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Inhibitors of thermally induced burn incidents – characterization by microbiological procedure, electrophoresis, SEM, DSC and IR spectroscopy

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Calorimetric (DSC) and Thermogravimetric (TGA) investigations, Acetate Electrophoresis (CAE), Fourier-transform infrared spectrometry (FTIR), Scanning Electron Microscopy analysis (SEM) and Microbiological procedure, were all carried out after heating the samples to a temperature simulating a burn incident. In particular, the purpose of the present study was analysed the effect of antioxidants: fucoidan from brown seaweed and flame-retardant cyclic organophosphates and phosphonates on organic chicken skin changed by a burn incident. DSC was considered a useful tool in assessing temperature-mediated cross-linking *in vitro*, there is an innovative analytical conclusion obtained from the studies described on the paper. FTIR tests revealed that heating a dry organic chicken skin to boiling point leads to the disappearance of a wide band in the 1650–1550cm^{-1} area or the conversion of a band, which may be attributed to the intermolecular β-sheet aggregates. Fucoidan from brown seaweed and flame-retardant cyclic organophosphates and phosphonates probably bind with the collagen changed by a burn (and also the influence of antioxidant solutions on samples a blank-not boiled organic chicken skin) incident forming a polymer film with collagen of chicken skin surface (SEM analysis), decrease the process of aggregation and recovery of native collagen. Good bacteriostatic properties were determined for samples of fucoidan from brown seaweed and flame-retardant cyclic organophosphates and phosphonates against the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. So, it was observed that fucoidan incorporated into collagen films can be used as a therapeutically active biomaterial that speeds up the process of wound healing.

Introduction

Heat-shock proteins (HSPs) are among new proteins induced in cells under stress conditions. They are also called "stress proteins" and belong to a group of molecules that play a fundamental role in maintaining cellular integrity. HSP families include both constitutive and stress-inducible members whose primary function is to interact with native and denatured proteins to prevent the aggregation of aberrantly folded proteins, facilitate the folding of native proteins, facilitate the refolding of denatured proteins, and to aid intracellular protein trafficking[1]. Although these functions underscore the importance of intracellular HSPs in the maintenance of cellular homeostasis and in promoting cell survival in response to stressful cellular conditions, accumulating evidence suggests that HSPs are actively secreted and have important extracellular functions.

Hypoxia is a microenvironmental stress in wounded skin, where it supports wound healing by promoting cell motility[2]. The mechanism of the hypoxia action remained speculative. Hypoxia plays a critical role in the pathophysiology of a variety of human disorders, such as ischemic cardiovascular disease, stroke, chronic lung disease, acute skin wounds and cancer[2]. After acute injury, the microenvironment of a skin wound is hypoxic, likely due to vascular disruption and high oxygen consumption by cells at the edge of the wound and in granulation tissue[3]. This hypoxic environment induces increased synthesis and secretion of growth factors and HSPs. Although the mechanisms were unclear, these observations suggest that acute hypoxia plays a positive role in early skin wound healing[4]. HSPs are also actively secreted by the cells and have important extracellular functions, such as activation of the immune system and anticancer action[5-6].

Protein glycation is a non-enzymatic reaction between amino groups of proteins and reducing sugars. Glycation is commonly known as the Maillard reaction[7]. In this reaction, the carbonyl group of a sugar interacts with the nucleophilic amino group of the amino acid, producing N-substituted glycosylamine (Schiff base) which is labile and may undergo two sequential rearrangements, yielding a reasonably stable aminoketose – the Amadori product. The Amadori products could subsequently cyclise, forming pyranose or furanose carbohydrate adducts. Further modifications in these early-stage glycation products, such as rearrangement, oxidation, polymerization and cleavage, give rise to irreversible conjugates, called advanced glycation end products (AGEs). These AGE modified proteins form cross-links, which result in aggregation and insolubility[8]. The relation between the conformational properties of albumin and intermolecular interactions under effect of temperature has been the object of several biophysical studies[9-10]. Protein glycation can be observed both *in vivo* and *in vitro*. A

good deal of data is available on glycation of collagen[11-12]. *In vivo*, these two influences (glucose concentration and time) translate to degree and duration of hyperglycemia, and result in the slow and irreversible process of glycation during the life of the protein[13]. *In vitro* glycation of serum albumin[14] and collagen has also been investigated under experimental conditions using different species and concentrations of carbohydrates in variable environments (ionic strength, temperature, duration of exposure). Modifying collagen with polysaccharide solutions was meant to obtain not only an antibacterial dressing, but one that would also reveal antitumor activity. Fucoidan and especially, partially hydrolysed fucoidan isolated from different species have been extensively studied on account of their varied biological properties, including antioxidant, anticoagulant and antitumor effects. This study continues earlier analyses of structural changes in fucoidan from *F. vesiculosus* L., examined by means of cellulose acetate membrane electrophoresis and Fourier transform infrared spectroscopy spectroscopy[15].

Initial experiments were aimed at determining whether heat shock at physiological/pathophysiological temperatures stimulates the release of HSPs. The objective of the present study was to monitor the effect of temperature on collagen from organic chicken skins, both pure and modified by antioxidants and glucose. The analyses will be carried out after heating the samples to a temperature simulating a burn incident.

Experimental

Abbreviations: pure chicken skin (PS); chicken skin heated to boiling point for 60 seconds (HS); chicken skin in presence of glucose (GS); chicken skin heated to boiling point for 60 seconds in presence of glucose (GHS); chicken skin in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); chicken skin heated to boiling point for 60 seconds and incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); chicken skin in presence of fucoidan from brown seaweed (FBS); chicken skin heated to boiling point for 60 seconds and incubated in presence of fucoidan from brown seaweed (FBS); human serum albumin (HSA); Differential Scanning Calorimetry (DSC); Termogravimetric Analysis (TGA); Acetate Electrophoresis (CAE), Fourier-transform infrared spectrometry (FTIR); Scanning Electron Microscopy analysis (SEM); cellulose acetate systems (CA-SYS-MINI).

Chemicals and materials

The following materials and reagents were used in the examination: reference crude fucoidan from *Fucus vesiculosus* L. (CAS Number 9072–19–9, Sigma-Aldrich, Poland); brown seaweed (non-commercial samples); flame-retardant cyclic organophosphates and phosphonates (commercial samples, Clarian, Switzerland).

