# Properties and Antioxidant Capacity of Anchovy (*Engraulis encrasicholus*) By-Product Protein Films Containing Thyme Essential Oil

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**Summary**

In this study, some properties and antioxidant capacity of anchovy (*Engraulis encrasicholus*) by-product protein films with added 0.5, 1.0 and 1.5 % of thyme essential oil were investigated. The films with thyme essential oil had higher elongation at break, water vapour permeability and oxygen permeability, lower solubility and tensile strength than control film (p<0.05). The incorporation of thyme essential oil affected transparency values of the films, but only the addition of 1.5 % of thyme essential oil significantly reduced the transparency (p<0.05). In the film matrix, molecular organisation and intermolecular interaction were changed by thyme essential oil addition. The films with thyme essential oil had a heterogeneous surface and a relatively smooth cross-section structure. Slightly higher phase transition and lower glass transition temperatures were observed in films with thyme essential oil. The antioxidant capacity of the films was improved by incorporating thyme essential oil depending on its volume fraction.

*Key words:* anchovy by-product protein film, thyme essential oil, water vapour permeability, mechanical properties, antioxidant capacity

## Introduction

Due to the environmental impact of the excessive quantity of non-degradable waste materials, packaging studies have recently focused on developing biodegradable and/or edible packaging films obtained from natural sources, such as by-products of agriculture, fishing or livestock raising (*1*). Among these natural sources, proteins from fish processing by-products have been successfully utilised in the development of edible films, as reported by various researchers (*2–4*). A small sea fish, anchovy (*Engraulis encrasicholus*) is nearly 12 cm long with the highest catch rate among the marine fish found in Turkey (*5,6*). Fish head, frame and viscera, as by-products, are generated by anchovy processing, and have been generally turned into by-products with low value (for example fish flour powder to be added to animal feed) or directly dis-posed into the environment, which in turn causes pollution problems (*6*). Therefore, it is important that fish processing by-products are effectively evaluated for the prevention of environmental pollution and obtaining high value-added products (*4*).

Food products can be protected by edible films and coatings against moisture migration or diffusion of the gases that have substantial role in food deterioration, such as $O_2$ or $CO_2$. Moreover, films and coatings can act as carriers for antioxidants, antimicrobials, nutrients, spices and herbs (*1*). However, due to their hydrophilic character, fish protein films are generally insufficient barriers to prevent water vapour migration, as reported by various researchers (*2,3,7,8*). Water vapour permeability (WVP) and antioxidant properties of the films can be improved by adding active compounds such as essential oils (*2,3*,

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*9,10*). Among these essential oils, thyme essential oil is a key essential oil containing active compounds such as thymol and carvacrol with known antioxidant capacity (*11*). Various properties of chitosan films containing thyme essential oil were investigated by Altiok *et al.* (*12*), and they indicated that oxygen transmission and WVP rate of the film barely increased, while its mechanical properties decreased with the addition of thyme essential oil. Moreover, they observed an increase in the antioxidant capacity of films with the addition of thyme essential oil. Pires *et al.* (*2*) studied the characteristics of biodegradable films containing thyme essential oil and hake proteins. Their study showed that the addition of thyme essential oil to the films reduced the WVP and increased the antioxidant activity. Kavoosi *et al.* (*13*) found that the addition of thyme essential oil to gelatin films led to an important decrease in tensile strength (TS), an increase in water solubility, elongation at break (EAB) and WVP. In addition, they indicated that the films containing thyme essential oil showed perfect antioxidant properties. Jouki *et al.* (*14*) found that thyme essential oil addition to quince seed mucilage films increased the WVP and oxygen permeability (OP), whereas decreased the glass transition temperature.

As seen from the above results, the incorporation of thyme essential oil into chitosan, hake protein, gelatin or quince seed mucilage films affected WVP, OP, water solubility, mechanical properties, glass transition temperature and antioxidant capacity. As far as we know, there is no data on the addition of thyme essential oil to anchovy by--product protein films. Therefore, this work aims to evaluate the effect of thyme essential oil, at various volume fractions, on the properties and antioxidant capacity of the anchovy by-product protein films. Thickness, solubility, transparency, WVP, OP, mechanical and other properties were determined by differential scanning calorimetry, scanning electron microscopy and Fourier-transform infrared spectroscopy. Ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity values of the anchovy by-product protein films containing thyme essential oil were also assessed.

