Food Packaging and Shelf Life 24 (2020) 100478

Contents lists available at ScienceDirect

Food Packaging and Shelf Life

journal homepage: www.elsevier.com/locate/fpsl

Novel ABTS-dot-blot method for the assessment of antioxidant properties of food packaging

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ARTICLE INFO

Keywords:
Antioxidant activity
ABTS
Gelatin film
Cellulose film
Berry juices
Green tea

ABSTRACT

The new ABTS-dot-blot method for the direct determination of antioxidant activity of active packaging that is in contact with foodstuffs has been developed. The usefulness of the new method was verified with the use of agarose, pork gelatin, bacterial cellulose and cellulose-chitosan films with incorporated standard antioxidant – Trolox or plant phytochemicals derived from three types of berry juices (chokeberry, blue-berried honeysuckle, rowanberry) or green tea. The plant components used for preparation of films were characterized by antioxidant profiling with the use of HPLC coupled with post-column derivatization with ABTS. The most abundant antioxidants were polyphenols, mainly anthocyanins, hydroxycinnamates and flavan-3-ols. The antioxidant properties of different types of films studied were evaluated by typical cuvette spectrophotometric ABTS test or by the novel method employing ABTS radical stabilised in an agarose gel. The results obtained for the Trolox containing films showed quantitative linear relationship between antioxidant content and the degree of ABTS bleaching in agarose gel. The results of both standard spectrophotometric method as well as ABTS-dot-blot approach indicated that gelatin, bacterial cellulose and chitosan on their own possessed antioxidant activity, which was substantially increased (6–10 times) by the addition of phytochemicals during film preparation. The highest antioxidant activity was observed for microbial cellulose films, which were prepared on the basis of material obtained during the kombucha drink production.

1. Introduction

Oxidative processes may cause degradation of food proteins, lipids as well as pigments, which limits the shelf life of products containing them. Since antioxidants delay these deleterious effects, their incorporation into packaging materials has gained popularity (Suppakul, Miltz, Sonneveld, & Bigger, 2003; Yingyuad et al., 2006). Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been widely used in food packaging to prevent food oxidation. Due to safety concerns associated with synthetic redox active compounds, extensive research has been undertaken to find appropriate natural reducing compounds that could serve as the alternative to synthetic ones. Currently, there is growing interest in incorporating natural antioxidants such as tocopherols, polyphenols, plant extracts and essential oils to active packaging materials (Yildirim et al., 2018). The assessment of antioxidant activity of these different types of films was performed with the use of popular spectrophotometric tests using DPPH or ABTS radicals as indicators. However, the major obstacle in such measurements is difficulty with obtaining transparent reaction mixtures needed for absorption measurements. The films rather do not mix directly with the solutions of mentioned radicals, so in the first step, the antioxidants had to be extracted or dissolved in water or organic solvent (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013; Li, Miao, Wu, Chen, & Zhang, 2014; Pastor, Sánchez-González, Chiralt, Cháfer, & González-Martínez, 2013; Siripatrawan & Harte, 2010; Tongnuanchan, Benjakul, & Prodpran, 2013; Wu et al., 2013). After centrifugation, the collected supernatants were mixed in most cases with organic solvent solution of indicatory reagents. In such cases, the measured changes in indicator absorbance delievers the information on antioxidant properties of the extracts containing released from films redox active compounds. In this approach the information about antioxidant activity of film forming materials (*e.g.* gelatin, cellulose) is lost, because they are separated as solid residue during centrifugation of the extract. The determined values may depend also on solubility of reducing substances, their extraction yield, thus may be strongly influenced by the method of extract preparation. In addition, the use of organic solvent solution of radical reagents in final reaction mixture can cause precipitation of remains of

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https://doi.org/10.1016/j.fpsl.2020.100478
Received 5 April 2019; Received in revised form 3 January 2020; Accepted 29 January 2020
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the film forming material that impacts solution transparency and impairs absorption measurements.

These disadvantages of batch spectrophotometric methods prompted us to develop a procedure that bypasses film extraction step. Moreover, we attempted to mimic the processes in the contact area, where the antioxidant protection take place in real conditions. The aim of this work was to develop the alternative method, named here ABTS-dot-blot, which enables the assessment of antioxidant potential of packaging materials on the basis of observation of their interactions with the surface of medium simulating food product. In our method, as an indicator we used ABTS radicals stabilized in an agarose gel. If the tested material showing anti-radical properties is placed on such a gel, then its green colour will disappear quantitatively depending on the activity of the tested films. The concentration dependence in the proposed approach was initially examined with the use of films containing incorporated known amounts of Trolox. The applicability of this method for antioxidant activity determination was then checked for different types of films: pork gelatin films with the addition of rowanberry, blue-berried honeysuckle or chokeberry juices as a source of redox active phytochemicals and for cellulose–chitosan films enriched in green tea phytochemicals. The juices and green tea infusion used for the preparation of films were characterized on the basis of antioxidant profiles performed with the use of HPLC coupled with post-column derivatization with ABTS reagent. The results of proposed innovative ABTS-dot-blot method obtained for different types of films were compared with the results obtained by batch spectrophotometric ABTS test performed for film extracts.

