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Analytical Methods

Molecularly imprinted hydrogels as functional active packaging materials

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ABSTRACT

This paper describes the synthesis of novel molecularly imprinted hydrogels (MIHs) for the natural antioxidant ferulic acid (FA), and their application as packaging materials to prevent lipid oxidation of butter. A library of MIHs was synthesized using a synthetic surrogate of FA, 3-(4-hydroxy-3-methoxyphenyl)propionic acid (HFA), as template molecule, ethyleneglycol dimethacrylate (EDMA) as cross-linker, and 1-allylpiperazine (1-ALPP) or 2-(dimethylamino)ethyl methacrylate (DMAEMA), in combination with 2-hydroxyethyl methacrylate (HEMA) as functional monomers, at different molar concentrations. The DMAEMA/HEMA-based MIHs showed the greatest FA loading capacity, while the 1-ALLP/HEMA-based polymers exhibited the highest imprinting effect. During cold storage, FA-loaded MIHs protected butter from oxidation and led to TBARs values that were approximately half those of butter stored without protection and 25% less than those recorded for butter covered with hydrogels without FA, potentially extending the shelf life of butter. Active packaging is a new field of application for MIHs with great potential in the food industry.

1. Introduction

Lipid oxidation, together with microbial spoilage and browning reactions, is a major cause of food deterioration and a great challenge for food manufacturers. Lipid oxidation is a relevant quality problem in the processing, packaging and storage of foodstuffs with high fat content. Spontaneous reaction of lipids with atmospheric oxygen (autooxidation) is the main process involved in degradation of dietary lipids. It proceeds via a series of free-radical chain reactions that can result in the development of off-flavors and odors, changes in color and texture, destruction of valuable nutrients, or even generation of toxic compounds (Kanner & Rosenthal, 1992). Therefore, lipid oxidation is a decisive factor in determining shelf life, especially for foods with high fat content, such as butter (Mallia, Escher, Dubois, Schieberle, & Schlichtherle-Cerny, 2009; Mallia, Escher, & Schlichtherle-Cerny, 2008).

The food industry applies several methods to prevent oxidation reactions. Vacuum or inert atmosphere packaging, using materials with adequate barrier properties against gas or radiation, and incorporation of antioxidants are the most popular (Becker, Nissen, & Skibsted, 2004). However, this is a challenging issue for many foodstuffs. For example, the use of vacuum or inert atmospheres in fresh meat packaging may favor the development of pathologic anaerobic microorganisms, or changes in pigmentation, which have a negative impact on product quality and safety. The efficiency of inert atmospheres also decreases when the packaging is opened and stored on several occasions.

The use of antioxidants may overcome some of these shortcomings. These compounds interact with reactive oxygen species, protecting lipids from oxidation (Shahidi & Zhong, 2005), and retain flavor, color and freshness for extended periods of time. Currently, there is a trend for incorporating antioxidants in the packaging material with a dual purpose; packaging and protecting foods through controlled release of the antioxidant. Special interest is being focused on the development of active materials incorporating antioxidants for such application, with emphasis on those of natural origin, such as tocopherol, oregano or rosemary extracts (Barbosa-Pereira, Angulo, Lagarón, Paseiro-Losada, & Cruz, 2014;

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Barbosa-Pereira, Aurrekoetxea, Angulo, Paseiro-Losada, & Cruz, 2014; Barbosa-Pereira et al., 2013; Bentayeb, Rubio, Batlle, & Nerín, 2007; Blanco-Fernandez, Rial-Hermida, Alvarez-Lorenzo, & Concheiro, 2013; de Abreu, Losada, Maroto, & Cruz, 2011; Gimenez, Gomez-Guillen, Perez-Mateos, Montero, & Marquez-Ruiz, 2011; Gemili, Yemenicioglu, & Altinkaya, 2010), due to concerns raised about the safety of synthetic antioxidants (Dopico-Garcia et al., 2011).

Ferulic acid (FA) is a phenolic member of the cinnamic acid family, commonly found in seeds and leaves, both in free form and covantly conjugated to the plant cell wall polysaccharides, glycoproteins, polyamines, lignin and hydroxy fatty acids (Kumar & Pruthi, 2014). It is well known for its high antioxidant capacity, similar to that of superoxide dismutase, one of the most potent antioxidants (Gupta, Sukhida, & Bhatia, 1979). Although rarely found in free form, the ester form of FA is attached to polysaccharides such as arabinoxylans, pectins and xyloglucans, and proteins (Figueroa-Espinoza, Poulsen, Borch, Zargahi, & Rouau, 2002). Thanks to its low toxicity, FA is used in the food industry as a preservative and cross-linker for the preparation of gels and edible films (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). Compared to other antioxidant phenolic compounds, FA shows a higher antioxidant activity, with a lower dependence on pH, and it can also inhibit the growth of bacteria, fungi and yeasts (Heinonen, Lehtonen, & Hopia, 1998).