## Materials and Methods

### Anchovy by-product protein powder and essential oil preparation

Anchovy (*Engraulis encrasicholus*) processing by-products (head, frame and viscera) used as material in the film production were obtained from SASTAS A.S. (Samsun, Turkey) and minced with an electronic meat mincer (model MEW 613; Mado Primus, Eilenburg, Germany). Anchovy by-product proteins were prepared, with some modifications, as reported by Zavareze *et al.* (*4*). Firstly, minced by-products were mixed with water at 1:2 ratio (by mass per volume) in a blender (Model SHB 3062; Sinbo, Istanbul, Turkey) for 1 min. For protein solubilisation, 2 M NaOH (pH=11.2) were added and the mixture was homogenised by continuous agitation with a magnetic stirrer (model HS15-03P; MTOPS, Yangju City, Kyunggi-do, Korea) for 1 h. The sample was centrifuged with a laboratory centrifuge (model NF 800R; Nuve, Ankara,

Turkey) at 9000×*g* and 4 °C for 30 min in order to obtain three phases (soluble proteins, insoluble proteins and lipids). The supernatant (soluble proteins) was collected and acidified with 2 M HCl (pH=5.35) for protein precipitation. Then, it was stirred for 5 min and centrifuged with a laboratory centrifuge again at 9000×*g* and 4 °C for 15 min. Finally, the precipitated proteins were oven-dried with forced air (model JSOF-50; JSR, Gongju City, Korea) at 35 °C for 24 h, milled and stored in glass jars at 4 °C until film preparation. The protein mass fraction of anchovy by-product protein powder was 68.58 %, determined according to standard AOAC method 960.52 (*15*).

Dried thyme (*Thymus vulgaris* L.) was obtained from a local market (Samsun, Turkey), powdered in a coffee grinder (model 2909; Sinbo) and hydrodistilled for 3 h with 50 g of dry plant material and 500 mL of distilled water in a Clevenger-type device (Sesim Kimya Laboratuvar, Ankara, Turkey). Amber coloured vials were used to collect the extracted essential oil, which was dehydrated using anhydrous sodium sulphate, then stored in the dark at 4 °C until use.

### Preparation of films

The films were prepared according to Limpan *et al.* (*16*), with some modifications. A mass of 4.0 g of anchovy by-product protein powder was mixed with 100 mL of distilled water, the solution pH was set to 11.5 using 5 M NaOH, and glycerol (Merck, Darmstadt, Germany) was then added at 40 % (by mass of anchovy by-product protein) as plasticiser. The solution was homogenised for 5 min at 11 600×*g* with an Ultraturrax homogeniser (model T25; IKA, Staufen, Germany). Afterwards, it was gently stirred with a magnetic stirrer at 85 °C for 60 min to completely dissolve anchovy by-product protein powder, centrifuged at 2900×*g* for 10 min and filtered to eliminate undissolved residuals and cooled to (40±2) °C. Tween 80 (Merck) at 0.05 % (by volume) of essential oil was combined with the film-forming solution to help the dissolution of the essential oil, then thyme essential oil was added at 0 (as control), 0.5, 1.0 and 1.5 % (by volume). These solutions were emulsified for 3 min at 11 600×*g* with an Ultraturrax homogeniser. The film-forming emulsion (50 g) was placed into acrylic plates 15 cm in diameter, and then oven-dried (model JSOF-50; JSR) for 24 h at 40 °C under air circulation. Samples of dried films were taken out of the plates and conditioned to 54 % relative humidity (by using a solution of saturated magnesium nitrate) within a desiccator at room temperature for 3 days. All samples were prepared in triplicate.

### Determination of physicochemical properties

A digital micrometer (model 3101-25A; Insize, Jiangsu, PR China) reading to the nearest 0.001 mm was used to determine the film thickness. Values of each triplicate film sample were measured at 10 different locations, and then water vapour permeability, mechanical properties and transparency value of the films were calculated based on the mean thickness value.

A method used by Gennadios *et al.* (*17*) was slightly modified and applied to determine the film solubility in water. In order to determine initial dry mass, precondi-

tioned film pieces (20 mm×20 mm) were left to dry for 24 h at 105 °C. Following the agitation for 24 h in 50 mL of distilled water with sodium azide (0.1 %, by mass per volume) at room temperature, the insoluble matter was separated carefully and left to dry for 24 h at 105 °C for the determination of final dry mass. The following equation was used to calculate the solubility in water:

$$\text{Solubility} = \left( \frac{m_{\text{initial}} - m_{\text{final}}}{m_{\text{initial}}} \right) \cdot 100 \qquad /1/$$

where $m_{\text{initial}}$ and $m_{\text{final}}$ are the initial and final dry mass (g), respectively. All tests were carried out in triplicate.

### Determination of optical properties

Transparency value of the film was calculated by measuring the absorbance at 600 nm using UV-Vis spectrophotometer (model Cary 60; Agilent Technologies, Mulgrave, Australia) according to Kurt and Kahyaoglu [18]. The samples were cut into rectangular pieces and placed in the spectrophotometer test cell. The reference value was determined from an empty test cell. The following equation was used to determine the transparency value of the film:

$$\text{Transparency value} = \frac{A_{600\,\text{nm}}}{\delta} \qquad /2/$$

where $A_{600\,\text{nm}}$ is the absorbance at 600 nm, and $\delta$ is the mean film thickness (mm). All measurements were performed in triplicate at 5 different locations.