2. Materials and methods

2.1. Materials

HPLC grade methanol and formic acid (98–100 %) were obtained from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and agarose were from Sigma-Aldrich (Saint Louis, MO, USA). The stock solution of ABTS was prepared in aqueous $Na_2S_2O_8$ solution (2.45 mmol/L) to reach a concentration of 7 mmol/L and left in the dark overnight at 21 °C. Our experience shows, that under such conditions, the concentration of ABTS radical reaches the maximum after 6 h and is stable for at least 2 days. *Gluconacetobacter xylinus* subsp. xylinus LOCK89 was purchased from Institute of Fermentation Technology and Microbiology (Lodz University of Technology) Culture Collection. Chitosan samples with 96.7 % deacetylation degree and molecular weight of 456 kDa were obtained from krill chitin in the Sea Fisheries Institute in Gdynia according to the method described by Kołodziejska, Wojtasz-Pająk, Ogonowska, and Sikorski (2000).

2.2. Plant material preparation

Chokeberry (*Aronia melanocarpa* (Michx.) *Elliott*), blue-berried honeysuckle (*Lonicera caerulea* L. var. *edulis*), and rowanberry (*Sorbus aucuparia* L.) originated from local plantations (Northern Poland). To obtain juices, the plant material was washed and placed in the juice extractor (Omega 8004, USA) operated according to the producer's manual. The green tea infusion was prepared by the addition of boiling water (1 L) to 6 g of commercially available green tea leaves (distributed by Posti, Poland). After 5 min, the green tea leaves were strained and the infusion was cooled for further use.

2.3. On-line profiling of antioxidants in fruit juices and green tea

To obtain profiles of antioxidants present in plant samples, the HPLC-DAD system (Agilent Technologies, Wilmington, DE, USA) was connected with a Pinnacle PCX Derivatization Instrument (Pickering Laboratories Inc., Mountain View, California, USA) and UV–VIS detector (Agilent Technologies, Wilmington, DE, USA). The conditions of chromatographic separation of bioactive compounds present in fruit juices were as follows: column Agilent Eclipse XDB (C-8, 4.6 × 150 mm; 3.5 µm); mobile phase: A - 4.8 % of formic acid, B - methanol; elution program: 0 min - 5 % B; 30 min - 50 % B; flow rate – 0.8 mL/min; injection volume - 4 µL. The post-column derivatization procedure was carried out according to Kusznierewicz, Piasek, Bartoszek, and Namieśnik (2011) and Kusznierewicz, Piasek, Bartoszek, and Namieśnik (2011). The groups of phytochemicals were identified on the basis of UV-VIS spectra and classified as ascorbic acid (AA), hydroxycinnamates (HCA), iridoids (IR), anthocyanins (AA), catechins (CT), flavonols (FV) and polar fractions (PF) embracing unidentified compounds. The antioxidant activity of identified groups of phytochemicals was quantitated as a sum of areas under the negative peaks recorded during analyses by HPLC with post-column derivatization with ABTS reagent.

2.4. Preparation of Trolox–agarose films

The reference films with known amount of standard antioxidant were prepared by combining Trolox solutions with agarose, which does not exhibit antioxidant activity. These films were produced by mixing 9 mL of hot (55 − 60 °C) agarose solution (30 mg/mL) with 1 mL of methanolic solution of Trolox (concentration range 0.2–1.2 mg/mL) and while still liquid poured onto Petri dishes (60 mm). After 24 h of drying at 21 °C, the solidified dry films with different contents of Trolox were collected and weighed.

2.5. Preparation of gelatin films

The pork gelatin solution was prepared at a concentration of 50 mg/mL. The 30 g of obtained solution was then mixed with 1.8 mL of juice from rowanberry (G + RB), blue-berried honeysuckle (G + BBH) or chokeberry (G + CHB). The solutions were poured onto square plastic plate (11.6 × 11.6 cm) to form a film about 3 mm thick and left to solidify. The control films (G) were prepared in the same way, but with the addition of water instead of juice.

2.6. Preparation of cellulose films

2.6.1. Preparation of chitosan and glycerol containing cellulose films

The broth containing glucose (10 g/L), yeast extract (5 g/L), peptone (5 g/L), Na_2HPO_4 (2.7 g/L), citric acid (1.15 g/L) was sterilized at 115 °C for 15 min and then ethanol was added to attain the concentration of 1 % (v/v). To 200 mL of broth, liquid culture of *G. xylinus* was added (200 µL weekly) and the culture was incubated at 28 °C for 7 days. During this time, a cellulose membrane was formed on the medium surface. To purify the membrane from microorganisms and residues of the medium, cellulose was washed out in tap water, boiled in 5 % NaOH solution for 1 h and rinsed again in tap water until neutralized. Purified material was immersed in distilled water, autoclaved at 121 °C for 20 min and stored refrigerated until use. Sterile cellulose was ground in MixSy® (Zepter International, Poland) grinder for 2 min, frozen at − 20 °C, then dried by sublimation (24 h). The dried cellulose was again ground for 2 min in MixSy®.