Molecular imprinting allows the preparation of synthetic materials, which can be processed in a variety of formats and with tunable sorption/release behavior, with the ability to recognize specific molecules for a variety of applications (Whitcombe, Kirsch, & Nicholls, 2014). The advantage of MIPs as active-molecule delivery systems is that they can regulate the release rate according to an affinity-based mechanism, increasing the residence time of the target molecule within the polymer matrix by means of either covalent or non-covalent bonding in specific binding sites. The creation in the polymer network of ad hoc binding sites for a specific molecule also leads to enhanced loading efficiency and enables more precise control of the performance of the network when the release is intended to be triggered by specific stimuli, such as the concentration of a given substance or a change in a physical variable (Alvarez-Lorenzo & Concheiro, 2004). The interest in MIPs relies on their low cost, the ease of synthesis, and their relatively high stability against temperature, pressure, organic solvents, and acid and alkaline conditions. However, to the best of our knowledge, MIPs have not yet been evaluated as components of active substance-eluting food packages.

In this work we describe the application of molecularly imprinted hydrogels (MIHs), loaded with FA as an active antioxidant film for the packaging of oxygen-sensitive foods. The release of FA into food does not result in a toxicological risk, which is an advantage over the use of synthetic antioxidants. The surrogate template approach was applied to prevent co-polymerization of FA during MIHs synthesis. To do that, 3-(4-hydroxy-3-methoxyphe nyl)propionic acid (HFA) was synthesized and used as a template for the preparation of MIHs using the con-covalent imprinting approach. Two functional monomers, namely 1-allylpiperazine (1-ALPP) and 2-(dimethylamino)ethyl methacrylate (DMAEMA), in combination with 2-hydroxyethyl methacrylate (HEMA) as co-monomer and ethyleneglycol dimethacrylate (EDMA) as cross-linker, at different molar concentrations, were evaluated for MIH synthesis (Fig. S2, Supplementary material). The HFA-imprinted hydrogels were characterized by IR and equilibrium rebinding assays, determining their cross-reactivity to FA by HPLC with diode array detection (HPLC-DAD). The MIHs compositions provided higher FA loading capacities and lower non-selective bindings, when compared to the respective non-imprinted hydrogels (NIHs), and were characterized further by monitoring their antioxidant and antibacterial properties. The effectiveness of the new antioxidant active packaging FA-MIH to retard lipid oxidation was tested in butter samples.

2. Experimental

2.1. Materials

All solvents were chromatographic grade and used without further purification. Pure water from a Milli-Q water purification system (Millipore, Bedford, USA) was used. Ferulic acid (FA, 99%), 1-allylpiperazine (1-ALPP, 96%), 2-hydroxyethylmethacrylate (HEMA, 97%), ethylene glycol dimethacrylate (EDMA, 98%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 99%), dimethyldichlorosilane (99.0%), 4-(2-hydroxyethyl)-1-piperazinee thanesulfonic acid (HEPES, ⩾99.5%) and 2-(*N*-morpholino) ethanesulfonic acid (MES, ⩾99.5%) were all from Sigma–Aldrich (St. Louis, USA). Trifluoroacetic acid (TFA, peptide peptide grade, >99%) was from Fluorochem (Hadfield, UK). Trichloroacetic acid PA-ACS (TCA, 99.5%) and trichloromethane stabilized with 50 ppm of amylene PA-ACS (99.8%) were provided by Panreac Química S.L.U. (Barcelona, Spain). 2-Thiobarbituric acid (TBA, ⩾98%) and 1,1,3,3-tetraethoxypropane (TEP, ⩾96%) were purchased from Sigma–Aldrich (Steinheim, Germany).

The chromatographic system consisted of a HP-1100 series high performance liquid chromatograph from Agilent Technologies (Palo Alto, USA) equipped with a quaternary pump, on-line degasser, autosampler, automatic injector, column thermostat and diode-array (DAD) detector. Chromatographic separation of FA and HFA was performed on an AQUA™ C18 analytical column (250 mm × 4.6 mm, 5 μm) from Phenomenex (Torrance, USA). An isocratic program was used with the mobile phase combining 60% of solvent A (H_2O with 0.3% TFA) and 40% of solvent B (acetonitrile, ACN, with 0.3% TFA) at a flow rate of 1 mL min^{-1}. The absorbance wavelength was set at 320 nm.

2.2. Synthesis of hydroferulic acid (HFA)

A solution of 0.5 g of FA in 50 mL absolute methanol (MeOH) was circulated through the H-Cube™ hydrogenator (Thalesnano Nanotechnology Inc.) through a 10% Pd/C catalyst (Fig. 1). The flow rate was set at 1 mL min^{-1} and the temperature at 25 °C (full hydrogen mode). The process was repeated three times to obtain HFA with purity over 95%. Product formation was confirmed by FTIR-ATR spectrometry (Perkin Elmer, UK) and 1H NMR analysis (Bruker Avance DPX 300 MHz-BACS60). The peaks of HFA in $CDCl_3$ were assigned as follows: δ (ppm): 6.84 (d, J = 7.3 Hz, 1H), 6.71–6.69 (m, 2H), 3.87 (s, 3H), 2.90 (t, J = 7.7 Hz, 2H), 2.65 (t, J = 7.7 Hz, 2H) (Fig. S2, Supplementary material).