### Determination of mechanical properties

A texture analyser (TA-XT2 texture analyser; Stable Micro Systems Co., Ltd., Godalming, UK) was utilised to determine the tensile strength (TS) and elongation at break (EAB) of the films, based on ASTM D882-12 standard method [19]. Sample films were sliced into strips (1 cm×4 cm) and conditioned in 54 % relative humidity (RH) for 3 days. During the extension of the strips, force and distance were determined at 1.5 mm/s up to break. The load at break was divided by the film cross-sectional area to calculate TS. Cross-sectional area was calculated based on the mean thickness of film strips. Elongation at the moment of rupture was divided by the initial gauge length, and then multiplied by 100 in order to calculate the EAB (%). The results show the average of five samples for each type of films.

### Determination of barrier properties

The water vapour permeability (WVP) values were determined according to ASTM E96 / E96M-10 standard method [20]. The films (*d*=14 mm) were sealed with paraffin on glass permeation cups containing silica gels (0 % RH) and left in desiccators containing distilled water (100 % RH) at 25 °C. The mass of the glass permeation cups was measured for 8 h at 1-hour intervals and the following equation was used to determine the WVP ((g·mm)/ (m²·h·kPa)) of the films:

$$\text{WVP} = \left( \frac{m}{t} \right) \cdot \left( \frac{\delta}{\Delta p \cdot A} \right) \qquad /3/$$

where $m$ is the mass gain (g), $t$ is the time of gain (h), $\delta$ is the film thickness (mm), $\Delta p$ is the partial pressure difference through the film at 25 °C (kPa) and $A$ is the film area exposed to moisture transfer ($1.539 \cdot 10^{-4}$ m²). The $m/t$ was determined *via* linear regression ($R^2 > 0.99$) based on the mass of water absorbed by the system at the steady state. All values were determined in triplicate for each film type.

Oxygen permeability (OP) was determined based on the peroxide value (PV) using AOCS Cd 8-53 method [21]. Briefly, a 25-mL tube was filled with 10 mL of sunflower oil, covered with films containing thyme essential oil, sealed using paraffin and sticky tape, and stored at a controlled temperature (60 °C) for 10 days. The PV of the sunflower oil samples was determined by titration with sodium thiosulfate. All values were determined in triplicate for each film type.

### Fourier-transform infrared spectroscopy measurements

Fourier-transform infrared (FTIR) spectra of the samples were measured using a FTIR spectrometer (model Spectrum Two; PerkinElmer, Akron, OH, USA) at 4 cm⁻¹ resolution within a range of 650–4000 cm⁻¹. Before the analysis, the samples were left at room temperature in a desiccator containing silica gel for two weeks to obtain the highest dehydration. For each spectrum 32 scans were run.

### Scanning electron microscopy determination

Scanning electron microscope (model JSM-7001F; JEOL, Tokyo, Japan) was utilised at 10 kV to determine the surface and cross-section characteristics of the samples. Before the visualisation process, sputter coater with gold/palladium (model SC7620; Quorum Technologies, Laughton, UK) was used to coat the films, and surface and cross-section photographs were taken at 1500 and 4000× magnification, respectively.

### Differential scanning calorimetry measurements

Thermal properties of the samples were measured *via* a differential scanning calorimeter (model DSC 4000; PerkinElmer). Before the analysis, film samples were stored at room temperature for two weeks in a desiccator with silica gel to dehydrate. A sample weighing 5 mg was sealed in aluminium pans, and then scanned under a nitrogen atmosphere at 10 °C per min over the range of −50 to 120 °C.

### Antioxidant capacity determination

Antioxidant capacity of anchovy by-product protein films containing thyme essential oil was determined using the ferric reducing antioxidant power (FRAP) method [22] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity method [23].

For the FRAP determination, 1.5 mL of methanol was used to dissolve 0.15 g of anchovy by-product protein film, and then 50 µL of film extract solution were added to 0.95 mL of FRAP reagent prepared by mixing 300 mM acetate buffer (pH=3.6), 20 mM $FeCl_3$ and a solution of 10 mM ferric-2,4,6-tripyridil-*s*-triazine (TPTZ) in 40 mM HCl at the ratio of 10:1:1. The absorbance was determined at 593 nm by UV-Vis spectrophotometer (Model Cary 60;

Agilent Technologies). Trolox standard curves were used to calculate FRAP values, which were expressed in mg of Trolox per mL of film.

For the DPPH scavenging activity determination, 10 µL of film extract solution, obtained by dissolving 0.15 g of film in 1.5 mL of methanol, were added to 1 mL of DPPH in methanol solution (60 µM) and 40 µL of methanol. After firmly shaking the mixture, it was left at room temperature in the dark for 30 min. The absorbance was determined at 517 nm. All reagents, except for the sample, were used to prepare a negative control. Following equation was employed to calculate the DPPH scavenging activity:

$$\text{DPPH scavenging activity} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \cdot 100 \qquad /4/$$

where $A_{\text{control}}$ is the absorbance of the control, and $A_{\text{sample}}$ is the absorbance of the sample.

