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Study of apple juice preservation by filtration through silica microparticles functionalised with essential oil components

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Abstract

Novel non-thermal preservation technologies are needed given consumers' demand for minimally processed high quality food products with a long shelf life. In this work, novel filtering materials based on silica microparticles functionalized with essential oil components (eugenol and vanillin) were applied as an alternative preservation method for apple juice. After confirming the immobilisation of the antimicrobial compounds on particles' surface, the capacity of the filtration system to pasteurise apple juice inoculated with *Escherichia coli* was proven (a reduction of at least 5-log). The influence of filtration on the physico-chemical (pH, acidity, soluble solids, colour) and microbiological (mesophilic, psychrophilic and mould and yeast) parameters of fresh apple juice was evaluated throughout refrigerated storage, as well as the potential leaching of the immobilised bioactive compounds. The results showed the capability of the functionalised particles to completely remove the native flora of fresh apple juice and to preserve the product's microbial stability throughout the study period (> 120 days). Filtering juice through the eugenol-functionalised particles did not strongly affect the physico-chemical properties, except for the colour attribute. The use of immobilised vanillin significantly affected some product characteristics, probably due to the partial release of compounds to the food matrix. Therefore, the immobilisation methodology should be optimised before applying this support to a real scenario. Nevertheless, the immobilisation of these bioactive compounds on silica microparticles allowed filtration technology to be developed as a promising alternative to existing pasteurisation technologies to provide microbial stable and quality acceptable food products.

Keywords: naturally-occurring antimicrobial compounds; cold pasteurization; covalent immobilization; filtration; silica microparticles; apple juice.

1. Introduction

Apple juice can be contaminated by certain pathogens, such as *Escherichia coli* O157:H7, *Salmonella enterica* or *Cryptosporidium parvum,* given their ability to adapt to cooling temperatures and low pH. Therefore, the consumption of fresh apple juice has been linked to various outbreaks of diarrhoea and haemolytic uremic syndrome (Martínez-Gonzáles & Castillo, 2016). In addition to pathogenic microorganisms, juice can also contain altering microorganisms, such as yeasts, mainly *Saccharomyces spp*., and moulds like genera *Penicillium* and *Aspergillus,* and lactic acid bacteria like *Lactobacillus* and *Leuconostoc spp.* (Choi & Nielsen, 2005; Ferrario & Guerrero, 2016). Juice contamination is prevented by pasteurisation methodologies, done to ensure a 5-log reduction in the microorganisms that can cause spoilage and pose public health problems, according to the US FDA (Food and Drug Administration, 2001).

Traditionally, thermal pasteurisation has been the gold standard of preservation treatment. Thermal processing involves heating up food, which results in some degree of nutritional loss, undesirable sensorial changes and loss of some functional properties (Choi & Nielsen, 2005). Due to the negative effect of thermal pasteurisation on key juice parameters and the growing consumer demand for minimally processed foods, alternative non-thermal pasteurisation techniques have appeared in recent years. These methods should ensure that pathogenic microorganisms are absent and they should prevent the growth of spoilage microorganisms to extend the product's shelf life and to guarantee a minimum impact on nutritional and sensory food properties. New non-thermal technologies, such as pulsed light, ultraviolet radiation, ultrasound, high hydrostatic pressure, pulsed electric field and dense phase carbon dioxide, have been developed. However, they imply several limitations that prevent their industrial application, including limited antimicrobial efficacy (Chemat, Zill-E-Huma, & Khan, 2011), impact on food properties (Ahmed & Ramaswamy, 2003), and high implementation costs (Morris, Brody, & Wicker, 2007).

Filtration is an important non-thermal process used for the clarification, concentration and microbial stabilisation of different foods (Papafotopoulou-Patrinou et al., 2016). Deep-bed filtration using sand or diatomaceous earth is used to eliminate organic matter and microorganisms, but does not offer sufficient efficiency and presents regeneration problems (Devi, Alemayehu, Singh, Kumar, & Mengistie, 2008). Membrane filtration offers selective filtration that reduces microbial contamination without having to use heat treatment (Lipnizki, 2010). However, the retention of food components, membrane fouling and cleaning requirements are critical factors that limit the extensive application of this technology (Fuenmayor, Lemma, Mannino, Mimmo, & Scampicchio, 2014).

Filtration as a methodology is continuously progressing and alternative materials can be proposed to design new filtration systems to be used as a food preservation technology. Among these novel materials, it is worth highlighting supports functionalised with antimicrobial molecules. Essential oil components (EOCs), as natural antimicrobials with recognised antimicrobial and antifungal activity (Burt, 2004), can be immobilised on silica supports to create new antimicrobial particles, and to prevent the negative effects associated with their use in a free form (strong smell and taste, high volatility or instability) (Tyagi, Gottardi, Malik, & Guerzoni, 2014). The inhibitory effect of immobilised EOCs has been recently demonstrated *in vitro* and in food matrices (Ruiz-Rico et al., 2017). Immobilisation of EOCs on silica particles has provided improved antimicrobial properties of bioactive compounds (García-Ríos, Ruiz-Rico, Guillamón, Pérez-Esteve, & Barat, 2018) with a significantly reduced impact on aromas of treated foods (Ribes et al., 2017).

By taking these antimicrobial supports as starting point, this work aims to apply EOC-functionalised silica microparticles as filtering aids for the cold pasteurisation of fresh apple juice, and to evaluate the influence of the filtration process on the physico-chemical and microbiological properties of apple juice during refrigerated storage.

2. Materials and Methods

2.1. Chemicals

Eugenol, (3-aminopropyl)triethoxysilane (APTES), sodium borohydride, sodium hydroxide, chloroform, n-butanone and silica microparticles (mean size of 50 μm) were provided by Sigma-Aldrich (Madrid, Spain). Vanillin was purchased from Ventós (Barcelona, Spain). Acetonitrile, n-hexane, methanol, potassium hydroxide, sulphuric acid, dispersive solid phase Extrabond® QuEChERS and microbiological media were provided by Scharlab (Barcelona, Spain).

