

Development of antimicrobial active packaging materials based on gluten proteins

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ABSTRACT

BACKGROUND: The incorporation of natural biocides agents into protein-based bioplastics, a source for biodegradable polymeric materials, manufactured by thermo-mechanical method is a way to contribute to sustainable food packaging industry.

RESULTS: This study assesses the antimicrobial activity of ten different biocides incorporated into wheat gluten-based bioplastics. Additionally, the effect that formulation, processing, and further thermal treatments exert on the thermo-mechanical properties, water absorption characteristics and rheological behaviour of these materials is studied.

CONCLUSIONS: Bioplastics containing six of the ten examined bioactive agents have demonstrated suitable antimicrobial activity at 37°C after their incorporation into the bioplastic. Moreover, the essential oils are able to create an antimicrobial atmosphere within Petri dish. Depending on the selected biocide, its addition may alter the bioplastics protein network in a different extent, which leads to materials exhibiting less water uptake and different rheological and thermo-mechanical behaviours.

Keywords: Protein, bioplastic, antimicrobial agents, water absorption viscoelasticity, processing.

INTRODUCTION

Traditional packaging materials derived from petroleum oil are neither readily recyclable nor environmentally sustainable and are associated with a number of health risks, for instance, the migration of harmful additives. Bioplastics are a quite novel alternative to substitute conventional plastics in packaging of products characterized by their short shelf life.¹ Among other biopolymer sources for bioplastics, wheat gluten proteins have been proposed in previous studies because they are renewable, biodegradable and readily available at a reasonable price (around 1.3–1.4 €/kg).^{2,3} Likewise, this type of bioplastics can be obtained through of mechanical method, or thermoplastic processing, which consists of mixing proteins (biopolymer) and plasticizer to obtain a dough-like material.^{4,5}

In this context, extrusion, blowing, injection, and thermo-compression are feasible alternatives due to their energy-efficient combined with their high productivity.⁶⁻⁸

Moreover, biopolymer materials may serve as carriers of antimicrobials and control their release, maintaining high concentrations on food surfaces with a low migration of active substances. The so-called antimicrobial packaging is a promising and rapidly emerging technology in which antimicrobial agents are incorporated into the polymeric package (or coated or adsorbed on the polymer surfaces) to inhibit or retard the proliferation of microorganisms in foods, thus extending the shelf life of the product.⁹⁻¹¹ Regarding biocides, natural antimicrobial compounds are also gaining scientific and technological interest focused on extending the shelf life of foods, reducing or eliminating pathogenic bacteria, and increasing product's overall quality.¹²⁻¹⁴ Several studies on the antimicrobial and antifungal efficiency of essential oils are available in the scientific literature.^{12,15-17} For instance, edible, medicinal and herbal plants and

spices such as oregano, rosemary, thyme, sage, basil, turmeric, ginger, garlic, nutmeg, clove, mace, savory, and fennel, have been successfully used alone or in combination with other preservation methods.¹⁸⁻²¹ The incorporation of essential oils, as well as their individual chemical constituents, into packaging materials has been developed recently, being another way in which essential oils can be used.^{19, 22-24} It is worth noting that such antimicrobial agents could affect food organoleptic properties. Their selection would depend on the type of food to be preserved, trying to reduce their impact on its organoleptic characteristics and consumer's perception.

However, a major limitation for the use of natural biocides in polymer-based packaging arises from their high volatility and thermal susceptibility. During extrusion process, the high pressure and temperature in the extruder barrel can affect the chemical stability of the embedded antimicrobial compounds (which generally are heat-sensitive and thermally unstable) and reduce their efficacy on food-borne spoilage microorganism.²⁵ Interestingly, the low processing temperatures required for the manufacture of protein-based bioplastics may successfully be used in the development of biodegradable antimicrobial food packaging. In this regard, wheat gluten based bioplastics has successfully been employed in the development of antimicrobial active packaging materials due to their ability to retain and release active compound.²⁶

The overall objective of this work was the development, by means of a thermoplastic process, of protein-based bioplastics with suitable antimicrobial and thermo-mechanical characteristics to be used as a packaging material in food or non-food applications. With this aim, this study assessed the antimicrobial activity of 10 different natural biocides incorporated into wheat gluten-based bioplastics. Additionally, the effect that formulation, processing, and further thermal treatments exert on the thermo-mechanical

properties, physico-chemical characteristics and rheological behaviour of these materials was studied.

MATERIALS AND METHODS

Wheat gluten (WG) was provided by RIBA S.A. (Spain) with 830 g kg⁻¹ protein and its moisture content was 80 g kg⁻¹ on dry basis. Glycerol (G), from Panreac Química, S.A. (Spain), was used as protein plasticizer.

Ten antimicrobial agents were incorporated into the bioplastics (eight essential oils and two active agents, carvacrol and cinnamaldehyde, main compounds of the essential oils), which were supplied by Sigma-Aldrich (U.S.A.) (Table 1). All the antimicrobial agents evaluated in this study are listed as generally recognised 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).