### Statistical analysis

All experiments were performed in triplicate and the values were presented as mean±standard deviation. Data of physical, optical, mechanical and barrier properties, and antioxidant capacity were determined using the analysis of variance (ANOVA) and the comparison of the mean values was done using Duncan's multiple range test. SPSS statistical package program v. 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was utilised with a significance level at p=0.05.

## Results and Discussion

### Physicochemical properties of films

Table 1 shows the physicochemical properties of anchovy by-product protein films containing thyme essential oil at various volume fractions. The sample thickness ranged from 0.197 to 0.202 mm, and the addition of thyme essential oil did not have a significant (p>0.05) effect on the resulting film thickness. Altiok *et al.* (*12*) reported similar results in the chitosan films with thyme essential oil, and Bahram *et al.* (*10*) also obtained similar results in the whey protein films prepared with 0.8 and 1.5 % cinnamon essential oil. However, Jouki *et al.* (*14*) showed that the addition of thyme essential oil increased the thickness of quince seed mucilage films, but significant increase was only obtained at the highest thyme essential oil level used (2 %). These different results could be attributed to the solid content of film-forming solutions.

Solubility is one of the important properties of edible films, due to its effect on the film resistance to water, in particular in humid environments (*24*). As seen in Table 1, the films containing thyme essential oil were less soluble than the control film (p<0.05), but differences between solubility values of films containing different volume fractions of thyme essential oil were not significant (p>0.05). A decrease in the hydrophilic nature of the sample may have led to these results. According to Kavoosi *et al.* (*13*), the type and concentration of compounds and their inherent hydrophilicity and hydrophobicity indices alter their additive effect on the film solubility. Decreased solubility with the addition of different essential oils was also determined by various researchers (*3,10,24–26*).

### Optical properties of films

Consumer preference is directly affected by optical properties of edible films used as food packaging materials (*27*). As seen in Table 1, the addition of thyme essential oil affected the transparency values of anchovy by-product protein films, and only the addition of 1.5 % thyme essential oil reduced transparency (p<0.05), which was verified by higher transparency value. The decrease in transparency could be attributed to the increase in light scattering caused by oil droplets in the film network. In addition, the colour components in thyme essential oil probably contributed to the decrease of film transparency and films containing thyme essential oil became less transparent. Similar results were obtained by Pires *et al.* (*2*) for hake protein films containing thyme essential oil, by Tongnuanchan *et al.* (*28*) for fish skin gelatin films containing plai, turmeric and ginger essential oils and by Hosseini *et al.* (*29*) for fish gelatin chitosan biocomposite films containing oregano essential oil.

### Mechanical properties of film samples

Mechanical properties of the samples were determined based on the TS and EAB values, the key indicators of film flexibility and strength (*25*). Table 1 shows the TS and EAB values of anchovy by-product protein films

Table 1. Physicochemical, optical, mechanical and barrier properties of anchovy by-product protein films with thyme essential oil

| Property | $\varphi$(thyme essential oil)/% | | | |
| --- | --- | --- | --- | --- |
| | 0 (control) | 0.5 | 1.0 | 1.5 |
| Thickness/mm | $(0.20\pm0.01)^a$ | $(0.199\pm0.002)^a$ | $(0.197\pm0.006)^a$ | $(0.202\pm0.003)^a$ |
| Solubility/% | $(63.4\pm0.4)^a$ | $(59.2\pm1.5)^b$ | $(59.16\pm0.03)^b$ | $(56.2\pm2.7)^b$ |
| Transparency | $(1.28\pm0.01)^b$ | $(1.27\pm0.06)^b$ | $(1.31\pm0.03)^b$ | $(1.46\pm0.02)^a$ |
| Tensile strength/MPa | $(1.46\pm0.04)^a$ | $(0.55\pm0.09)^b$ | $(0.47\pm0.01)^b$ | $(0.52\pm0.02)^b$ |
| Elongation at break/% | $(51.5\pm2.2)^b$ | $(79.6\pm7.2)^a$ | $(83.4\pm6.8)^a$ | $(80.1\pm4.8)^a$ |
| Water vapour permeability/((g·mm)/(m²·h·kPa)) | $(1.5\pm0.1)^b$ | $(2.08\pm0.07)^a$ | $(2.12\pm0.02)^a$ | $(2.0\pm0.2)^a$ |
| Oxygen permeability/(mmol/kg) | $(14.0\pm1.1)^b$ | $(16.6\pm0.2)^a$ | $(16.5\pm2.6)^a$ | $(15.92\pm2.04)^a$ |