To prepare one-component solution (C), dried cellulose powder was suspended in deionized water to achieve the final concentration of 2 % (w/v). Cellulose-chitosan films (2:1 by weight) (C + Ch) were obtained by mixing the equal volumes of 2 % suspension of cellulose with 1 % suspension of chitosan suspended in deionized water adjusted to pH 6 using 0.5 M HCl solution. The resulting mixtures were homogenized for 3 min at 10000 rpm using Silent Crusher M D-91126 (Heidolph Instruments GMbH&Co, Schwabach, Germany). In all experiments, 23 g of suspensions were cast on Petri dishes 120 × 120 mm, then spread manually to the outside borders. The films were dried at room

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temperature for 24 − 48 h at 35–45 % relative humidity. Some films were additionally plasticized with glycerol at a concentration of 7 % (C + Ch + Gly1) or 16 % (C + Ch + Gly2) of the dry mass of films.

2.6.2. Preparation of green tea containing cellulose films

The cellulose containing green tea (C + GT) was obtained during the kombucha drink production process. The green tea infusion (1 L) was mixed with 35 g of sucrose and then 20 g of wet kombucha membrane from previous culture was added. Cellulose was formed during incubation for 10 days at room temperature. After this time cellulose membranes were collected, immersed in distilled water and autoclaved at 121 °C for 20 min. Sterile cellulose was air dried at room temperature.

2.7. Antioxidant activity of films by spectrophotometric method

The antioxidant activity of films studied was determined by spectrophotometric method with the use of ABTS radicals as described by Miller and Rice-Evans (1996) with slight modifications. The stock solution of ABTS (7 mM) was diluted before measurements with water to display the absorbance of 0.7 at 734 nm. The diluted ABTS solution (4.5 mL) was combined with pieces of films (10 mg). In the case of films containing green tea (C + GT), smaller portions of the samples were used (3 mg). After 20 min allowed for reaction, the mixtures were centrifuged at 4 °C for 10 min at 3000x*g* (Thermo Scientific Heraeus Megafuge, Karlsruhe, Germany). The final clear solutions were transferred to cuvettes and their absorbance was measured at 734 nm with the use of a spectrophotometer (Jenway, 6305, Essex, UK). The amount of ABTS radicals scavenged by 1 g of the tested films was calculated similarly as described in Baranowska et al. (2018) from the Beer–-Lambert–Bouguer Law (Beer's Law) according to the equation:

$$Sc_{ABTS} = \frac{V_{ABTS} \bullet (A_0 - A_f) \bullet 1000}{\varepsilon \bullet l \bullet m} \quad (1)$$

where Sc_{ABTS} is the amount of scavenged ABTS (μmol); A_0 is the initial absorbance of the ABTS solution; A_f is the absorbance of the radical solution after reaction time; ε is the molar extinction coefficient of the ABTS - 16,000 $M^{-1}\,cm^{-1}$ at 734 nm (Re et al., 1999); l is the cuvette optical path (1 cm) and m is the mass of tested film (g).

2.8. Antioxidant activity of films by novel ABTS-dot-blot method

2.8.1. Preparation of indicatory ABTS-gels

The hot solution of agarose (225 mL, 30 mg/mL) was mixed with ABTS aqueous solution (25 mL, 7 mM) and poured onto a rectangular plastic plate (11 × 20 cm). After 1 h, the gel was ready for experiments. To stabilize ABTS radicals, ABTS-gels were covered with plastic foil and stored in a dark place to minimize exposure on light and air. Under such conditions, the ABTS radicals were stable over 48 h at 21 °C, as indicated by the unchanged colour of ABTS-gels measured in ChemiDoc™ XRS + System (BioRad Richmond, CA, USA).

2.8.2. ABTS-dot-blot procedure

The circles (Ø 15 mm) were cut out from all studied films. The circles were flattened on the surface of ABTS-gel. After 20 min, the film circles were removed and gel was scanned in ChemiDoc™ XRS + System. The extent of ABTS-gel discolouring was analysed with Image Lab 4.0 software. During the test, it is recommended to avoid high temperatures (> 30 °C) and intense light. The antioxidant activity of films studied was expressed as amount of ABTS radical scavenged by 1 g of foil and calculated from the following equation:

$$Sc_{ABTS} = \frac{(CI_0 - CI_1) \bullet \pi \bullet r^2 \bullet h \bullet V_{ABTS} \bullet C_{ABTS}}{CI_0 \bullet V_{gel} \bullet m \bullet 10} \quad (2)$$

where Sc_{ABTS} is the amount of scavenged ABTS (μmol); CI_0 is the colour intensity of agarose ABTS-gel; CI_1 is the colour intensity of bleached spot after incubation with a circle of film whose antioxidant activity is to be determined; ($\pi\ r^2\ h$) is the volume of gel under the tested film where r is the radius of film circle (cm) and h is the thickness of ABTS gel (cm); V_{ABTS} is a volume of stock solution of ABTS added to the gel (mL); C_{ABTS} is the molar concentration of stock solution of ABTS (mM); V_{gel} is the volume of agarose gel with ABTS solution (mL); m is the mass of tested film (g).

2.9. Statistical analysis

All analyses were carried out in three parallel replications and mean ± SD (standard deviation) were calculated for the values obtained. Correlation between results of spectrophotometric and ABTS-dot-blot method was examined using Pearson's coefficient. Statistical differences between control films (without additives) and films with additives (fruit juices, green tea, chitosan) were tested using analysis of variance (ANOVA) with Dunnett's multiple comparison test (GraphPad Prism 8).

