



Solid fat influences sorbic acid partitioning and enhances the preservation effect on *C. guilliermondii* in biphasic food model systems

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ABSTRACT

Preservation of emulsions relies on factors including pH, temperature, structure and the application of carboxylic acid preservatives, such as sorbic and benzoic acid. Organic acid preservatives tend to migrate to the lipid phase of emulsions. Taking into account the fact that organic acid in the aqueous phase is solely responsible for antimicrobial activity, this partitioning behavior is considered as a loss of preservative. The influence of microstructure properties on sorbic acid distribution and preservation effect was investigated in model food systems comprising of aqueous phase, and liquid oil and solid fat as lipid phase, which represent major constituents of water-oil emulsions. The aqueous phase of the food model systems was comprised of Yeast Nitrogen Broth (YNB) in phosphate buffer and buffered at pH 4.5, 5.5, 6.5. Sorbic acid (100 mg/L) in the form of potassium sorbate was added to the aqueous phase. *Candida guilliermondii* (2 log CFU/mL) was inoculated in the aqueous phase to resemble industrial post-contamination CFU levels. Growth parameters, generation time (GT) and lag phase (λ) of *C. guilliermondii* in the aqueous phase of the food model systems were quantified during 1 month at 7 °C and maximum 80 h at 22 °C. HPLC analyses were performed to evaluate total sorbic acid content in each food model system. Sorbic acid inhibited growth of *C. guilliermondii* in YNB + SA at pH 4.5 at 7 °C and 22 °C. The presence of liquid oil caused partitioning of sorbic acid into the lipid phase in YNB + SA|oil at pH 4.5 at 7 °C and 22 °C, reducing its inhibitory effect. Adding solid fat into the model food systems significantly prolonged lag phase duration in the YNB + SA|oil + fat system at pH 4.5 at 22 °C and growth was inhibited at 7 °C. HPLC analysis showed a final aqueous sorbic acid concentration of 34 mg/L and 44 mg/L in YNB + SA|oil system at pH 4.5 at 7 °C and 22 °C after 1 month and 168 h, respectively. In YNB + SA|oil + fat system at pH 4.5, 87 mg/L of aqueous sorbic acid was measured after 1 month at 7 °C and 168 h at 22 °C, indicating that the presence of solid fat retards sorbic acid partitioning. Results show that structural components such as liquid oil and solid fat have an effect on the aqueous sorbic acid concentration and its preservation effect. The presence of solid fat reduces the tendency of sorbic acid to partition into the lipid phase, which is reflected in the inhibitory effect of sorbic acid on *C. guilliermondii*.

1. Introduction

Food preservatives play a significant role in food production processes and storage by inhibiting the growth of spoilage microorganisms and extending the shelf life of food (Cheng et al., 2010). Survival and growth of microorganisms in food is affected not only by the presence of preservatives and storage conditions, but also by the structure of food (Wilson et al., 2000). Food structure can have an impact on the shelf-stability of food products by constraining (limiting) the mechanical distribution of water (Pragalaki et al., 2013), the chemical distribution of organic acids (including food preservatives), and by physically constraining the mobility of microorganisms (Brocklehurst and Wilson,

2000). The lipid phase of an emulsion can be partly or completely crystalline in structure, which influences the stability of water droplets or air bubbles dispersed in the emulsion and provides the required texture (Robins, 2000). In food systems, preservatives are soluble in the water and lipid phases, and are not soluble in hydrocarbons, proteins and fibre (Cheng et al., 2010). As microorganisms only grow in aqueous environments, the concentration of a preservative in the water phase is directly related to the growth potential of microorganisms present in the food system. The amount of a preservative dissolved in the lipid phase implies a loss of the preservative effect. Therefore, the exact quantitative determination of the concentration(s) of preservatives in the aqueous phase is crucial for evaluating the safety and quality of

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food products (Davidson et al., 2005; Lück and Jager, 1997). This information is particularly useful for determining the minimum inhibitory concentrations (MICs) of preservatives applied in biphasic products. MIC values of preservatives are usually determined in synthetic water-based media and expressed as aqueous phase concentrations (Arroyo-López et al., 2008). Exact quantification of aqueous phase sorbic acid concentration and knowledge about its partitioning behaviour help determine the true effective inhibitory dose in emulsions.

Sorbic acid (E200) is a valuable preservation agent, the use of which is regulated by EU Regulation 1333/2008 in the European Union. The limit of use is 1000 mg/kg and 2000 mg/kg in fat emulsions with either more or < 60% of fat, respectively. Its inhibitory effect is mainly due to the undissociated form of the acid (Lund et al., 2000). It is particularly effective in preventing mould and yeast growth (Sperber and Doyle, 2009). Due to its low solubility in water (0.16 g/L), sorbic acid is usually applied in the form of its salt derivatives, predominantly as potassium sorbate (E202). The concentration of undissociated acid in the aqueous phase, $[HA]_{aq}$, at a given pH can be calculated from the formula:

$$[HA]_{aq} = \frac{[HA]_T}{1 + 10^{(pH - pK_a)}} \quad (1)$$

where $[HA]_T$ is the total acid added in the system, and pK_a is the acid dissociation constant.

The distribution behaviour of sorbic acid in different systems has been investigated by several researchers (Deuel et al., 1954; Lubieniecki-von Schelhorn, 1967; Sofos, 1989). Denyer and Baird (2007) found that partitioning of preservatives in emulsions depends on the type and amount of fat, pH, surface interfacial area, droplet size and other ingredients. Despite the several studies that have been performed to date, none have comprehensively investigated the influence of type of lipid (oil and/or fat) in an emulsion on the partitioning of sorbic acid. The experimental investigation of partitioning of sorbic acid in water, oil and fat systems is fundamentally valuable for optimising its applications in emulsions.