The results represent mean values±standard deviation of three replicates. Values in the same row with different letters in superscript are significantly different (p<0.05)

containing thyme essential oil at different volume fractions (0.5, 1.0 and 1.5 %). According to the results, control film had the highest TS and the lowest EAB (p<0.05) compared to others. On the other hand, no changes were observed in the TS and EAB values in films containing 0.5, 1.0 and 1.5 % thyme essential oil (p>0.05). Results showed that a 64 % reduction in the TS values (1.46–0.52 MPa) was obtained by the addition of 1.5 % thyme essential oil to the anchovy by-product protein films. The addition of essential oils into films may reinforce the development of heterogeneous film matrix, which may in turn lead to discontinuity in the film network (9,28). Addition of essential oils to protein-based films could prevent protein-protein interaction and produce flexible domains within the film (9). Moreover, these changes could be attributed to plasticising effect of the thyme essential oil (14). As a result, films containing thyme essential oil became weaker, with lower TS, but more stretchable. Jouki *et al.* (14) observed an increase in the EAB and a decrease in TS of quince seed mucilage films containing thyme essential oil, which is in line with the results of this study. Similarly, Shojaee-Aliabadi *et al.* (26) found that the addition of *Satureja hortensis* essential oil into κ-carrageenan film caused a significant (p<0.05) decrease of TS and increase of EAB. Increases of EAB and decreases of TS are typical results of essential oil addition, which have been largely handled in the research on various edible films (9,25,27–29).

### Barrier properties of films

Water and oxygen are important compounds whose migration needs to be controlled, because physical and chemical changes in foods are usually created by the transfer of moisture and oxygen between the food and its surrounding atmosphere (29). Table 1 shows the WVP values of anchovy by-product protein films containing various volume fractions of thyme essential oil. The addition of thyme essential oil to anchovy by-product protein films increased the WVP values from 1.5 to 2.12 (g·mm)/(m²·h·kPa) (p<0.05), but significant differences were not detected between the films containing various volume fractions of thyme essential oil (p>0.05). Many factors such as the ratio of hydrophilic and hydrophobic film components, film thickness, water sensitivity and crystallinity can change the WVP values of the films (14). The increase in the WVP values of the films containing essential oil can be attributed to the discontinuities, caused by lipid droplets in the polymer network leading to a loss in film cohesion and therefore enhancing the transport of water vapour through the film (29,30). Additionally, this may also be due to the ratio of hydrophilic and hydrophobic film constituents, directly influencing the transfer of water vapour through the film network (29). Altiok *et al.* (12) showed that WVP of chitosan films containing thyme essential oil barely increased compared to the control film, Jouki *et al.* (14) found a significant increase in the WVP of quince seed mucilage films containing thyme essential oil and in another study, Hosseini *et al.* (29) found that the addition of oregano essential oil into fish gelatin/chitosan films also increased WVP values of the films, all of which is consistent with the present results. In contrast, some researchers found that the incorporation of essential oils into edible films improved WVP of the films (3,9,25,26). These differences could be attributed to the above-mentioned factors.

In this study, OP value of the control film, measured as PV, was determined to be 14.0 mmol of $O_2$ per kg. The incorporation of thyme essential oil into the anchovy by-product protein films significantly increased (p<0.05) this value, which can be explained by higher oxygen solubility in non-polar oil phase that contributes to the increase of the transfer rate of oxygen molecules into plasticised polymer matrix (14). Additionally, this behaviour could be attributed to the irregularities in the film matrix. Altiok *et al.* (12) reported similar results in chitosan films containing various amounts of thyme essential oil, Jouki *et al.* (14) also found similar results in quince seed mucilage films containing 1.5 and 2 % thyme essential oil, and Ghasemlou *et al.* (25) reported similar results in corn starch films containing 3 % *Zataria multiflora* Boiss essential oil.

