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Rapid evaluation of ergosterol to detect yeast contamination in fruit juices

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Abstract
Foods and beverages are nutrient-rich systems prone to a rapid development of microorganisms that hamper their long-period storage. Particularly, yeasts are strong fermenters of fresh and processed fruits and vegetables; hence, they are often accountable for their spoilage and production of off-flavor. This work provides a quick and easy tool to recognize and count the spoilage of juices with ergosterol as distinctive biomarker of molds and yeasts. Melon juice was reconstituted at natural physical–chemical parameters according to legislation, and *Saccharomyces cerevisiae* was selected as yeast to contaminate the juice. Chemical and enzyme tests were performed on the fresh juice to ensure its authentic properties. Ergosterol was then evaluated using a spectrophotometric method that was proven against the official plate count test. The study showed linear and consistent results and, therefore, the ergosterol molecule may be indicated for testing molds and yeasts in contaminated beverages, replacing the common and time-consuming analysis.

Keywords Ergosterol · Food safety · Marker · Melon juice · *Saccharomyces cerevisiae* · Microbiological test

Introduction

The contamination of fruit juices by fungal spoilage is still a challenge today, although industries have used many strategies to control foodborne microorganisms. When beverages undergo deterioration caused by microorganisms, yeast and mold are the most common among all spoilage microbes and, in such a case, they alter odors, appearance, taste, and texture, which can lead to low-quality products [1].

The food industry has traditionally used yeasts and molds to manufacture fermented products (e.g., alcoholic beverages and bakery products) and mature cheeses. In spite of these positive actions, uncontrolled or accidental development of microorganisms can lead to cross-contamination that will likely result in the production of toxins weakening food safety. From the consumer's perspective, if beverages are no longer attractive, it restricts their trade even if they are still safe. Quality issues caused by spoilage can occur at any stage of the production chain of juices, from the raw material, before harvest, during handling and processing, and in final packaging [1–4].

Fermentative spoilage that occurs in beverages and vegetable products by molds and yeasts is mainly due to the natural juice pH from 4.5 to 7.0, and to the availability of water (water activity, a_w) that in natural juices gets to 0.97 [2, 3]. Microorganisms remain alive only in aqueous phases where they carry out their proper metabolic activities. Yeasts often join with spoilage of beverages and vegetable fluids, because unicellular microbes may easy diffuse in liquids.

Saccharomyces, Zygosaccharomyces, Candida, and Pichia are the yeast genera that commonly contaminate high sugar, high salt, and high acid products, such as sugar cane, sugar syrups, honey, concentrated fruit juices, jams, jellies, and dried fruits [2]. Also, products preserved with weak acids and/or long-term frozen products are susceptible to yeast deterioration [2, 5]. Out of the approximately 1500 yeast species recognized in taxonomy, only a few hundred species (100–150) of yeasts are generally isolated from foods and beverages [2, 5]. In particular, *Saccharomyces cerevisiae*

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is above all recognized for its strong fermentative potential that is implicated in the spoilage of high-sugar foods and processed fruit beverages, where contamination may reach final amount of 10^7–10^8 CFU/mL. After sugar breakdown by yeast, foods become cloudy, gassy, and a sediment is formed, all clear sign of spoilage [2, 6].

Several assays for monitoring yeasts have been reported in previous studies, including the standard colony-count assay (CFU isolation and enumeration), cell counting using a haemocytometer, dielectric spectroscopy, immunological techniques (ELISA), microscopic methods and molecular approach using PCR-based methods [4–7]. Most of these techniques are unpractical for a certain determination of yeast in routine recognition of microbiological quality of food and beverages due to their low repeatability and because they are time-consuming techniques [7, 8]. Some other methods have shown applicability towards medium complexity, for instance when microbial biomass cannot be easily separated from a solid substrate. Therefore, new reliable assays for a good and fast detection of yeast and molds have been developed lately by measuring chemical components of these microbes [8]. Several tests analyze constituent components, others examine indicator products extracted from molds and yeasts. Different assays have been reported for specific determination of sterols, volatile organic compounds (VOC), β-D-glucan, mycotoxins, allergens, and extracellular polysaccharides [8]. Recently, the ergosterol molecule of exclusively fungal cells has been recognized as a more sensitive indicator than others for revealing yeasts and molds colonization in food [8–11]. Ergosterol is the main sterol component of fungal cell membranes, playing a key specific role, while it is absent in vascular plants, bacteria and animals. Thus, its presence and detection in food specifically denotes spoilage and may be indicated as a significant quality parameter. Despite the abundance of literature on its chemical determination so far carried out, its accepted use for estimating fungal biomass in food and drink is still being questioned. The doubt about its application is mainly related to the presence of two forms of ergosterol one free and one esterified, which are prevalently located on plasma membrane and on cytosolic particles of fungi, respectively [9]. For these reasons, different assays for ergosterol have reported until now, mainly the highly sensitive and quick chromatographic determination [9].

