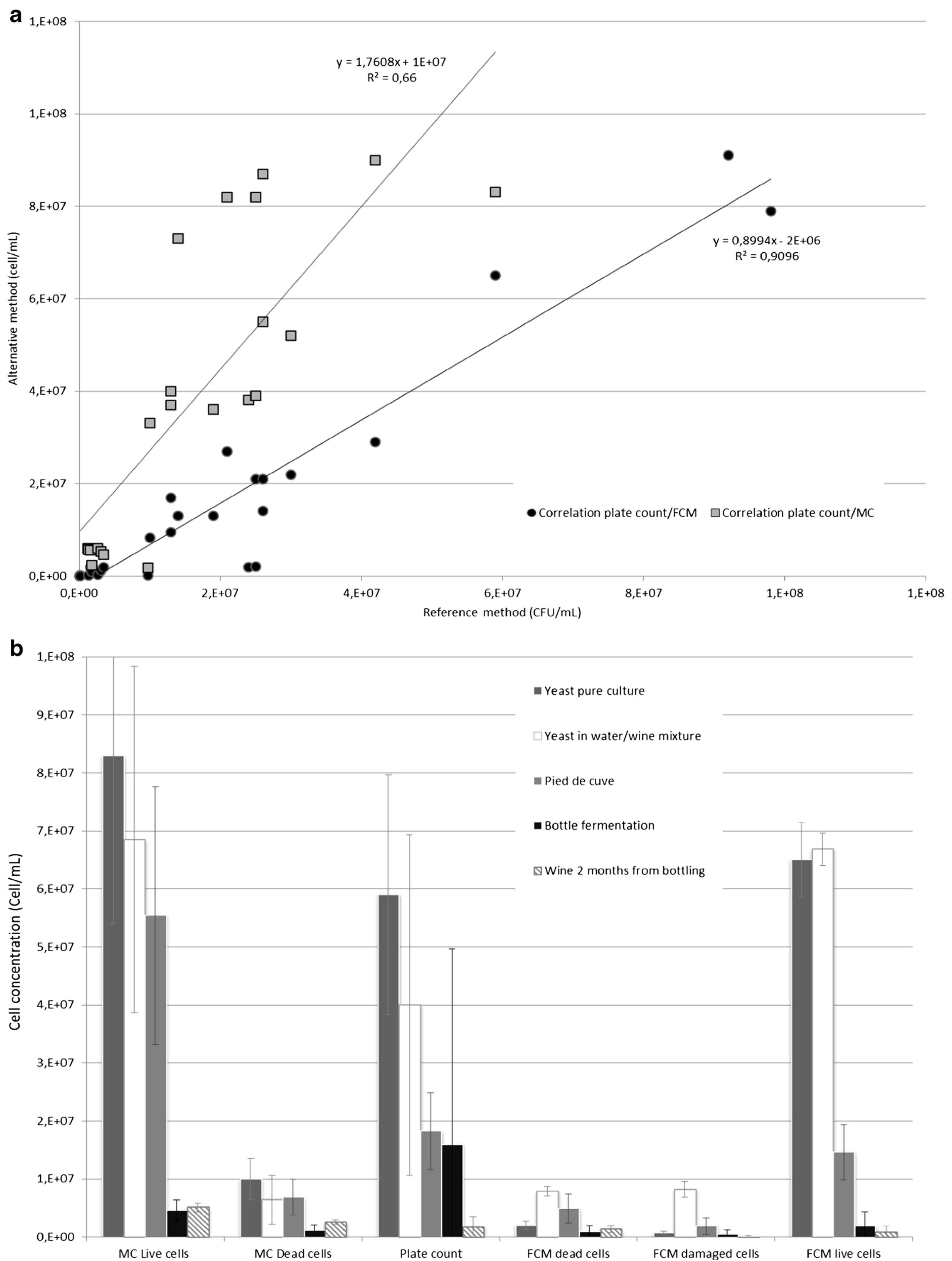


**Fig. 8** **a** Comparison of measurements performed using flow cytometry (FCM), plate counts (PC), and microscopic counts (MC) **b** Evolution of cell concentrations through the sparkling wine production process