### Results of Fourier-transform infrared spectroscopy

Anchovy by-product protein and thyme essential oil interaction was investigated with FTIR spectroscopy. The FTIR spectra of control film and films containing 0.5, 1.0 and 1.5 % thyme essential oil are shown in Fig. 1. The peak around 1043 cm⁻¹ was observed in all films, corresponding to the OH group, primarily from glycerol incorporated as a plasticiser (28,29). Increasing thyme essential oil volume fraction probably resulted in the dilution of glycerol and decrease of the peak amplitude. Tongnuanchan *et al.* (28) also found similar spectra for fish skin gelatin films containing ginger, turmeric and plai essential oils, where OH group was observed at the wavenumber ($v$) of 1031–1035 cm⁻¹. All films had major peaks at $v$=1624–1628 cm⁻¹ (amide I, representing C=O stretching/hydrogen bonding coupled with COO), 1542–1547 cm⁻¹ (amide II, resulting from bending vibration of NH groups and stretching vibrations of CN groups) and 1235–1237 cm⁻¹ (amide III, illustrating the in-plane bending vibrations of CN and NH groups of bound amide or vibrations of $CH_2$ groups of glycine) (9,28). Similar results were also found by Arfat *et al.* (9) for fish protein isolate/fish skin gelatin films with zinc oxide nanoparticles and basil leaf essential oil, where amide I, amide II and amide III peaks were observed at $v$=1631, 1537 and 1238 cm⁻¹, respectively. In general, the
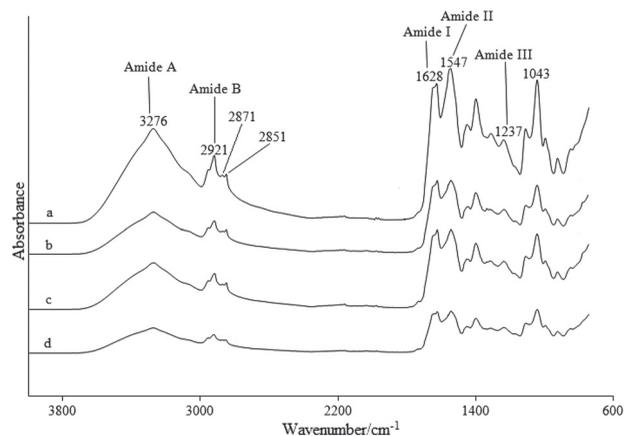


**Fig. 1.** FTIR spectra of anchovy by-product protein films containing thyme essential oil $\varphi$(TEO)/%: a=control, b=0.5, c=1.0 and d=1.5

amplitudes of the films decreased as the thyme essential oil volume fraction increased and the highest amplitudes for amides I, II and III were determined in control films, probably because of the highest protein content.

For all film samples, an amide A peak (representing the NH stretching coupled with hydrogen bonding) was found at $v$=3273–3276 cm$^{-1}$ and an amide B peak (representing CH stretching and NH$_3^+$) was found at $v$=2919–2923 cm$^{-1}$. The amplitude of amide A peak decreased when thyme essential oil was added to the films, especially at 1.5 % volume fraction. This certified the lower interaction among anchovy by-product proteins, shown by the lower TS of the film with the increasing EAB, when higher volume fractions of thyme essential oil were added. Similar results for fish skin gelatin films containing ginger, turmeric and plai essential oils were also reported (*28*). Peaks at $v$=2851 cm$^{-1}$ (methylene symmetric CH stretching) and 2871 cm$^{-1}$ (methyl asymmetric CH stretching) were also found in all film samples. The amplitude of the peak at $v$=2871 cm$^{-1}$ decreased, while the amplitude of the peak at $v$=2851 cm$^{-1}$ increased when thyme essential oil was incorporated into films. It was reported that symmetric methylene stretching bands approx. at $v$=2851 cm$^{-1}$ were present in most of the lipids, while asymmetric methyl stretching bands approx. at $v$=2871 cm$^{-1}$ were present in most of the proteins (*9,28,31*). Similar results in gelatin films containing ginger, turmeric and plai essential oils and in fish gelatin films containing basil and citronella essential oils have been reported (*28,31*). Hence, the FTIR spectrum results indicated that the addition of thyme essential oil into anchovy by-product protein films affected the molecular organisation and intermolecular interaction in polymer matrix.

### Results of scanning electron microscopy observations

Scanning electron microscopy (SEM) images of the surface and cross-section of anchovy by-product protein films containing thyme essential oil are shown in Fig. 2. The control film showed a homogeneous and continuous surface (Fig. 2a), while the anchovy by-product protein films containing thyme essential oil had a heterogeneous

surface (Figs. 2b–d). In addition, many granules were observed on the surface of anchovy by-product protein films containing thyme essential oil, and in general, the size of the granules increased as the essential oil volume fraction increased. This showed that the essential oil droplets were still unstable in emulsion systems stabilised by Tween 80. Consequently, the permeability of the surface containing granules was facilitated and resulted in an increase in WVP and OP values of anchovy by-product protein films containing thyme essential oil.

The control film had a heterogeneous, rough and compact cross-section structure (Fig. 2e), while films containing thyme essential oil had a relatively smooth cross-section structure (Figs. 2f–h). With the addition of thyme essential oil, cross-section structure probably softened, which could be responsible for the higher WVP and OP values of the films containing thyme essential oil. Various researchers reported that the surface and cross-section structure of edible films were affected by both addition and volume fraction of essential oils (*12,29,30*). Jouki *et al.* (*14*) also reported similar results when thyme essential oil was added into the quince seed mucilage films.