2.2. Synthesis of the EOC-functionalised silica microparticles

Antimicrobial supports were prepared by the covalent immobilisation of vanillin and eugenol on the surface of commercial silica microparticles following the synthetic procedure of García-Ríos et al. (2018). For eugenol, the aldehyde derivative was prepared to add a second reactive moiety in the molecule. The aldehyde derivative of eugenol and unmodified vanillin were reacted with APTES to obtain alkoxysilane derivatives capable of being attached to silica microparticles' surface. Finally, the imine bond formed between the aldehyde group of bioactive compounds and the amino group of the alkoxysilane moiety was reduced to stabilise the immobilised antimicrobial compounds.

2.3. Materials characterisation

The commercial silica microparticles and the EOCs-functionalized supports were characterised by standard techniques. Zeta potential analysis was performed in a Zetasizer Nano ZS (Malvern Instruments, UK) using previously sonicated particle suspensions in distilled water (1 mg/mL). Zeta potential values were obtained by applying the Smoluchowski model. The degree of functionalisation was determined by thermogravimetric (TGA) and elemental analyses. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc.,

Schwarzenbach, Switzerland), with a heating program that consisted of a heating ramp of 10 °C/min from 25°C to 1,000°C in an oxidant atmosphere (air, 80 mL/min).

2.4. Assessment of the EOCs-functionalised supports as filtering materials for juice cold pasteurisation

To test the effectiveness of the EOC-functionalised supports used as filtering materials to microbiologically stabilise juices, the preservation method should be able to reduce by at least 5 logarithmic cycles of pathogenic microorganisms to ensure the safety of the treated product (Kiskó & Roller, 2005). Therefore, this assay was performed using commercial juice inoculated with *Escherichia coli.* The non-pathogenic strain *Escherichia coli* K12 (CECT 433, Colección Española de Cultivos Tipo, Valencia, Spain) was used as a surrogate of *E. coli* O157:H7, which can mimic the pathogen's survival and growth properties (Kim & Harrison, 2009). Tryptic soy broth (TSB) and plate count agar (PCA) were used to grow the bacterium. The bacterial strain was reconstituted following the CECT instructions and bacterial stock was stored at 4°C in PCA before being used. To obtain an inoculum with an approximate microbial density of 10^9 cells/mL, the cells from a colony were transferred to a test tube with 10 mL of TSB to be incubated at 37°C for 24 h. After incubation, the cell concentration of the inoculum was checked by determining optical density at 600 nm in a Helios Zeta UV-VIS instrument (Thermo Scientific, Hampton, New Hampshire, USA). Then, the inoculum was centrifuged at 4,000 rpm for 10 min and the precipitated cells were resuspended in 1 L of apple juice to obtain a final *E. coli* density of 10^6 cells/mL.

The filtration assays were performed in a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) connected to an Erlenmeyer flask to collect the sample. The filtering bed placed in the funnel consisted of three layers: (i) cellulosic paper on the manifold base; (ii) a bed of bare EOCs-functionalised supports (layer of 0.5-1 cm thickness); (iii) another cellulosic membrane as the covering top. For the filtration tests, the microbial retention

capability of the EOCs-functionalised supports was assessed after filtering 100 mL of inoculated apple juice through the bed of particles. Then the collected apple juice was plated in selective media Tryptone Bile X-glucuronide (TBX) agar, and plates were incubated at 37°C for 24 h. The values of the counts were logarithmically transformed and expressed as $\log_{10}$ CFU/mL.

Assays were performed in triplicate. Two control samples, namely the non-filtered juice and the juice filtered through a bed of bare (non-functionalised) silica particles, were included in the assays to quantify the microbial count in the absence of treatment and after filtration through bare supports.

2.5. Influence of the filtration process on the shelf life of fresh apple juice

After establishing the capability of the EOCs-functionalised supports as filtering elements to cold pasteurise commercial inoculated juice, the influence of the filtration process on the different physico-chemical and microbiological parameters of apple juice during refrigerated storage was evaluated. Besides, the potential leaching of the immobilised molecules and the influence of the treatment on the sensory properties after filtration were determined.

2.5.1. Apple juice preparation

Apple juice was prepared with 'Braeburn' apples (*Malus domestica)*. After purchasing apples in a local supermarket, juice was prepared in a Thermomix TM31 (Vormerk MSL, Madrid, Spain), where the peeled and chopped apples were mixed with distilled water (1 L of water per kg of apples). The mixture was filtered through a stainless steel strainer without pressure to eliminate fruit pulp and to obtain fresh juice.

Fresh apple juice (150 mL) was filtered under sterile conditions through the layer (0.5 cm thickness, 2 g) of the bare or EOCs-functionalised supports and was stored in proportions of 20

mL in Falcon tubes at 4°C to be analysed at different times. The assay was done in triplicate, including the above-mentioned control samples.

2.5.2. Analytical methods for shelf life evaluation

Different analytical determinations were carried out during the shelf life of both fresh juice and filtered and refrigerated juice. The microbial analyses were carried out on days 0, 3, 6, 9, 12, 30, 60, 90 and 120. The physico-chemical parameters were determined on days 0, 3, 6, 9 and 12. All the analyses were performed in triplicate.

The microbial analyses consisted of enumerating the native flora of apple juice, including mesophilic aerobic, psychrophilic and mould and yeast. Peptone water 10-fold dilution aliquots of juice were prepared and plated on selective media according to the target microorganism. For the enumeration of the mesophilic aerobic populations, samples were plated in PCA to be incubated at 30°C for 72 h. The psychrophilic bacteria were determined on PCA plates incubated at 4°C for 10 days. The mould and yeast populations were counted on potato dextrose agar plates and incubated at 25°C for 72 h (Ferrario & Guerrero, 2016). The CFU/mL were determined (detection limit of 5 CFU/mL) and were logarithmically transformed and expressed as log CFU/mL.