Preparation of antioxidant solutions

The dried algae from brown seaweed and flame-retardant phosphonates (0.5 g) were extracted with 15 mL of barbital buffer and maintained at 60°C for an hour with constant mechanical stirring. The extract was collected. All the reagents used were of analytical grade.

Preparation of organic chicken skin samples

The samples of organic chicken skin were collected, washed thoroughly and stored at deep freezer until used. First, they were washed twice in 10 wt.% of NaCl solutions to remove unnecessary proteins on the surface by stirring the solution for 48 h. Then, chicken skins were extracted with 0.4 M HCl for 30 min. All the preparative procedures were performed at 4°C. Chicken skins were extracted with 0.5 M acetic acid for two days, and the extracts were centrifuged for 30 min. Finally, the samples of organic chicken skin were collected, washed thoroughly and stored at deep freezer until used.

DSC and TGA analysis

Differential Scanning Calorimetry (DSC) investigations were carried out with a TA Instruments equipped with a MDSC Calorimeter 2920 and RCS cooling system. The samples of chicken skin were heated at a rate of 10°/min from –50°C to 290°C (atmosphere N_2, flow 40 cm^3/min; standard aluminium pans). The weights of the examined samples amounted to about 5 mg. Registration sensitivity was above 0.2 μW. The enthalpies and characteristic temperatures of transitions were calculated by means of Universal V2.6D TA Instruments software.

Termogravimetric Analysis (TGA) investigations were performed using Thermogravimetric Analyzer TA Instruments Q500. Measurements were done in a temperature range from 30°C

to 800°C with the heating rate of 10°/min in nitrogen atmosphere (flow 40 ml/min). At a temperature of 800°C, the inert gas was switched to the air (3 min) for burning the organic remains of the sample.

Agar dilution method

The samples of organic chicken skin were examined. They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive *S. aureus* and Gram-negative *E. coli*. Physiological salt (2 cm^3) was poured into two sterile test tubes. Using a sterile (red hot) inoculation loop, *E. coli* sample was taken from its culture on enriched agar (used for growing particularly demanding bacteria strains), inserted into one of the test tubes and diluted in the salt. Using a pipette, 3 drops of the suspension were transferred onto enriched agar; then, using a cooled sterile bacteria spreader, they were spread all over the agar surface. After that, the spreader was sterilized again and a part of skin was placed in the centre of the Petri plate. The same procedure was repeated for *S. aureus*, which was placed on mannitol salt agar (containing 7.5% NaCl for inhibiting the growth of other bacteria). The Petri plates were subsequently placed in a tube and then kept in a laboratory heater at 37°C for 24 and 48 h. The samples of organic chicken skin were exposed to the same bacteria: *E. coli* on MacConkey agar (containing salts of bile acids and crystal violet inhibiting the growth of Gram-positive bacteria) and *S. aureus* on mannitol salt agar (with high concentration of NaCl inhibiting the growth of other bacteria). The samples were kept in a laboratory heater at 37°C for 24 h, and then photos were taken (Table 1).

Electrophoretic analysis

The samples were subjected to electrophoresis on a strip of cellulose acetate membrane (CA-SYS-MINI) in barbital buffer (pH 8.6) at 6 mA, maximum 200 V for 0.5 h. The strips were stained with 0.5% toluidine blue in 3% HOAc solution and then rinsed in distilled water and air-dried. The strips were stained with 0.5% amido black in 5% HOAc solution and then fixed in methanol, HOAc and water (18:4:18); then rinsed in 5% HOAc solution and distilled water and air-dried. Semi-quantitative analysis of the proteins content in the samples was also conducted using GELSCAN v.1.45 software (Kucharczyk T.E., Poland).

IR spectroscopic analysis

FTIR spectroscopic analysis was performed using a Nicolet 6700 Fourier-transform spectrophotometer (Thermo Scientific, USA) using OMNIC 7.0 software and equipped on diffusion accessory EasiDiff (Thermo Nicolet Industries) (spectral region: 4000–500 cm^{-1}, resolution: $4cm^{-1}$, number of scans: 160) of the solid samples (fragments of the samples of pure organic chicken skin). Spectra of three repacked subsamples of each individual sample were averaged to one spectrum. All spectra were performed using a linear baseline and pre-processed with the Fourier smoothing (Grams 32 AI software, Galactic Industries); smoothing degree: 50%.

Scanning electron microscopy analysis

Chicken skin surface was examined using a JSM 5500LV scanning electron microscope supplied by JEOL. The samples were mounted on aluminum stubs and coated with gold (JFC 1200 Jeol). Secondary electrons (SE) and back-scattered electrons (BSE) observations were conducted, with the accelerating voltage of 10 kV. Microphotographs were taken at magnifications ranging from 50× to 1000×.

Results and discussion

‘‘Fucoidan and other antioxidant solutions can be used as therapeutically active biomaterials that speed up the process of wound healing.’’ This thesis, present in many ongoing studies worldwide, was the basis of this investigation.

Burn injury is a complex traumatic event with various local and systemic effects, affecting several organ systems beyond the skin. Prolonged exposure to temperatures higher than 40°C leads to denaturation of proteins and finally loss of their plasma membrane integrity. This process is rapid and may only take a second when exposed to temperatures higher than 60°C, i.e. flame burns[16].

Microbiological procedure, SEM, FTIR, DSC and CAE were all carried out after heating the samples to a temperature simulating a burn incident.

The results of scanning electron microscopy analysis are shown in Figures 1–2, being an example of representative analyses of a series of tests. The images reveal clear differences in the morphology of the surface of the samples tested, including even non-specific damage to the skin surface. The differences can be seen for subsequent stages of thermal processing. For samples of skin heated to boiling point for 60 s, for 1 hour at 45°C and for 30 s at 55°C (Figs. 1bd and 2a), surface bulges and blisters were found, while Figure 1c (skin heated to boiling point for 5 min) indicates the smoothing of the skin surface caused by prolonged exposure to high temperature. For samples shown in Fig. 2bcd, a membrane covering the surface of the damaged skin appears. These photos illustrate the effect of the solutions used as shielding antioxidants: fucoidan (extracted from brown algae), and flame-retardant cyclic organophosphates and phosphonates[17].