### Differential scanning calorimetry analysis

Film transitions were determined by differential scanning calorimetry (DSC) when thermal changes occurred in the film (*31*). DSC thermograms of anchovy by-product protein films containing thyme essential oil are shown in Fig. 3. All thermograms showed endothermic peaks at the phase transition temperature ($t_m$) ranges of 53.53–58.04 °C. These peaks are probably related to the denaturation temperature of myosin and actin proteins (*7*). Da Rocha *et al.* (*7*), in their studies with Argentine anchovy (*Engraulis encrasicholus*) protein isolate, observed the endothermic peak at 62.2 °C. Altiok *et al.* (*12*) reported that chitosan films with or without thyme essential oil had wide endothermic peaks nearly at 35–140 °C. Temperature differences of endothermic peaks could be attributed to the type of film or essential oil, the oil quality and volume
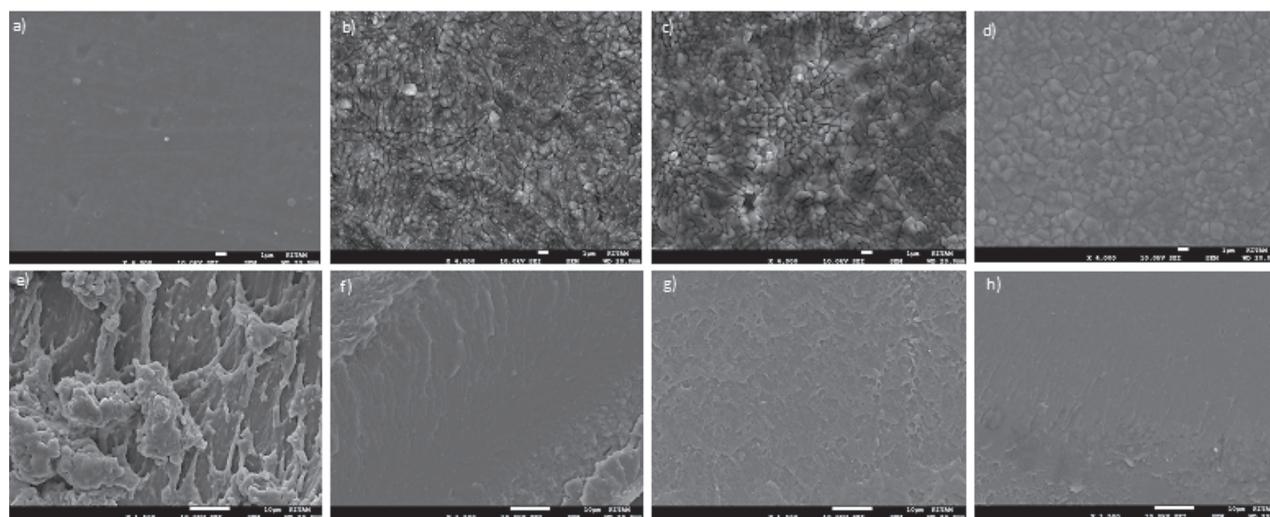


**Fig. 2.** SEM images of the surface: $\varphi$(TEO)/%: a) control, b) 0.5, c) 1.0 and d) 1.5, and cross-section: e) control, f) 0.5, g) 1.0 and h) 1.5 of anchovy by-product protein films containing thyme essential oil (TEO)
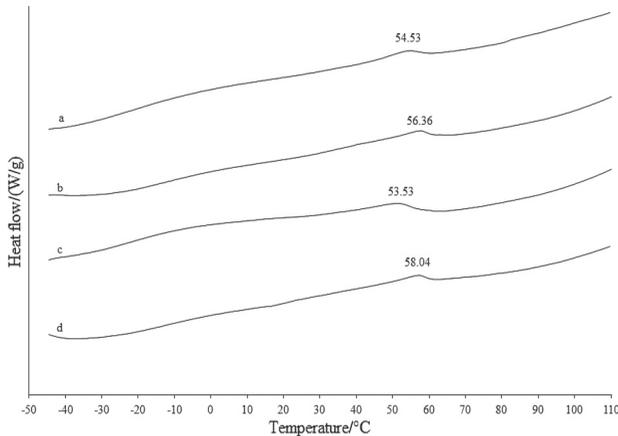
**Fig. 3.** Differential scanning calorimetry thermograms of anchovy by-product protein films containing thyme essential oil $\varphi$(TEO)/%: a=control, b=0.5, c=1.0 and d=1.5

fraction, and heating conditions. Except for the film containing 1.0 % thyme essential oil, anchovy by-product protein films with thyme essential oil had slightly higher $t_m$ than the control film, which might be attributed to more hydrophobic nature and larger molecular mass of thyme essential oil. Jouki *et al.* (*14*) found similar results in quince seed mucilage films containing thyme essential oil, and Tongnuanchan *et al.* (*31*) also observed similar results for fish gelatin films containing basil and citronella essential oils.

