Development of protein-based bioplastics with antimicrobial activity by thermo-mechanical processing

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ABSTRACT

This study focuses on the development of new bio-active protein-based bioplastics through a thermo-mechanical processing, which involves a first compounding step followed by compression-moulding of the resulting material into the desired shape. Two types of proteins, wheat gluten and egg-white albumen, and two different bioactive agents, formic acid and oregano essential oil, were selected. The effect of biocide addition on the material rheological response, its antimicrobial activity and biocide release behaviour have been assessed. Rheological tests demonstrated that formulation and processing may exert a notable effect on the material linear viscoelasticity. Kirby-Bauer tests carried out on four selected types of microorganisms revealed that oregano essential oil into a wheat gluten-based matrix may be suitable for applications where the active agent must be progressively delivered (for up to 7 days). Moreover, this biocide can inhibit microorganisms' growth even if the bioplastic is not in direct contact with the substrate.

Keywords:

Bioplastic

Biocide

Protein

Controlled-release

Rheology

1. Introduction

Replacement of synthetic polymers with new biodegradable materials is becoming an important challenge nowadays. The growing demand of petroleum along with the political circumstances in many of the most important producer countries have increased its price far away from those of previous decades (Shawkat and Huimin, 2004). Biopolymers, derived from agricultural sources (Irissin-Mangata et al., 2001; De Graaf, 2000), seem to be a promising alternative. Different vegetable (corn, wheat gluten, soy, etc.) and animal (milk, albumen, collagen, gelatin, etc.) proteins have been used to manufacture bioplastics (Jerez et al., 2007a, 2007b; Pommet et al., 2003). Protein-based biomaterials may be an efficient way to produce biodegradable materials with a large range of functional properties. These applications include packaging, matrix for enzyme immobilization or controlled-release, etc. (Yu and Min, 2006; Suda et al., 2000).

A protein-based material could be defined as a stable three-dimensional macromolecular network stabilized and strengthened by hydrogen bonds, hydrophobic interactions and disulfide bonds (Pommet et al., 2003). However, as proteins themselves do not have sufficient plasticity to be handled, a plasticiser is required. Plasticisers are molecules with low molecular weight and volatility, which modify the three-dimensional structure of proteins by reducing the intermolecular forces and increasing the polymer chains mobility (Gennadios, 2002). Moreover, a plasticiser may reduce the bioplastic glass transition temperature (Pouplin et al., 1999; Irissin-Mangata et al., 2001; Matveev et al., 2000). Plasticizer incorporation into the protein matrix can be performed by following two different methods: a) physico-chemical or “casting” method, using a chemical reactant to disrupt disulfide bonds (Gontard et al., 1993); and b) thermoplastic processing, which consists of mixing proteins and plasticizer by a combination of heat and shear (Attenburrow et al., 1999; Hernandez-Izquierdo et al., 2008) and, depending on protein,

of an additional stage involving further thermo-mechanical treatments (e.g. compression moulding) is required to achieve a suitable material (Song and Zheng, 2008; Min et al., 2008).

Studies on active packaging have been largely developed in the last recent years. Quintavalla and Vicini (2002) defined an active packaging material as ‘‘a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food’’. Principal active packaging systems involve oxygen scavenging, moisture absorption, carbon dioxide or ethanol generation and, finally, antimicrobials (Coma, 2008). Antimicrobial packaging reduces, inhibits, or retards the growth of pathogen microorganisms in packed foods (Vermeiren et al., 1999) through: a) volatile and non-volatile antimicrobial agents incorporated into polymers; b) coating or adsorbing antimicrobials onto polymer surfaces (Appendini and Hotchkiss, 2002). A variety of compounds, including organic acids, enzymes such as lysozyme, natural antimicrobials such as spices, etc. have been proposed for active food packaging (Tharanathan, 2003; Weng and Hotchkiss, 1992). However, the essential oils, which contain high concentrations of phenolic compounds such as carvacrol, eugenol and thymol, show the strongest antibacterial activity against foodborne pathogens (Burt, 2004; Lamber et al., 2001). Their antimicrobial properties have been demonstrated in numerous studies (Avila-Sosa et al., 2010; Emiroglu et al., 2010; Seydim and Sarikus, 2006). In addition, various organic acids and their salts (formic acid, sorbates, benzoates and propionates) are used to inhibit the microbial growths (Over et al., 2009; Ricke, 2003) and to increase the shelf-life of fresh dough together with cooling, because these products are packaged without thermal treatment.

With the aim of finding novel biomaterials with antimicrobial activity, the present work studies the thermo-rheological response, biocide release behaviour and the inhibitory

effects against four selected pathogens of protein-based bioplastics in which two types of active agents (formic acid and oregano essential oil) were incorporated. Selected proteins (wheat gluten or egg-white albumen) would also allow for taking advantage of the low solubility and high swelling in water of the resulting bioplastics in order to develop controlled-release matrix for bioactive agents. In this regard, previous works on bioplastic from these proteins have shown enhanced water resistance (protein solubility below 5%) and high water absorption capability (up to 300%) depending on material formulation and processing (Jerez at al., 2007b; Zarate-Ramírez et al., 2011).

2. Materials and methods

2.1. Materials

Two proteins from different sources were used in this research: a) wheat gluten (WG), provided by RIBA S.A. (Spain), with 83 wt.% protein; b) egg white (EW) albumen, supplied by OVOSEC S.A. (Spain), with 73 wt.% protein. Glycerol (G), from Panreac Química, S.A. (Spain), was used as protein plasticiser. On the other hand, formic acid (FA) and oregano essential oil (OEO), from Panreac Química, S.A. (Spain) and Destilerías Muñoz Gálvez, S.A. (Spain), respectively, were used as antimicrobials. Both oregano essential oil and formic acid (in packaging) are listed as generally recognized as safe (GRAS) by the Food and Drug Administration. In addition, their uses as food additives and in packaging are regulated by EU (EC-No 1333/2008 and EC-No 1935/2004 regulations).

2.2. Samples preparation

Bioplastics compounding was performed in a Polylab torque-rheometer equipped with a Rheomix 3000p kneading tool (Thermo-Haake GmbH, Germany). This device allowed evolution of mixing temperature and torque to be monitored. Neither heating nor cooling was supplied to the kneading chamber (filled to 85% of its full capacity) during compounding. The procedure consisted in mixing protein (fine powder) with both biocide and plasticiser (liquids), by means of two rollers counter-rotating at 50 rpm. The mixing time applied, which depended on the type of protein, varied between 10 and 30 min. Subsequently, the resulting dough-like biomaterials were compression-moulded into rectangular specimens (50 mm length, 10 mm width and 3 mm thick) by applying, a gauge pressure of 100 bar, for 10 min, at two selected temperatures of 90 and 120ºC (Jerez et al., 2005a, 2005b). Finally, specimens were allowed to cool down to room temperature inside the hot-plates press before removing from the mould. Afterwards, bioplastics were stored at 53% relative humidity (RH) before testing.