The determined physico-chemical parameters were titratable acidity, pH, °Brix and colour. An acidity analysis was done by titration with NaOH (0.1 N) in the presence of phenolphthalein. A pH-meter Crison micropH 2001 (Crison Instruments S.A., Alella, Barcelona, Spain) was used to measure the samples' pH value. Soluble solids (°Brix) determination was made with a refractometer RFM300 (Bellingham & Stanley Ltd, Kent, UK). Juice colour was measured by a colorimeter Minolta CM 3600D (Minolta, Osaka, Japan) under a standard illuminant D65 and 10° observer. The CIEL*a*b* uniform colour space was selected to calculate the colour parameters: L* (brightness), a* (red–green) and b* (yellow–blue). The total colour difference between the unfiltered sample and the samples treated on day 0 was also calculated.

2.5.3. Leaching the immobilised EOCs

Besides the microbiological and physico-chemical parameters, the possible leaching of the immobilised bioactive compounds was determined by gas chromatography-mass spectrometry (GC-MS). The QuEChERS procedure was followed for the extraction of EOCs based on an extraction phase with an organic solvent and different salts, and a clean-up step of the organic extract by dispersive solid-phase extraction (d-SPE). Vanillin was extracted from the juice samples (5 mL) using 10 mL of acetonitrile in the presence of sodium chloride and magnesium sulphate. After vigorous stirring, samples were centrifuged for 3 min at 3,300 rpm. The organic layer was transferred to the d-SPE tube (PSA and magnesium sulphate). The tube was vortexed for 1 min and centrifuged at 3,300 rpm for 3 min. The liquid extract was collected and evaporated under reduced pressure. Finally, the obtained extracts were suspended in 2 mL of methanol and were analysed by GC-MS.

The analysis was performed in a 6890/5975 inert GC-MS (Agilent Technologies, USA), equipped with an HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature was held at 60°C for 3 min before being raised to 100°C at 10°C/min, to 140°C at 5°C/min, and finally to 240°C at 20°C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperatures were set at 250°C and 230°C, respectively. The parameters for the MS analysis were the EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40-550 amu. EOCs were identified by matching the mass spectra with the standard mass spectra from the NIST MS Search 2.0 library and comparing the mass spectra of the pure compounds. EOCs were quantified according to the external standard method, in which a calibration curve (0.5, 1, 5, 10, 25, 50, 100, 250 and 500 mg/L) of the peak area was used against the compound concentration (Ribes, Fuentes, Talens, & Barat, 2016).

2.5.4. Sensory evaluation

A sensory test was run to determine the consumer acceptability of the apple juice filtered through the eugenol-functionalised supports as the most effective filtering material in accordance with the results obtained with previous analyses. The juice filtered through the non-functionalised particles was also included as the control samples. The panel involved 44 untrained panellists (29 females, 15 males), whose ages ranged from 21 to 48 years. Four sensory parameters (appearance, colour, odour and general acceptance) were judged on a 9-point hedonic scale (1 = very much dislike, 9 = very much like) (UNE-ISO 4121:2003). Judges also evaluated the typical odour of juices on a 5-point hedonic scale (1 = not typical, 5 = very typical). Individual juice samples (10 mL) were presented to the panellists at room temperature in a capped transparent glass phial with random 3-digit code.

2.6. Statistical analysis

Data were statistically analysed using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The effect of filtration and storage time on the physico-chemical and microbiological parameters of apple juice was analysed by a multifactor analysis of variance (multifactor ANOVA). The sensorial test was examined by a one-way ANOVA to compare samples according to sensory parameters. The LSD (least significant difference) procedure was used to test the differences between averages at the 5% significance level.

3. Results and Discussion

3.1. EOC-functionalised supports characterisation

The silica microparticles functionalised with the antimicrobial compounds were characterised to confirm the immobilisation of EOCs on the particles' surface. Table 1 shows the amount of antimicrobial compounds anchored to the supports and the surface charge of particles. Grafting was more efficient for vanillin as functionalised silica particles were obtained with the organic

matter content anchored to the supports being 3-fold higher than for eugenol. The immobilisation process changed the surface charge of the supports, as indicated by the Zeta potential results. The non-functionalised silica microparticles gave negative Zeta potential values due to the presence of silanol moieties on the supports' surfaces. In contrast, the supports functionalised with the bioactive compounds gave positive Zeta potential values because of the grafting of the EOC-alkoxysilane derivatives, which thus reaffirms the efficiency of functionalisation.

Table 1. Content of the immobilised organic matter (α) and Zeta potential values for the bare and functionalised silica microparticles.

SiO_2 support	**α (mg compound/g SiO_2)**	**Zeta potential (mV)**
Bare	-	-28.6 ± 3.2
Eugenol	35.8	30.9 ± 5.6
Vanillin	114.9	28.6 ± 1.0

3.2. Capability of the EOCs-functionalised supports used as filtering materials to cold pasteurise apple juice

Figure 1 shows the *E. coli* K12 counts after the inoculation of the commercial apple juice, and also after filtering juice through a bed of silica particles (0.5 cm thickness, 2 g) (layer I) for both the bare support and for the support functionalised with either eugenol or vanillin. With vanillin, the experiment was also carried out with a bed of particles (1 cm thickness, 4 g) (layer II). As seen in Figure 1, filtering juice through the non-functionalised support did not significantly reduce the inoculated bacterium (6.84±0.13 and 6.74±0.14 log CFU/mL for the unfiltered juice and the juice filtered through the control silica, respectively). In contrast, filtering apple juice through the EOC-functionalised supports significantly removed the *E. coli* load of the beverage ($p<0.05$). The use of the eugenol-functionalised support resulted in an approximate reduction of 5 logarithmic cycles. With vanillin, filtering juice through layer I of particles (0.5 cm thick) led

to a 3-log reduction, which was not enough to reach the required pasteurisation level. For this reason, the experiment was also carried out with a thicker bed of particles (1 cm; layer II), which resulted in a reduction of 5.5 log cycles. These results reveal that the removal capability of the EOC-functionalised particles' bed can be attributed to the interaction of the immobilised EOCs with the microbial cells to result in microbial retention. Free EOCs affect microbial cell membrane permeability, which leads to the leakage of cell contents and, in turn, results in irreversible damage and cell death (Burt, 2004). As recently reported, the immobilisation of the EOCs on a support's surface allowed their antimicrobial properties to be preserved (Ruiz-Rico et al., 2017). After immobilisation, the antimicrobial molecules at a high local concentration on the supports' surface could interact with microorganisms during the filtering process, which resulted in microbial damage. Besides, the efficiency of EOCs as preservatives in juice matrices has been previously demonstrated for eugenol (Ghosh, Mukherjee, & Chandrasekaran, 2014; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2008) and vanillin (Char, Guerrero, & Alzamora, 2009; Moon, Delaquis, Toivonen, & Stanich, 2006).