As the consumer's demand for high-quality fresh fruit juices increases, here was verified the suitability of ergosterol as a biomarker for rapid identification and estimation of contamination by yeasts. This particular fresh melon juice was used experimentally because it is one of the excellent sources of vitamin A, vitamin C, and microelements among the juices. Moreover, melon is really appreciated by consumers for its sweetness, texture, and antioxidant molecules, such as polyphenols and flavonoids [12, 13].

The paper covers a range of issues from juice extraction, biochemical characterization of the fresh juice, to the formulation of melon juice sample with a proximate composition corresponding to the EU official directive, to the microbiological analysis. Then, the extraction and the fast quantification of the ergosterol molecule, carried out through spectrophotometric technique, which in turn allowed to identify juice pollution from *S. cerevisiae* (chosen as a model of cell fungi) using ergosterol as biomarker.

Materials and methods

Reagents and standards

All analytical grade solvents and reagents were purchased from Sigma Aldrich S.r.l. (Milan, Italy).

Plant material and melon juice preparation

Eminenza was the Italian cultivar of cantaloupe melon (*C. melo* L. var. *reticulatus*) utilized in this study. It is a variety with limited storability, rapid overripen, loss of firmness, and desiccation. Eminenza was hand-harvested in Villa Literno (Caserta province, Italy; 41°0′33 N–14°4′34 E) at commercial maturity condition. Pulps (about 500 g) from three melons were collected and random pieces were utilized for juice preparation. Briefly, melon samples were blended with a mixer followed by filtration through cheesecloth, obtaining the melon juice. All the following analyzes were performed on freshly prepared melon juice reconstituted to regular °Brix content as recommended from the Council Directive 2001/112/EC relating to fruit juices in Annex V, which establishes the minimum °Brix level for reconstituted fruit juice and reconstituted fruit purée.

Physical–chemical analyses

The total soluble solids (TSS) content was measured with a digital refractometer and expressed as °Brix at 20 °C. Titratable acidity (TA) was quantified by titrating 10 mL of melon juice with NaOH (0.1 N) to an endpoint of pH 8.1 and expressed as citric acid g/100 mL [14]. Three replicates were carried out for all measurements.

Bioactive compounds and antioxidant activity

Bioactive compounds, total polyphenols, *ortho*-diphenols, and flavonoids were extracted using 2 g of melon juice, as previously reported by Vella et al. [15] and were tested in triplicate.

Total polyphenols were determined by spectrophotometry according to the Folin–Ciocalteu method [16]. In

brief, amount of 150 µL of juice was mixed with 750 µL of Folin–Ciocalteu reagent and 600 µL of 7.5% (w/v) Na_2CO_3. After incubation, the absorbance was read at 765 nm. Polyphenols amount was calculated from a calibration curve with gallic acid as standard and results were expressed as µg of gallic acid equivalents (GAE) per mg of juice.

Ortho-diphenols content was evaluated by Arnow assay [17]. Briefly, amount of 400 µL of juice was mixed with 400 µL of 0.5 M HCl, 400 µL of 1.45 M $NaNO_2$–0.4 M Na_2MoO_4 and 400 µL of 1 M NaOH. The absorbance was recorded at 500 nm and *ortho*-diphenols were determined by a calibration curve obtained using caffeic acid (CA) as standard. Results were expressed as µg of CA equivalent (CAE) per mg of juice.