Bioplastic compounds were mixed in a Polylab torque-rheometer equipped with a Rheomix 600p kneading tool (Thermo-Haake GmbH, Germany). This device allowed the 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 process 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 t_{mix} was always estimated as 1.5 t_{peak} for the sake of comparison. Subsequently, the resulting dough-like biomaterials were compression-moulded into rectangular specimens (50 mm length, 10 mm width 3 mm thick) by applying, a gauge pressure of 100 bar for 10 min at a selected temperature of 90°C.^{27,28} 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 contained a 670 g kg⁻¹ protein, 230 g kg⁻¹ glycerol and 100 g kg⁻¹ of the different biocides (Table 1). The selected biocide concentration was found as optimal for this type of materials in previous work.²⁹

Additionally, viscosity of the resulting dough-like biomaterial was measured after mixing process in a capillary rheometer (Rheocap S20, Thermo-Haake, Germany). The flow measurements were performed at 90°C, ranging apparent shear rates ranged from 10 to 1000 s⁻¹. Capillary dies (1 mm radius) and tubes with L/D=20 and L/D= 30 ratio were used. For each condition, pressure drop against time was monitored. The apparent wall shear stress was corrected for entrance and exit pressure losses, using Bagley's correction, to obtain the true wall shear stress. Similarly, the apparent wall shear rate was corrected for non-Newtonian melt flow, by using the Rabinowitsch-Weissenberg equation, to calculate the true shear rate at the wall.³⁰ All tests were performed at least twice.

Dynamic Mechanical Thermal Analysis (DMTA) experiments were performed with a Seiko DMS 6100 (Seiko Instruments, Japan), using 50x10x3 mm³ samples in double cantilever (bending) mode, according to the ASTM standard method D5023-01.³¹ Temperature sweep tests from 30 to 170 °C were carried out, within the linear viscoelastic region, at constant frequency (1 Hz) and heating rate of 2°C/min. During analysis, the storage modulus (E'), the loss modulus (E'') and loss tangent, tan (δ), were recorded and plotted versus temperature. The complex modulus can be calculated as, $|E^*|^2 = |E'|^2 + |E''|^2$, measured in tension or flexure. Each sample was analyzed at least twice.

Pure biocides volatility and bioplastics samples were studied by thermogravimetric analysis (TGA), with a Q50 (TA Instruments, USA). Tests were carried out in a range of temperature from 30 to 250 °C for pure biocides and from 30 to 500°C for bioplastics at a heating rate of 10°C/min, in N₂ atmosphere. Experiments were carried out in duplicate. TGA tests conducted on pure biocides (Table 1) and biocide-containing bioplastics showed a biocide loss during processing lower than 5 wt.% for most of the samples, except for bioplastics containing white thyme or red thyme which had a weight loss 16 wt.% and 22 wt.% respectively.

The water absorption (Ab) was calculated as follows ³²:

$$Ab = \frac{(W_1 - W_0 + W_{sol})}{W_0} \times 100 \quad [1]$$

Where W₁, W₀ and W_{Sol} are the weights of the specimen containing water, the dried specimen, and water-soluble residuals, respectively.

The Kirby–Bauer test ³³ was used to determine the antimicrobial activity of the active bioplastics prepared. Square pieces (1×1cm²) of bioplastics containing 100 g kg⁻¹ of different biocides, were set on a solidified agar culture medium inoculated with 100μl of a selected microorganism suspension containing 2.0×10⁸ CFU/ml in sterilized Petri dishes 90 mm in diameter a selected microorganism. The four microorganisms studied, usually involved in food preservation and water pollution control processes, were: “*Aspergillus niger*” (fungus–mould) (wild strain) ATCC 16404, “*Candida albicans*” (fungus–yeast) (wild strain) ATCC 10231, “*Staphylococcus aureus*” (bacteria gram-positive) CECT131 and “*Escherichia coli*” (bacteria gram-negative) CECT434. Subsequently, the plates were incubated at 37°C for 24-48 hours in the cases of *E. coli* & *S. Aureus* and incubated at 22°C for 48-78 hours in the cases of *C. albicans* & *A.*

niger, in the appropriate incubation chamber. 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 active bioplastics tested can be quantitatively calculated by measuring the diameter of the inhibition zone, that is, the clear halo around the sample. The data were presented as mean \pm standard deviation (SD) of three determinations. A probability value of $p < 0.05$ was considered significant.

Alternatively, bioplastic antimicrobial activity was tested by placing the specimen under the lid of the Petri dish, so that the material was 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.

RESULTS/DISCUSSION

Thermoplastic processing

The manufacture of bioplastics requires complete mixing of all the components during the thermoplastic processing in order to obtain a material with suitable mechanical properties. Ten antimicrobial agents were tested as additives to wheat gluten-based bioplastic. However, for sake of clarity, mixing profiles of four protein-plasticizer-biocide blends (containing red thyme, clove, cinnamon essential oil and cinnamaldehyde) were selected as examples to describe the evolution of torque and temperature during mixing process (Figure 1). Biocide addition was found to decrease the mixing torque (below 25 N·m) with respect to the reference bioplastic (40 N·m) and the evolution of torque exhibited different regions depending on the antimicrobial agent. Thus, mixing torque curve for the reference bioplastic presented three regions, during the first 10 minutes, a non-significant increase in torque can be seen. Then, a significant

increase in torque up to a maximum value about 40 N·m was observed in the second region. Finally, a torque decay was obtained. Temperature evolution also displayed different zones during the mixing process, which were related to the torque regions (Figure 1B). The sample containing red thyme, clove and cinnamon produced three regions in both mixing torque and temperature curves. However, clove and cinnamon addition shortened the time interval previous to the torque exponential and red thyme addition showed a significant decrease in torque values at the maximum (15 N·m). Temperature evolution during processing for blends containing cinnamon and red thyme tended to constant values after 20 min, although cinnamon reached higher values (up to 68°C). An exponential increase in temperature curve up to 70°C for bioplastic containing clove is observed, this temperature was slightly higher than the one reached for the reference bioplastic. On the other hand, the sample containing cinnamaldehyde produced only two regions, cinnamaldehyde addition led to an instantaneous increase in the torque (up to 20 N·m) followed by a continuous decay whilst temperature increases up to 85°C. Balaguer et al.,^{35,36} reported that cinnamaldehyde promoted the formation of intermolecular covalent bonds between polypeptide chains and therefore reticulates the protein matrix. Consequently, this interaction might produce an exothermic reaction during the mixing process of this material.