All the formulations studied present a 67 wt.% protein and 33% plasticizer+biocide blend, with the relative quantities of glycerol and biocide being adjusted so as to obtain a biocide content of 0, 5, 10 or 33 wt.% over the total weight of the bioplastic.

2.3. Testing procedures

Dynamic Mechanical Thermal Analysis (DMTA) tests were conducted on 50×10×3 mm^3 samples with a DMS 6100 (Seiko Instruments Inc., Japan) in double cantilever (bending) mode. In these tests, the storage modulus E' (elastic response) and loss modulus E'' (viscous response) of polymers are measured as a function of temperature (or frequency) as the polymer is deformed under an oscillatory load (stress). The complex modulus can be calculated as, $|E^*|^2 = |E'|^2 + |E''|^2$, measured in tension or flexure. Temperature sweep

tests from 30 to 170 ºC were carried out, within the linear viscoelasticity (LVE) region, at selected frequency and heating rate of 1 Hz and 2ºC/min, respectively.

Pure biocides volatility was studied by means of thermogravimetric analysis (TGA). Tests were carried out in a Q-50 analyser (TA Instruments, USA), on 5-10 mg samples which were heated from 30 to 250 ºC at 10 ºC/min, in N_2 atmosphere.

The Kirby-Bauer test (Boyle et al., 1973) was used in order to determine the antimicrobial activity of the two biocides proposed (formic acid and oregano essential oil). Round paper disks soaked in pure biocide or its corresponding 10 vol.% aqueous solution were set on a solidified agar culture medium inoculated with a solution of a selected microorganism. The four microorganisms studied, usually involved in food preservation and water pollution control processes, were: *Aspergillus niger* (fungus-mold) (wild strain), *Candida kefyr* (fungus-yeast) (wild strain), *Bacillus cereus* (bacteria gram-positive) CECT131 and *Escherichia coli* (bacteria gram-negative) CECT434. Antimicrobial test were carried out by disc diffusion method using 100 µl of bacterial suspension containing $2.0x10^8$ CFU/ml in sterilized Petri dishes 90 mm in diameter. The paper discs (6mm in diameter) were impregnated with 20 µl of each biocide dilution. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. The growth inhibition level exhibited by a selected microorganism for every different biocide tested can be quantitatively calculated by measuring the diameter of the inhibition zone, that is, the clear halo around the disk. Thus, K-B tests were carried at room temperature for 48 h, after which an average inhibition diameter was calculated for every combination of microorganism and biocide tested. The data were presented as mean ± standard deviation (SD) of three determinations. A probability value of $p<0.05$ was considered significant.

A similar procedure was followed for the active bioplastics prepared, but replacing the paper discs by a square piece (1x1 cm^2) of that material. Alternatively, bioplastic

antimicrobial activity was tested by placing the specimen under the lid of the Petry dish, so that the material is not in contact with the inoculated agar. In that case, inhibition would arise from the development of an antimicrobial atmosphere. Experiments were carried out in triplicate.

The transport rate, from the bioplastic samples into water, of the two active agents studied was evaluated by immersion of 1 g bioplastic in 100 mL water and taking samples of water at 24, 48, 72 h, and finally after 1 week at room temperature. Formic acid release was determined by acid/base titration and, as for OEO release, concentration in water of phenolic compounds (thymol and carvacrol) contained in this biocide was determined by means of a colorimetric assay involving the Folin-Ciocalteu reagent (FCR), and expressed in gallic acid equivalent (mg/L).

3. Results and discussion

3.1. Biocides antimicrobial activity

In order to guarantee biocides antimicrobial effectiveness on the microorganisms selected, the Kirby-Bauer test was firstly carried out on their pure or 10 vol.% aqueous solution samples. As previously mentioned, if bacteria are susceptible to a particular antimicrobial, an area of clearing called “inhibition zone” (where bacteria are not capable of growing) surrounding the paper disk soaked in the biocide arises. As an example, Figure 1a illustrates the effect of oregano essential oil, pure and diluted, on *Bacillus cereus*.

Table 1 presents the inhibition zone diameters corresponding to the different microorganisms studied for the two types of active agents. Biocides clearly demonstrate larger values of inhibition zone diameters, and so, a more effective antimicrobial activity, when they are used as pure substances. Thus, diameters always result notably reduced

when using a 10 vol.% aqueous solution. However, except for *Aspergillus niger*, a certain degree of antimicrobial activity is still appreciated after dilution. This fact reveals that, when dealing with *Candida kefyr*, *Bacillus cereus* or *Escherichia coli*, a 10 vol.% solution lies above the minimum inhibitory concentration (MIC), a parameter which marks the threshold above which no growth is observed. In addition, for diluted samples of biocides, *Bacillus cereus* and *Escherichia coli* are found to show the lowest resistance (largest diameters) to oregano essential oil. Hence, these microorganisms are expected to present the smallest oil MIC values. Similarly, *Bacillus cereus* would present the smallest acid MIC value. With respect to *Aspergillus niger*, an aqueous solution with a biocide content above 10 vol.% must be used for microbial growth to be inhibited. Thus, it seems to be the most resistant microorganism to both biocides, as no inhibition zones were detected in their corresponding tests with diluted solutions. This result would correlate with larger oil/acid MIC values for this specific type of bacteria.

In consequence, this procedure provides valuable information on the appropriate antimicrobial type and concentration to combat a particular microorganism, and will be further used in the formulation of the active protein-based bioplastics.

3.2. Study on formic acid-containing bioplastics

3.2.1. Thermoplastic processing

Mixing during the bioplastic compounding has proven to be a key factor in determining the final quality of the resulting biomaterial (Jerez et al., 2007a, 2007b). On these grounds, Figure 2 shows the evolution of mixing torque and temperature during the manufacture of formic acid-containing bioplastics with a constant plasticiser/protein ratio of 0.5. As previously explained, the relative quantities of glycerol and biocide were properly adjusted so as to obtain biocide contents of 0 (reference sample), 5 or 10 wt.% over the total weight of the bioplastic.

In relation with WG-based bioplastics, three different regions may clearly be distinguished in the reference (0 wt.% formic acid) sample: a) during the 15 first min, approximately, torque does not increase significantly, as this region stands for an induction period, which was seen to decrease as mixing speed was raised (not shown here); b) in the second region, which extends over a time interval of 5 min, torque undergoes an exponential increase up to a maximum value of about 40 N·m; c) torque finally decays, whilst seems to tend to a constant value. Analogously, three different zones have also been identified from the sigmoid function which relates temperature and time: a) a nearly isothermal step; b) a further exponential increase up to 65ºC; c) a very slight increase in temperature, which corresponds to the final torque decay.