Three series of semi-quantitative studies of skin samples placed directly on agars or on paper discs are discussed here (Table 1). Antibacterial properties of the modified skin samples were compared with a reference sample of pure chicken skin (PS) and chicken skin heated to boiling point for 60 s (HS) and also in presence of glucose (GHS). For chicken skin modified with fucoidan from brown seaweed (FBS) and flame-retardant cyclic organophosphates and phosphonates (FRP), the zone of the growth inhibition for Gram-positive (*S. aureus*) bacteria was bigger than 1 mm. The microbiological procedure of analysing skin, as discussed above, can serve as a kind of monitoring to find their antibacterial properties or the effect of the solutions used as shielding antioxidants: fucoidan (extracted from brown algae), and flame-retardant cyclic organophosphates and phosphonates.

IR and Raman are able to investigate the bimolecular changes in the skin. The following papers[18-26] are examples of such analysis. Generally, it is well known that heat and chemical denaturant - induced protein aggregates form the intermolecular β-sheet structure. Moreover, FTIR spectroscopy is diagnostically useful to examine the β-sheet structure, which is one of the major structures of the aggregates. Indeed, recent studies[27] on thermal aggregation process suggest that the first step of aggregation consists in a partial opening of the protein native conformation. The amide I band (1649–1659 cm^{-1}) in the infrared spectrum is usually assigned to α-helix structure[28], the two shoulders[29] that appear at 1620 cm^{-1} and 1680 cm^{-1} may be attributed to the intermolecular β-sheet aggregates.

The absorption peaks of PS, HS, FRP and FBS can be found in Figure 3. While heating the organic chicken skin to boiling point for 60 s (HS) leads to the disappearance (Fig. 3b) of a wide band in the 1650–1550 cm^{-1} area (Fig. 3a), incubating it in fucoidan solutions or flame-

retardant phosphonates (FRP) leads to the reemergence of the band (Fig. 3de). The most diagnostic peaks (amide I and amide II bands) in the IR spectra of collagen have the following locations: PS – 1651, 1608, 1572 cm^{-1}; FRP – 1645, 1605, 1576 cm^{-1}; FBC – 1649, 1602, 1578 cm^{-1}. So, the peak frequency of the amide band shifts to a lower frequency.

One drawback of infrared spectroscopy of aqueous solutions is the strong absorbance of water in the mid-infrared spectral region (near 1645 cm^{-1})[31] which overlaps the important amide I band of proteins and some side chain bands. A considerable number of water molecules remains associated with the dried proteins. Nevertheless, approximately a quarter of all N-H and C=O groups are not hydrogen bonded when the proteins are dry[31].

For samples incubated with pure organic chicken skin and heated to boiling point, glucose was added to initiate protein glycation (Fig. 3c). For a sample incubated *in vitro* in a fucoidan solution containing glucose, glycation was confirmed in a FTIR examination[30], occurring in collagen obtained from chicken skins. The sensitivity of the amide I vibration to secondary structure makes it possible to study protein folding, unfolding and aggregation with infrared spectroscopy[31]. In the collagen glycation assay, while glucose reacts with collagen and forms cross-linked aggregates, fucoidan and flame-retardant phosphonates probably decrease the process of aggregation and recovery of native collagen (Fig. 3de). This suggests that a decrease of the α-helical component induces cooperatively the formation of the heat-induced and solvated aggregates. While the folded protein exhibits a structured amide I spectrum after band narrowing techniques have been applied[31], the unfolded protein shows a broad, featureless amide I band centred near 1650 cm^{-1} which is characteristic of unordered structure. In contrast, aggregated protein often shows a band near 1623 - 1641cm^{-1} which is characteristic of intermolecular β-sheets. These preliminary infrared spectroscopy examinations complement earlier electrophoretic observations regarding the incubation of collagen in fucoidan solutions[28, 30].

The FTIR study was also designed to evaluate the influence of antioxidant solutions on samples a blank organic chicken skin incubated with glucose and the antioxidants but not boiled. Wavelengths analyses between 1500 cm^{-1} and 1700 cm^{-1} revealed two peaks, corresponding to the region of amide bound protein. The sensitivity of the amide I vibration to secondary structure makes it possible to study protein folding, unfolding and aggregation with infrared spectroscopy[31]. The absorption peaks of PS, GS, FBS and FRP can be found in Fig. 4a-e. The band positions given in the Fig. 4a-e should be regarded only as guidelines for the interpretation of spectra. The main bands, height of the band and their relative band width below the main peaks are shown in Table 2. These bands are shown in detail in Fig. 5a-d,

resolved into Gaussian-shaped bands using GRAMS software. The most diagnostic peaks (amide I and amide II bands in Fig. 5a-d) in the IR spectra of collagen have the following locations (Table 2): PS – 1653, 1626, 1543 cm^{-1}; FRP – 1666, 1634, 1543 cm^{-1}; FBS – 1665, 1634, 1551cm^{-1}; GHS – 1662, 1626, 1548 cm^{-1}. So, the peak frequency of the amide band shifts as the chicken skin is incubated in presence of the antioxidants. The antioxidants probably bind with the collagen forming a polymer film. Besides band position and band intensity, the third spectral parameter, the band width, is also informative. As the band position for a given vibration usually is slightly different for every conformer, heterogeneous band broadening is the consequence. Flexible structures will thus give broader bands than rigid structures and the band width is a measure of conformational freedom[31]. For molecules that bind to proteins, the restriction of conformational freedom is a natural consequence of binding; this (Table 2- for example, the sample FBS) often reduces the band width[31].

The study concentrating on cellulose acetate membrane electrophoresis was designed to evaluate the influence of antioxidant solutions on HSA incubated pure organic chicken skin heated to boiling point, then incubated at 60°C for 60 min, simulating a burn incident. For the sample of HSA in presence of chicken skin heated to boiling point for 60 s and incubated in presence of fucoidan from brown seaweed (Fig. 6c), a range is identified with intensity and R_f comparable to native samples (Fig. 6af), which proves the shielding effect of algae fucoidan on collagen subjected to thermal stress[27]. For samples in Figure 6bde, an additional oligomer band is visible on the electropherogram at the starting line. The presence of this band is probably related to the secretion of HSPs during the conditioning of samples at 60°C for 60 min. Research in this area remains at an early stage and will be continued.