The maximum temperature reached at the end of the mixing process, T_{\max} , fell between 40-60°C for most of the blends (Table 1). Therefore, no loss of biocides by evaporation was expected during the mixing stage. These results show that, only blends containing carvacrol, cinnamaldehyde and clove (88, 85 and 75°C respectively) reached a temperature higher than the reference bioplastic (72°C) during the mixing process. This temperature is enough to cause denaturation of the wheat gluten.³⁶

Antimicrobial agents seem to significantly affect torque and temperature behaviour and, therefore, the specific mechanical energy (SME). It is evident that the effect of an essential oil depends on their capacity to interact with the protein. Table 1 gathers the mixing process parameters for formulations of bioplastic containing 10 different biocides. The SME values for the bioplastic samples studied were calculated as follows³⁷:

$$SME = \frac{\Omega}{m} \int_0^{t_{mix}} M(t) \partial(t) \quad [2]$$

where ω (in rad/s) is the mixing speed, m (in g) the sample mass, $M(t)$ (in Nm) the torque and t_{mix} (in s) the mixing time.

As can be seen in Table 1, biocide addition led to a decrease in SME for all blends. This fact was more evident for the sample containing rosemary and lemon which exhibited the lowest SME values, followed by carvacrol and cinnamaldehyde. These samples also reached maximum torque within a time, t_{max} , lower than 6.5 min. As this suggests, the samples in the latter group undergone a rapid increase in torque until they reached the maximum, and as a result, they failed to produce a first region during the torque evolution, unlike the rest of the samples, which all did. Sample containing cinnamon exhibited nearly SME values to the reference sample, followed by clove and white thyme. These samples also reached maximum torque within a time higher 20 min.

Finally, once the mixing stage was finished, the resultant dough-like biomaterials were compression-moulded into rectangular specimens at 100 bar and 90°C for 10 min.

Antimicrobial activity

The effectiveness in killing microorganisms was tested by two ways: first, by placing the bioplastic on the substrate, so that the microbial inhibition was assessed in contact with the culture medium; and second, by placing the bioplastic on the inside of the lid of the Petri dish, separated from the culture medium. The latter may be useful in non-contact food applications. As a result, the microbial inhibition was assessed in contact with the culture medium (by biocide release and diffusion) and under non-contact conditions developing an antimicrobial atmosphere (by biocide release and evaporation). Microbiological tests confirmed that cinnamon, clove and red thyme inhibited the growth of two selected bacteria (*E. coli* and *S. aureus*) under both contact and non-contact conditions (Figure 2). Similarly, different studies have reported the effectiveness of clove and cinnamon oils against both Gram-positive and Gram-negative groups.^{19,38-40} Furthermore, Figure 3 presents the antimicrobial activity under contact conditions of biocide-free bioplastics and of those with added cinnamaldehyde. As may be seen, a wide and well-defined inhibition zone around bioplastics containing biocide is clearly noticed. If compared to previous biocide-containing bioplastics, those formulated with cinnamaldehyde exhibited the strongest antimicrobial effects against the four microorganisms evaluated (*E. coli*, *S. aureus*, *A. niger*, *C. albicans*). Cinnamaldehyde has been reported as an antimicrobial agent that exerts antimicrobial activity against a wide range of microorganism including bacteria, yeasts, and fungi.^{14,}

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Table 2 presents the inhibition diameter (d) for bioactive bioplastics containing 10 different biocides in their formulation. The antimicrobial effectiveness of those bioactive agents was evaluated by measuring the diameter of the inhibition area, that is, the area clear or not colonized around the sample. As aforementioned, the blend containing cinnamaldehyde exhibited the greatest inhibition against all the studied

microorganisms. Bioplastics containing cinnamon, clove, white thyme and carvacrol also have demonstrated antifungal and antimicrobial activity, although they seem to exert a stronger antimicrobial effect against to *A. niger* than any other microorganisms (Table 2). Phenolic compounds of biocides, which contain a high percentage of eugenol, carvacrol and/or thymol, are primarily know because of their activity against bacteria,^{46,47} fungi^{46,48} and yeasts.^{46,49} Even though red and white thyme contain the same active compounds (thymol/carvacrol), in this study, it was white thyme which exerted higher ability to inhibit the growth of the selected microorganisms. Sample containing peppermint only presented low effectiveness against *A. niger*. Finally, all microorganisms evaluated were resistant when bergamot lemon and rosemary were employed as biocide, no inhibition area was observed.

Thermomechanical properties and viscosity

Biocide addition into a protein-based bioplastic is expected to produce a very broad range of thermomechanical properties. According to the results presented here, bioplastic containing cinnamon, clove, red thyme, carvacrol, cinnamaldehyde and white thyme showed optimal antimicrobial effectiveness against all the microorganisms evaluated and were therefore chosen for further studies of their thermomechanical properties, viscosity and water uptake behaviour.