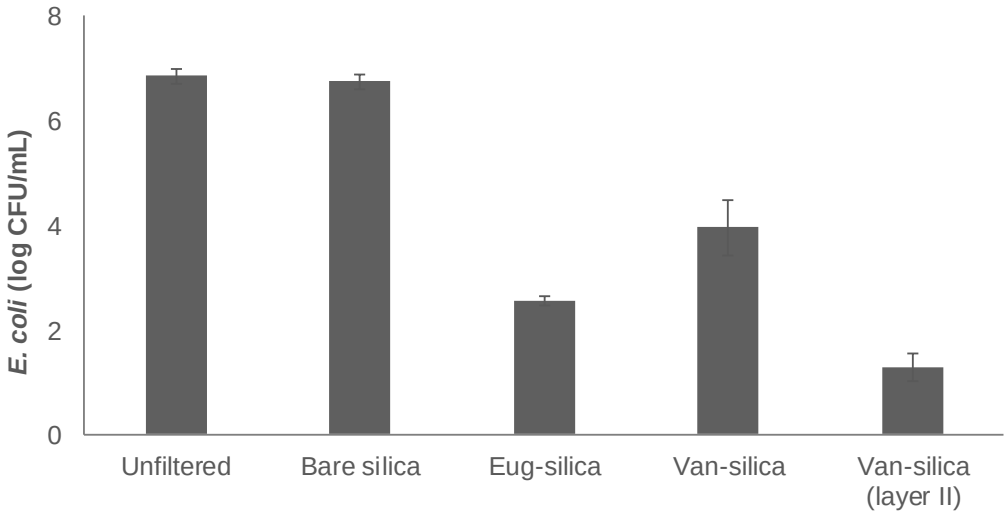

Figure 1. *E. coli* counts (log CFU/mL) in the inoculated apple juice before treatment and after filtering juice through a 0.5-cm bed of the bare silica microparticles and EOCs-functionalised supports. Layer II indicates a bed thickness of 1 cm. Mean value±SD (n=3).

Filtration through the EOC-functionalised supports played a significant role in the elimination of the *E. coli* inoculated in the apple juice samples (5-log reduction in the microbial count), which confirms the suitability of this system as cold pasteurisation technology. This treatment allowed microorganisms to be removed at room temperature similarly to other emerging non-thermal technologies. Exposing the inoculated apple juice to pulsed light resulted in poor *E. coli* inactivation (ca. 3 log cycles), but this technology combined with ultrasound treatment led to an acceptable 5.9 log reduction (Ferrario & Guerrero, 2016). The inactivation of *E. coli* O157:H7 in the apple juice samples treated by pulsed electric fields resulted in a 4.5 log reduction in the work reported by Evrendilek et al. (2000). The combined use of ultraviolet radiation and pulsed electric fields gave a reduction of 6-7 log cycles, similarly to conventional heat treatment (Noci et al., 2008). High hydrostatic pressure has also provided promising results by achieving total *E. coli* inactivation (more than 5 log cycles) in fruit juice (Lavinas, Miguel, Lopes, & Valente Mesquita, 2008). Besides, a reduction of 7 log cycles in *E. coli* in apple juice has been achieved by dense phase carbon dioxide combined with mild heat treatment (Liao, Hu, Liao, Chen, & Wu, 2007).

3.3. Influence of filtration on the apple juice quality parameters during storage

After confirming the capability of the EOC-functionalised supports used as filtering elements to cold pasteurise apple juice, the effect of filtration on the microbiological and physico-chemical properties of apple juice during refrigerated storage was evaluated.

3.3.1. Microbiological parameters

Figure 2 shows the evolution of the microbial counts of mesophilic aerobic, psychrophilic and mould and yeast of the untreated and treated apple juices throughout the storage period. The results on day 0 indicated that the filtration process produced a microbial stabilisation of the juice samples filtered through the EOCs-functionalised supports. It resulted in the total inhibition

of native flora, whereas the unfiltered juice and the juice treated with the bare support had a microbial load of 1-2 log cycles of different microorganisms. These results agree with those obtained for the commercial apple juice inoculated with *E. coli* K12 (Section 3.2).