The purpose of this study (Fig. 6) was also to initiate protein glycation with temperature. Glycation of proteins in the body increases in states of increased sugar content in blood, known as the oxidative stress condition (burn, inflammation, wounds of various origins, etc.). In the collagen glycation assay, while glucose reacts with collagen and probably forms cross-linked aggregates, fucoidan decreases the process of aggregation[27] and recovery of native collagen (Fig. 6c). For samples in Figure 5de, incubated in propolis solution and flame-retardant cyclic organophosphates and phosphonates, the mechanism of this phenomenon has not been fully recognized and is still under investigation. However, it is worth mentioning that the flame retardant (FRP) has antioxidant properties and can dehydrate the modified material. It also allows a thin insulating layer to be created on the surface of the material[32].

Then, DSC, TG and DTG analyses were carried out after heating the samples to a temperature simulating a burn incident. Figure 7 shows the temperature of phase transition for native collagen from chicken skin and for chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates. Transition enthalpy (ΔH, area under curve) provides information on the organization structure of its matrix.

When analysing the DSC curve (Fig. 7a) for the unmodified sample (chicken skin heated to boiling point for 60 s; HS) tested immediately after its preparation and drying with a filter paper, two characteristic endothermic peaks can be seen. The first of them (specific enthalpy ΔH=1023 J/g) represents the melting of the frozen water that is not integrally bound with the sample, while the other one (specific enthalpy ΔH=1023 J/g) is associated with a gradual release (evaporation) of the moisture contained in the sample. When air-dried for 4 hours (Fig. 7b), the same material does not reveal the excessive water peak, and the intensity of the other peak is reduced by nearly 20 times (ΔH=52,1 J/g).

In contrast, the DSC curve (Fig. 7c) recorded for the modified sample (chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates for 0.5 h; FRP), which was tested after about 2.5 hours of air-drying, reveals two more endothermic effects, unseparated and having their local minimums at 137.7 °C and 140.4°C, respectively. These additional effects can be attributed to the influence of the modification on the kinetics of the moisture released from the skins as they are heated during the calorimetric measurement.

Additional effects reflecting the influence of the modification on the behaviour of the chicken skins during their thermal dissociation were sought using thermogravimetric examination.

TGA analysis (curves: of weight loss as a function of temperature and derivative of weight loss as a function of temperature – DTG, respectively) in the temperature range of 200°C–500°C, corresponding to the area of proper thermal destruction of the samples tested (about 70% total weight loss), indicate that for unmodified samples (Fig. 8ab), regardless of the degree of their dryness, decomposition is a one-step process, as indicated by only one local maximum on the DTG curve, corresponding to the temperature of maximum weight-loss rate.

For modified skins (Fig. 8c), their thermal decomposition is a two-step process, as indicated by two peaks on the DTG curve, at 327.1°C and 358.1°C, respectively. Another result of the modification is a slightly earlier decomposition, and there is about 8% more char (residue

after degassing the sample in an inert gas atmosphere to 800°C) produced, as compared with the unmodified material.

In general, DSC is considered a useful tool for assessing *in vitro* cross-linking of proteins modified with glucose[33] and other compounds[34]. Its potential as a diagnostic tool in biological samples is small due to the relative difficulty of obtaining representative samples and the biological variance between individual samples.

Conclusions

All analyses were carried out after heating the samples to a temperature simulating a burn incident. This process is highly intensified both by the temperature and time of incubation. For a sample incubated *in vitro* in solutions of antioxidants containing glucose, glycation was confirmed. In the collagen glycation assay, while glucose reacts with collagen and forms cross-linked aggregates, fucoidan and other antioxidants decrease the process of aggregation and recovery of native collagen. Additionally, the electrophoretic examination seems to suggest that the heat shock proteins (HSPs) can be identified in this way. Analysing them will be the subject of further research.

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Fig. 1. Scanning electron microscopic images of the surface of skin samples: (a) untreated skin (×50); (b) skin heated to boiling point for 60 s (×50); (c) skin heated to boiling point for 5 min (×50); (d) skin heated for 1 hour at 45°C (×1000).

Fig. 2. Scanning electron microscopic images of the surface of skin samples: (a) skin heated for 30 s at 55°C (×500); (b) skin heated to boiling for 10 s and incubated with brown seaweed (×100); (c) skin heated to boiling for 10 s and incubated with flame-retardant phosphonates (×500); (d) skin heated to boiling for 10 s and incubated with flame-retardant phosphonates (×50).

Fig. 3a–e. The fragment of the FTIR spectra: (a) pure chicken skin (PS); (b) chicken skin heated to boiling point for 60 s (HS); (c) chicken skin heated to boiling point for 60 s in presence of glucose (GHS); (d) chicken skin heated to boiling point for 60 s and incubated in presence of fucoidan from brown seaweed (FBS); (e) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP).

Fig. 4a–e. The fragment of the FTIR spectra: (a) the spectrum illustrating a proper signal to noise ratio; (b) pure chicken skin (PS); (c) chicken skin incubated in presence of glucose (GHS); (d) chicken skin heated incubated in presence of fucoidan from brown seaweed (FBS); (e) chicken skin heated incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP).

Fig. 5a-d. The fragment of the FTIR spectra resolved into Gaussian-shaped bands at about 1700–1500 cm^{-1} attributed to amide I and amide II bands of: (a) pure chicken skin (PS); (b) chicken skin heated incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); (c) chicken skin heated incubated in presence of fucoidan from brown seaweed (FBS); (d) chicken skin incubated in presence of glucose (GHS).

Fig. 6. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of HSA samples. From left to right: (a) untreated HSA; (b) HSA in presence of skin heated to boiling point for 60 s in presence of glucose, then incubated at 60°C for 60 min; (c) HSA in presence of chicken skin heated to boiling point for 10 s and incubated in presence of fucoidan from brown seaweed (FBS); (d) HSA in presence of chicken skin heated to boiling point for 10 s and incubated in presence of propolis solution; (e) HSA in presence of chicken skin heated to

boiling point for 10 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); (f) untreated HSA.

Fig. 7. DSC curves for: (a, b) chicken skin heated to boiling point for 60 s; (c) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates for 0.5 h.

Fig. 8. TG (abc) and DTG (a'b'c') curves for: (a, b) chicken skin heated to boiling point for 60 s; (c) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates for 0.5 h.