Figure 4 shows the results of DMTA tests in double cantilever mode conducted, over a temperature interval from 30 to 170°C, on bioplastics specimens containing an antimicrobial agent and moulded at 100 bar and 90°C. The evolution of the viscoelastic functions for a WG/G sample²⁸ showed a remaining thermosetting potential above 110°C, deducible from the apparent increase in E^* with temperature (Fig 4A) and from

the minimum in loss tangent at ca. 150°C (Figure 4B). This behaviour may be related to an incomplete protein denaturation after the material processing.³⁶ Compared to the reference sample, biocides addition did not produce any noticeable change in the thermomechanical response below 80°C, only a slightly increase in E^* for samples containing carvacrol and cinnamaldehyde and a plasticizing effect (with slightly lower values of E^*) if clove essential oil is added. However, a decrease in loss tangent ($\tan \delta$) values was observed by the addition of biocides, leading to more elastic materials. Above that temperature, samples show different behaviour depending on the biocide. Thus, an increase E^* values in a temperature range between 95 and 120°C for samples containing clove, cinnamon, red and white thyme was found. This increase was more evident in the sample containing cinnamon. On the other hand, E^* showed a broad plateau in temperature interval that extends from 110°C in the case of sample containing cinnaldehyde and 100°C for sample containing carvacrol up to the end of the measurement. Thereby, carvacrol and cinnamaldehyde seemed to exert a clear influence on broadening the temperature interval over which the rubbery-like plateau extends and consequently, on decreasing the bioplastic temperature susceptibility. Furthermore, a significant increase in the $\tan \delta$ peak was observed for the sample with cinnamaldehyde (~60°C) and (~45°C) for the sample with carvacrol. Both compounds seemed to act such as a crossliker of this protein-based bioplastic.^{34,35}

Figure 5 shows viscous flow curves at 90°C for wheat gluten/glycerol samples containing cinnamon, clove white and red thyme, collected just after the mixing process. The flow behaviour for blends containing cinnamaldehyde and carvacrol was not characterized due to both samples did not flow through of the capillary.

If compared to the biocide-free bioplastic, samples containing cinnamon, red thyme and white thyme showed similar viscosity to the reference sample in a wide range of shear rates. On the other hand, clove led to a significant increase in the sample viscosity. In all cases, the viscosity of these protein-based materials was higher than the well-known low density polyethylene (LDPE) at 160°C.

As seen in Figure 5, a shear-thinning behaviour was observed for all samples, which was fitted to the power-law model:

$$\eta = k\dot{\gamma}^{n-1} \quad [2]$$

The consistency (k) and the flow index (n) obtained from a power law fittings are shown in Table 3. As can be observed, biocides addition led to an increase in the consistency index and a decrease in the flow index for samples. These results point out biocide addition increases the shear-thinning character (and the development of the protein network) of the dough-like material obtained just after mixing. Moreover, the observed changes in rheological behaviour might affect subsequent processing operations at which these bioactive materials could be subjected, e.g. thermomoulding, injection moulding etc.

Water absorption properties

Previous studies have pointed out that both processing conditions and bioplastic formulation may lead to materials with a wide range of mechanical responses and underlying microstructures.^{28,32,49} As a result, different swelling capabilities should be expected. Table 3 gathers the water absorption values after 24 h for wheat gluten/glycerol blends containing six different antimicrobial agents. It can be observed that biocide addition led to a lower water uptake values as compared to the reference blend (980 g kg⁻¹). The best results were found for samples containing cinnamaldehyde

and carvacrol (560 and 540 g kg⁻¹ respectively) which agree with the previously thermomechanical behaviour observed (Fig 4). Cinnamaldehyde consists of a phenyl group attached to an unsaturated aldehyde which can act as a crosslinking agent for proteins^{34,35} and therefore it might be responsible for the significant reduction in water absorption. On the other hand, carvacrol is a phenolic compound which has highly hydrophobic nature, and has proven to reduce water uptake because of its chemical structure.^{50,51} The results obtained for samples containing white thyme, cinnamon, clove and red thyme exhibited water uptake values between 740 to 800 g kg⁻¹, being white thyme the lowest and red thyme the highest values of this group of samples, even though both of them contain thymol/carvacrol. Furthermore, no difference was found in the water uptake for samples containing cinnamon and clove (740 g kg⁻¹). In the case of cinnamon this reduction in the water uptake is due to cinnamaldehyde which is its major compound.

In the present study, protein-based bioplastics with very broad rheological characteristics can be obtained as result of the selected biocide and material processing. Bioplastics containing six of the ten examined bioactive agents demonstrated suitable antimicrobial activity after incorporation into the bioplastic, the activity depending on the actual biocide. Moreover, the essential oils were able to create an antimicrobial atmosphere within a packaging. More precisely, bioplastics formulated with cinnamon, clove, carvacrol, Cinnamaldehyde and white thyme demonstrated suitable activity on all microbial agent tested. Among these biocides, the addition of cinnamon, clove or white thyme seemed to less affect the protein network and, therefore, the processability and mechanical properties of the bioactive bioplastic. Additionally, such biocides lead to a significant reduction in material water absorption.