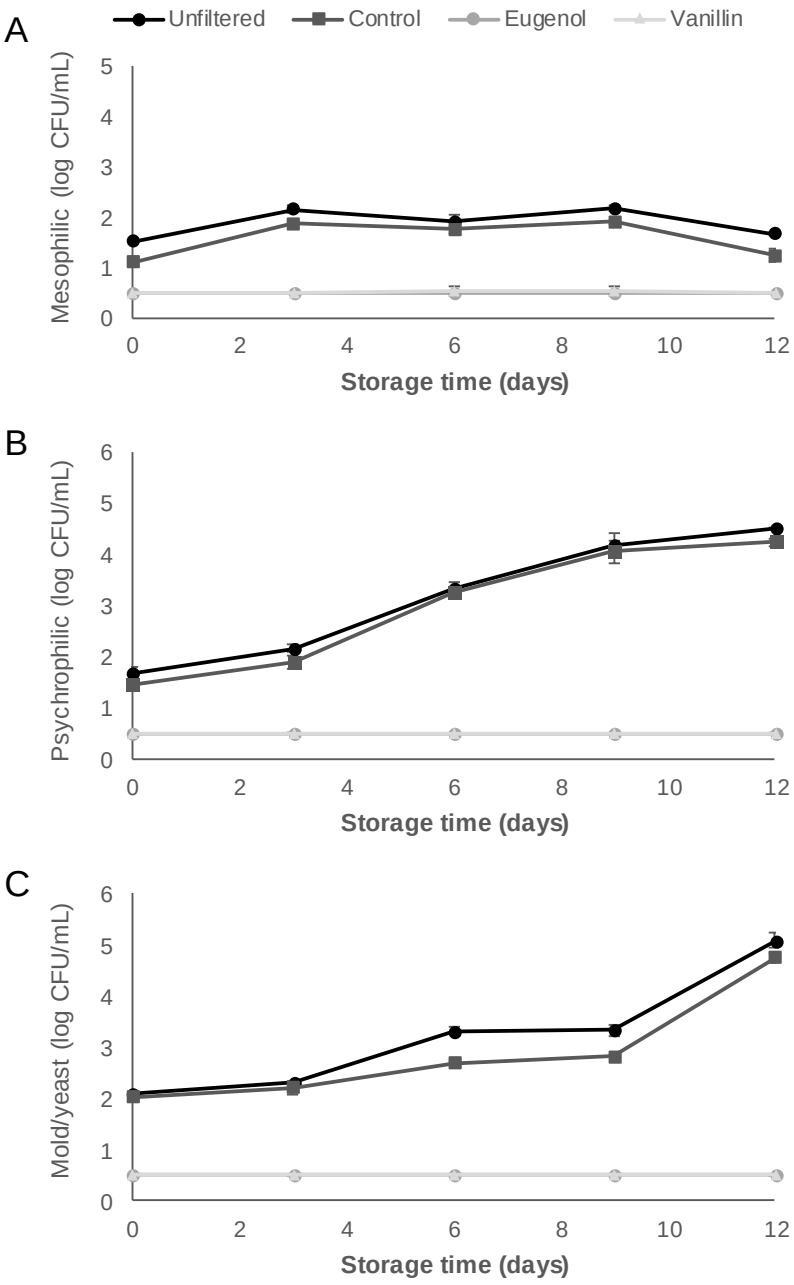

Figure 2. Microbial counts (log CFU/mL) of mesophilic aerobic (A), psychrophilic (B) and mould and yeast (C) of the unfiltered apple juice and the juice filtered through the non-functionalised particles (control) and EOC-functionalized supports during refrigerated storage. Mean values±SD (n=3).

Regarding juice evolution during refrigerated storage, an increase in microbial counts was observed in response to storage time for both the unfiltered juice and the juice filtered through the non-functionalised supports. There was significant difference in the mean counts of the juice samples before and after filtering through bare particles ($p<0.05$). This partial microbial retention (less than 0.5 log cycles) endorses the very limited removal effect of the unmodified silica microparticles due to physical adsorption by weak forces on the filtering bed (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas, 2016). In contrast, the juice samples filtered through the EOCs-functionalised supports led to the absence of microbiota throughout the 12 storage days. After 12 storage days, the unfiltered juice and that filtered with the unmodified support exceeded the acceptability limit of 100 CFU/g of the total counts established as a safety criterion for unpasteurised fruit and vegetable juices, according to Regulation (EC) No. 2073/2005 of the European Commission (European Union, 2005). Conversely, international guidelines recommend an acceptable level of aerobic mesophilic microorganisms values to be 10^4 CFU/g for pasteurised juices (Health Protection Agency, 2009).

Therefore, if we consider the product to be pasteurised juice, no sample exceeded the recommended limit after 12 storage days. In order to establish the shelf life of juices, the microbiological parameters were monitored until 120 days of apple juice storage at 4°C. On day 30, the unfiltered juice and that filtered with the unmodified support gave a microbial count of ca. 4 log CFU/mL, which resulted in a shelf life of less than 30 days (Table 2). Conversely, the samples filtered through the EOC-functionalised supports sustained a total inhibition of native flora until day 120, when 1.4 log CFU/mL of mesophilic aerobic was enumerated for the juice treated with immobilised vanillin. Thus after filtering samples with the EOC-functionalised supports, the juices displayed microbial stability for more than 120 days, unlike the juices not treated with the immobilised bioactive compounds with a major increase in microbial load after 30 days.

Table 2. Microbial counts (log CFU/mL) of mesophilic aerobic, psychrophilic and mould and yeast of the unfiltered apple juice and the juice filtered through the non-functionalised particles (control) and EOC-functionalised supports from day 30 to 120 of refrigerated storage. Mean values±SD (n=3).

Sample	Day	Mesophylic	Psychrophilic	Mould/yeast
Unfiltered	30	4.1 ± 0.0	5.2 ± 0.1	5.6 ± 0.1
	60	5.2 ± 0.2	6.5 ± 0.0	5.7 ± 0.2
	90	5.6 ± 0.1	6.3 ± 0.1	5.8 ± 0.2
	120	5.8 ± 0.3	6.5 ± 0.6	6.4 ± 0.6
Control	30	3.8 ± 0.1	5.3 ± 0.1	5.3 ± 0.3
	60	5.8 ± 0.0	6.6 ± 0.1	6.4 ± 0.0
	90	6.8 ± 0.2	6.9 ± 0.2	6.7 ± 0.3
	120	7.0 ± 0.2	7.3 ± 0.0	6.9 ± 0.1
Eugenol	30	nd	nd	nd
	60	nd	nd	nd
	90	nd	nd	nd
	120	nd	nd	nd
Vanillin	30	nd	nd	nd
	60	nd	nd	nd
	90	nd	nd	nd
	120	1.4 ± 1.2	1.6 ± 1.1	1.2 ± 1.1

nd (no detected, <5 CFU/mL)

These are remarkable results compared with other non-thermal treatments, such as microfiltration (Campos et al., 2002), pulsed electric field processing (Evrendilek et al., 2000) and high hydrostatic pressure treatment (Lavinas et al., 2008), and provide a shelf life that lasts up to 60 days stored at 4°C. Moreover, the apple juice treatment done with pulsed light combined with ultrasound exceeded 4 log CFU/mL after 10 days of refrigerated storage (Ferrario & Guerrero, 2016). Consequently, the filtration using the EOCs-functionalised supports proves to

be a preservation potential for developing 'fresh-like' apple juice with an equivalent or longer shelf life than thermal treatment in microbiological characteristics terms.