Tab. 1. Resistance of *Escherichia coli* and *Staphylococcus aureus*.

Tab. 2. Analysis of bands at 1700–500 cm^{-1} in FTIR spectra obtained using GRAMS software.

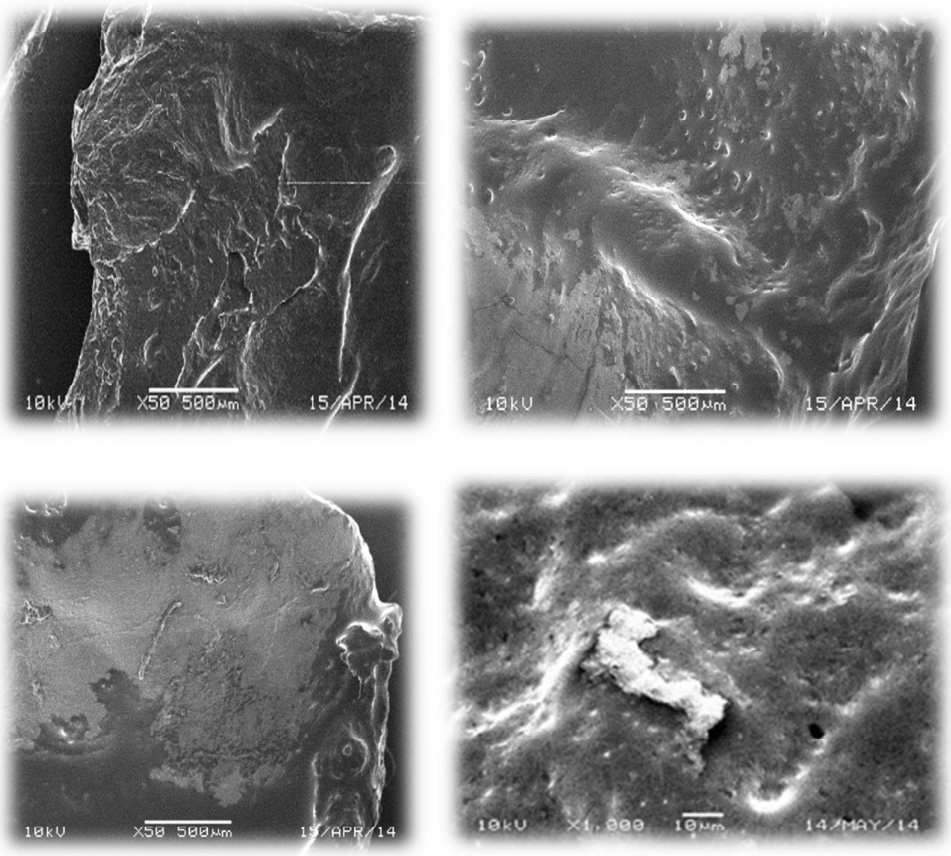

Fig. 1. Scanning electron microscopic images of the surface of skin samples: (a) untreated skin (×50); (b) skin heated to boiling point for 60 s (×50); (c) skin heated to boiling point for 5 min (×50); (d) skin heated for 1 hour at 45°C (×1000).

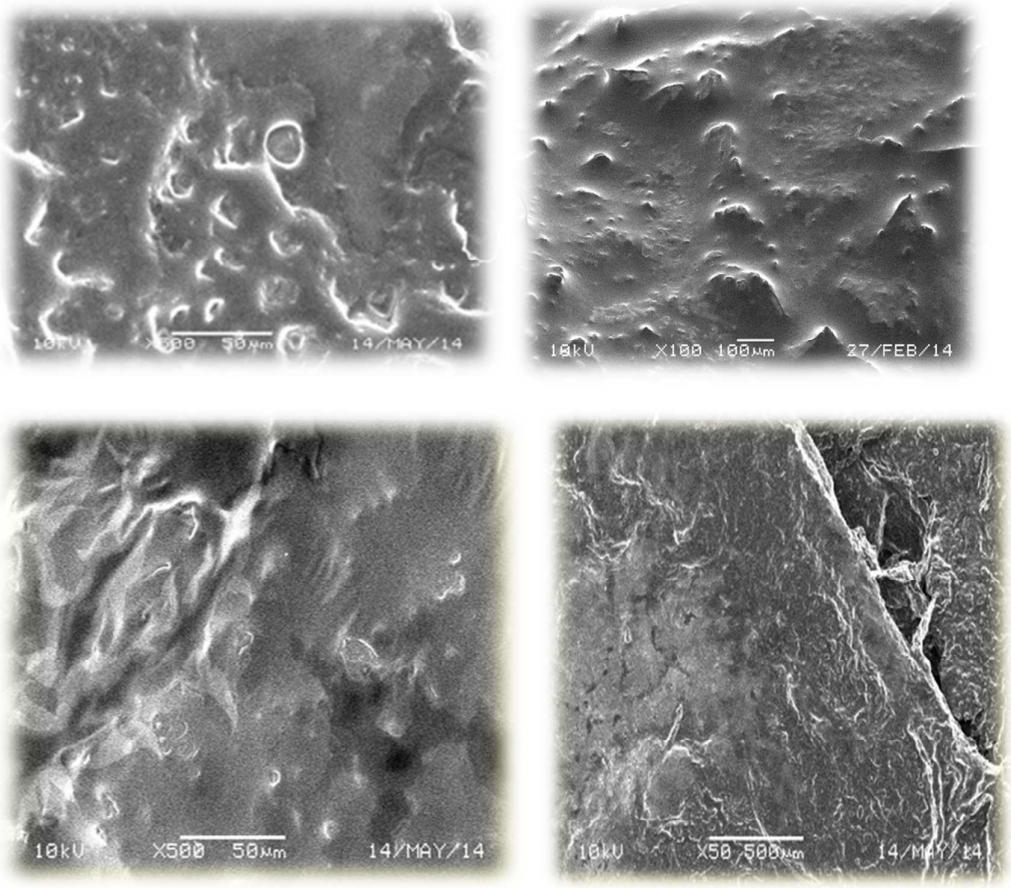

Fig. 2. Scanning electron microscopic images of the surface of skin samples: (a) skin heated for 30 s at 55℃ (×500); (b) skin heated to boiling for 10 s and incubated with brown seaweed (×100); (c) skin heated to boiling for 10 s and incubated with flame-retardant phosphonates (×500); (d) skin heated to boiling for 10 s and incubated with flame-retardant phosphonates (×50).