In this work, the influence of sunflower oil (liquid oil) and a palm stearine hardstock (solid fat) on the distribution behaviour of sorbic acid in emulsions was experimentally determined by means of HPLC analysis and validated by challenge tests with *Candida guilliermondii*. The aqueous phase consisted of phosphate buffer and nutritive media, buffered at pH values of 4.5, 5.5, 6.5, to simulate the typical pH values of the aqueous phases of emulsions. *C. guilliermondii* was chosen as a model spoilage yeast. It has been isolated as a spoilage microorganism in soft drinks, dairy products, butter and olive oil (Mushtaq et al., 2007; Zullo et al., 2010). Preliminary tests (results not shown) showed that *C. guilliermondii* was completely inhibited by 300 mg/L sorbic acid. As the aim of the study was to demonstrate the effect of sorbic acid on the lag phase and growth rate of *C. guilliermondii*, 100 mg/L of sorbic acid was chosen as the concentration that would permit growth and enable quantification of the growth parameters.

2. Materials and methods

2.1. Yeast strain and culturing conditions

Candida guilliermondii, strain NP566U + pURA5-GFP, was kindly provided by Dr. N. Papon of the Biomolécules et Biotechnologies Végétales, Université François-Rabelais de Tours, Faculté de Pharmacie, Tours, France. Stock cultures were prepared as follows. Firstly, subcultures were prepared by incubating 5 mL Yeast Nitrogen Broth (YNB) inoculated with *C. guilliermondii* for 48 h at 22 °C. Thereafter, these subcultures were streak plated on Yeast Nitrogen Agar (YNA), and incubated at 30 °C for 5 days to obtain the stock cultures which were subsequently stored at 4 °C until required. Stock cultures were prepared monthly. YNB consisted of 6.7 g Yeast Nitrogen Base without amino acids (Sigma Aldrich, USA) and 20 g of saccharose (Sigma Aldrich) in

1 L of distilled water. YNA had the same composition as YNB, with 20 g of bacteriological agar (Sigma Aldrich) added to solidify the medium. YNB was filter sterilized through a Rapid Flow Bottle Top Filter (Nalgen, USA) with a pore diameter of 0.45 µm. YNA was sterilized by autoclaving for 15 min at 121 °C.

2.2. Preparation of inoculum for challenge tests

Candida guilliermondii was grown in 5 mL YNB at 22 °C for 48 h while shaking at 200 rpm on a plate shaker (IKA Werken, Germany). Shaking (agitation) had previously been determined to increase the cell yield by ca. 1 log CFU/mL in comparison to non-shaken (static) cultures. Cell counts of 8 log CFU/mL were obtained in these stock suspensions after 48 h of incubation. The inoculum for challenge tests was prepared by dilution of the stock suspension in buffered YNB to ca. 3 log CFU/mL.

2.3. Preparation of aqueous phase

As mentioned previously, the aqueous phase consisted of phosphate buffer supplemented with nutrients. Phosphate buffers of pH 4.5, 5.5, 6.5 were prepared according to European Pharmacopoeia (Council of Europe, 2004). All materials used to make phosphate buffers were obtained from Chem-Lab NV, Belgium. Yeast Nitrogen Base without amino acids (6.7 g/L) as well as saccharose (20 g/L) were added to the buffers. Potassium sorbate (VWR, USA), 134 mg/L, corresponding to 100 mg/L (100 ppm) of sorbic acid (SA), was added to each buffer in the experiments investigating the effect of sorbic acid on the growth of *C. guilliermondii*. The pH of these buffers was checked and adjusted after the addition of the nutrients and potassium sorbate with H₃PO₄ (6 M) and NaOH (10 M) (Merck, USA). The pH of the buffers was measured and adjusted at 20 °C with the aid of a digital pH meter (S220 SevenEasy, Mettler-Toledo, USA).

2.4. Preparation of the food model systems

A ratio of 40% aqueous phase and 60% lipid phase by weight was used in all conditions. None of the samples were emulsified, as the addition of the emulsifier would influence sorbic acid behaviour. The aqueous phases (pH 4.5, 5.5, 6.5) were with the diluted stock suspension of *C. guilliermondii* to achieve an initial concentration of ca. 2 log CFU/mL. Thereafter, 15 g of sunflower oil (Vandemoortele NV, Belgium) was added to 10 mL of each aqueous phase. Food model systems consisting of aqueous phase, liquid oil and solid fat were prepared as follows. 7.5 g of sunflower oil and 7.5 g of a solid interesterified hard stock fat (Vandemoortele NV) were heated up to 60 °C and mixed at 10000 rpm for 3 min by means of an Ultra Turrax (IKA T25 Digital, Germany). The lipid phase mixture was then allowed to cool to 37 °C before placing it onto the inoculated aqueous phase in order to prevent potential thermal inactivation of the inoculum. All food model systems were prepared in triplicate and incubated at 7 °C and 22 °C for 1 month and maximum 80 h, respectively. A schematic representation of the model systems can be seen in Fig. 1.

2.5. Sampling and enumeration of *C. guilliermondii*

Sampling was performed periodically during storage by aseptically drawing 100 µL aliquots from the aqueous phase of each replicate. This small sample volume was drawn from the replicates to ensure a suitably large volume of aqueous phase remained in the Falcon flasks to permit further sampling. It should be noted that an equivalent volume of oil was also removed to maintain the 40/60 ratio of aqueous to lipid phase. Serial decimal dilutions were prepared in Peptone Physiological Salt (PPS, 8.5 g NaCl + 1 g neutralized bacteriological peptone per L). In the case of the systems containing the mixture of sunflower oil and solid hard stock fat, repeated sampling from the same recipient was not

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