Flavonoids content was determined according to colorimetric method based on the formation of flavonoid–aluminum compounds, modified according to Vella et al. [15]. In the assay, amount of juice was mixed with distilled water and $NaNO_2$ (5% w/v) up to 1 mL. After 5 min, $AlCl_3 \times 6H_2O$ were added and the reaction was stopped by adding 1 M NaOH and distilled water. The absorbance was read at 510 nm and (+)-catechin was used to create the standard curve. The results were expressed as µg of catechin equivalents (CE) per mg of juice.

Total tannins were assessed as reported by Vella et al. [15] incubating samples with BSA at 30 °C for 1 h. The supernatant, representing the non-tannin fraction, was collected by centrifugation at 4 °C and was analyzed using Folin-Ciocalteu method. Tannins were measured by difference from the polyphenols first determined and the ones after BSA precipitation. Tannins were expressed as µg of gallic acid equivalents (GAE) per mg of juice.

The antioxidant activities were evaluated by means of two in vitro assays, the Ferric Reducing Antioxidant Power (FRAP) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity. All samples were analyzed in triplicates according to the two tests. Freshly prepared FRAP reagent was added to juice and the absorbance was recorded at 593 nm incubation. The antioxidant activities were calculated from the calibration curve of L-ascorbic acid and results were expressed as µg of ascorbic acid equivalents (AAE) per mg of juice. The free radical-scavenging activity (RSA) of the juice was assessed according to the procedure of Blois [18]. In brief, different concentrations of juice were mixed with DPPH methanolic solution. The absorbance reduction at 517 nm of the DPPH was determined continuously. The RSA was calculated as a percentage of DPPH discoloration, using the following equation:

$$\%RSA = \left[\frac{(A_{\mathrm{DPPH}} - A_s)}{A_{\mathrm{DPPH}}}\right] \times 100 \quad (1)$$

where A_S is the absorbance of the solution when the juice was added and A_{DPPH} is the absorbance of the DPPH solution. The EC_{50} value was obtained from the graph of %RSA against the juice concentrations in mg/mL.

Enzyme activities

Polyphenol oxidase (PPO), peroxidase (POD), pectin methylesterase (PME) and polygalacturonase (PG) were the enzymes evaluated in the fresh juice. PPO and POD activities were determined using a modified method of Chisari et al. [19]. Amounts of juice (2–5 gr) were homogenized in phosphate buffer at pH 7.5, with 1 mM dithiothreitol (DTT). PME and PG activities were evaluated using the same above procedure but first with extraction in 1 M NaCl. The mixtures were centrifuged at 4 °C and the supernatants were used as crude extracts. All activities were expressed as enzyme units (U), defined as the quantity of enzyme required to produce 1 µmole of product per minute. Moreover, for each enzyme, the specific activity (U/µg of protein) was calculated using Bradford method [20], with BSA as standard.

PPO assay was performed in 100 mM phosphate buffer (pH 6.8) and 0.1 M catechol. The mixture was incubated at RT and after the juice (10–50 µL) was added. The reaction was monitored at 412 nm [19].

POD activity was determined by means of method of Chisari et al. [21]. Briefly, 3% hydrogen peroxide was mixed with 4% guaiacol, in 100 mM phosphate buffer (pH 6.8) and added to an aliquot (10–50 µL) of juice. After incubation at RT, the reaction was monitored at 470 nm for 2 min.

PME activity was determined by a modified method of Hagerman and Austin [22, 23]. An aliquot of juice (from 1 to 10 µL) was added to 1 mL of mixture composed by 0.1% of pectin solution from citrus peel (galacturonic acid > 74%, methoxy groups > 6.7%) and 0.1 g/L solution of bromothymol blue. The absorbance decrease was monitored for 2 min at 620 nm.

PG activity assay was based on the quantification of reducing groups produced, via the spectrophotometric method of Miller [24], using dinitrosalicylic (DNS) acid reagent. Briefly, an aliquot of juice (1–10 µL) was incubated with 0.5% polygalacturonic acid (MW 25,000–50,000) solution in 50 mM phosphate buffer, pH 7.0. After incubation at RT, DNS reagent was added to the tube and the mixture was boiled for 5 min. The reaction was stopped in ice and the absorbance was measured at 546 nm.