3.3.2. Physico-chemical parameters

Figure 3 shows the evolution of the physico-chemical parameters (pH, acidity and °Brix) of the untreated and treated apple juice samples over a 12-day refrigerated storage period. After filtration (day 0), the unfiltered juice presented an average pH value of 3.6, and the juice filtered through the non-functionalised silica and eugenol-functionalised support obtained similar values (homogenous groups, $p<0.05$). In contrast, a significant difference was observed after filtering the apple juice through the vanillin-functionalised support, which had a higher pH value of 4.4. This value fell beyond the normal range of apple juice, which could lead to a different perception of taste and, consequently, to diminished consumer acceptability. This variation may be due to either the partial release of the immobilised bioactive compound (Table 3) or the presence of APTES moieties without reacting with the antimicrobial molecules on the supports' surfaces to provide the larger amount of alkoxysilane derivative immobilised on the support (Table 1). Regarding the evolution of the juice stored at 4°C, the pH values remained stable throughout the study period, and only slightly lowered during the storage of all the samples.

In acidity terms, a significant statistical difference was observed according to sample treatment ($p<0.05$), as shown in Fig. 3B. All the filtered samples presented significant differences with the untreated juice, but the acidity values obtained for the unfiltered juice and the juice filtered through the bare or eugenol-functionalised supports were similar. However, filtering juice through the vanillin-functionalised support modified this parameter more as acidity decreased. These results agree with the obtained pH values, as explained above.

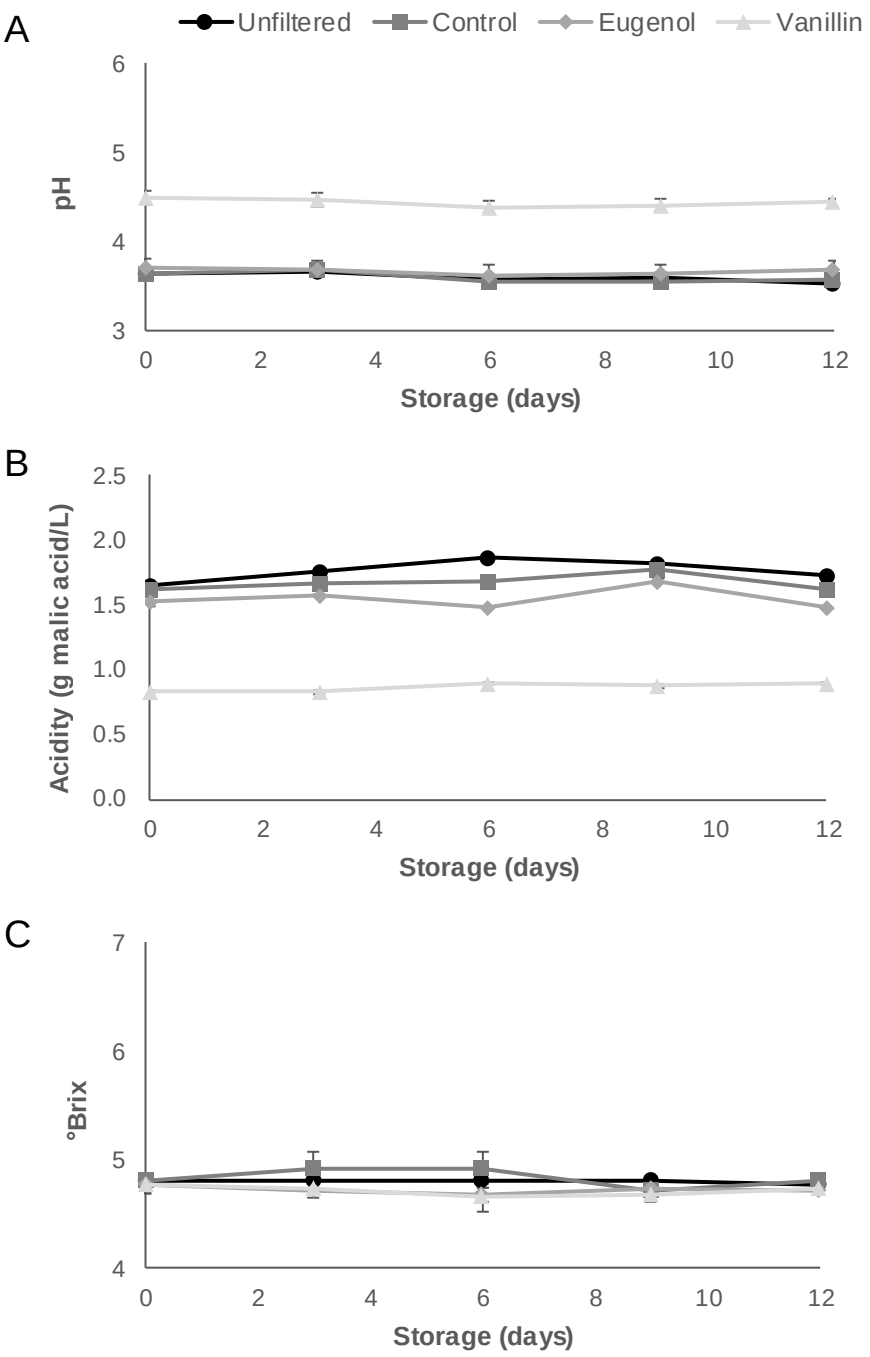

Figure 3. The pH, titratable acidity and °Brix values of the unfiltered fresh apple juice and after filtration through a bed of control silica support and the EOCs-functionalised supports during refrigerated storage. Mean values±SD (n=3).

Similarly to the acidity results, the processed juices significantly differed from fresh juice in soluble solid content terms ($p<0.05$), but these differences were minor and fell within the 4.7

and 4.8 °Brix range. In addition, the °Brix values of the different samples remained stable throughout the refrigerated storage.