2.3. Synthesis of imprinted (MIH) and non-imprinted (NIH) hydrogels

The HFA imprinted and non-imprinted hydrogels were prepared following a previously reported procedure (Ribeiro et al., 2011). The composition of the MIH and NIH libraries is shown in Table S1 (Supplementary material). In a typical procedure, the prepolymerization mixture was purged with a stream of Ar for 10 min

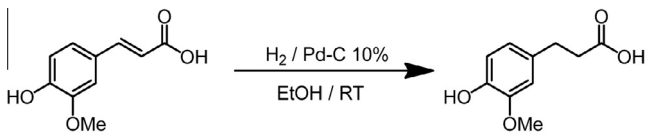

Fig. 1. Reaction scheme for HFA synthesis from FA.

and injected into the polymerization molds formed by two glass plates (10 × 10 cm), previously treated with dimethyldichlorosilane, held together and separated by a 0.9 mm thick silicone spacer (1 cm wide) (Alvarez-Lorenzo et al., 2002). The molds were placed in an oven at 50 °C for 12 h and then at 70 °C for 24 h. After polymerization the imprinted and non-imprinted membranes were removed from the molds and immersed into boiling water for 15 min, to extract the unreacted components. Finally, the membranes were cut into 10-mm diameter discs and subsequently rinsed with EtOH 96% until removal of the template was >99.9%, as checked by HPLC-DAD. Finally, the discs were dried in a vacuum oven at 45 °C for 48 h and stored in the dark at room temperature.

2.4. Physical and structural characterization of MIHs/NIHs

IR spectra were recorded using a FTIR-ATR (Perkin Elmer, UK) in the wave-number range of 400–4000 cm^{-1}. DSC scans of dried hydrogels (5–10 mg) were carried out in a in a DSC Q100 (TA Instruments, New Castle, USA) with a refrigerated cooling accessory. The samples were heated from 25 up to 150 °C, cooled down to −10 °C, and then heated again to 300 °C, always at 10 °C min^{-1}. Nitrogen was used as purge gas at a flow rate of 50 mL min^{-1}. All experiments were performed in duplicate. Degree of swelling in water was calculated, in triplicate, as follows (Eq. (1)):

$$Q\,(\%) = \frac{w_t - w_0}{w_0} \cdot 100 \quad (1)$$

where w_0 is the weight of the dry hydrogel sample, and w_t is the weight of the hydrogel after being immersed in water for a time t. Oxygen permeability (Dk) and transmissibility of hydrogels previously swollen in a 0.9% NaCl solution were measured in triplicate at room temperature using a Createch permeometer (model 210T, Rehder Development Company, Castro Valley, USA) fitted with a flat polarography cell and in a chamber at 100% of relative humidity.

2.5. FA loading and sorption kinetics

To evaluate the loading capacity in different solvents, dry hydrogels (approximately 90 mg) were immersed in 5 mL of FA 68 μM solutions prepared in HEPES 0.1 M (pH 7.5), HEPES 0.1 M (pH 7.5)/ACN (75/25; v/v), MES 0.1 M (pH 5.7) or distilled water and incubated in a shaker, at 150 rpm, for 48 h at 25 °C. After incubation, the supernatant was collected and injected into the HPLC-DAD instrument. For equilibrium binding experiments, the polymers were incubated with FA in a concentration range between 50 and 4000 μM in water. After 48 h, the hydrogels were removed and the concentration of FA in the supernatant was monitored by HPLC-DAD. The amount of FA bound to the polymer was calculated as μg of FA g^{-1} of dry hydrogel. The experimental data were fitted to a Freundlich isotherm (FI) model (Umpleby et al., 2004). All experiments were carried out in triplicate.

2.6. Antimicrobial activity of the FA-loaded MIHs

Antimicrobial activity of MIHs with and without FA were assessed against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, and total aerobic mesophilic flora isolated from chicken roti. The evaluation of MIHs with T2 formulation was performed using qualitative (agar diffusion) and quantitative (ASTM E 2149) methods, as described in Supporting information.

2.7. Antioxidant effectiveness of FA-loaded MIHs as butter packaging

Pieces (10 cm) of FA-loaded MIHs and non-loaded NIHs (used as controls) were placed on the top surface of butter in cans containing 250 g butter, kindly supplied by Feiraco (Ames, Spain). Butter cans, prepared under the same conditions, without placing film on the surface of the butter were used as another control. The cans were covered with a plastic tape (with a gap to the surface of either the butter or the covering film of 1–2 cm, which imitates common use conditions) and stored at 4 °C during six weeks. Butter samples were collected at selected times for analysis. Three different trials were performed to evaluate the effectiveness of the FA-loaded MIHs in reducing lipid oxidation and the influence of the waiting time between subsequent sample collections on butter oxidation. Sampling was carried out every two days in the first trial (0, 2, 4, 7, 9, 11 and 14 days), every week in the second trial (0, 1, 2, 3, 4, 5, and 6 weeks), and after three weeks in the third trial (0 and 3 weeks). At each time of sampling, the films were removed from the surface and 3.0 ± 0.1 g of butter collected in a screw-capped test tube. Since the diameter of the butter cans was 10 cm, the amount (3 g) of butter collected represents the removal of a maximum depth of one millimeter. After sampling, the FA-loaded MIH and the non-loaded NIH films were placed back on top of the butter until the next measurement.