Yeast growth and juice contamination

Saccharomyces cerevisiae was obtained from the strain collection of National Research Council (CNR). Stock culture was maintained at 4 °C on MEA (malt extract 30 g/L;

peptone 5 g/L, agar 15 g/L). Inocula were obtained from overnight cultures on MEA plates at 28 °C. *S. cerevisiae* was grown in ME (malt extract 30 g/L; peptone 5 g/L) for 24 h, shaking at 120 rpm at 28 °C. The growth of *S. cerevisiae* was monitored both by measuring the absorbance at 600 nm and by counting on plates (CFU/mL). A linear relation was determined using OD and CFU/mL ($y = 0.0000002\ x$; $R^2 = 0.9583$).

Contamination of juice with suspension of *S. cerevisiae* was carried out with fresh melon juice, to a final concentration of 10^4 CFU/mL. Samples of adulterated juice were evaluated at three points (24 h, 48 h, and 72 h), and centrifuged at 4 °C. The pellets were used to extract the ergosterol. The molecule was determined through the above linear regression equation, between ergosterol (expressed as µg) and the CFU/mL.

Ergosterol extraction and quantification

Ergosterol was obtained according the procedure reported by Vella and Laratta [11], with minor modifications. Briefly, extraction from yeast cells was performed by saponification, adding 25% potassium hydroxide solution in ethanol. The mixture was incubated in a water bath at 80 °C for 1 h. The mixture was extracted twice by adding distilled water and petroleum ether in a separating fennel. The upper phases that contained Ergosterol were evaporated to dryness and efficient extractions were monitored by means of thin layer chromatography (TLC), using petroleum ether/ethyl ether 6:4 (v/v) as solvent system. TLCs were revealed under UV light, carbonizing with cerium (IV) sulfate ($Ce(SO_4)_2$) reagent. The molecule was identified by means of TLC ($R_f = 0.3$) and through the UV spectrum carried out between 240 and 300 nm, as reported by Vella and Laratta [11]. The UV absorption spectrum of ergosterol shows two major peaks, at 274 nm and at 282 nm, a minor peak at 290 nm and a shoulder at 262 nm, corresponding to the characteristic spectra. Ergosterol was calculated by means of a standard curve, recording the absorbance at 282 nm, according to the formula $y = 0.0179 \times$ ($R^2 = 0.9986$).

Statistical analysis

All samples were analyzed in triplicates and results were expressed as mean ± standard deviation (SD). Means, SD, calibration curves and linear regression analyses (R^2) were determined using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

Results and discussion

The juice is a particular and complex environment whose compositional and physical features promote the growth of microbes, because acidity, oxygen, and nutrients affect also food preservation conditions. As a result, at the beginning of the work the physicochemical properties, bioactive compounds and antioxidant activity were evaluated in the reconstituted juice. The work then focused on contamination of melon juice with *S. cerevisiae* and on the assembling the new microbiological test. Figure 1 outlines a block diagram of the experimental design approach.

The quality of the cantaloupe melon juice was examined by analyzing the following parameters: pH, titratable acidity (TA), total soluble solids (TSS), bioactive compounds, and ripening enzymes.

Depending on the fruit juice, the acidity results from the contribution of various organic acids such as citric, malic, fumaric, acetic, ascorbic, and galacturonic [25]. Concerning pH, the melon juice showed a good value of 6.57 ± 0.02, as reported in Table 1. This is usually consistent with data of literature with the exception of Fundo et al. [13] that reported a lower pH value of approximately 6.14, possibly because the study was carried out on different melon cultivars. The TA parameter is rather linked to the total concentration of free protons that can be neutralized by a strong base. Melon juice showed a value of TA of 0.0683 ± 0.0037 g per 100 mL citric acid that agrees with literature data reported previously [19].

Total soluble solids (measured in °Brix) represent the balance of sugars and acids present in a vegetal matrix and is universally accepted as an index of fruits maturity. This is a key parameter because it affects the flavor of fruit and therefore needs to be protected in processed industrial products such as juice, jam and ice cream. In melon cultivars, the optimal values reported for TSS at commercial maturity vary in the range of 10–14°Brix [19, 26]. In this study, TTS is 13.65 ± 0.35°Brix, meaning that this melon juice owns a considerable sugar content.