The highest glass transition temperature ($t_g$) of –24.27 °C was obtained in control film and the addition of 0.5 % thyme essential oil into the anchovy by-product protein films caused a decrease in $t_g$ to –32.82 °C (data not shown). However, $t_g$ of films containing 1.0 and 1.5 % thyme essential oil most likely became too low, thus could not be observed in the tested temperature range. Lower $t_g$ is better for film flexibility, and results showed that films containing thyme essential oil had lower $t_g$, indicating better flexibility than the control sample. Moreover, Jouki *et al.* (*14*) found that pure quince seed mucilage film had $t_g$ of –35.2 °C, which is higher than of the same film containing thyme essential oil ($t_g$=–43 to –57 °C).

*Antioxidant capacity of films*

FRAP and DPPH scavenging activity values of anchovy by-product protein films containing various volume fractions of thyme essential oil are shown in Fig. 4. The lowest FRAP value (0.91 mg/mL, expressed as Trolox equivalents, Fig. 4a) was observed in the control film, and it significantly increased (p<0.05) with the increase of the volume fraction of thyme essential oil from 0.5 to 1.5 %. Like FRAP value, the lowest DPPH scavenging activity value (0.06 %) was determined in the control film, and it significantly increased (p<0.05) with the increase of the thyme essential oil volume fraction. However, no significant differences were detected between the films containing 1.0 and 1.5 % thyme essential oil (p>0.05) (Fig. 4b). Lower FRAP and DPPH scavenging activity values could be attributed to free sulfhydryl groups and amino acids of anchovy by-product protein such as tryptophan, methionine and tyrosine (*32*). Effect of thyme essential oil on the antioxidant capacity of chitosan films was discussed by Altiok *et al.* (*12*) and Bonilla *et al.* (*30*), by Espitia *et al.* (*33*) in edible açai films and Jouki *et al.* (*14*) in quince seed mucilage films. Similar results were also shown for hake protein films containing thyme essential oil (*2*). The antioxidant capacity of thyme essential oil may be explained by its thymol and carvacrol content, two phenolic compounds with known antioxidant capacity (*11*). The results indicated that anchovy by-product protein films containing thyme essential oil could be used for the packaging of foods as active films with antioxidant activity.

**Conclusions**

Addition of thyme essential oil affected the solubility, transparency, mechanical properties, water vapour permeability (WVP), oxygen permeability (OP), FTIR spectra, texture and thermal properties of the films. The addition of thyme essential oil into anchovy by-product protein films decreased the solubility and tensile strength values, but increased the elongation at break, WVP and OP values. The increase in WVP and OP values of the films might limit their application in foods. However, increasing the volume fraction of thyme essential oil did not lead to significant changes in these properties. Film containing 1.5 % thyme essential oil became more opaque
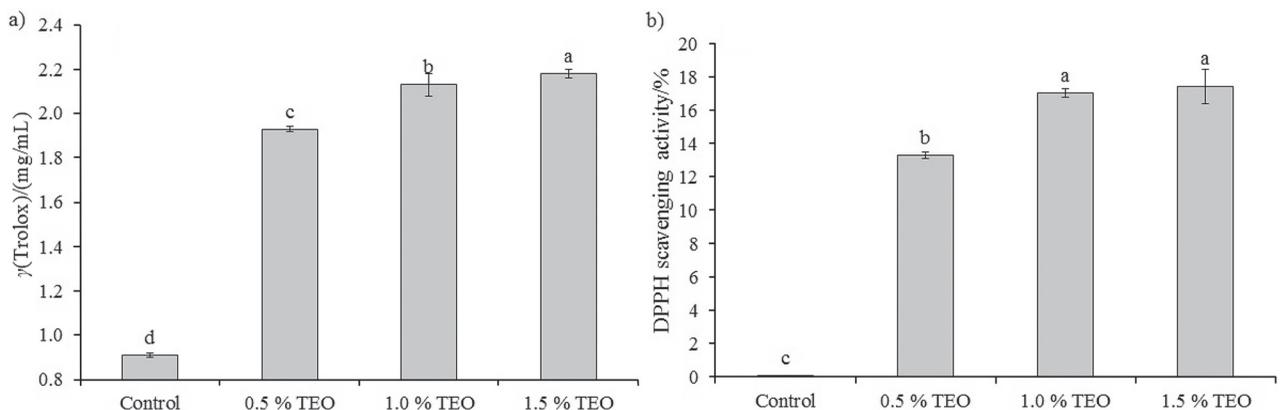


**Fig. 4.** Antioxidant capacity of anchovy by-product protein films containing thyme essential oil (TEO) measured by: a) FRAP as Trolox equivalents, and b) DPPH scavenging activity. Bars represent mean values±standard deviation of three replicates, different letters on the bars indicate significant differences (p<0.05)

than other films. The antioxidant capacity of anchovy by-product protein films was considerably improved by the addition of thyme essential oil. These results showed that the addition of thyme essential oil into the anchovy by-product protein films is suitable for developing antioxidant films. However, research on the assessment of the antimicrobial properties of these films is also required for their practical applicability.

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