When formic acid is added to the WG-based formulations, no induction period is observed (see Figure 2). However, after a notable initial increase, both mixing torque and temperature evolve as the reference sample does and tend to a very similar constant value. Moreover, no significant differences are found when 5 and 10 wt.% samples are compared, although slightly higher mixing temperatures for the lowest acid concentration may be noticed.

Regarding the EW-based reference sample, low and nearly constant values of torque and temperature are observed over the 10 first minutes. In contrast, formic acid addition leads to an instantaneous increase in torque (up to more than 90 N·m) followed by a rapid decay, whilst temperature suddenly increases up to more than 100ºC. This high value of temperature is due to the large heat flux dissipated by friction during blending of the high viscosity resulting material. However, if the reference sample processing is prolonged beyond 10 min, a powdery-like instead of a dough-like material is obtained. In the same way, acid addition did not allow a material with suitable features to be achieved. As a conclusion, excessive energy input or acid addition provokes important changes in this

240 globular protein microstructure, which may significantly increase its sensitivity to shear.
241 Thus, the resulting material cannot be further transformed into a bioplastic by means of a
242 thermo-mechanical treatment and will not be considered for study.

243 In any case, irrespectively of the type of protein used (EW or WG), FA exhibits
244 an active role by promoting shear induced crosslinking effects. WG/GL and EW/GL
245 blends initially exhibit native pH in absence of FA, which in both cases is close to the
246 isoelectric point (pI). The isoelectric point of spray-dried egg albumen has been estimated
247 to be close to pH 5.4 by z-potential tests (Riddick, 1968). As for wheat gluten the protein
248 concentrate used in this work typically shows a pI around pH 6 (Bengoechea et al., 2006)

249 Under such conditions the average net charge of protein surfaces is not far from
250 zero and random aggregation among protein segments is favoured. Somehow, FA seems
251 to inhibit physical aggregation among protein segments. Thus, addition of FA provokes
252 a reduction of pH that leads to a displacement from zero to positive net charge of protein
253 surfaces, which in turn tends to inhibit random aggregation. As a result, more active sites
254 exposed to the surrounding medium, thus being readily available for crosslinking. In such
255 a case, torque and temperature mixing profiles suggest that the induction period is not
256 required, either for WG or EW based systems, to carry out shear-induced disruption of
257 protein aggregates previously to the development of crosslinking reactions.

258

259 **3.2.2. Thermo-mechanical behaviour**

260 Figure 3 shows the results of DMTA tests in double cantilever mode, over a temperature
261 interval from 30 to 170ºC, carried out on WG-based bioplastic specimens prepared by
262 compression-moulding at 90ºC.

263 For the reference sample, |E*| decreases with increasing temperature up to 100ºC,
264 followed by a short temperature interval where |E*| levels off. This rubbery-like plateau

265 extends from 100 up to 110ºC. Finally, the flexural complex modulus starts increasing
266 again, evidencing an apparent thermosetting potential even after compression-moulding.
267 This outcome may most probably be the result of a part of the wheat gluten protein yet to
268 denature after dough post-treatment at 90ºC (Jerez et al., 2007a; González-Gutiérrez et
269 al., 2010). In fact, as previously shown, this viscoelastic growth region becomes more
270 apparent when the dough is processed at temperature lower than 90ºC or dampens and
271 eventually vanishes by processing at higher temperature (Zárate-Rámirez et al., 2011).
272 Formic acid addition also seems to exert a clear and pronounced influence on broadening
273 the temperature interval over which the rubbery-like plateau extends, and consequently,
274 on decreasing the bioplastic temperature susceptibility. This plateau region has been
275 extensively described in polymer rheology in terms of an entanglement network formed
276 by the simple topological interaction of polymer chains. It arises at frequencies higher
277 (timescale shorter) than the lifetime of the topological entanglements (Ferry, 1980).
278 However, for these protein-based systems, it may be attributed to a situation that falls
279 between a temporary entangled network and covalent crosslinking (Ross-Murphy, 1995),
280 as hydrophobic interactions usually act not at a point on the chain as covalent crosslinks
281 do, but involve more extended “junction zones”. This effect becomes more important
282 with increasing acid content, as it may be increasing the number of sites available for
283 interactions among chains, probably by inhibiting protein aggregation, as previously
284 mentioned.
285 In order to compare the mechanical properties of these WG-based bioplastics with a
286 standard synthetic polymer, DMTA results for a commercial LDPE have also been
287 included in Figure 3. It can be seen that WG-based bioplastics with a plasticiser/protein
288 ratio of 0.5 and compression-moulded at 90ºC, show lower values of |E*| over the
289 temperature interval comprised between room temperature and 120ºC. However, whilst

290 the bioplastics did not experience any melting event upon heating, LDPE complex
291 modulus was seen to greatly decay with increasing temperature increases, with values
292 rapidly going down as temperature approached its corresponding melting point (around
293 120ºC).

294 **3.2.3. Antimicrobial activity**

295 In an analogous manner to the biocides, the antimicrobial effectiveness of the active WG-
296 based bioplastics containing formic acid was ascertained by measuring the inhibition zone
297 diameter in the Kirby-Bauer test. As an example, Figure 1b shows the antimicrobial
298 activity, on the selected microorganism *Escherichia coli*, of WG-bioplastics containing 5
299 and 10 wt.% formic acid (A_2 and A_3, respectively). In order to establish a comparative
300 analysis, K-B tests were also conducted on the WG-bioplastic without addition of the
301 biocide. They revealed the absence of antimicrobial activity (a biocide-free bioplastic, A_1,
302 is included in Figure 1b).

303 Results in Table 2 show that, even after being incorporated into the bioplastic, formic acid
304 still demonstrates an adequate antimicrobial activity, which becomes higher as acid
305 concentration increases. *Aspergillus niger* and Escherichia coli are the most resistant
306 microorganism when formic acid is employed as biocide, as shown by the lowest
307 inhibition zone diameters at 5 wt.% FA.

308 **3.2.4. Evaluation of the controlled-release potential**

309 Evaluation of active agent releasing rate from biopolymer matrix has received
310 outstanding attention over the last years, as they may find promising applications in a
311 broad spectrum of fields, ranging from food packaging to biomedical, pharmaceutical,
312 environmental or agricultural engineering. Some specific examples can be water pollution
313 control or long-term food preservation, where a slow diffusion results of major
314 importance in order to assure a suitable product performance. In that sense, Han (2005)