Among bioactive compounds in melon, the largest group of polyphenols consist mainly of flavonoids and phenolic acids. The content and type of biomolecules differ mainly among the melon cultivars considered as it was reported previously [15]. In particular, the total polyphenols content found in this study for melon juice was 2.80 ± 0.07 µg GAE/mg, the *ortho*-diphenols were 0.84 ± 0.01 µg CAE/mg, the flavonoids were 0.51 ± 0.01 µg CE/mg, and tannins were 0.68 ± 0.08 µg GAE/mg, as listed in Table 1. Hence, the polyphenols result higher than those reported by Fundo et al. [15] and Miller et al. [27] in a study on cantaloupe melon pulps. These variations could be attributed to several factors, including cultivar,

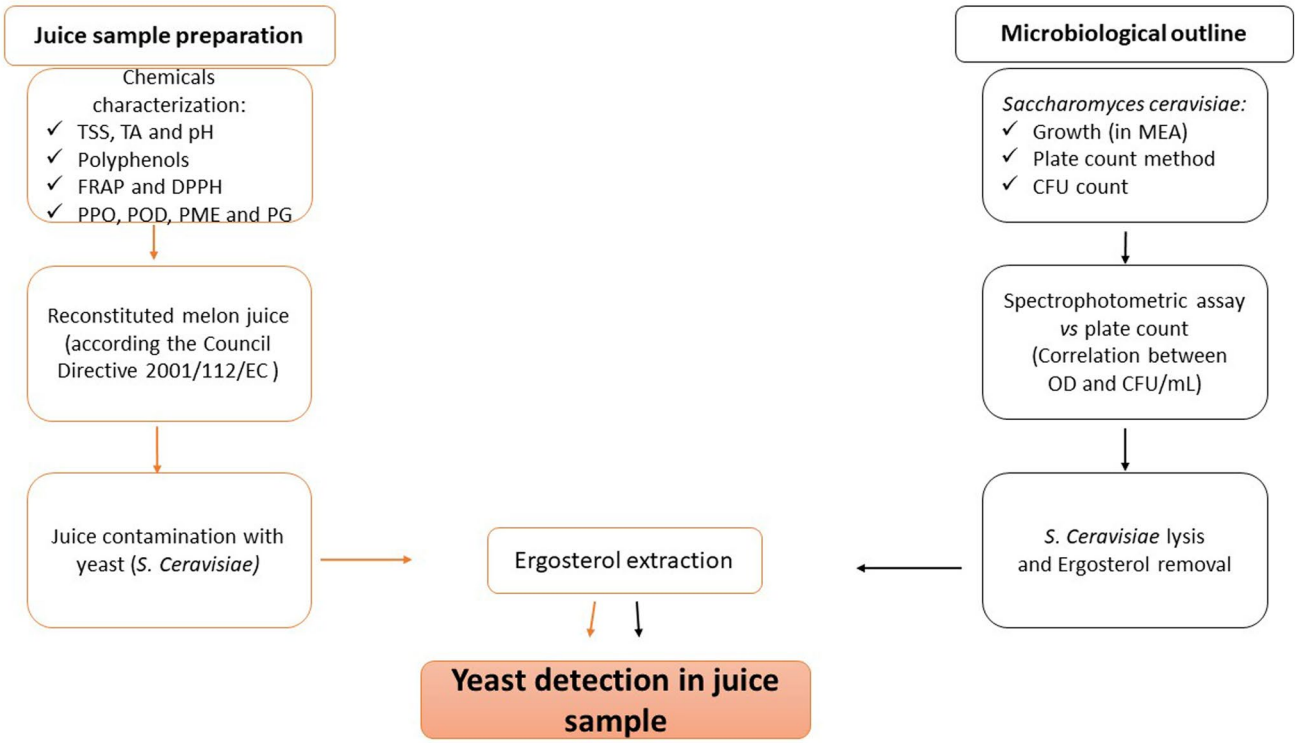

Fig. 1 Block diagram of the experimental design

Table 1 Physicochemical properties, bioactive compounds and antioxidant activity of melon juice

pH	6.57 ± 0.02
TSS (°Brix)	13.65 ± 0.35
TA (g citric acid/100 mL)	0.0683 ± 0.0037
Polyphenols (μg GAE/mg)	2.80 ± 0.07
Ortho-diphenols (μg CAE/mg)	0.84 ± 0.01
Flavonoids (μg CE/mg)	0.51 ± 0.01
Tannins (μg GAE/mg)	0.68 ± 0.08
Antioxidant power (μg AAE/mg)	1.40 ± 0.04
Antioxidant activity (EC_{50}—mg/mL)	13.70 ± 0.94