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REFERENCES

1. Guilbert, S.; Gontard, N. Agro-polymers for edible and biodegradable films: review of agricultural polymeric materials, physical and mechanical characteristics. In *Innovations in Food Packaging*; Han, J. H., Ed.; Academic Press: New York. 2005
2. Zhong, N.; Yuan, Q. Preparation and properties of molded blends of wheat gluten and cationic water-borne polyurethanes. *Journal of Applied Polymer Science*. 2013, 128, 460-469.
3. Domenek, S.; Feuilleley, P.; Gratraud, J.; Morel, M.H.; Guilbert, S. Biodegradability of wheat gluten based bioplastics. *Chemosphere*. 2004, 54, 551-559.
4. Attenburrow, G.; Barnes, D.J.; Davies, A.P.; Ingman, S.J. Rheological properties of wheat gluten. *Journal Cereal Science*. 1990 12, 1-14.
5. Kokini, J.L.; Cocero, A.M.; Madeka, H.; De Graaf, E. The development of state diagrams for cereal proteins. *Trends in Food Science & Technology*. 1994, 5, 281-288.
6. Pellissari, F.; Yamashita, F.; García, M.A.; Martino, M.; Zaritzky, N.; Grossmann, M.V. Constrained mixture design applied to the development of cassava starch–chitosan blown films. *Journal of Food Engineering*. 2012, 108, 262–267
7. Flores, S.K.; Costa, D.; Yamashita, F.; Gerschenson, L.N.; Grossmann, M.V. Mixture design for evaluation of potassium sorbate and xanthan gum effect on properties of tapioca starch films obtained by extrusion. *Materials Science and Engineering: C*. 2010, 30, 196–202.
8. Thunwall, M.; Boldizar, A.; Rigdhal, M. Compression molding and tensile properties of thermoplastic potato starch materials. *Biomacromolecules*. 2006, 7, 981–986.
9. Han, J.H. Antimicrobial packaging systems. In *JungH. Han(Ed), Innovations in food packaging*. Amsterdam: Elsevier Academic Press. 2005, pp.81 – 107.
10. Suppakul, P.; Miltz, J.; Sonneveld, K.; Bigger, S.W. Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications. *Journal of Food Science*. 2003, 68, 408-420.
11. Appendini, P.; Hochkiss. Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*. 2002, 3, 113-126.
12. Tajkarimi, M.M.; Ibrahim, S.A.; Cliver, D.O. Antimicrobial herb and spice compounds in food. *Food Control*. 2010, 21, 1199-1218.
13. Del Nobile, M.A.; Lucera, A.; Costa, C.; Conte, A. Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*. 2012, 3, 287. <http://dx.doi.org/10.3389/fmicb.2012.00287>.
14. Burt, S.A. Essential oils: their antibacterial properties and potential applications in foods: a review. *International Journal of Food Microbiology*. 2004, 36, 162-167.
15. Burt, S.A.; Reinders, R.D. Antimicrobial activity of selected plant essential oils *Escherichia coli* O157:H7. *Letter in Applied Microbiology*. 2003, 36, 162-167.
16. Delaquis, P.J.; Stanich, K.; Girard, B.; Mazza, G. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*. 2002, 74, 101-109.

17. Mejholm, O.; Dalgaard, O. Antimicrobial effects of essential oils on the seafood spoilage microorganism *Photobacterium phosphoreum* in liquid media and fish products. *Letter in Applied Microbiology*. 2002, 67, 199-202.
18. Du, H.; Li, H. Antioxidant effect of cassia essential oil on deep-fried beef during the frying process. *Meat Science*. 2008, 78, 461-468.
19. Gutierrez, J.; Barry-Ryan, C.; Bourke, P. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*. 2008, 124, 91-97.
20. Burt, S. A., Der Zee, R. V., Koets, A. P., De Graaff, A. M., Van Knapen, F., Gaastra, W., et al. Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*. 2007, 73, 4484-4490.
21. Holley, R.A.; Patel, D. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology*. 2005, 22, 273-292.
22. Abou-taleb, M.; Kawai, Y. Shelf life of semi fried tuna slices coated with essential oil compounds after treatment with anodic electrolyzed NaCl solution. *Journal of Food Protection*. 2008, 71, 770-774.
23. Fisher, K.; Phillips, C. Potential antimicrobial uses of essential oils in food: Is citrus the answer? *Trends in Food Science and Technology*. 2008, 19, 156-164.
24. Gaysinsky, S.; Weiss, J. Aromatic and spice plants: Uses in food safety. *Stewart Post Harvest Review*. 2007, 4, 1-9.
25. Del Nobile, M.A.; Conte, A.; Buonocore, G.G.; Incoronato, A.L.; Massaro, A.; Panza, O. Active packaging by extrusion processing of recyclable and biodegradable polymers. *Journal of Food Engineering*. 2009, 93 1–6.
26. Gómez-Martínez, D.; Partal, P.; Martínez, I.; Gallegos, C. Gluten-based bioplastics with modified controlled-release and hydrophilic properties. *Industrial Crops and Products*. 2013, 43, 704-710.
27. Jerez, A.; Partal, P.; Martínez, I.; Gallegos C.; Guerrero, A. Bioplástico y métodos para su preparación. *Oficina Española de Patentes y Marcas*. Patent, P200501556. 2005.
28. Jerez, A.; Partal, P.; Martínez, I.; Gallegos C.; Guerrero, A. Rheology and processing of gluten based bioplastics. *Biochemical Engineering Journal*. 2005, 26, 131-138.
29. Martinez, I.; Partal, P.; García-Morales, M.; Guerrero, A.; Gallegos, C. Development of protein-based bioplastics with antimicrobial activity by thermo-mechanical processing. *Journal of Food Engineering*. 2013, 117, 247–254.
30. Keith, S. *Mechanics* (Third ed.). Addison-Wesley. ISBN 0-201-07392-7. 1971.
31. Standard test method for Plastics: Dynamic Mechanical Properties: In Flexure (Three-Point Bending). Designation D 5023-01. In *Annual book of ASTM standards* Philadelphia, PA: American Society for Testing and Materials. 2001.
32. Gómez-Martínez, D.; Partal, P.; Martinez, I.; Gallegos, C. Rheological behaviour and physical properties of controlled-release gluten-based bioplastics. *Bioresource Technology*. 2009, 48, 1828-1832
33. Boyle, V.J.; Fancher, M.E.R.; Ross, Jr. W. Rapid, modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial disks. *Antimicrob. Agents Chemother*. 1973, 3, 418-424.
34. Balaguer, M.P.; Gomez-Estaca, J.; Gavara, R.; Hernandez-Munoz, P. Functional properties of bioplastics made from wheat gliadins modified with