315 reported that the efficiency of active packaging is based on an antimicrobial releasing
316 rate, from the film to food, which matches with the growth kinetics of the target
317 microorganism.
318 Figure 4a illustrates the cumulative curve for formic acid concentration in water over
319 time. As expected, a higher formic acid content greatly increases the biocide
320 concentration in water. It can also be seen that concentration remains constant over the
321 time interval tested. This means that formic acid release entirely occurs within the 24 first
322 hours (first measurement), after which diffusion rate drops down to zero. WG-based
323 matrix is not capable of regulating a progressive release of this substance, which
324 consequently would limit the suitability of this active bioplastic for applications where
325 the active agent can be delivered straightaway. On the other hand, compression-moulding
326 temperature seems to produce some differences, as a lower formic acid release is noticed
327 if compression-moulding is performed at 120ºC (compare curves for 10 wt.% FA).
328 Related to this, TGA results have shown the mass loss of formic acid is complete at 100ºC.
329 Thus, even though post-treatment is carried out at high pressure, bioplastic specimens
330 containing different quantities of formic acid are expected at each temperature (90 and
331 120ºC). As a result, this fact must be taken into consideration during material
332 manufacture. In this sense, the selection of proteins which may be processed using milder
333 temperatures might be a key factor for the development of biodegradable/bioactive
334 materials containing volatile antimicrobial agents. It is possible to calculate the biocide
335 fraction released (mg biocide into water/mg biocide initial in the protein matrix) at every
336 bioplastic concentration. These calculations show that just about 39% and 73% of the
337 initial biocide added is released for samples containing 5 and 10 wt.% FA, respectively.
338 The remaining quantity of biocide may have gone during processing or may be reacted
339 with the protein matrix.

3.3. Study on oregano essential oil-containing bioplastics

3.3.1. Thermoplastic processing

The evolution of mixing torque and temperature during the manufacture of bioplastics containing oregano essential oil is illustrated in Figure 5. In contrast to formic acid, no significant differences are noticed when the essential oil is added to the WG-based formulations. Mixing torque curves still present three different regions: a) induction (period over which torque does not increase significantly); b) torque exponential increase; and c) final decay. Thus, biocide addition just seems to slightly shorten the time interval previous to the torque exponential increase. On the other hand, 10 wt.% oregano essential oil is shown to decrease the mixing torque with respect to the reference bioplastic, although the same torque value is eventually reached. Instead, if oregano essential oil concentration is further raised up to 33 wt.% (and no glycerol is added), the resulting mixing operation presents values of torque which remain close to zero and no plasticizing process is noticed. Whilst the glycerol-involved materials (with up to 10 wt.% oregano) obtained after mixing can be already considered as bioplastics with suitable mechanical properties, the mixing process of the glycerol-free material (i.e. with 33 wt.% oregano) gives rise to a dough-like blend, which requires of further thermo-mechanical treatments, by compression-moulding at 120ºC, to obtain the bioplastic.

Similarly, for the EW-based formulations, mixing torque does not undergo any changes on the reference sample after essential oil addition. Thus, very low and nearly constant values of torque are still monitored during the bioplastic compounding. However, the quality of the material obtained after kneading allowed DMTA testing specimens to be prepared via compression-moulding. Interestingly, if compared to formic acid, oregano

essential oil has shown to exert quite a non-significant influence on altering the compounding process of the bioplastics.

3.3.2. Thermo-mechanical behaviour

Figure 6 shows the results of DMTA tests in double cantilever mode, over a temperature interval from 30 to 170ºC, carried out on essential oil-containing bioplastics prepared by compression-moulding at 90ºC. As can be seen, biocide addition in concentrations of up to 10 wt.% does not produce any remarkable change in the thermo-mechanical response of the reference WG-based bioplastic. It just seems to very slightly extend the temperature interval over which the rubbery-like plateau appears. The thermosetting potential, characterised by a further increase in |E*| and corroborated by an important decrease in the tanδ values at the highest temperatures, remains (Figure 6a). However, the glycerol-free sample, with an essential oil content of 33 wt.%, is strikingly seen to modify the bioplastic rheological behaviour after compression-moulding at 120 ºC. This system is characterized by an apparent plateau region, which extends over a temperature interval of about 40ºC, and the absence of thermosetting potentials. Moreover, it exhibits a notable increase in |E*| of more than one order of magnitude (comparable to LDPE). According to previous results by Zárate-Ramírez et al. (2011) an increase in the temperature of processing (i.e. from 90 to 130ºC) yielded an increase in the DMA profile which was much less pronounced than that shown by the 33% OEO-containing bioplastic (moulded at 120ºC) with reference to the 33% glycerol-containing sample (moulded at 90ºC). Thus, although the increase in moulding temperature plays a role, most of the enhancement found in flexural properties must be attributed to the replacement of the plasticizer itself. As seen in Figure 5, oregano essential oil alone does not exert any plasticizing effect, so that it is expected that natural material moisture would play the only plasticizing role

during protein thermal denaturation, which eventually results in a lack of polymer chain mobility and high modulus values after bioplastic moulding at 120 ºC.

Regarding the reference EW-based bioplastic, a monotonous decrease in |E*| with increasing temperature, as well as a significant change in the decay rate giving rise to a plateau region, can be appreciated. This event, which coincides with the maximum in tanδ at 75ºC, is known to be related to a gel-glasslike transition temperature of the plasticized egg white protein (Nakamura et al., 1999; Sepe, 1997; Takushi, 1998). Oregano essential oil addition in concentrations of up to 10 wt.% does not bring about any substantial changes in the thermo-mechanical response under small deformations of the reference EW-based bioplastic. However, some differences are noticed, e.g. the plateau starts at lower temperatures, which is evidenced by a shift of the gel-glasslike transitions towards lower temperatures (compare tanδ peaks in Figure 6b). Moreover, addition of 5 wt.% essential oil generally induces an enhancement in |E*| values, whereas a further increase up to 10 wt.% produces a notorious reduction in |E*| leading to values even lower than those shown by the OEO-free system at the lowest temperature interval studied.

Finally, large differences in |E*| values are noticed if two similar formulations containing either wheat gluten or egg-white proteins are compared. Despite the low mixing torque values registered during the compounding of the EW-based bioplastics, much higher values of |E*| are found for them (comparable to LDPE). This result demonstrates, on the one hand, that post-treatment (for example, compression-moulding) is of major importance in the manufacture of suitable EW-based bioplastics and, on the other hand, the EW-based dough achieved after kneading presents a high thermosetting potential.