3. Results and discussion

3.1. Composition of antioxidants in plant samples

The characterisation of redox active phytochemicals in plant material used for preparation of films was very important for proper interpretation of final results and verification of reliability of the proposed new method. In this research, the composition of antioxidants in juices from berry fruits: blue-berried honeysuckle, rowanberry and chokeberry and also green tea infusion used for preparation of films was analyzed by HPLC post-column derivatization. In the upper panels of Fig. 1, the chromatograms of analytes detected at 270 nm in plant samples studied are presented. The chlorogenic acids and flavonols were dominating constituents in rowanberry juice, which is consistent with the data reported in literature (Gil-Izquierdo & Mellenthin, 2001; Kylli et al., 2010). In blue-berried honeysuckle and chokeberry juices, anthocyanins were the dominant phenolic components. The content of hydroxycinnamates in chokeberry juice was about 4 times higher than in blue-berried honeysuckle juice. In the case of flavonols, their amount was similar in the latter two juices. These data are in accordance with those published by Piasek et al. (2011). Similar profiles of polyphenols, but different contents of individual analytes were reported for extracts from pomace obtained after juice extraction from rowanberry, chokeberry and blue-berried honeysuckle (Staroszczyk et al., 2020). The chromatograms obtained for green tea infusion showed mostly the presence of flavonoids belonging to group of flavan-3-ols, as reported previously (Zuo, Chen, & Deng, 2002). Bottom panel of Fig. 1 presents chromatographic profiles of antioxidants obtained with chemical post-detection with the use of ABTS radicals and indicated which individual components were responsible for the antioxidative potential of plant samples and to what extent. The antioxidant activity of fruit juices and green tea samples was assessed as a sum of areas under negative peaks of identified groups of phytochemicals and was presented as a bar graph (Fig. 1). The highest antioxidant activity exhibited chokeberry juices where about 73 % of the calculated antioxidant activity was attributed to anthocyanins. This group of polyphenols represented also the major antioxidants in the case of blue-berried honeysuckle (60 %). The obtained results revealed that main contributions to the total antioxidant activity of rowanberry juice came from hydroxycinnamates and vitamin C (46 and 32 %, respectively). In the case of green tea infusion, its antioxidant activity resulted from the presence of catechins. The total antioxidant activity of extracts used to prepare films could be assembled in a series CHB > GT > RB > BBH.

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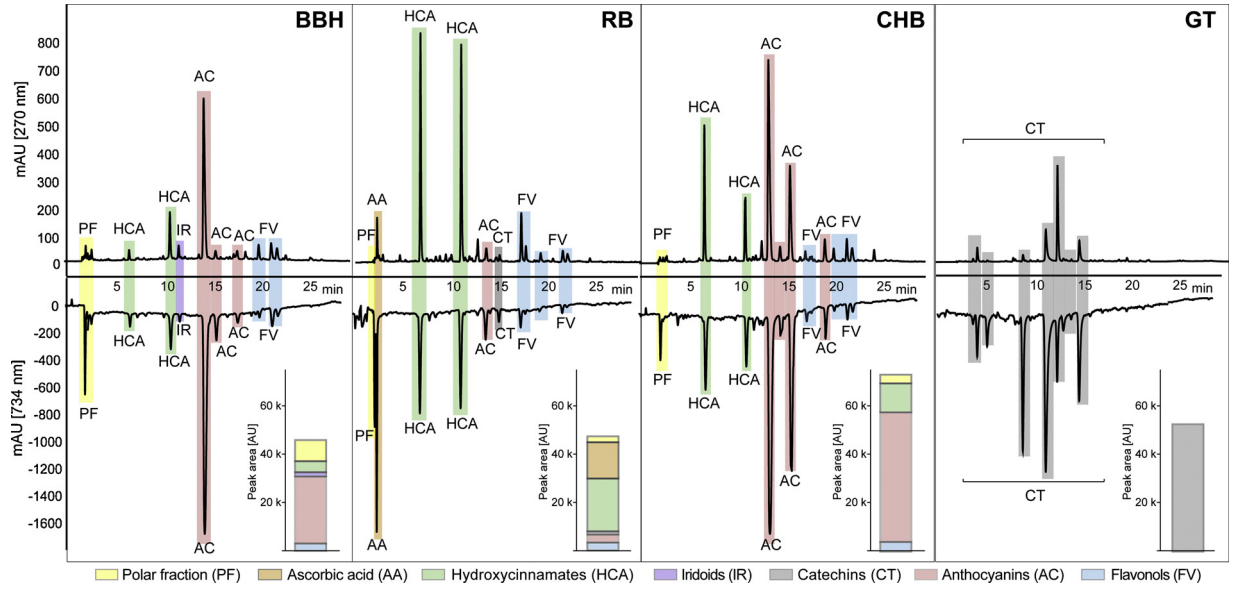

Fig. 1. Sample HPLC chromatograms of rowanberry juice (RB), blue-berried honeysuckle juice (BBH), chokeberry juice (CHB) and green tea infusion (GT) (top chromatograms at 270 nm) along with profiles of antioxidants detected online with ABTS reagent (bottom chromatograms at 734 nm). The groups of phytochemicals were identified on the basis of UV–vis spectra. The antioxidant activity of samples was assessed as a sum of areas under the negative peaks of identified groups of phytochemicals recorded during post-column derivatization with ABTS reagent and is presented as a bar graphs.