Lipid oxidation was evaluated applying the 2-thiobarbituric acid reactive substances (TBARs) method slightly modified (Ayar, Özcan, Akgül, & Akin, 2001). Butter was melted at 70 °C for 10 min and filtered under vacuum before use. An aliquot of filtered butter (0.3 ± 0.05 g) was weighed in a screw-capped test tube and extracted with 5 mL of 10% TCA solution. Samples were vigorously shaken in a vortex. After that, 3 mL of an aqueous TBA solution (0.5%) were added to the test tube, which was sealed with a screw cap, homogenized using a vortex shaker and incubated in an oven at 100 °C for 40 min. Then, the samples were cooled to room temperature and the fat was removed by shaking with 3 mL of chloroform followed by centrifugation. Finally, the absorbance was measured at 530 nm against a blank containing 5 mL of TCA solution and 3 mL of TBA reagent. All the analysis were performed in triplicate. The concentration of malondialdehyde (MDA), a relatively stable end product generated during secondary lipid oxidation, was calculated from a standard curve obtained using 1,1,3,3-tetraethoxypropane (TEP), a precursor of MDA, at concentrations between 0 and 2 mg L^{-1}. Results were expressed as milligrams of MDA per kilogram of butter.

Lipid oxidation data were compared using analysis of variance (ANOVA) and significant differences between the butter cans packaging conditions (control, NIHs and MIHs) were assessed by the Duncan's multiple range test at $P < 0.05$ using the IBM SPSS Statistics 20.0.0 software package. TBARs data are presented as the mean and the standard deviation.

3. Results and discussion

3.1. MIHs preparation

All polymers were prepared with HEMA as functional co-monomer in order to obtain safe, non-toxic, hydrophilic networks that when in contact with food release the active compound. The monomers HEMA and DMAEMA and the cross-linker EDMA are authorized for the manufacture of plastic materials intended to come into contact with foodstuff according to the EU legislation (Commission Regulation, 2011). This is not the case for 1-ALLP; however, this functional monomer was tested as well because it is able to interact through hydrogen bonding with the template molecule. It should be noted that preliminary studies of migration of residual monomers from the hydrogel films led to values below the limits established for food contact materials (Otero-Pazos et al., 2014).

The use of FA as a template for polymer preparation was discarded as this molecule contains a reactive double bond in position 2 that may lead to the incorporation of FA into the polymer

network, blocking the binding sites in the resulting material. To overcome this shortcoming and produce high quality binding sites, FA was hydrogenated and the resulting hydrofeluric acid (HFA) was used instead as template for MIHs synthesis (Fig. 1; ^{1}H NMR in Fig. S2). As shown in Fig. S3 in the Supplementary material, hydrogen bonding interaction of the HFA template with the functional monomers 1-ALPP and DMAEMA was confirmed by ^{1}H NMR. No signal shifts were observed in the ^{1}H NMR spectra of HFA upon addition of HEMA, the structural monomer added to the polymerization mixture to endow the polymeric film with hydrophilicity.

For polymer preparation, HFA was directly dissolved in the monomers mixture. The composition of the MIH and NIH libraries is shown in Table S1. The resulting MIHs were translucent in the dry form, but transparent when hydrated, irrespective of their composition. DSC scans of the dry MIHs and NIHs (data not shown) were very similar. The dried networks exhibited just one glass transition at 92–93 °C, which is slightly below that recorded for pHEMA networks. These results confirm the good miscibility of the monomers used for MIH synthesis (namely, random polymerization occurred) and that the presence of HFA during polymerization did not affect the stiffness of the network.

3.2. Swelling kinetics

The swelling profiles of the MIHs and NIHs in water are shown in Fig. 2. The MIHs swelled faster and to a larger extent than the NIHs for all the compositions tested, except for the 1-ALLP/HEMA polymers. This behavior has been attributed to the higher porosity of the imprinted networks as a result of the different propagation rate of the radical polymerization reaction in the presence of the template (Byrne, Park, & Peppas, 2002). MIHs containing exclusively HEMA as functional monomer (Ai) showed greatest swelling. In contrast, all NIHs showed similar swelling profiles regardless of the comonomer used.

Water diffusion coefficients D_w were calculated by fitting the following solution of Fick's law to the relative degree of swelling (Eq. (2)) (Ribeiro et al., 2011):

$$\frac{W_t}{W_\infty} = 4\left(\frac{D_w t}{\pi h^2}\right)^{\frac{1}{2}} \quad (2)$$

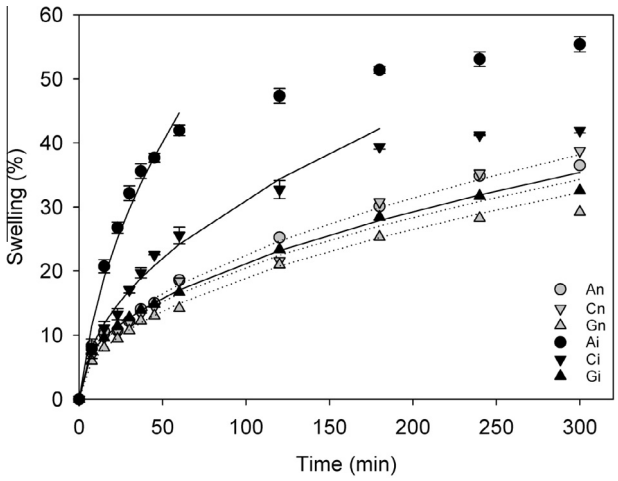

Fig. 2. Swelling profiles of the NIHs and MIHs synthesized with HEMA (An and Ai), DMAEMA/HEMA (Cn and Ci) and 1-ALPP /HEMA (Gn and Gi) as functional monomers ($n = 3$). Lines show the fitting to Eq. (2) (continuous lines refer to MIHs, and dotted lines to NIHs).