3.3.3. Antimicrobial activity

The inhibition zone diameters for active bioplastics containing two different oregano essential oil concentrations (5 and 10 wt.%) are presented in Table 2. As previously

414 explained, K-B tests conducted on the WG-bioplastic without addition of the biocide
415 revealed the absence of antimicrobial activity. However, bioplastics containing this
416 bioactive agent have demonstrated a suitable antimicrobial activity, which becomes
417 higher as its concentration increases. Furthermore, at 5 wt.% OEO, the biocide
418 effectiveness is clearly higher when this is incorporated into a WG-based bioplastic.
419 Previous results revealed that a suitable combination of formulation and processing
420 conditions may lead to materials with different microstructure, which was seen to
421 ultimately control their linear viscoelastic response. In the same manner, microstructure
422 might also influence the way a specific active agent diffuses out of the bioplastic matrix.
423 Hence, inhibition diameters shown in Table 2 verify that WG-based matrix would be
424 based on a microstructure which seems to facilitate the biocide migration.
425 Finally, it can be observed that, on the contrary to formic acid, oregano essential oil seems
426 to exert a greater antimicrobial effect on *Aspergillus niger* after being incorporated into
427 the bioplastic. *Escherichia coli* and *Candida kefyr* are the most resistant microorganism
428 under the action of oregano essential oil at 5 wt.% OEO and for the EW matrix.
429 Interestingly, essential oil may also inhibit the growth of microorganisms even if it is not
430 in direct contact with them, by promoting the formation of an antimicrobial atmosphere
431 within the container. Thus, the antimicrobial activity of an oregano oil-containing
432 bioplastic sample placed under the Petri dish lid is illustrated in Figure 1c. This outcome
433 may be of relevant importance in the development of matrix for non-contact food
434 preservation. For the sake of comparison, the antimicrobial activity of OEO-free LDPE
435 is also shown in Figure 1c. As seen, no antimicrobial activity was found.
436
437 **3.3.4. Evaluation of the controlled-release potential**

EW-based bioplastics have turned out to be unable to control the release of the essential oil when material is immersed in water. On the contrary, diffusion rate of essential oil corresponding to WG-based bioplastics, which showed to have the strongest capability of growth inhibition, has been evaluated. Figure 4b illustrates the evolution with time of the cumulated oregano essential oil concentration, expressed in terms of gallic acid equivalent (mg/L), in water. Results demonstrate that, in contrast to formic acid, oregano oil release occurs progressively with time. Thus, its rate decreases but the cumulated concentration increases with time. Consequently, WG-based matrix seems to be suitable in the manufacture of active oregano oil-containing bioplastics for long-term applications. Compression-moulding temperature does not produce any significant differences in the oregano oil release rate (compare curves at 5 wt.%). On these grounds, TGA results carried out on a sample of pure oregano oil reveal no massive substance loss at any of the two compression-moulding temperatures. Compared to formic acid, which hardly resists temperatures close to 100ºC, EOE shows a mass loss lower than 5 wt.% at such temperature. Moreover, even lower mass loss during compression-moulding should be expected as volatility decreases with increasing pressure.

4. Conclusions

Protein-based (wheat gluten or egg-white albumen) bioplastics containing two different active agents (formic acid or oregano essential oil) have demonstrated a suitable antimicrobial activity when tested on four selected types of microorganisms.

Studied protein-based bioplastic are not able to control the formic acid migration to water. In contrast, oregano oil release occurs progressively from the WG-based matrix, being suitable this material for long-term applications. Moreover, bioplastics containing essential oil may also inhibit the growth of microorganisms even if they are not in direct contact with them, by promoting the formation of an antimicrobial atmosphere within the

container. In consequence, some of the formulations studied may find successful applications in food packaging or water pollution control.

5. Acknowledgements

This work is part of a research project sponsored by OX-CTA and by "Ministerio de Economía y Competitividad" (Ref. MAT2011-29275-C02-01). The authors gratefully acknowledge their financial support.

6. References

Appendini, P., Hotchkiss & J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Sci. Emerging Technol*., 3, 113–126.

Attenburrow, G., Barnes, D., Davies, A.P. & Ingman, S.J. (1990). Rheological properties of wheat gluten. *J. Cereal Sci*., 12, 14-31.

Avila-Sosa, R., Hernandez-Zamoran, E., Lopez-Mendoza, I., Palou, E., Munguia, M.T.J., Nevarez-Moorillon, G.V. & Lopez-Malo, A. (2010). Fungal inactivation by Mexican orégano (Limpia berlandieri Schauer) essential oil added to amaranth, chitosan, or starch edible films. *J. Food Sci.*, 75, M127–M133.

Bengoechea, C., Cordobés, F. & Guerrero, A. (2006). Rheology and microstructure of gluten and soya-based o/w emulsions. *Rheol. Acta*, 46, 13-21.

Boyle, V.J., Fancher, M.E. & Ross Jr., R.W. (1973). Rapid, modified Kirby–Bauer susceptibility test with single, high-concentration antimicrobial disks. *Antimicrob. Agents Chemother.*, 3, 418–424.

Burt, S. A. (2004). Essential oils: their antibacterial properties and potential applications in foods: a review. *Int. J. Food Microbiol*., 94, 223–253.

Coma, V. (2008). Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Sci*., 78, 90–103

De Graaf, L.A. (2000). Denaturation of proteins from a non-food perspective. *J. Biotechnol*., 79, 299-306.

Emiroglu, Z.K., Yemis, G.P., Coskun, B.K. & Candogan, K., (2010). Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. *Meat Sci.,* 86, 283–288.

Ferry, J.D. (1980). *Viscoelastic Properties of Polymers*. John Wiley and Sons, Inc., Toronto, ON.

Gennadios, A. (2002). *Protein based films and coatings*. CRC press, New York, pp. 66-115.

Gontard, N., Guilbert, S. & Cuq, J.L. (1993). Edible Wheat Gluten Films: Influence of the main process variables on film properties using response surface methodology. *J. Food Sci.,* 57, 190-196.

Gonzalez-Gutierrez, J., Partal, P., Garcia-Morales, M. & Gallegos, C. (2010). Development of highly-transparent protein/starch-based bioplastics. *Bioresour. Technol*., 101, 2007-2013.

Han, J.H. (2005). Antimicrobial packaging systems, in Han, H.J. (Ed.), *Innovations in Food Packaging*. Elsevier Academic Press. Amsterdam, The Netherlands. pp. 80–107

Irissin-Mangata, J., Gérard, B., Bernard, B. & Gontard, N. (2001). New plasticizers for wheat gluten films. *Eur. Polym. J.*, 37, 1533-1541.

Hernandez-Izquierdo, V. M., Reid, D. S., McHugh, T. H., Berrios, J. D., & Krochta, J. M., (2008). Thermal Transitions and Extrusion of Glycerol-Plasticized Whey Protein Mixtures. *J. Food Sci.*, 73(4), 169-175.