FCM (Fig. 8b). The live cell population remained up to $10^6$ cells/mL for both samples during the preparation of the pied de cuve and bottle fermentation; cell concentration subsequently decreased rapidly, resulting in less than $10^4$ cells/mL two months after bottling. This trend is consistent with the evolution occurring within the sparkling wine production process previously described by other authors (Ribéreau-Gayon et al. 2006). The ratio between live and dead yeast cell populations remained below 1 until the end of alcoholic fermentation, defined as stability in pressure inside bottles for at least two weeks. Despite the value of single measurements, the ratio between live and dead cells (RLD) or compromised cells (RLC) showed an interesting trend, illustrating the evolution of the yeast population (Table 4). In the pure yeast culture, the RLD reached a value of 32.5, indicating a negligible presence of dead cells. This value decreased dramatically in adapting to the wine environment at the beginning of the phase carried out with a water/wine mixture. After pied de cuve preparation, the RLD value once again reached very high values (about 15), but successive inoculation in wine and bottling progressively reduced the RLD through the fermentation process, with a final value close to 1 at the end of alcoholic fermentation. The behaviour of the RLC was similar to that observed for the RLD, although the second index reflected a higher "sensitivity" to the variation in physiological state, showing a larger difference in terms of the mean value in subsequent phases of the production process. Another observation concerned the variability associated with the different methods of analysis in the same step of the production process (Fig. 8b). In the case of ADY count, the data obtained using FCM were characterized by less variability than that observed by plate or microscopic count, enabling enhanced monitoring of the fermentation process and targeted interventions on the wine microflora.

## Discussion

Today, thanks to the availability of highly automated analytical methods, it is possible to closely monitor the chemical variables involved in the process of winemaking (Di Egidio et al. 2010; Buratti et al. 2011; Romera-Fernandez et al. 2012). The latest advances in this field have even proposed a complete automation of winemaking, with sensors that are able to recognise the progress of evolution from grape must to wine (Frohman and de Orduna 2012). This is not the case in the evolution of microbiota during oenological fermentation. Microbiological controls performed in oenology are still carried out using traditional techniques that effectively relegate microbiological analysis to the laboratory, preventing the continuous and careful control of the evolution of microbiota throughout the production process. Flow cytometry may represent a means to enhance levels of microbiological control during winemaking (Malacrino et al. 2001; Diaz et al. 2010; Branco et al. 2012; Quiros et al. 2012). With the intention of contributing to the dissemination of FCM in the wine industry, we reported on two "case studies" for the direct application of FCM during wine production. The first section of the work concerned the validation of a method to quantify live and dead yeast cells inside active dry yeast (ADY) using FCM. As with previous works having a similar purpose (Attfield et al. 2000), the experiment was designed following the guidelines as established by the International Organization for Standardization and the International Electrotechnical Commission (ISO/IEC 17025 1999), and considered a large number of ADYs, in an effort to define a standard method for the use of flow cytometry in oenology. The experiments were performed considering more than 80 ADYs purchased on the Italian market in 2012 and 2013, providing a representative overview of the "state of the art" of yeast cultures for oenological use available on the market today. Given that the International Organisation of Vine and Wine (Codex Oenologique International 2014a) has established $10^{10}$ cells/g as the minimum permissible concentration of viable yeast cells in ADY, we designed our work around this cell concentration level (Table 2). The analysis of yeast using FCM is essentially based on two successive steps. In the first, we set the parameters of the FCM apparatus using a standard yeast culture. We regulated the voltage of physical parameters (FSC and SSC) to distinguish the yeast

**Table 4** Evolution of cell viability during the production process of sparkling wine monitored by flow cytometry (FCM). The ratio between live and dead cells (RLD) or compromised cells (RLC) showed an interesting trend, reflecting the stress effect of media composition on the yeast population

| | FCM dead cells (cells/mL) | FCM damaged cells (cells/mL) | FCM live cells (cells/mL) | RLD | RLC |
|---|---|---|---|---|---|
| Pure yeast culture | 2.0E+06 | 7.3E+05 | 6.5E+07 | 32.5 | 89.0 |
| Yeast in water/wine mixture | 7.8E+06 | 8.2E+06 | 6.7E+07 | 8.5 | 8.2 |
| Pied de cuve | 4.9E+06 | 1.9E+06 | 1.5E+07 | 3.0 | 7.7 |
| Bottle fermentation | 8.8E+05 | 5.1E+05 | 1.9E+06 | 2.2 | 3.8 |
| Wine two months after bottling | 1.4E+06 | 1.5E+05 | 8.0E+05 | 0.6 | 5.4 |