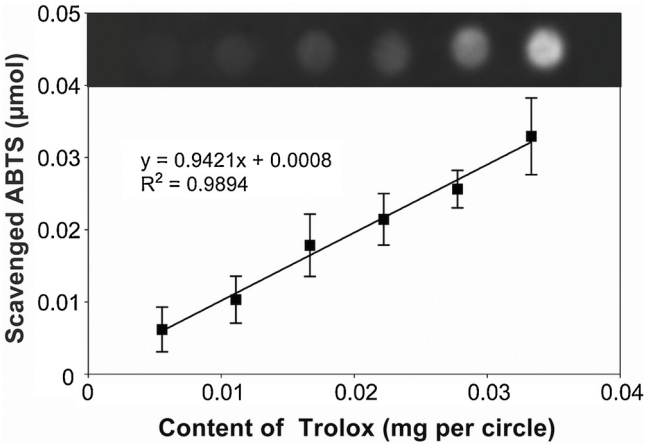

Fig. 2. The scan of agarose ABTS-gel with spots bleached by circles of standardized agarose films with different contents of Trolox (5–33 µg per circle) along with the calibration line generated on the basis of amount of ABTS scavenged (µmol) by these films in ABTS-dot-blot method. The linear regression was calculated for a mean y values derived from three independent repetitions.

3.2. Antioxidant activity of Trolox-agarose films

In this study, we assumed that there is possibility of determining the antioxidant activity of active packaging on the basis of their effects detected on the surface imitating the food product. The proposed ABTS-dot-blot method involved the use of medium, which could simulate the food product surface, while the presence of an indicator in this medium would make it possible to quantify the antioxidant barrier offered by the tested packaging. In any universal test, it is important that indicatory material does not contain any additional components that could interfere with the measured property (redox activity in this case), that it can be prepared in a reproducible way, and that it is not expensive. For these reasons, we have chosen combination of agarose gel not displaying redox activity *per se* and ABTS radical widely used for the detection of food antioxidants. Initially, at the stage of indicator selection, three reagents most popular in spectrophotometric methods were tested: ABTS, DPPH and Folin-Ciocalteu. It turned out, that only in the case of aqueous solution of ABTS radical, it was possible to obtain agar gel with the stable and homogenous colour. By selecting the right concentration of ABTS radical in agarose and the proper thickness of

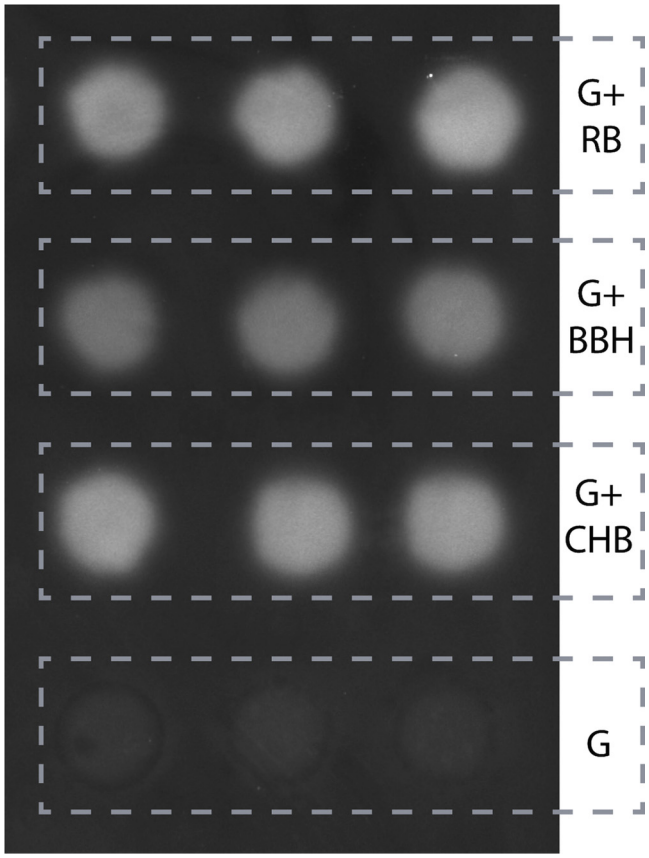

Fig. 3. The scan of agarose ABTS-gel with spots (triplicates) bleached by circles of gelatin control film (G), and gelatin films with addition of blue-berried honeysuckle (G + BBH), rowanberry (G + RB), or chokeberry (G + CHB) juices.