511 Jerez, A., Partal, P., Martínez, I., Gallegos, C. & Guerrero, A. (2005a). Rheology and
512 processing of gluten based bioplastics. *Biochem. Eng. J*., 26, 131-138.
513 Jerez, A., Partal, P., Martínez, I. & Gallegos, C. (2005b). Bioplástico y método para su
514 preparación. Oficina Española de Patentes y Marcas. Patent, P200501556.
515 Jerez, A., Partal, P., Martinez, I., Gallegos, C. & Guerrero, A. (2007a). Egg white based
516 bioplastics developed by thermomechanical processing. *J. Food Eng*., 82, 608-617.
517 Jerez, A., Partal, P., Martinez, I., Gallegos, C. & Guerrero, A. (2007b). Protein-based
518 bioplastics: effect of thermo-mechanical processing. *Rheol. Acta*, 46,711-720.
519 Lamber, R.J.W., Skandamis, P.N., Coote, P.J. & Nychas, J.E. (2001). A study of the
520 minimum inhibitory concentration and mode of action of oregano essential oil, thymol
521 and carvacrol. *J. Appl. Microbiol*., 91, 453–462.
522 Matveev, Y., Grinberg ,V. & Tolstoguzov, V. (2000). The plasticizing effect of water on
523 proteins, polysaccharides and their mixtures. Glassy state of biopolymers, foods and
524 seeds. *Food Hydrocolloids*, 14, 425-437.
525 Nakamura, A., Hara, K., Hiramatsu, N. & Matsumoto, A. (1999). Low frequency Raman
526 peak and elastic anomaly of dehydrated heat-treated egg-white gel. *Physica B*, 263–264,
527 327–329.
528 Over, K. F., Hettiarachchy, N., Johnson, M. G., & Davis, B. (2009). Effect of organic
529 acids and plant extracts on Escherichia coli O157:H7, Listeria monocytogenes, and
530 Salmonella Typhimurium in broth culture model and chicken meat systems. *J. Food Sci.*,
531 74, 515–521.
532 Pommet, M., Redl, A., Morel, M-H & Guilbert, S. (2003). Study of wheat gluten
533 plasticization with fatty acids. *Polymer*, 44, 115-122.
534 Pouplin, M., Redl, A. & Gontard, N. (1999). Glass transition of Wheat Gluten Plasticized
535 with Water, Glycerol, or Sorbitol. *J. Agric. Food Chem*., 47, 538-543.

536 Quintavalla, S. & Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat*
537 *Sci*., 62 373–380
538 Min Z., Song Y. & Zheng Q. (2008). Influence of reducing agents on properties of
539 thermo-molded wheat gluten bioplastics. *J. Cereal Sci.* 48, 794–799.
540 Ricke, S. C. (2003). Perspectives on the use of organic acids and short chain fatty acids
541 as antimicrobials. *Poultry Science*, 82, 632–639
542 Riddick. T. M. (1968). *Control of colloid stability through zeta potential*. Zeta-meter,
543 Inc., Vol. 11. New York, USA.
544 Ross-Murphy, S.B. (1995). Structure–property relationships in food biopolymer gels
545 and solutions. *J. Rheol*., 39, 1451–1463.
546 Shawkat, H. & Huimin, L. (2004). The impact of the Asian on the behaviour of US and
547 international petroleum prices. *Energy Economics*, 26, 135-160.
548 Sepe, M.P. (1997). Thermal analysis of polymers. *Rapra Rev. Rep* 8 (11), p. 120.
549 Seydim, A.C. & Sarikus, G., (2006). Antimicrobial activity of whey protein based edible
550 films incorporated with oregano and garlic essential oils. *Food Res. Int.*, 39, 639–644.
551 Suda, K., Wararuk, C. & Manit, S. (2000). Radiation modification of water absorption of
552 cassava starch by acrylic acid/acrylamide. *Radiation Physics and Chemistry*, 59, 413-427.
553 Song, Y. & Zheng, Q. (2008). Improved tensile strength of glycerol-plasticized gluten
554 bioplastic containing hydrophobic liquids. *Bioresour. Technol.*, 99, 7665–7671
555 Takushi, E. (1998). Existence of gel-glasslike transition point in biopolymer gels.
556 *Thermochim. Acta*, 308, 75–76.
557 Tharanathan, R. N. (203). Biodegradable films and composite coatings: past, present and
558 future. *Trends Food Sci. Technol*., 14, 71–78.
559 Vermeiren, L., Devlieghere, F., van Beest, M., de Kruiif, N. & Debevere, J. (1999).
560 Developments in the active packing of foods. *Trends Food Sci. Technol*., 10, 77–86.

561 Weng, Y. M. & Hotchkiss, J. H. (1992). Inhibition of surface molds on cheese by
562 polyethylene containing the antimycotic imazalil. *J. Food Prot*., 55, 367–369.
563 Yu, C. & Min, H. (2006). Crosslinked carboxymethylchitosan-g-poly(acrylic acid)
564 copolymer as a novel superabsorbent polymer. *Carbohydr. Res*., 341, 887-896.
565 Zárate-Ramírez, L., Romero, A., Martínez, I., Partal, P. & Guerrero, A. (2011). Wheat
566 gluten-based materials plasticized with glycerol and water by thermoplastic mixing and
567 thermomoulding. *J. Sci. Food Agric*., 91, 625- 633
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Figure captions

Figure 1. Kirby-Bauer tests pictures: a) pure and 10 vol.% aqueous solution oregano essential oil, on *Bacillus cereus*; b) WG-based bioplastics containing formic acid (A_1: 0 wt.%; A_2: 5 wt.%; A_3: 10 wt.%), on *Escherichia coli*; c) WG-based bioplastic specimen containing oregano essential oil (10 wt.%) and LDPE on *Escherichia coli* (both of them, not in contact with the culture medium).

Figure 2. Evolution with time of mixing torque (a) and temperature (b) for formic acid-containing bioplastics.

Figure 3. Temperature dependence of the dynamic flexural complex modulus for formic acid-containing bioplastics.

Figure 4. Diffusion rate tests. Evolution with time of formic acid (a) and gallic acid equivalent (b) concentrations in water.

Figure 5. Evolution with time of mixing torque (a) and temperature (b) for oregano essential oil-containing bioplastics.

Figure 6. Temperature dependence of the dynamic flexural complex modulus (a) and tanδ (b) for oregano essential oil-containing bioplastics.

586

587 **Table 1.** Antimicrobial activity of the bioactive agents.

	Inhibition diameter (cm) *			
	Oregano Essential Oil		**Formic Acid**	
	Pure biocide	**Aqueous solution (10 vol.%)**	**Pure biocide**	**Aqueous solution (10 vol.%)**
Aspergillus niger	5.52± 0.16	-	2.58±0.18	-
Candida kefyr	3.06±0.26	1.16±0.14	3.29±0.26	1.45±0.16
Bacillus cereus	4.06±0.21	1.97±0.16	3.94±0.12	2.09±0.13
Escherichia coli	2.95±0.10	1.99±0.17	4.13±0.25	1.50±0.12

588 *Values are mean inhibition zone (cm) ± S.D. of three replicates (p=0.05)

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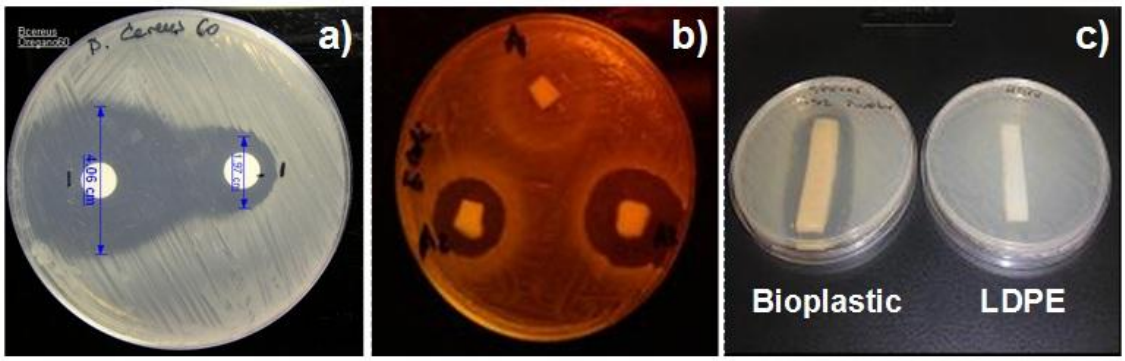
590

Table 2. Antimicrobial activity of the active bioplastics.