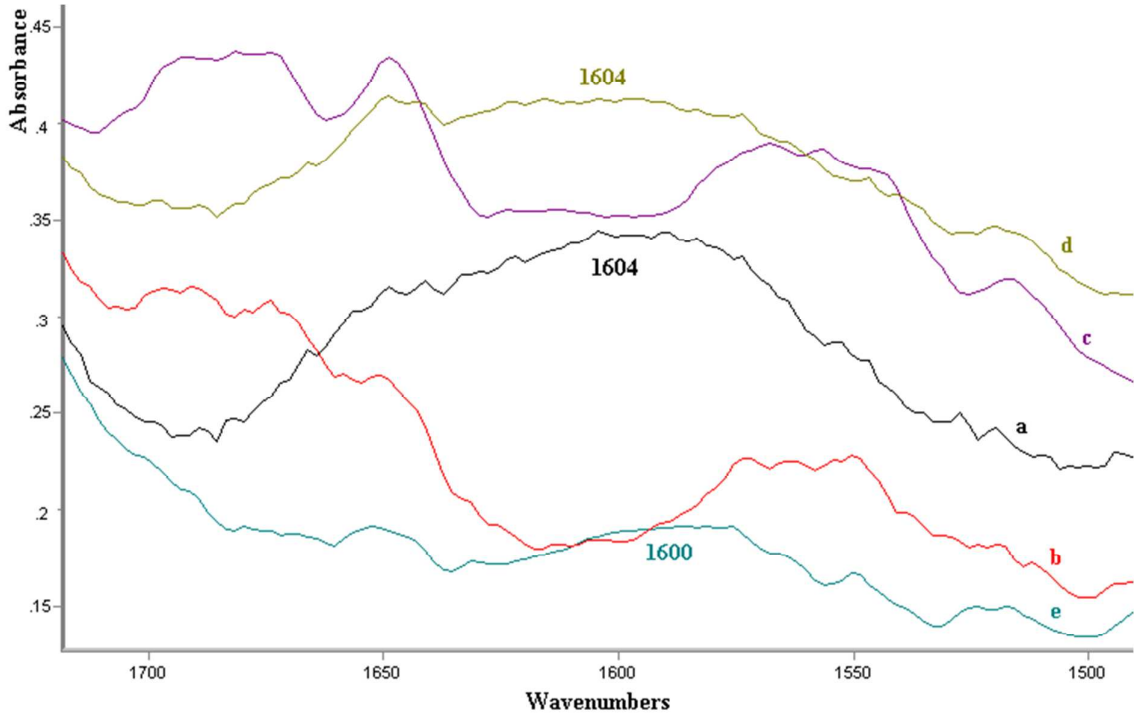

Fig. 3a–e. The fragment of the FTIR spectra: (a) pure chicken skin (PS); (b) chicken skin heated to boiling point for 60 s (HS); (c) chicken skin heated to boiling point for 60 s in presence of glucose (GHS); (d) chicken skin heated to boiling point for 60 s and incubated in presence of fucoidan from brown seaweed (FBS); (e) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP).

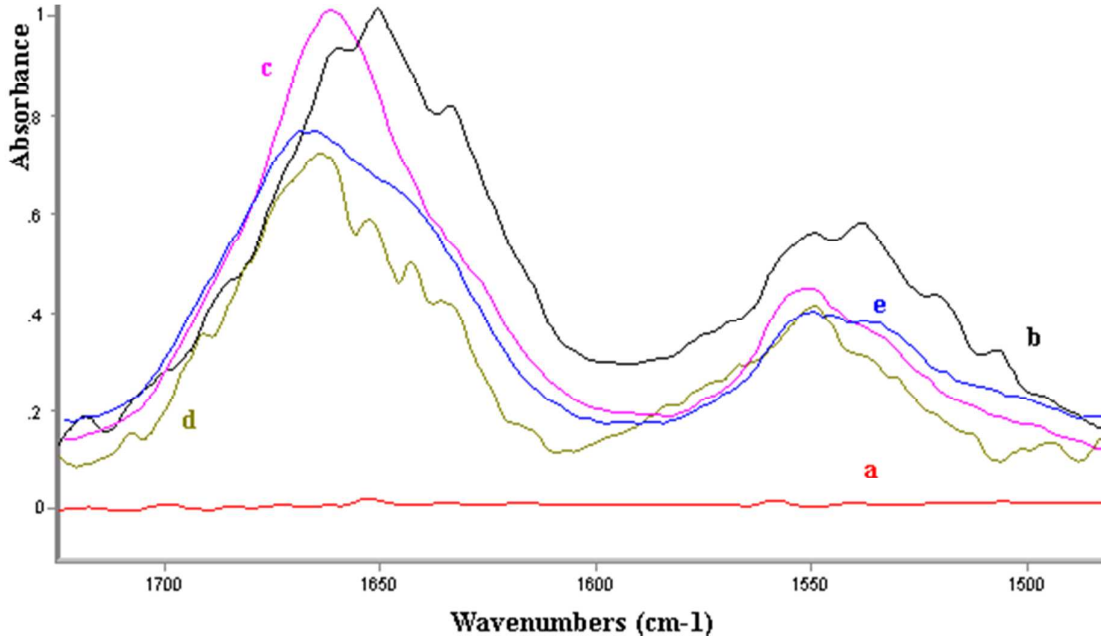

Fig. 4a–e. The fragment of the FTIR spectra: (a) the spectrum illustrating a proper signal to noise ratio; (b) pure chicken skin (PS); (c) chicken skin incubated in presence of glucose (GHS); (d) chicken skin heated incubated in presence of fucoidan from brown seaweed (FBS); (e) chicken skin heated incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP).