degree of ripening, and environmental issues, such as climatic conditions and geographical origin [12, 15]. To the best of our knowledge, the data on *ortho*-diphenols, flavonoids and tannins have never been reported in melon juice until now. The antioxidant capacity of juice was assayed by means of the FRAP method and displayed a value of 1.40 ± 0.04 μg AAE/mg. Moreover, it is reported in Table 1 the EC_{50} of 13.70 ± 0.94 mg/mL, calculated with the DPPH assay. These results unequivocally demonstrate that juice retains an effective antioxidant power that is consistent with previous work on pulps from some cantaloupe cultivars [12, 15].

The enzymes extracted from melon juice are enzymes linked to senescence and ripening processes in vegetable tissues. Results of enzyme activities are reported in Fig. 2A and B.

The enzymatic browning of juices is mostly related to the high activity of oxido-reductive enzymes POD and PPO [21]. POD activity in melon juice was 2.16 ± 0.11 μM, with a specific activity of 0.52 ± 0.03 U/μg of protein. Furthermore, PPO activity was 4.41 ± 0.22 μM, showing a related specific activity of 2.68 ± 0.13 U/μg of protein, as depicted in Fig. 2A.

The loss of cloud in juice and the consequent decrease in consumer acceptability is generally attributed to enzymes PME and PG [28, 29]. These two enzymes showed activities of 0.0064 ± 0.0003 μM and 0.0538 ± 0.027 μM, respectively. In Fig. 2B, the specific activities for PME 0.0054 ± 0.0002 U/μg of protein and for PG 0.0454 ± 0.0022 U/μg of protein are reported, respectively. At the best of our knowledge, this is the first time that degrading and antioxidant enzymes were studied in cantaloupe juice, with the exception of Chisari et al. [19] that studied variation of these enzymes at different time of maturity in a diverse cultivar of melon. The values found in these work are in accordance with the activities studied in fruits of other varieties of cantaloupe, muskmelon, and pineapple [19, 21, 23].

Moreover, the activities of PPO, POD, PME and PG, are all fundamental parameters for monitoring the thermal

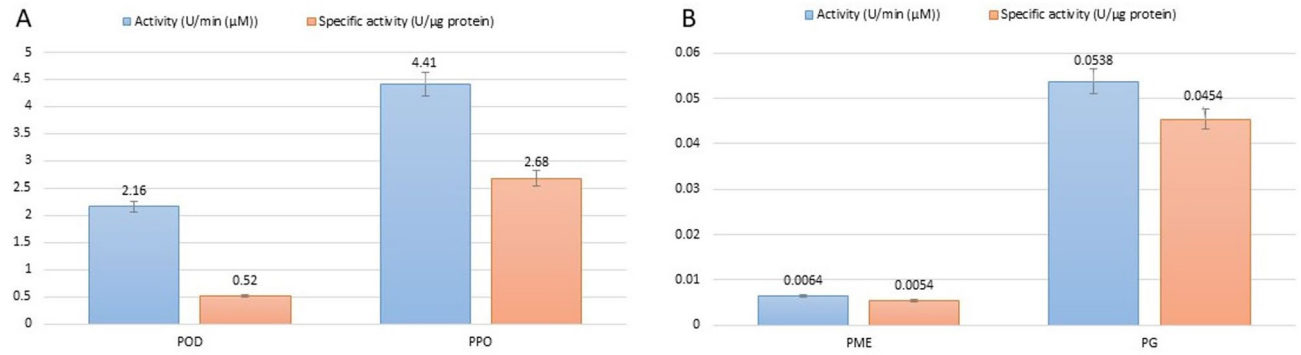

Fig. 2 Activities of enzymes: POD and PPO (**A**); PME and PG (**B**)

stability of juices during industrial processing, as well as for preserving the shelf life of fresh fruits and vegetables [19]. In fact, in a study conducted on residual PME activity in a pasteurized juice was demonstrated that at nearly about 10^{-3} units order the juice may display a cloud precipitate [23]. Therefore, a mild heat treatment is indicated to render these enzymes inoperative. As a result, after enzymatic inactivation, melon juice becomes more appetizing and pleasing to consumers' taste.