	Inhibition diameter (cm)					
	Oregano Essential Oil				**Formic Acid**	
	5 wt.%		**10 wt.%**		**5 wt.%**	**10 wt.%**
	EW	**WG**	**EW**	**WG**	**WG**	**WG**
Aspergillus niger	++	++	+++	+++	+	++
Candida kefyr	+	++	++	++	++	++
Bacillus cereus	++	++	++	++	++	++
Escherichia coli	+	++	++	++	+	++

Inhibition diameter:
+++ (d >50 mm)
++ (20 mm< d <50 mm)
+ (d < 20 mm)

600 

601

602 Figure 1.

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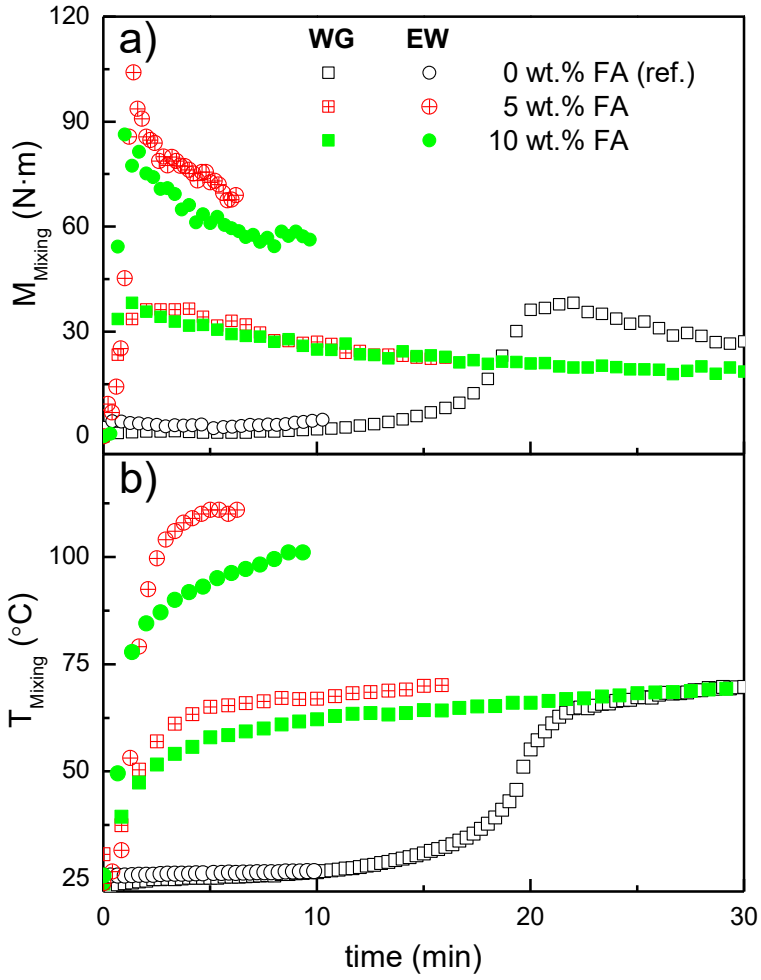

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605 Figure 2.

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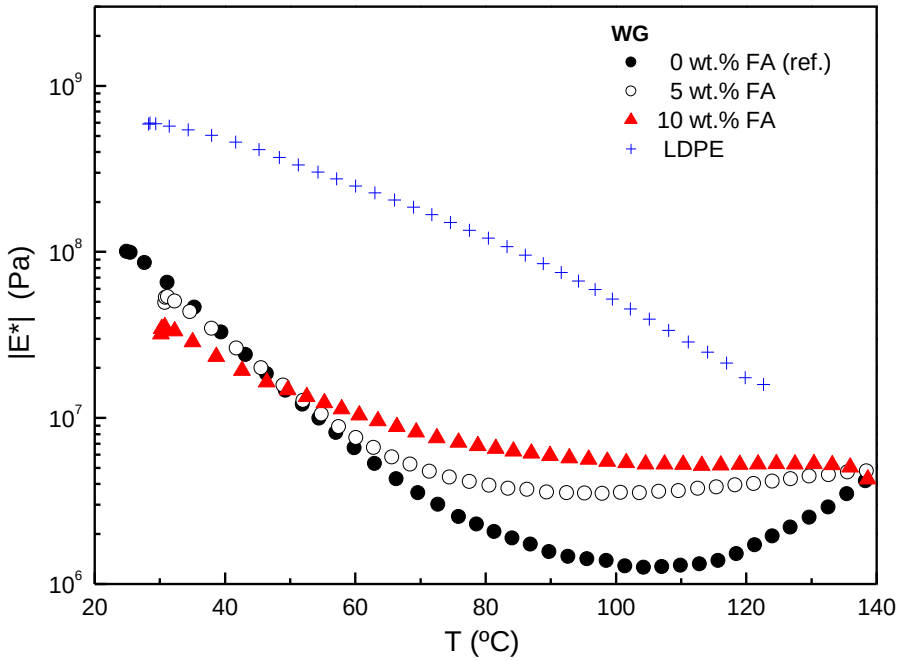

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611 Figure 3.