- cinnamaldehyde. *Journal of Agricultural and Food Chemistry*. 2011, 59, 6689-6695.
35. Balaguer, M.P.; Gomez-Estaca, J.; Gavara, R.; Hernandez-Munoz, P. Biochemical properties of bioplastics made from wheat gliadins cross-linked with cinnamaldehyde. *Journal of Agricultural and Food Chemistry*. 2011, 59, 13212-13220.
 36. Jerez, A.; Partal, P.; Martínez, I.; Gallego, C.; Guerrero, A. Egg white-based bioplastics developed by thermomechanical processing. *Journal of Food Engineering*. 2007, 82, 608- 617.
 37. Redl, A.; Morel, M.H.; Bonicel, J.; Vergnes B.; Guilbert S. Rheological properties of gluten plasticized with glycerol: dependence on temperature, glycerol content and mixing conditions. *Rheologica Acta*. 1999, 38, 311-320.
 38. El-Seedi, H.R.; Khatlab, A.; Gaara, A.H.M.; Mohamed, T.K.; Hassan, N.A.; Elkattan, A.E. Essential oil analysis of *Micromeria nubigena* H.B.K. and its antimicrobial activity. *Journal of Essential Oil Research*. 2008, 20, 452-456.
 39. Kim, T.J.; Weng, W.L.; Stojanovic, J.; Lu, Y.; Jung, Y.S.; Silva, J.L. Antimicrobial effect of water-soluble muscadine seed extracts on *Escherichia coli* O157:H7. *Journal of Food Protection*. 2008, 71, 1465-1468.
 40. Lopes-Lutz, D.; Alviano, D.S.; Alviano, C.S.; Kolodziejczyk, P.P. Screening of chemical composition, antimicrobial and antioxidant activities of *Artemisia* essential oils. *Phytochemistry*. 2008, 69, 1732-1738.
 41. Balaguer, M.P.; Borne, M.; Chalier, P.; Gontard, N.; Morel, M.-H.; Peyron, S.; Gavara, R., Hernández-Muñoz, P. Retention and release of cinnamaldehyde from wheat protein matrices. *Biomacromolecules*. 2013, 14, 1493-1502.
 42. Xing, Y.; Li, X.; Xu, Q.; Yun, J.; Lu, Y. Antifungal activities of cinnamon oil against *Rhizopus nigricans*, *Aspergillus flavus* and *Penicillium expansum* in vitro and in vivo fruit test. *International Journal of Food Science & Technology*. 2010, 45, 1837-1842.
 43. Gutierrez, L.; Escudero, A.; Batlle, R.; Nerin, C. Effect of mixed antimicrobial agents and flavors in active packaging films. *Journal of Agricultural and Food Chemistry*. 2009, 57, 8564-8571.
 44. Lopez, P.; Sanchez, C.; Batlle, R.; Nerin, C. Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. *Journal of Agricultural and Food Chemistry*. 2007, 55, 4348-4356.
 45. Guarda, A.; Rubilar, J.F.; Miltz, J.; Galotto, M.J. The antimicrobial activity of microencapsulated thymol and carvacrol. *International Journal of Food Microbiology*. 2011, 146, 144-150.
 46. Periago, P.M.; Zuijlen, A.; Fernandez, P.S.; Klapwijk, P.M.; Steeg, P.F.; Corradini, M.G.; Peleg, M. Estimation of the non-isothermal inactivation patterns of *Bacillus sporothermodurans* IC4 spores in soups from their isothermal survival data. *International Journal of Food Microbiology*. 2004, 95, 205-218.
 47. Daferera, D.J.; Ziogas, B.N.; Polissiou, M.G. The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. *Crop Protection*. 2003, 22, 39-44.
 48. Arora, D.S.; Kaur, J. Antimicrobial activity of spices. *International Journal of Antimicrobial Agent*. 1999, 12, 257-262.
 49. Rouilly, A.; Orliaz, O.; Silvestre, F.; Rigal, L. New natural injection-moldable material from sunflower oil cake. *Bioresource Technology*. 2006, 97, 553-561.

50. Rubilar, J.F.; Cruz, R.M.S.; Silva, H.D.; Vicente, A.A.; Khmelinskii, I.; Vieira, M.C. Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. *Journal of Food Engineering*. 2013, 115, 466-474
51. Du, W.X.; Avena-Bustillos, R.J.; Hua, S.S.T.; McHugh, T.H. Antimicrobial volatile essential oils in edible films for food safety. In: Mendez-Vilas, A. (Ed.), *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances*. Formatex Research Center, Badajoz, Spain. 2011, pp. 1124–1134

Table 1. Processing parameters and water absorption of the protein-based bioplastics with antimicrobial agents and volatility of the pure biocides

Glycerol (g kg ⁻¹)	Antimicrobial agent (10 g kg ⁻¹)	Mixing process parameters			Water absorption (g kg ⁻¹)	volatility of the pure biocides at 100°C (g kg ⁻¹)
		T _{max} (°C)	t _{max} (min)	SME (KJ/Kg)		
33	none	75	21.9	2647	98.50±1.39	-
23	Red thyme	51	26.0	2027	80.00±1.13	28.05±2.22
23	Cinnamon	60	20.5	2539	75.25±1.06	6.35±0.62
23	Clove	72	17.4	2439	75.40±1.56	3.20±0.03
23	Peppermint	52	15.4	1257	-	16.15±0.01
23	Bergamot	45	14.8	1162	-	30.50±3.19
23	Rosemary	47	6.3	376	-	52.26±0.66
23	White thyme	53	22.9	1849	74.30±1.04	23.05±0.45
23	Lemon	40	6.5	385	-	49.25±3.78
23	Carvacrol	88	3.6	557	55.60±0.71	4.50±0.13
23	Cinnamaldehyde	85	6.0	727	56.35±0.79	3.35±0.03