where t is the swelling time, W_t is the weight of the sample after t min of incubation; W_∞ is the final weight of the sample and h is the average thickness of the hydrogel. Water uptake showed a linear dependency on $t^{½}$ ($r^2 > 0.98$) for all the polymers tested. Therefore, a Fickian behavior is observed in spite of polymer swelling, *i.e.*, water molecules easily penetrate into the free pore volume and polymer relaxation does not influence the sorption rate, as previously reported for other HEMA hydrogels (Gehrke, Biren, & Hopkins, 1994). Water diffusion coefficients in the MIHs were (Ai: $3.66 \pm 0.02) \times 10^{-6}$, (Ci: $1.97 \pm 0.01) \times 10^{-6}$, and (Gi: $0.94 \pm 0.01) \times 10^{-6}$ cm^2 s^{-1}. While the corresponding values in the NIHs were $(0.99 \pm 0.01) \times 10^{-6}$, $(0.68 \pm 0.01) \times 10^{-6}$ and $(1.13 \pm 0.01) \times 10^{-6}$ cm^2 s^{-1}, for An, Cn and Gn, respectively. These values are similar to those reported previously for HEMA-solely hydrogels in water (Peniche, Cohen, Vázquez, & San Román, 1997), but smaller than the self-diffusion coefficient of water, 2.8×10^{-5} cm^2 s^{-1}. This finding suggests that the diameter of the hydrogel capillaries are not much larger than the water molecule diameter (Refojo, 1965).

3.3. FA loading capacity and imprinting factor

FA rebinding assays of MIH/NIHs synthesized either with HEMA (A) or with mixtures of HEMA and DMAEMA (C) or 1-ALPP (G) (template:functional monomer 1:2 M ratio) were carried in water and in saline media. The polymers (90 mg) where incubated in 5 mL of a 0.068 mM FA for 48 h, which was sufficient for the networks to reach the binding equilibrium (see Fig. S4, Supplementary material). The highest FA uptake, both in water and HEPES buffer, was observed with the P(DMAEMA-co-HEMA-co-EDMA) hydrogels, while rebinding in the P(1-ALPP-co-HEMA-co-EDMA) networks was less, especially in the buffered media (Fig. 3).

The higher retention of FA in these networks in comparison to the P(HEMA-co-EDMA) polymers can be explained considering the ionic interaction of the antioxidant with the positively charged DMAEMA (pKa 8.4) (van de Wetering et al., 1998) or 1-ALPP (pKa 9.2) (Khalili, Henni, & East, 2009) monomers in the binding cavities at the sample pH. The presence of salts in the buffer decreased FA rebinding in all the polymers, which could be attributed to the screening of the ionic interactions at the binding sites as a result of the high ionic strength.

Increasing the template:functional monomer molar ratio from 1:2 to 1:4, in both the 1-ALPP/HEMA (Ii) and DMAEMA/HEMA (Mi) MIHs, resulted in a 2- to 3-fold increase in the uptake of FA by the MIHs. (Fig. 4a and b) The amount of FA rebound to the hydrogels was always lower for the MIHs prepared with 1-ALLP than for those with DMAEMA; however, the former showed a higher imprinting effect in water. A comparison of the elemental analysis data shown in Table S2 (Supplementary material) revealed a lower incorporation of 1-ALLP monomer, compared to DMAEMA, in the cross-linked network that could explain the decreased FA binding. The lowest FA loading capacity was observed with the 1-ALPP/HEMA NIHs, meaning that the network itself has a lower affinity for the antioxidant. In other words, the interaction of FA with randomly distributed 1-ALLP is weaker than with DMAEMA. Higher imprinting factors (IF) were also recorded for 1-ALLP-bearing MIHs (Ri vs. Rn; Fig. 4c), compared to DMAEMA-containing MIHs (Ti vs. Tn; Fig. 4d), when the concentration of both the template and the functional monomer raised while keeping 1:2 M ratio. Thus, the beneficial effects of the template polymerization became more evident for 1-ALLP-bearing polymers; i.e., the creation of imprinted cavities led to a marked increase in binding capacity. This finding is in agreement with previous reports that prove that higher imprinting factors are obtained using functional monomers with low-to-mid affinity for