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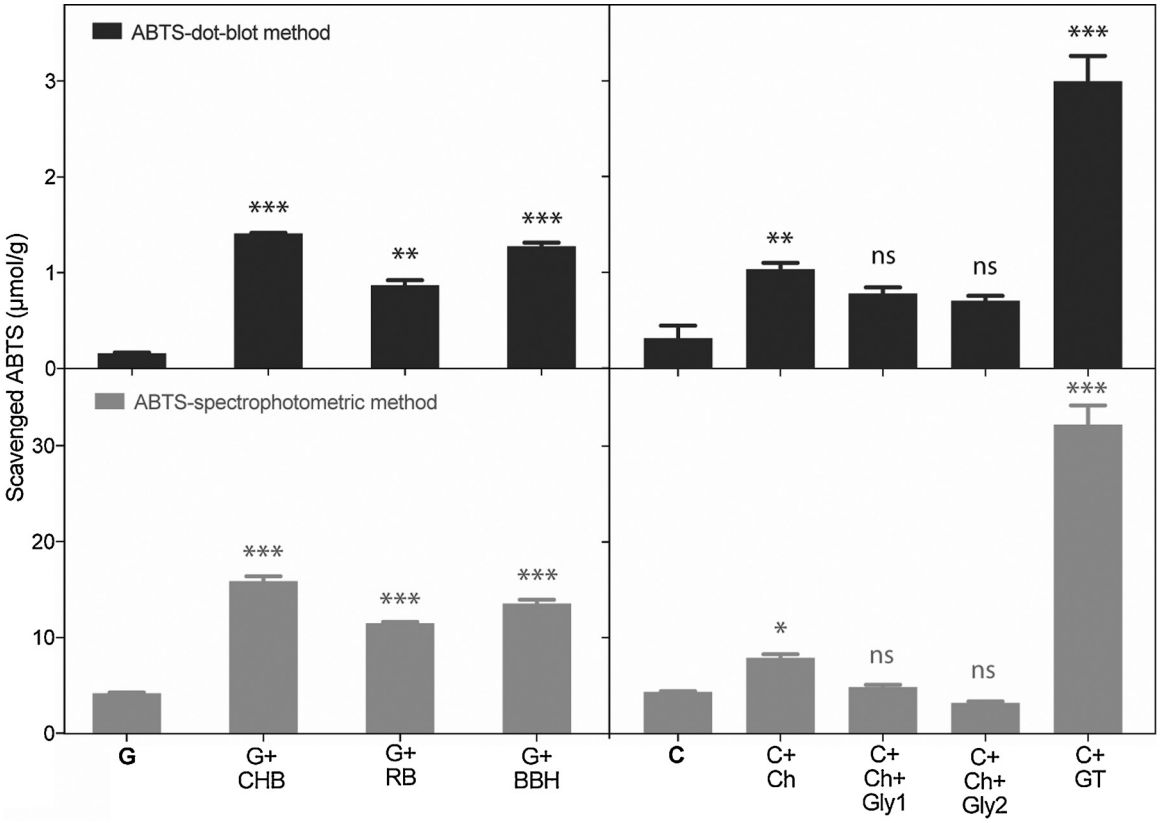

Fig. 4. The antioxidant activity of gelatin and microbial cellulose films determined by ABTS-dot-blot and ABTS-spectrophotometric methods. The film samples included: gelatin films without (G) and with addition of chokeberry (G + CHB), rowanberry (G + RB) and blue-berried honeysuckle (G + BBH) juices and microbial cellulose (C, 100 %), microbial cellulose with chitosan films (C + Ch, 67 %+33 %), microbial cellulose with chitosan and glycerol in two different concentrations (C + Ch + Gly1, 62 %+31 %+7 %), (C + Ch + Gly2, 56 %+28 %+16 %) and cellulose obtained during kombucha drink production with green tea (C + GT). Significantly different values between control films (without additives G and C) and films with additives determined by one-way analysis of variance (ANOVA) Dunnett's post hoc test are marked as * $p < 0.033$, ** $p < 0.002$, *** $p < 0.001$.

the gel, it is possible to adjust the test conditions suitable for individual films exhibiting different ranges of antioxidant activity. The materials with expected high antioxidant activity should be tested on quite thick agarose ABTS-gels with high concentration of ABTS radical, which ensures that after incubation with the tested sample, the obtained spot is not bleached in 100 %. In the case of presented experiments, the agarose ABTS-gels had the area of 11 × 20 cm and thickness of about 1 cm. The first step in the development of the method was to check if ABTS radicals stabilized in the agarose gel are bleaching proportionally to the concentration of antioxidants present in the films tested. For this purpose, the standardized films containing known amount of standard antioxidant – Trolox – were prepared. The agarose again was chosen as a material for standardized film preparation to make sure, that only Trolox caused bleaching of agarose ABTS-gel, not any other compound used for film forming. The dried Trolox-agarose films with different amounts of Trolox were used for testing the dose response relationship between bleaching of ABTS embedded in agarose gel and the concentration of antioxidant (Trolox) present in standardized films. The circles cut out from the standardized films were placed onto the surface of agarose ABTS-gel. After incubation, the circles of films were removed and agarose ABTS-gel was scanned and analysed by the intensity of colour of bleached spots. In Fig. 2, a sample picture of results of ABTS-dot-blot method for standardized films with different concentration of Trolox is presented. The linear dependence of antioxidant content on green colour intensity of ABTS-gel (calculated as amount of scavenged ABTS radicals) was observed that enabled the generation of calibration line. Fig. 2 presents the standard line based on quantitation of agarose ABTS-gel bleaching by Trolox-agarose films in ABTS-dot-blot method for three independent repetitions.