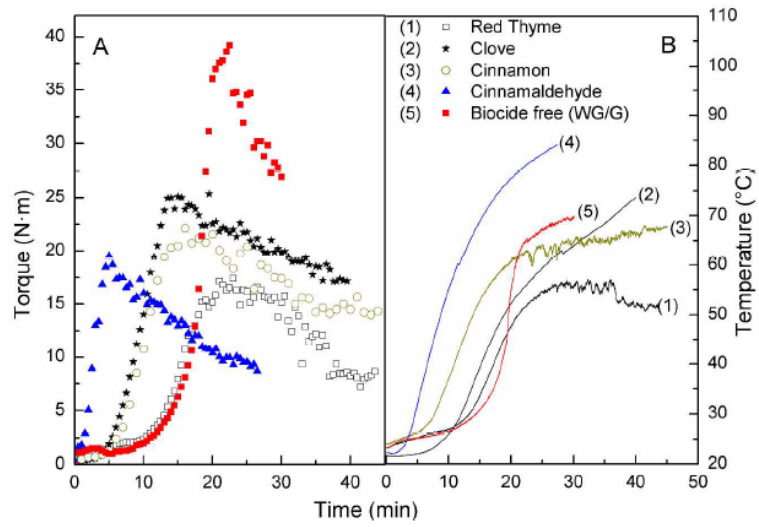
Table 2. Antimicrobial test results of bioplastic specimens.

Antimicrobial agent (10 g kg ⁻¹)	<i>E. coli</i> (Gran - bacteria)	<i>S. aureus</i> (Gran + bacteria)	<i>C. albicans</i> (Fungus)	<i>A. niger</i> (Fungus)
Bergamot	-	-	-	-
Cinnamon	++	++	++	+++
Clove	++	++	++	+++
Peppermint	-	-	-	+
Red thyme	+	+	+	+
Carvacrol	++	++	++	+++
Cinnamaldehyde	+++	+++	+++	+++
Lemon	-	-	-	-
Rosemary	-	-	-	-
White thyme	++	++	++	+++

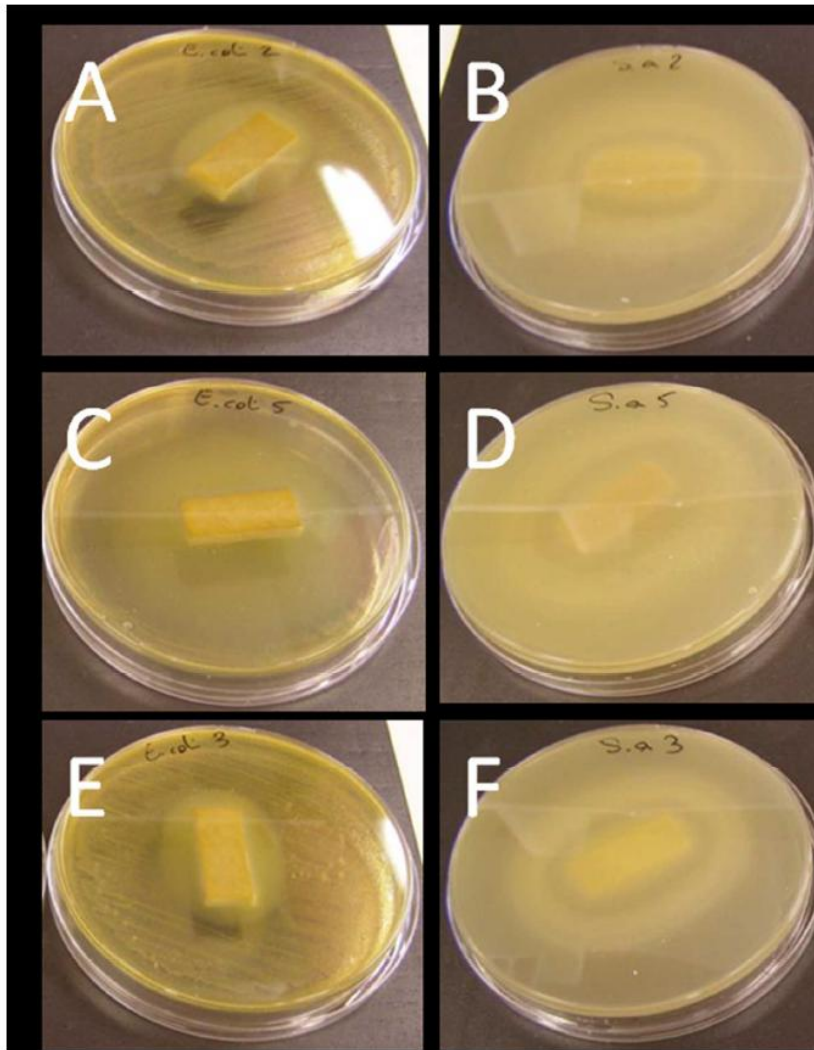
Inhibition diameter (d): +++ (70 mm < d), ++ (70 mm < d > 20 mm), + (d < 20 mm), Without activity

Table 3. Viscosity parameters of wheat gluten-based bioplastic containing different antimicrobial agents.