The influence of the conventional preservation techniques on the physico-chemical properties of fruit juices has been clearly stated, and justifies the need to develop mild preservation treatments. Thermal treatment has been reported to increase pH and is directly related with temperature, with pH values reaching ca. 4 under pasteurisation conditions, which can entail a shorter shelf life because higher pH values could favour microbial growth (Charles-Rodríguez, Nevárez-Moorillón, Zhang, & Ortega-Rivas, 2007). Similarly, thermal pasteurisation has been related with a significant decrease in acidity, which could be attributed to the degradation of organic acids with rising temperature (Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martin-Belloso, & Ortega-Rivas, 2007).

In contrast, the treatment of fruit juices using filtration technologies did not significantly affect their physico-chemical parameters, as studied in accordance with previous studies (Gialleli et al., 2016; Ortega-Rivas, Zárate-Rodríguez, & Barbosa-Cánovas, 1998). Similar results were achieved herein after filtering juice through the bare and eugenol-functionalised supports. The application of other non-thermal treatments, such as pulsed electric field, has been able to preserve pH, acidity and volatile compounds to a greater extent than thermal treatment (Aguilar-Rosas et al., 2007).

In addition, colour was studied like the other physico-chemical parameters defining juice quality. Figure 4 shows the evolution of L*, a* and b* of the apple juice samples during refrigerated storage. The statistical analysis revealed the significance of juice treatment and storage time on juice colour evolution. In general, the juice filtered with the EOC-functionalised supports was brighter (higher L* values) than the unfiltered juice or that treated with the bare supports, which became darker (lower L* values). The a* values mostly differed for the juice treated with the non-functionalised supports, but with a significant increase (more red components), while the samples treated with the EOC-functionalised supports were slightly ‘greener’ (lower a* values)

compared to the unfiltered samples. The b* values changed to a greater extent than the a* values, and resulted in more yellow components (higher b* values) for the treated samples than for the non-filtered juice (control <vanillin <eugenol). The total colour difference determination revealed the influence of the filtration process on the colour of the samples. The ΔE value for the samples filtered through the non-functionalised support *versus* the non-filtered samples was 2.17. This colour difference was at the same level as in previously reported studies into the thermal treatment of apple juice, in which heated juice obtained a ΔE of at least 2.69 compared to untreated juice (Krapfenbauer, Kinner, Gössinger, Schönlechner, & Berghofer, 2006). Filtering juice through the EOC-functionalised supports led to greater deviation, with ΔE values of 6.06 and 6.45 for the eugenol and the vanillin-functionalised supports, respectively.

Treating apple juice by ultrafiltration has led to significant browning in previous studies. Juice became darker according to the applied trans-membrane pressure, which could be attributed to enzymatic browning in relation to the difference in enzyme inactivation efficiency of techniques (Ortega-Rivas et al., 1998). In contrast, other non-thermal treatments, like pulsed electric field and ultraviolet radiation, have preserved apple juice colour after treatment and throughout its shelf life, and has resulted in the chromatic coordinates being maintained (Evrendilek et al., 2000; Noci et al., 2008). Therefore, treating apple juice with the EOC-functionalised supports resulted in significant colour differences, which is a weakness of this methodology compared to other techniques. Future studies should be conducted to assess how to overcome this limitation, probably by means of filtering many samples or with a previous washing with the food matrix.