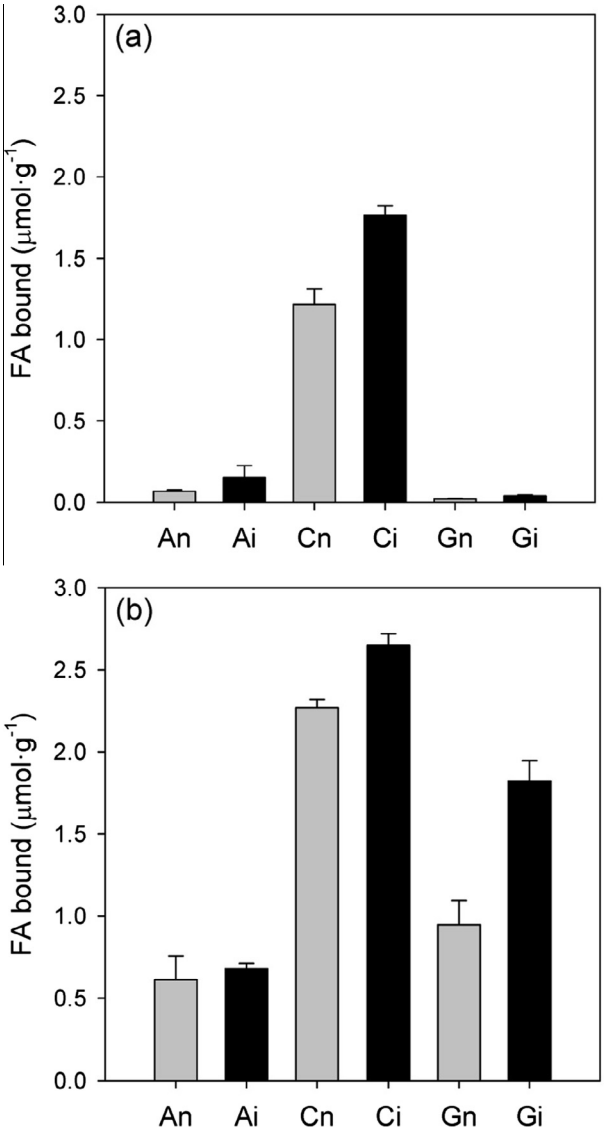

Fig. 3. FA rebinding in the MIHs (black squares) and NIHs (grey squares) after 48 h incubation in (a) HEPES (0.1 M, pH 7.5) and (b) water ($n = 3$, RSD < 12%). Hydrogel codes as in Table 1.

the template, which minimizes nonspecific interactions in the non-imprinted network (Hiratani & Alvarez-Lorenzo, 2004; Pardo, Mespouille, Dubois, Blankert, & Duez, 2014).

An excess of functional monomer (1:8 M ratio) did not increase further the loading capacity of the hydrogels, but even diminished the differences between imprinted and non-imprinted networks bearing 1-ALLP (Ji and Jn; Fig. 4a) or DMAEMA (Ni and Nn; Fig. 4b). This means that for MIHs prepared using 1:8 M ratio most functional monomers are randomly distributed as in the case of the NIHs (Tom, Schneck, & Walter, 2012).

3.4. FA sorption isotherms

The binding properties and homogeneity of the binding sites in the P(DMAEMA-co-HEMA-co-EDMA) and the P(1-ALPP-co-HEMA-co-EDMA) polymers were assessed in equilibrium binding experiments, using FA aqueous solutions in the 50–4000 μM range (Fig. S5). Once again the higher loading capability of DMAEMA-bearing networks was evidenced.

The adsorption isotherms were fitted to the Freundlich model (Eq. (3)):

$$\log\ B = m \cdot \log\ F + \log\ a \tag{3}$$

where B (μmol g^{-1}) and F (μM) are the concentrations of bound and free FA, respectively, and a and m are fitting constants that yield a measurement of physical binding parameters (Rampey et al., 2004; Umpleby et al., 2004). The preexponential factor a is a measure of the capacity (number of binding sites) and average affinity of the network. The constant m is a heterogeneity index; m values close to 1 indicate that all binding sites are identical from an energetic point of view, while m values near 0 indicate heterogeneous binding points (Rampey et al., 2004).

The fitting data are summarized in Table S3 (Supplementary material). Comparison of the heterogeneity parameter in the FI isotherm reveals a slightly higher heterogeneity for the MIHs than for the NIHs, which confirms that the presence of template during the polymerization contributes to the heterogeneity of the material, increasing the affinity of the binding sites by causing an adequate spatial arrangement of the monomers.

The affinity distribution of the binding sites (i.e., the plot of the number of sites N that have association constant K) was estimated using the following equation (Rampey et al., 2004):

$$N(K) = 2.303 \cdot a \cdot m \cdot \left(1 - m^2\right) \cdot e^{-2.303 \cdot m \cdot \log K} \tag{4}$$

within the limits $K_{min} = 1/F_{max}$ and $K_{max} = 1/F_{min}$. The affinity distributions showed the exponential decay that is characteristic of the isotherm region far from saturation (Umpleby et al., 2004). In the subsaturation region (low loadings), the differences between imprinted and non-imprinted networks became particularly evident. As expected from the m values, the affinity distributions shown in Fig. S6 indicate that the networks have association constants ranging from 290 to 80,000 L mol^{-1}, with predominance, in all polymers, of sites with low affinity. NIH In prepared with 1-ALPP functional monomer had the lowest number of binding points and with the lowest affinity. The corresponding MIH Ii showed a markedly greater number of binding sites and also with higher affinity. On the other hand, the networks prepared with DMAEMA as functional monomer (T1 and T2) possessed many more binding sites of high association constant. In this latter case, the contribution of the imprinting was smaller as DMAEMA can provide high affinity binding points even when polymerized in the absence of the template.

3.5. Antimicrobial activity of FA-loaded MIHs

MIHs did not cause growth inhibition against the tested bacteria neither in agar plates Fig. S5 (Supplementary material) nor in cell suspensions, which means that the amount of FA released was well below the minimal inhibitory concentration. In fact, bactericidal effect of FA was only observed for large concentrations (~3 mg ml^{-1} = 15,400 μM). Nevertheless, bacterial growth was not observed below the polymer (MIH/NIH) discs placed in direct contact with the bacterial culture surface. This finding can be attributed to a decrease in the oxygen concentration below the disc, which inhibits the growth of common food microbial contaminants *E. coli* and *S. aureus*. Thus, MIHs could contribute to food packaging materials not only with FA antioxidant performance, but also acting as a physical barrier for bacteria growth.