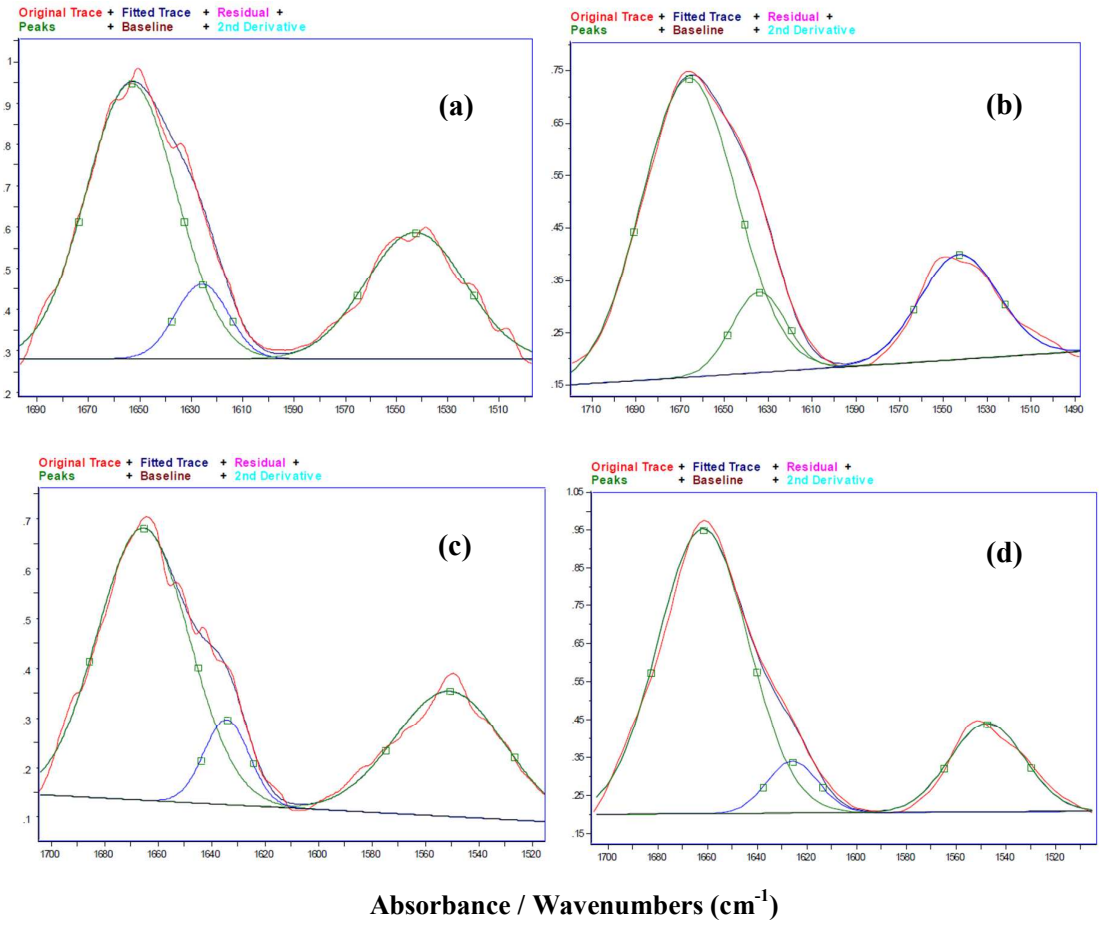

Fig. 5a-d. The fragment of the FTIR spectra resolved into Gaussian-shaped bands at about 1700–1500 cm^{-1} attributed to amide I and amide II bands of: (a) pure chicken skin (PS); (b) chicken skin heated incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); (c) chicken skin heated incubated in presence of fucoidan from brown seaweed (FBS); (d) chicken skin incubated in presence of glucose (GHS).

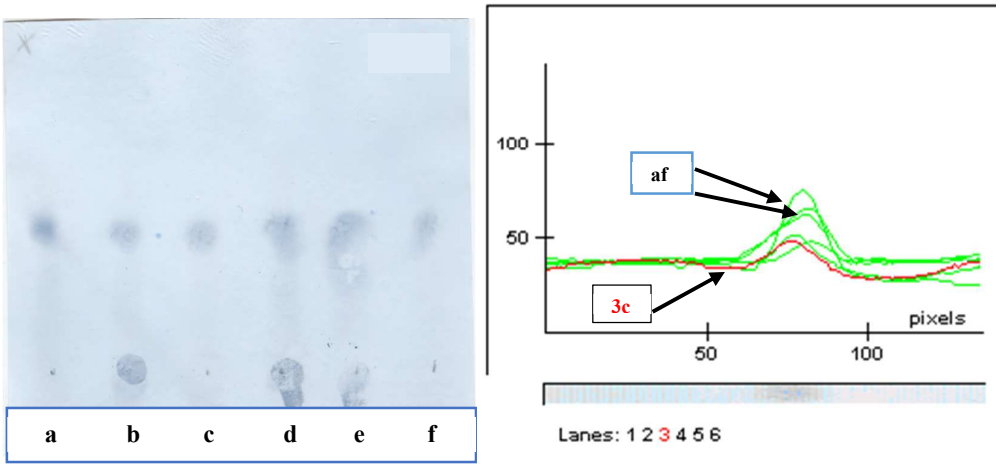

Fig. 6. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of HSA samples. From left to right: (a) untreated HSA; (b) HSA in presence of skin heated to boiling point for 60 s in presence of glucose, then incubated at 60°C for 60 min; (c) HSA in presence of chicken skin heated to boiling point for 10 s and incubated in presence of fucoidan from brown seaweed (FBS); (d) HSA in presence of chicken skin heated to boiling point for 10 s and incubated in presence of propolis solution; (e) HSA in presence of chicken skin heated to boiling point for 10 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates (FRP); (f) untreated HSA.