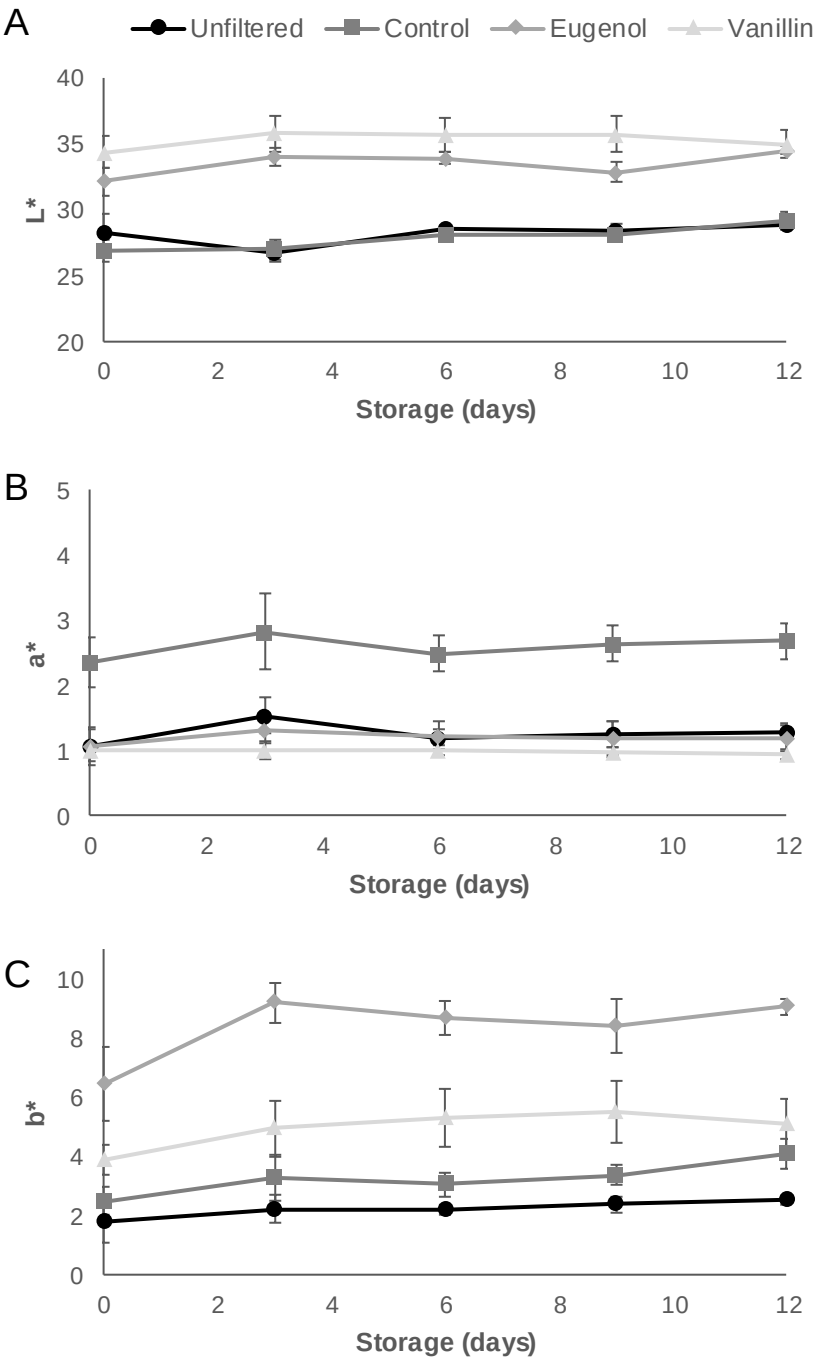

Figure 4. The CIEL*a*b* colour coordinates of the unfiltered fresh apple juice and after filtering through a bed of control silica support and EOCs-functionalised supports during refrigerated storage. Mean values±SD (n=3).

3.4. Leaching the immobilised EOCs

The potential leaching of the grafted bioactive compounds was quantified to define the stability of the immobilisation procedure and to determine the wash-out effects under the filtration conditions. Besides the amount of EOCs released from the supports, the percentage of leached compounds was calculated by considering the total EOCs attached to the supports (Section 3.1) and the amount of particles needed to prepare the bed of particles. Table 3 shows the amount of leached EOCs and the percentage of lixiviated compounds in juices after filtering through the EOC-functionalised supports. For immobilised eugenol, a zero wash-out effect was detected after filtering juice. In contrast, the juice filtered through the vanillin-functionalised support showed a 0.6% of the initial EOC's content grafted to particles' surface. Despite the low percentage of leached compounds, this partial release could be responsible for the impact of the treated juice on the physico-chemical properties and could negatively affect sensory properties.

Table 3. EOCs leaching (mg) and the relative percentage of the leached EOC after filtering 100 mL of apple juice through the bed of the EOC-functionalised supports. Mean values±SD (n=3).

SiO_2 support	**Amount of released compound (mg)**	**Relative percentage (%)**
Eugenol	nd	nd
Vanillin	1.7 ± 1.3	0.6 ± 0.4

nd: non detected

Notwithstanding, it is important to highlight that covalent immobilisation preserved the microbial stabilisation potential of the EOC-functionalised supports. In addition, the low percentage of leached EOCs ratifies the safety and longevity of filtration technology because it would allow the possibility of a repeated or continuous reuse of immobilised compounds. However, the immobilisation procedure should be optimised to ensure the zero release of

anchored molecules to prevent any influence on the physico-chemical and sensory properties of juice.

3.5. Consumer acceptability of the filtered apple juice

According to the results of previous sections, the juices filtered through the most suitable support to stabilise their microbiota without leaching the immobilised molecule from the support were sensory-evaluated. Table 4 shows the sensory evaluation results of the juices filtered through the non-functionalised and eugenol-functionalised supports. As we can see, the juice treated with the eugenol-functionalised support was well accepted ($p<0.05$) great for the attributes evaluated by the sensory panel, except for odour.

Treating juice with conventional preservation methodologies (thermal pasteurisation) causes sensory quality depletion and the appearance of odour and flavour defects (Manzocco, Plazzotta, Spilimbergo, & Nicoli, 2017). In contrast, the use of alternative non-thermal technologies, such as high-pressure carbon dioxide, pulsed electric field or high hydrostatic pressure, has resulted in the preservation of the sensory fresh-like features of treated juices (Kebede et al., 2018; Manzocco et al., 2017). Likewise, the apple juice subjected to filtration with the eugenol-functionalised support did not show any effect on the evaluated sensory attributes, which coincides with the results observed for the physico-chemical parameters (Section 3.3.2).

The use of the silica support can be the equivalent to using clarification agents, such as bentonite, to eliminate the sediments and dark pigments that appear during apple juice processing, and to obtaining similar sensorial acceptability (Lauret, Sartori, Imaizumi, Brunelli, & Filho, 2018). Conversely, filtration methodologies that involve very small pore sizes, such as microfiltration and ultrafiltration, result in significant sensory changes with little colour intensity and odour and flavour defects due to increase in the membrane retention of sugar, phenolic and other flavour components (Girard & Fukumoto, 1999).

Therefore, the sensory evaluation proved the suitability of the developed filtration treatment given the high attribute scores that confirm the preservation of the end product's major sensory attributes, as well as extending its shelf life beyond those obtained with conventional preservation methodologies.

Table 4. Scores of the different attributes evaluated in the apple juice filtered through the non-functionalised and eugenol-functionalised supports. Mean values±SD and median (n=44).

SiO_2 support	**Appearance**		**Colour**		**Odour**		**Acceptance**	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Control	5.8±1.5[a]	6	5.7±1.5[a]	6	6.7±1.5[a]	7	6.3±1.3[a]	6
Eugenol	7.2±1.0[b]	7	7.1±1.3[b]	7	6.9±1.5[a]	7	7.0±1.2[b]	7
α	*		*		ns		*	

Same letters in a column indicate homogeneous group membership. ns: non-significant, *p<0.05

4. Conclusions

In this study, silica microparticles functionalised with EOCs were applied as filtering materials for cold apple juice pasteurisation. The filtration process, through the EOC-functionalised supports, allows to: (i) clarify juice to avoid turbidity and sediment in the end product; (ii) microbiologically stabilise the food matrix to increase its shelf life. In fact treating fresh juice with this technology eliminated its native flora, and resulted in an apple juice with a much longer shelf life than that obtained by heat treatment.

The filtration process had a different influence on the physico-chemical parameters of juice according to the immobilised bioactive molecule. The use of the eugenol-functionalised particles proved to be the more adequate support as it removed the product's microbial load without affecting its physico-chemical parameters (pH, acidity, °Brix) or sensory profile, plus the zero release of the immobilised compound to the food matrix. Therefore, the proposed cold-

pasteurisation system has a high potential for treating fruit juices, as well as other liquid foods like milk, beer or wine.

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