S. cerevisiae: growth, ergosterol quantification and melon juice contamination

S. cerevisiae is a yeast that have traditionally established since antiquity a link to human activity. Thanks to the fermentative properties of yeast, many essential and beneficial foods and drinks, such as bread, wine and beer, have produced. On the other hand, the spoilage can cause adverse effects and, in some beverages like fruit juices, yeasts operate a decisive role in affecting the final quality [2–6]. There fore, a quick valuation of yeast spoilage is always a favorite choice.

The suitability of using ergosterol as indicator of yeast contamination in reconstituted fresh melon juice was explored. With this aim, melon juice was inoculated with a known concentrations of *S. cerevisiae*. Samples of inoculated juices, obtained after incubation at 24, 48 and 72 h, underwent to ergosterol extraction. Using the good correlation calculated between *S. cerevisiae* cells and OD ($R^2 = 0.9986$), for each incubation point was obtained the corresponding concentration of *S. cerevisiae* in CFU/mL. As reported in Table 2, *S. cerevisiae* reached 6.96×10^5 CFU/mL in melon juice after incubation at 24 h. After a period of 48 h yeast growth reached the value of 2.37×10^6 CFU/mL, rising up to 9.20×10^6 CFU/mL at 72 h of incubation. Results showed that ergosterol was extracted from juice efficiently and the analysis showed that the ergosterol content of *S. cerevisiae* from artificially inoculated melon juice increased linearly with the growth of cells over the incubation time from 24 to 72 h. This finding is in accordance with results reported by Porep [10] in a study conducted on grapes.

Table 2 CFU/mL of *S. cerevisiae* in fresh melon juice

(Time) h	CFU/mL	Erg (µg/mL)
24	6.96×10^5	3.48
48	2.37×10^6	11.85
72	9.20×10^6	46.00

Nowadays, the infections caused by yeasts are increasing and an early detection of contamination is critical in preventing allergies, gastroenteritis, or other effects that may be caused by the ingestion of polluted foods and drinks [5]. In specialized control laboratories for yeast determination indirect and direct methods are based on the recognition of some metabolic products or the direct observation of yeast cells, analyzing different parameters such as morphology, growth kinetics, and colony forming units. These techniques are high-sensitive and capable to detect up to small concentrations of microorganisms, but are time-consuming and laborious [30]. Other tests, microscopic, immunological, spectrometric and chemotaxonomic instead are not helpful for their high costs and the requirements of specialized staff [4, 5]. Recently, the use of DNA-based technique for the analysis of foodborne contamination has increased but results impracticable in industrial routine due to the price and complexity of equipment and staff [30]. Thus, the search for more rapid and efficient methods has led to use culture-independent technique, identifying components and/or metabolic products of microbes to be used as biomarkers.

Based on these considerations, ergosterol through spectrophotometric assay seems to be a reliable indicator to be used as microbiological quality marker in the routine detection of fungi. The proposed method results in rapid, efficient, and low cost features, permitting control purposes of foods

and drinks even in industrial laboratories. Interestingly, the test is capable of detecting the total yeast biomass present in a beverage, which comes from live and dead cells, even when has been subjected to heat treatment.

Conclusion

Growth of yeasts in foods and beverages is often difficult to detect because microbial biomass cannot be easily separated from a solid substrate or a cloudy drinks. Consequently, monitoring yeasts growth by measuring the concentration of a chemical compound of the yeast cell, the ergosterol, appears to be a helpful strategy in food safety. As well, ergosterol analysis has the exclusive advantage to be distinctive for fungi kingdom and to detect uncultivable form (dead forms or debride parts) that otherwise are uncountable. The test is speedy and cheap, since it is a simple spectrophotometric analysis, respect to more sophisticated or classic microbiological techniques. In addition, it has been shown to be effective when tested in a fresh cantaloupe juice.

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Author contributions B.L. and F.M.V. conceived the study and designed the experiments; F.M.V. and A.D.B. performed the experiments; R.C. managed software and statistical analysis; F.M.V and A.D.B. analyzed the data and prepared the figures; B.L. and F.M.V. wrote the draft; B.L., M.G., and F.M.V. reviewed and edited the manuscript. All the authors have read and agreed to the final version of the manuscript.

Declarations

Conflict of interest The authors reported no potential conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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