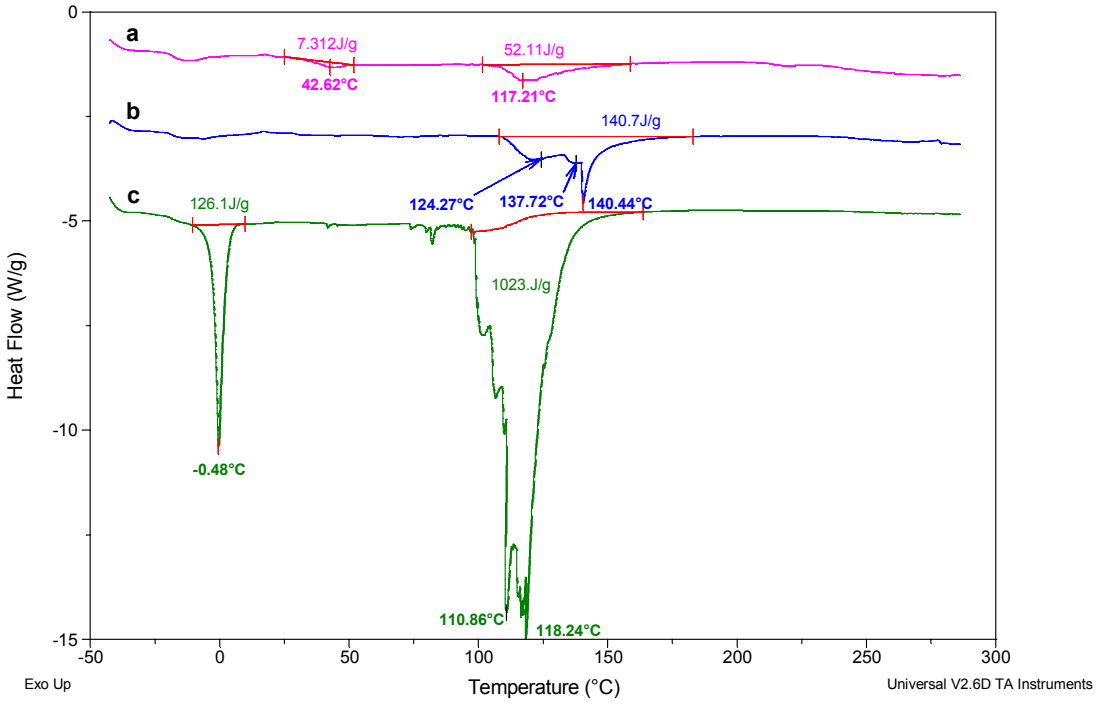

Fig. 7. DSC curves for: (a, b) chicken skin heated to boiling point for 60 s; (c) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates for 0.5 h.

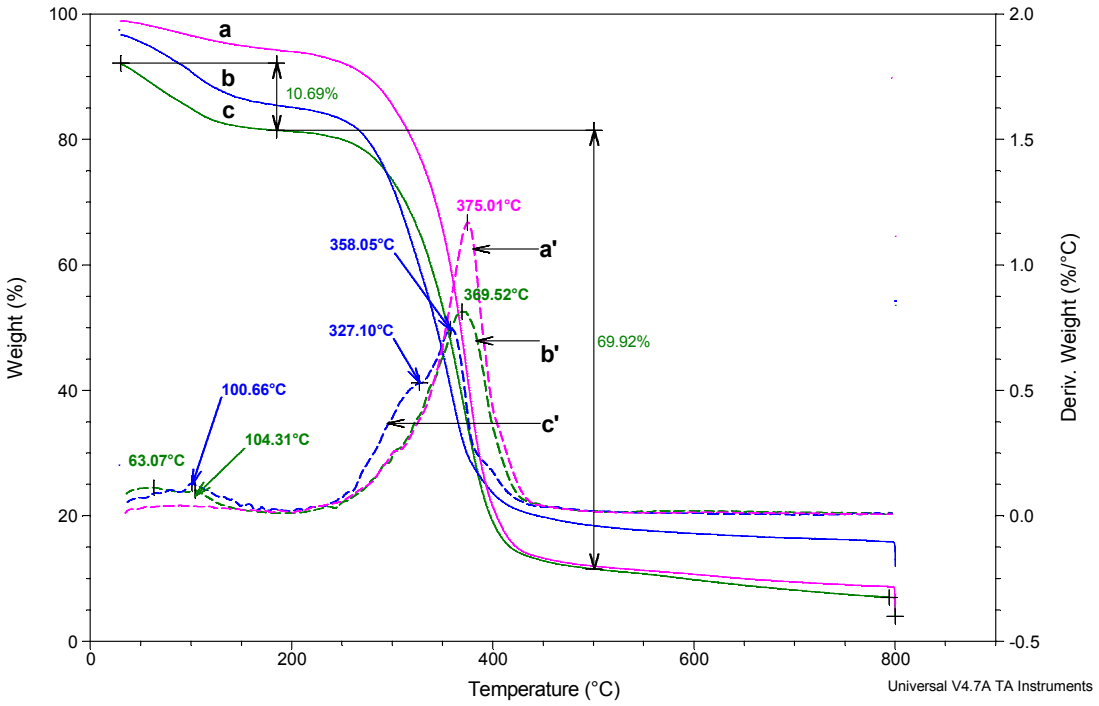

Fig. 8. TG (abc) and DTG (a'b'c') curves for: (a, b) chicken skin heated to boiling point for 60 s; (c) chicken skin heated to boiling point for 60 s and incubated in presence of flame-retardant cyclic organophosphates and phosphonates for 0.5 h.

Tab. 1. Resistance of *Escherichia coli* and *Staphylococcus aureus*.

Samples	Microorganisms	
	Escherichia coli	*Staphylococcus aureus*
Sample 1: pure chicken skin (PS)	-	-
Sample 2: chicken skin heated to boiling point for 60s (HS)	+	-
Sample 3: chicken skin heated to boiling point for 60s in presence of glucose (GHS)	-	+
Sample 4: chicken skin heated to boiling point for 60 s and incubated in presence of fucoidan from brown seaweed (FBS),	-	+
Sample 5: chicken skin heated to boiling point for 60 s and incubated in presence of flame retardant cyclic organophospates and phosphonates (FRP).	-	+

„ - „ lack of inhibition growth zone

„ + „ zone of the growth inhibition bigger than 1 mm

Tab. 2. Analysis of bands at 1700–500 cm^{-1} in FTIR spectra obtained using GRAMS software.

Sample	Band position (cm^{-1})	Height of the band	Relative band width	Statistical parameters*
(PS)	1653 1626 1543	0.68 0.18 0.31	41.4 24.1 45.4	0.00007 0.994 0.015
(FRP)	1666 1634 1543	0.57 0.15 0.20	50.4 29.0 41.8	0.00003 0.998 0.081
(FBS)	1665 1634 1551	0.55 0.17 0.25	40.4 19.8 48.1	0.00009 0.992 0.015
(GHS)	1662 1626 1548	0.75 0.14 0.23	42.6 24.2 35.0	0.00003 0.997 0.013

*Statistical parameters: RMS noise, corr., str error,