3.6. Antioxidant properties of FA-loaded MIHs in real samples (butter cans)

Several studies carried out in parallel during the present work confirmed that the FA-loaded MIHs (Otero-Pazos et al., 2014)

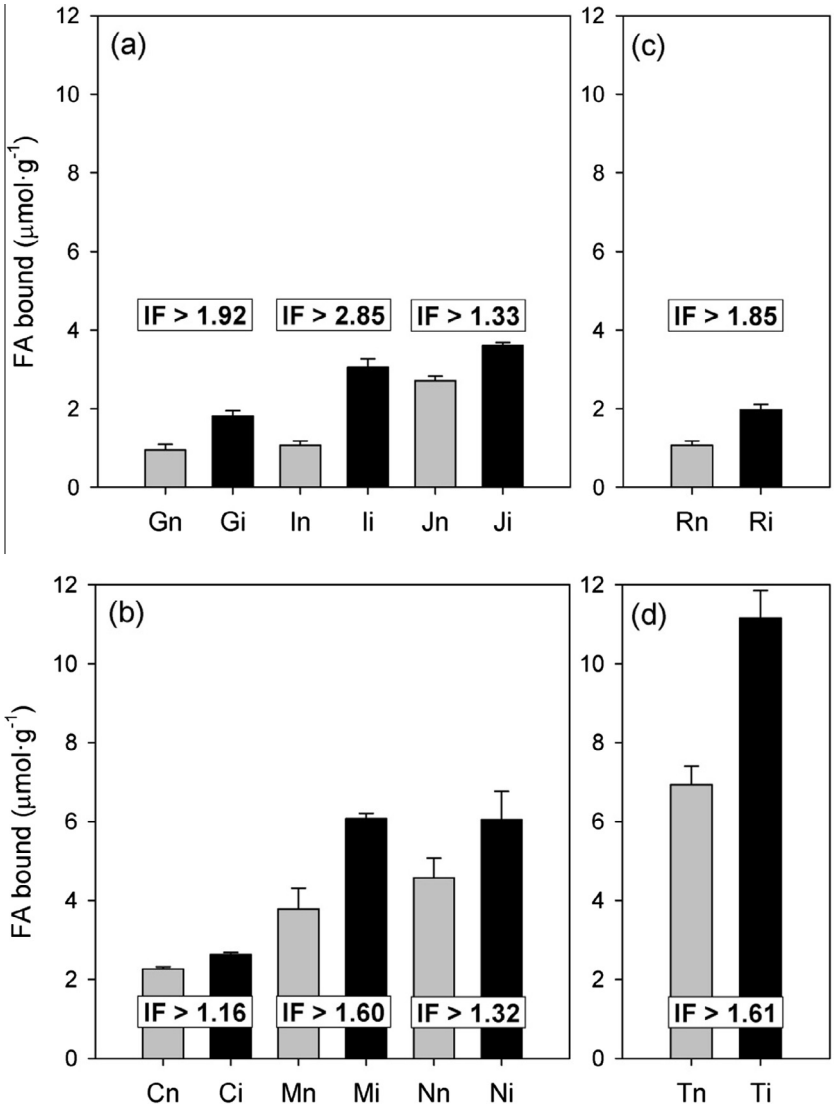

Fig. 4. Amount of FA rebound after 48 h incubation in 5 mL of 0.068 mM FA in water: (a) 1-ALPP/HEMA hydrogels and (b) DMAEMA/HEMA hydrogels (n = 3, RSD < 9%). MIHs (black squares) and NIHs (grey squares). Hydrogel codes as in Table 1.

comply with food contact materials legislation established by Commission Regulation (EU) No. 10/2011 (Commission Regulation, 2011).

Then, FA-loaded MIHs were tested for their suitability as antioxidant active packaging materials able to inhibit lipid oxidation of butter during storage. Antioxidant effectiveness was determined using the TBARs method, and the results are expressed as TBARs (mg MDA kg^{-1} of sample) (Table 1). The TBARs values were calculated for butter cans covered with the MIHs (Ti) loaded with FA and compared with those recorded for butter cans covered with the NIHs (Tn) without FA (for assessment of the effect of the polymer film itself without FA) and with those obtained for butter cans packaged in the absence of any film (controls opened to air).

The results show that the oxidation of the butter covered by the FA-loaded Ti MIH was, in most cases, significantly less intense than that recorded for butter covered with the film without FA (Tn) or for the control (without film). The greatest differences were observed in the first trial (sampling every two days) (Table 1a). During the first 11 days of storage at 4 °C, the lipid oxidation values obtained in the presence of the active films (Ti) were reduced around 40–60% with respect to the control without film and 20–25% with respect to the film control (NIH Tn). These differences may be attributed to the presence of oxygen that comes into contact with the butter whenever the can is opened. The headspace of the container is filled with air, leading to an increment in the oxidation process. These results are in agreement with those reported by Mallia et al. (2009) showing that the oxidation of conventional butter during storage, in the presence of oxygen, involves the conversion of unsaturated fatty acids to hydroperoxides, which decompose into secondary oxidation products. Films without FA in contact with the butter surface reduced lipid oxidation as a result of the protection against the oxygen present in the headspace of the container, when compared with the control without film.