3.3. Antioxidant activity of gelatin films

For the determination of antioxidant activity of gelatin films with and without addition of berry juices, two methods were employed. First one was based on standard spectrophotometric test with the use of ABTS radical reagent and the other was the newly developed ABTS-dot-blot method. To properly compare the results of both methods, the decrease in absorbance in the spectrophotometric method and the decrease in the intensity of the gel colour in the ABTS-dot-blot method were recalculated into the amount of ABTS scavenged by the gram of the film tested. For this purpose, the Eq. (1) was used in the case of spectrophotometric method and Eq. (2) in the case of ABTS-dot-blot method. In Fig. 3, the sample picture of results of ABTS-dot-blot method obtained for the gelatin films is presented. For this method the gelatin films without additives and enriched with the berry juices scavenged ABTS radicals in the range of 0.16–1.4 (µmol/g) (Fig. 4). The lowest antioxidant activity exhibited the gelatin films without any additive – control films (G). Most of the studies concerning gelatin-derived peptides in the area of food science and technology have dealt with their antioxidant activity. These peptides have repeated unique Gly-Pro-Hyp sequences in their structure, and the observed antioxidative properties have presumably been associated with this unique amino acid composition (Kim & Mendis, 2006). The addition of berry juices to the film forming solution caused about 5–9 times increase of antioxidant activity of gelatin films. The differences between antioxidant activities of films containing any of three kinds of juices did not exceed 38 %. The highest antioxidant activity was detected for films enriched with chokeberry juice, and then in order were films combined with blue-berried honeysuckle juice and rowanberry juice. In the case of total antioxidant

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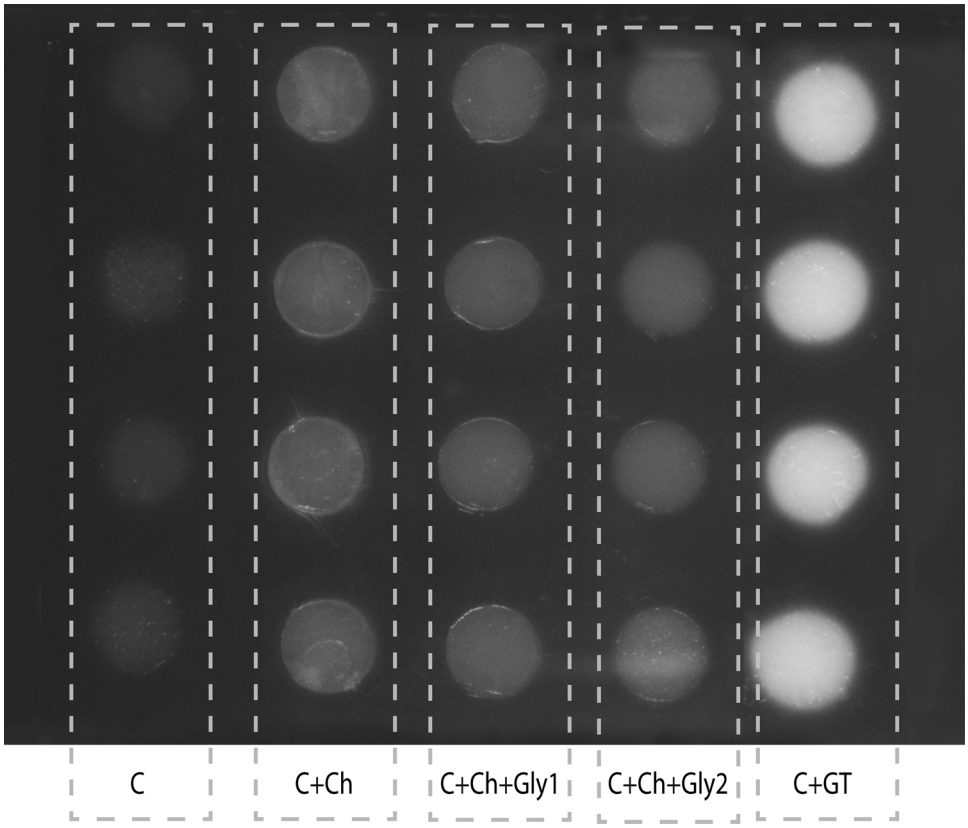

Fig. 5. The scan of agarose ABTS-gel with spots (four repetition) bleached by circles of microbial cellulose films (C, 100 %), microbial cellulose with chitosan films (C + Ch, 67 % +33 %), microbial cellulose with chitosan and glycerol in two different concentration (C + Ch + Gly1, 62 %+31 %+7 %) (C + Ch + Gly2, 56 %+28 %+16 %) and cellulose obtained during kombucha drink production with green tea (C + GT).

activities of pure juices, the highest value was determined also for chokeberry, however juices form rowanberry and blue-berried honeysuckle displayed similar antioxidant activity (Fig. 1, bar graphs). The lowest antioxidant activity of films with rowanberry juice could results from the degradation during film formation of unstable vitamin C, which accounted for 32 % of total antioxidant activity of added juice (Fig. 1). The presented results confirm that fruit juices can be valuable natural antioxidant components of functional films. Some of these incorporated phytochemicals also affected the colour of the gelatin films, especially when high concentration of anthocyanins was present in fruit juice. The use of concentrated plant extracts or preparations can result in much higher increases of antioxidant activity of films than these reported here (Li et al., 2014; Pires et al., 2013; Tongnuanchan, Benjakul, & Prodpran, 2012, 2013). In such cases, the ABTS-dot-blot method may need some optimisation relying in the selection of proper ABTS concentration and/or thickness of tester gel or time of its incubation with tested food packaging.