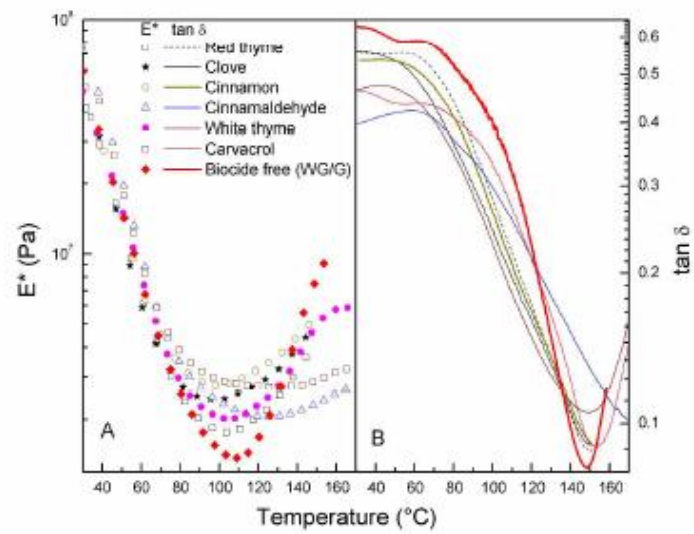
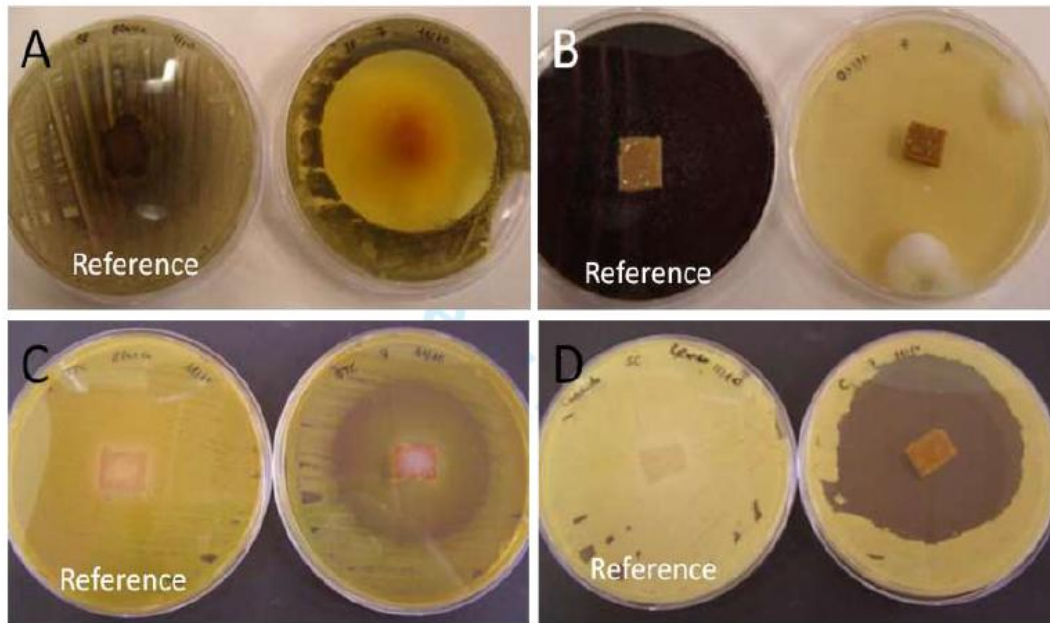
Glycerol (g kg ⁻¹)	Antimicrobial agent (10 g kg ⁻¹)	Parameters of Power-law model	
		<i>k</i> (Pa·s ^{<i>n</i>})	<i>n</i>
LDPE	Biocide free	7846	0.47
33	Biocide free	20361	0.36
23	Cinnamon	27134	0.30
23	Clove	30617	0.32
23	Red thyme	106864	0.11
23	White thyme	50691	0.21
23	Carvacrol	-	-
23	Cinnamaldehyde	-	-



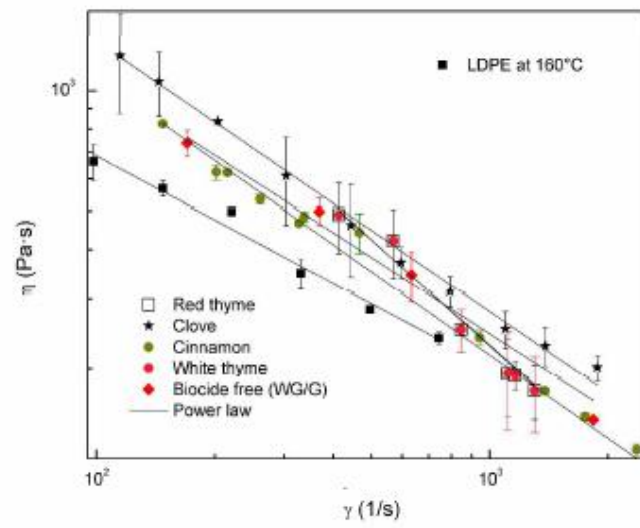
Evolution of torque and temperature during the mixing process for blends containing 10 wt.% clove and red thyme, 23 wt.% glycerol and 67 wt.% wheat gluten.
287x200mm (300 x 300 DPI)



Microbiological test pictures: Wheat gluten/glycerol containing 10 wt.%: cinnamon on A) *E.coli*; B) *S. aureus*; clove on C) *E.coli*, D) *S. aureus*; red thyme on red on E) *E.coli*, F) *S. aureus* (A, C, E are in contact and B, D, F are not in contact with the culture medium).
98x126mm (150 x 150 DPI)



Dynamic mechanical thermal analysis results, complex modulus (E^*) A) and $\tan \delta$ B) of specimens containing cinnamon, clove, carvacrol, cinnamaldehyde, white and red thyme.
287x201mm (300 x 300 DPI)



Viscous flow behaviour at 90°C, for blends containing cinnamon, clove, carvacrol, cinnamaldehyde, white and red thyme.
287x201mm (300 x 300 DPI)