In the second trial (weekly sampling), the differences between cans covered with MIH or NIH were less significant in the first weeks of storage, but after four weeks MIHs were more efficient in preventing butter oxidation (33–50% lower oxidation compared to uncovered cans). After 6 weeks storage, butter samples covered with FA-loaded MIHs presented lower TBARs values than those covered with non-loaded NIHs, and the corresponding controls without film (Table 1b).

In the third trial (sampling after three weeks), because the butter surface was exposed to oxygen for a more prolonged time than

Table 1
TBARs values (mg MDA kg^{-1} of sample) obtained in butter samples from cans without film (control) or covered with control film Tn (NIH) or with FA-loaded film Ti (MIH), determined in different sampling trials (a) every two days; (b) every week; (c) after three weeks, during storage at 4 °C. Mean values and, in parenthesis, standard deviations.*

Sample	(a) Time storage (days)						
	0	2	4	7	9	11	14
Control	0.75 (0.02)	0.98 (0.01)^a	1.03 (0.00)^a	1.03 (0.02)^a	0.75 (0.03)^a	0.72 (0.09)^a	0.67 (0.06)^a
Tn	0.75 (0.02)	0.77 (0.02)^b	0.75 (0.07)^b	0.52 (0.07)^b	0.50 (0.06)^b	0.54 (0.08)^b	0.60 (0.09)^a
Ti	0.75 (0.02)	0.58 (0.02)^c	0.58 (0.00)^c	0.41 (0.02)^c	0.35 (0.03)^c	0.43 (0.06)^b	0.49 (0.15)^a
	(b) Time storage (weeks)						
	0	1	2	3	4	5	6
Control	0.94 (0.12)	1.22 (0.01)^a	0.80 (0.15)	0.92 (0.14)	0.93(0.03)^a	1.08 (0.20)^a	0.83 (0.04)^a
Tn	0.94 (0.12)	1.24 (0.04)^a	0.90 (0.19)	0.88 (0.03)	0.66 (0.08)^b	0.75 (0.09)^b	0.72 (0.02)^b
Ti	0.94 (0.12)	0.90 (0.06)^b	0.77 (0.11)	0.88 (0.08)	0.62 (0.06)^b	0.54 (0.00)^b	0.54 (0.03)^c
	(c) Time storage (weeks)						
	0	3					
Control	0.70 (0.05)	1.46 (0.15)^a					
Tn	0.70 (0.05)	1.42 (0.23)^a					
Ti	0.70 (0.05)	0.53 (0.09)^b					

* Different lower case superscript letters (within the same time/column) denote significant differences between the mean values ($n = 3$) at $P < 0.05$.

in previous trials, oxidation recorded for MIHs were 60% lower than for control without film and for films without FA (Table 1c).

Several authors have evaluated the effect of different natural antioxidants and natural extracts to extend the shelf life of butter during cold storage. The use of these antioxidants, as food additives, reduced lipid oxidation with respect to the control tests during the period of storage. However, in such studies, the antioxidants were directly incorporated into the butter samples (Ayar et al., 2001; Dagdemir, Cakmakci, & Gundogdu, 2009; Ozturk & Cakmakci, 2006). In contrast, our MIH was designed to protect the first layers of butter and counteract the detrimental effects of air. Thus, FA was released slowly from the active film during storage, avoiding antioxidant additives in the bulk of the food. Studies performed by Otero-Pazos et al. (2014) showed that FA did not migrate to fat food simulants. However, we observed that FA-loaded MIHs showed high efficiency in prevention of lipid oxidation in butter. Overall, these results suggest that the protection provided by the active films is not due to migration of the active compound to the foodstuff, but the effect of FA on the headspace of the container. FA may act as scavenger of oxygen and/or radicals responsible for the oxidation process, which in turn leads to an extended shelf life of butter. These findings are in agreement with previous results shown in the literature for active packaging films containing natural polyphenols (Barbosa-Pereira, Aurrekoetxea et al., 2014; Nerín, Tovar, & Salafranca, 2008). Interestingly, the affinity of MIHs for FA ensures prolonged retention of the antioxidant in the film, while the limited migration to butter may avoid changes in the organoleptic properties of butter.

4. Conclusion

Antioxidant active films based on MIHs and FA (a natural antioxidant) were successfully developed. The use of molecularly imprinted polymers for this application is still unusual. However, MIHs represent a useful alternative to conventional packaging materials, such as ethylene–vinyl alcohol copolymer or polyvinyl alcohol, as their composition can be tuned to selectively bind, with a high loading capacity, the target antioxidant improving the antioxidant properties of the resulting active films. Moreover, many of the monomers usually applied for polymer synthesis are authorized for the manufacture of plastic materials intended to come into contact with foodstuff according to the EU legislation, which endorses the selection of the right combination for each application. MIHs preparation is simple and reproducible and the use of a dummy template, as an alternative to FA, for polymer synthesis avoids the problems associated to the presence of the antioxidant during radical polymerization. The FA-loaded MIHs were more effective as butter antioxidant materials than the non-loaded NIHs as oxidation was reduced by 25% after four weeks of butter storage. These results demonstrate the potential of MIHs for the development of antioxidant active packaging materials for use in the food industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.05.128.

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