population from the background and to eliminate autofluorescence in the sample (Fig. 2a). The second step, which involved the double-staining of yeast cells using fluorescein diacetate and propidium iodide (Fig. 2b), enabled the differentiation between live and dead cells. The two dyes proposed here are characterised by good sensitivity, as confirmed in our test results (Figs. 3), and offer a fast and easy means of staining that can be performed in a few minutes. More specifically, fluorescein diacetate is a cell-permeant esterase substrate that can serve as a viability probe for measuring both enzymatic activity, which is required to activate its fluorescence, and cell membrane integrity, which is required for intracellular retention of the fluorescent products (Jepras et al. 1995). Following hydrolysis using intracellular esterase, fluorescein emitted at a maximum of 518 nm allowed the discrimination of viable cells. Propidium iodide was used as a counterstain: it is not able to permeate live cells, while it colours cells with compromised membranes (Attfield et al. 2000). The maximum emission of propidium iodide that we observed was approximately 617 nm, allowing clear differentiation of the two signals, with little compensation of the FL1 signal (Table 1). Despite the ease with which analysis was performed, the qualitative descriptors of the FCM method (repeatability, reproducibility, and uncertainty) were comparable to, or in some cases better than, those typical of plate counts. General agreement of the results obtained was observed in terms of both live and dead cells, with the exception of samples having a low concentration of viable cells. In these cases, FCM seemed to overestimate the count compared to the culture method. This trend is not surprising, considering the difference in the nature of analytical data provided by the two methods. Cultures are often affected by underestimation of the number of cells present in the sample, both as a result of the aggregation of different cells in a single colony inside the Petri plate and the “viable but nonculturable” state of stressed cells (Salma et al. 2013). These risks are well-established, and are the basis of the expression of results of culture methods as “colony-forming units.” In contrast, FCM is based on direct counts of the cells present in the sample (Jepras et al. 1995; Gerbaux and Berger 2009), and therefore the analysis is not affected by these issues. Thus it is conceivable that FCM provides more accurate results, closer to the true value of cells present in the sample. In our experiments, the differences between the two analytical approaches were notable only in the samples with the lowest cell concentration, likely affected by cellular stress resulting from a lack of care with respect to the production process or inappropriate storage conditions. At values below the OIV limit ($1 \times 10^{10}$ cells/g), the difference between FCM and plate counts was not significant from either a statistical or technological point of view. In this interval of concentration, we found ADYs of high quality and therefore characterised by a negligible presence of damaged cells. In conclusion, this work begins by proffering a method for the analysis of ADYs using FCM, describing the validation process and the results obtained in the analysis of a large and representative population of ADYs available on the Italian market. On the basis of these results, it is possible to propose FCM as a valuable alternative to traditional methods for quality control of ADYs.

Considering also that *Saccharomyces cerevisiae* is one of the principal fermenting yeasts, it is reasonable that the analytical method described here could be extended to include the complete process of oenological fermentation performed using yeast. Therefore, in the second section of this work, we employed FCM technology to describe sparkling wine production from a microbiological perspective, and in particular the evolution of secondary fermentation in closed bottles. Although there have been some reports describing the monitoring of alcoholic fermentation using FCM, no previous works have reported data concerning the monitoring of yeast in sparkling wine production. This survey is critical in assessing the final quality of wines, given that during the process of fermentation in closed bottles, yeasts are subject to a large number of stress factors, combined in a way that is not generally observed in any other context (Penacho et al. 2012). For these reasons, the inoculation of yeast in wine before bottling is usually not initiated directly from ADYs, but rather follows a more complex trajectory involving the preparation of a pied de cuve, i.e., a mixture of wine, water, and grape must with a high cell charge already adapted to the fermentation environment (Kunkee and Ough 1966). The data in these experiments are consistent with those in the literature, indicating that during pied de cuve preparation, yeast biomass is subjected to intense selection due to the progressive increase in limiting factors such as ethanol (Casey and Ingledew 1986), high pressure (Gonzalez et al. 2008), and carbon dioxide (Cahill et al. 1980). FCM appears to represent a suitable approach for quantifying the cell concentration present in wine and cell stress during secondary fermentation by measuring dead and damaged cells (Figs. 7 and 8). These features of FCM, combined with the ease and speed of the analytical procedure, render flow cytometry a promising tool for use in monitoring the evolution of microbiota during winemaking.

## Conclusions

Today, the process of winemaking is based on rigorous control of the variables that influence the features of the wines. In efforts to progressively reduce the additives involved in wine production, and in particular the antiseptic compounds such as sulphur dioxide, the careful monitoring of microflora is an essential objective. This work contributes to the progression

towards this goal with a proposal of two applications for FCM in winemaking. The first section of the work provides a large base of data to define a standard method for the characterization of active dry yeast using FCM, demonstrating that FCM is clearly superior in terms of accuracy and reproducibility compared to plate counts. The second section of the work proposes FCM for monitoring the production of sparkling wine, one of the most valuable wine categories. Our results demonstrate the adaptability of FCM in the context of identification of yeast cell count after double-staining as a new indicator of the physiological state of yeast cells. In conclusion, the proposed FCM protocols of yeast monitoring ensure fast and accurate information regarding the fitness of yeast populations, preventing problems during alcoholic fermentation, and contributing to improvement in the quality of the wines produced.

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