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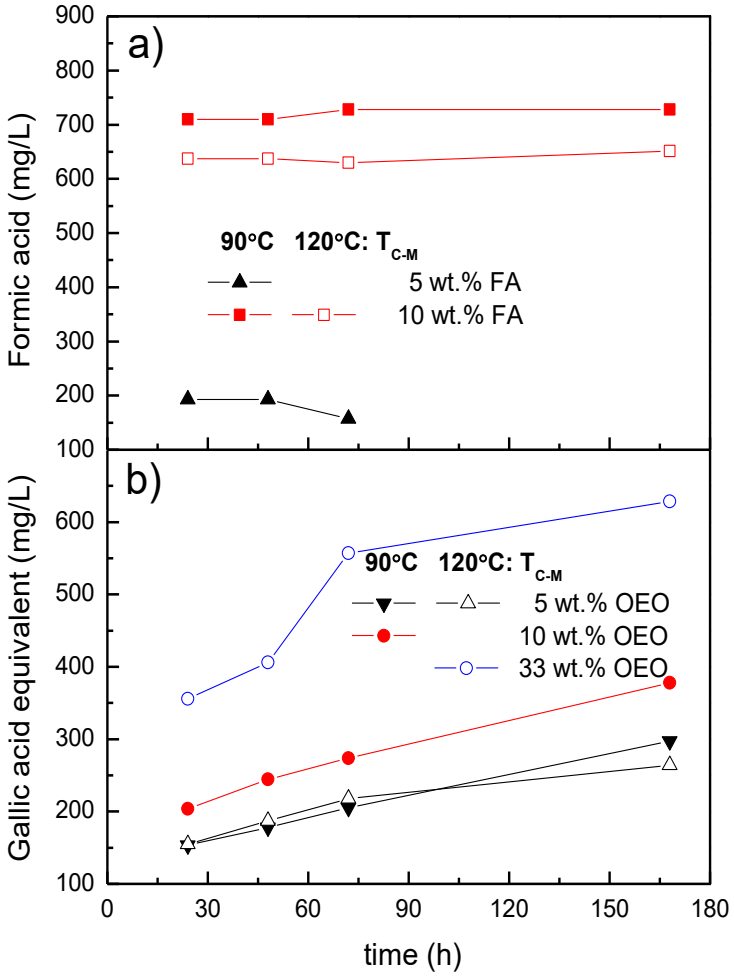
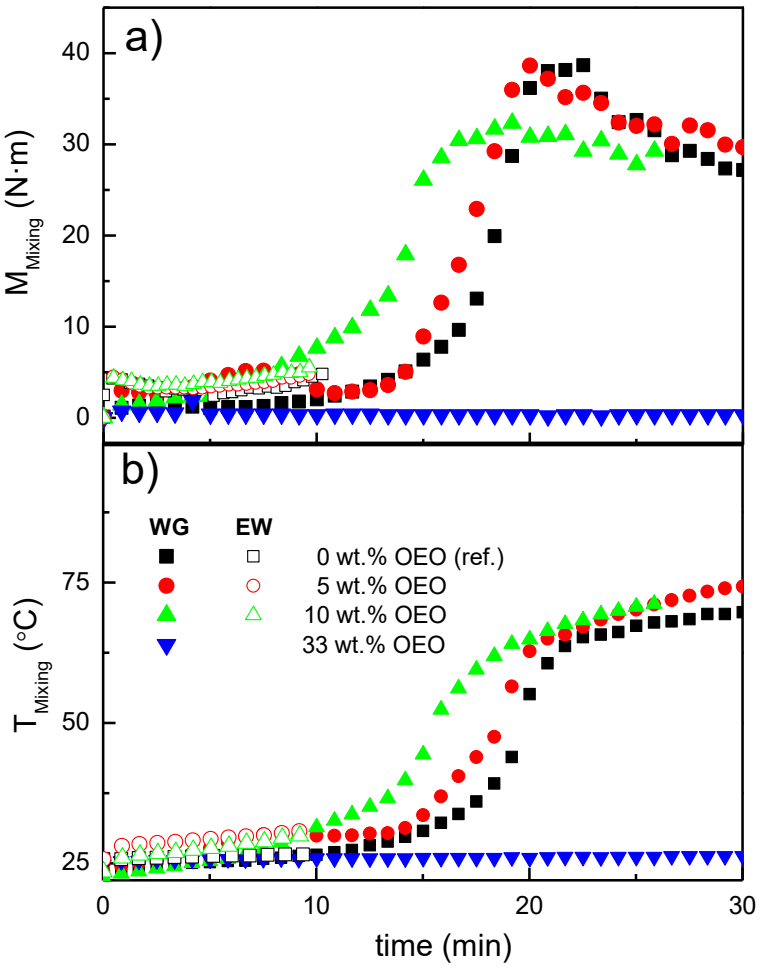

a)
Formic acid (mg/L)
900
800
700
600
500
400
300
200
100
90°C 120°C: T C-M
5 wt.% FA
10 wt.% FA
b)
Gallic acid equivalent (mg/L)
600
500
400
300
200
100
90°C 120°C: T C-M
5 wt.% OEO
10 wt.% OEO
33 wt.% OEO
0
30
60
90
120
150
180
time (h)

Figure 4.



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625 Figure 5.

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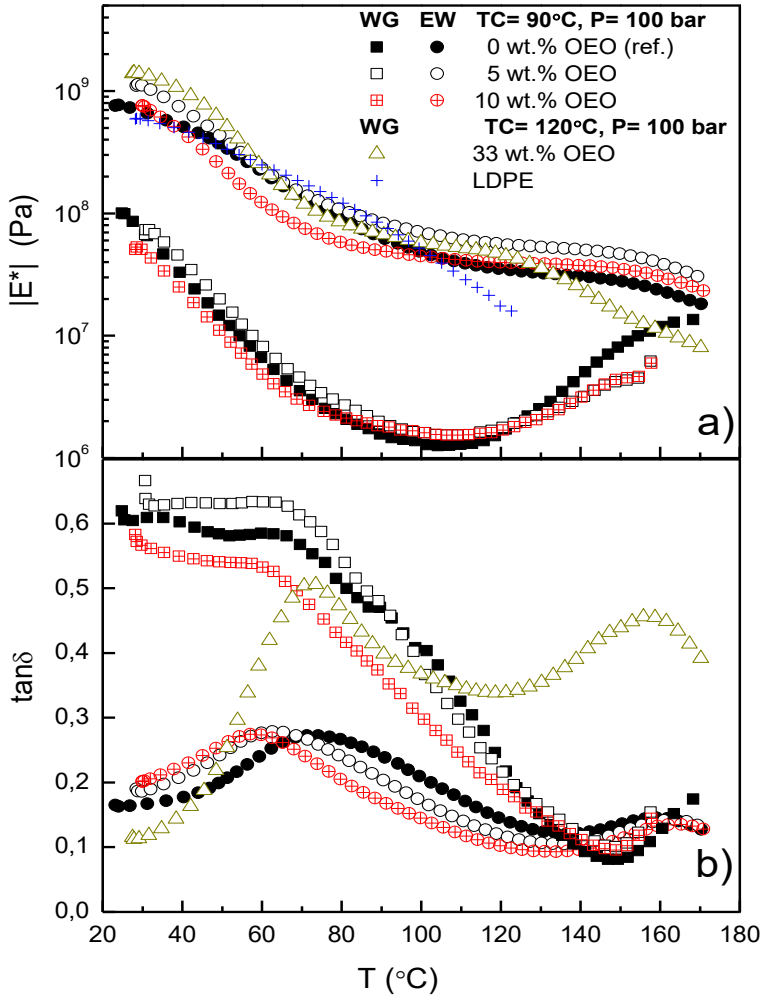

627

628 Figure 6.

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