The antioxidant activity of gelatin films studied was also determined by standard spectrophotometric test with ABTS reagent. In this case, the small pieces of films were submerged directly in the ABTS aqueous solution. After incubation, the mixture was centrifuged and absorption of clear supernatant was measured in a cuvette with the use of spectrophotometer. The results calculated also as the amount of scavenged ABTS by 1 g of films from Eq. (1) are presented in Fig. 4. The levels of antioxidant activities determined in spectrophotometric method represent the same trend as in the case of ABTS-dot-blot method (Pearson coefficient, r = 0.99), however the obtained in spectrophotometric test values were about 10 times higher. This is due to the obvious fact that more antioxidants have passed from the film to the aqueous ABTS solution due to better penetration of the film matrix by the solvent. In the case of ABTS-dot-blot method, only the interaction of antioxidants from the surface of films with ABTS incorporated to the gel within the contact surface of both materials and resulting from diffusion is observed.

3.4. Antioxidant activity of microbial cellulose films

The new ABTS-dot-blot method as well as standard spectrophotometric test were performed also for different kinds of films prepared on the basis of microbial cellulose.

In Fig. 5, the scan of agarose ABTS-gel with spots bleached by circles of different samples of microbial cellulose films is presented. The quantitative results of antioxidant activities of microbial cellulose samples studied are shown in Fig. 4. The lowest, but detectable antioxidant activity was observed for films containing only microbial cellulose. According to Wiegand, Elsner, Hipler, and Klemm (2006) such a kind of material possesses radical scavenging properties which can be increased for example by incorporation of collagen. In our study, the addition of chitosan to cellulose in 1:2 (w/w) ratio resulted in about 2–3 times higher antioxidant properties of obtained films. The scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH_2) groups to form stable macromolecule radicals, and the NH_2 groups can form ammonium (NH_3^+) groups by absorbing a hydrogen ion from the solution (Siripatrawan & Harte, 2010). The addition of glycerol to the cellulose-chitosan films caused the dilution of redox active component, so the antioxidant activity of films decreased with the increasing content of this plastificator. In the case of film containing green tea, which was prepared during the kombucha drink production, the ABTS scavenging effect was the highest. According to Srihari and Satyanarayana (2012), antioxidant activity of kombucha drink increases during fermentation. Unfortunately, the antioxidant activity of cellulose obtained during fermentation of kombucha (C + GT) has been rather rarely determined. In our study, such a type of microbial cellulose possessed about 8–10 times higher antioxidant activity than cellulose produced in a typical growth medium (C). The results obtained with the use of spectrophotometric test indicated the same tendency as ABTS-dot-blot method (Pearson coefficient, r = 0.98) and similarly to gelatin films, in the case of spectrophotometric method, we observed higher amount of

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scavenged ABTS radicals by films.

4. Conclusions

The presented innovative ABTS-dot-blot method as well as standard spectrophotometric ABTS test indicated that the combination of plant antioxidants with different natural polymers for production of films enhanced their antioxidant properties. The final properties of resulting films depended on the content and composition of reducing phytochemicals present in plant samples studied. The newly developed method based on the interaction of antioxidants present in packaging material and ABTS radicals stabilised in agarose gel seems to be a better alternative to typical spectrophotometric tests. Such an approach more reliably reflects the behaviour of film embedded antioxidants as protectants against oxidation of foods than spectrophotometric tests where films are placed in the reaction mixtures often with organic solvents. In the case of spectrophotometric methods, the obtained result may be overestimated due to the high degree of penetration of the entire volume of the tested film matrix by the solvent and thus higher efficiency of extraction of antioxidants into the solution. In the case of ABTS-dot-blot method, only the interactions between the tested material and the contact surface of matrix imitating food are observed. As in the spectrophotometric methods, also in the case of ABTS-dot-blot method, it is required to adjust the experimental conditions to the reducing activity of samples tested. In spectrophotometric methods, the appropriate concentration of radical and/or sample is determined so as to make measurements in the range of the linear response of the method. In the case of the ABTS-dot-blot method, depending on the activity of the tested materials, the appropriate concentration of the radical in the gel or/and the thickness of this gel should be adjusted. The performance of ABTS-dot-blot method requires neither sophisticated equipment nor expensive chemicals. It is easy to use and is very liable to optimisation depending on specific needs. Measurements of the colour intensity of the gel with ABTS can be made using both conventional densitometers and simple digital colorimeters available as standard computer software. The proposed ABTS-dot-blot method has great potential as a method of measuring the antioxidant activity of packaging materials and can also be helpful in determination of diffusion profile of active substances.

CRediT authorship contribution statement

Barbara Kusznierewicz: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Data curation, Writing - original draft, Project administration. **Hanna Staroszczyk:** Investigation, Resources. **Edyta Malinowska-Pańczyk:** Investigation, Resources. **Karol Parchem:** Investigation. **Agnieszka Bartoszek:** Writing - review & editing.

Acknowledgments

This work was supported by National Science Centre (Poland) in a programme MAESTRO 6 (application number 2014/14/A/ST4/00640) and under grant number NN 312255638.

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