Research Article

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Estimation of the total antioxidant potential in the meat samples using thin-layer chromatography

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Abstract: There is limited literature on the antioxidative properties of food of animal origin. Measurements of antioxidative properties are usually performed using the reaction of reduction of colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) radials. Changes of the DPPH color are tracked photometrically. These measurements are interfered by both, the tested samples and reduced DPPH. This study aims to demonstrate the ability to separate different forms of DPPH (DPPH$^{\bullet}$ and DPPH-H) by thin-layer chromatography (TLC). Further, it has been practically applied in the study of the determination of antioxidative properties of the meat samples. It was found that TLC can be used for the separation of different forms of DPPH as well as for measurement of TAP (total antioxidant potential) values related to the DPPH$^{\bullet}$. The strongest antioxidant properties were observed for pork neck extracted in buffer pH 2 and for smoked salmon fish extracted in acetone, the lowest for veal and turkey fillet extracted in methanol.

Keywords: thin-layer chromatography, meat, antioxidants, total antioxidant potential, DPPH

1 Introduction

Free radicals are molecules, ions, atoms or groups of atoms having, on the valence shell (or orbital) one, or more, unpaired electron, giving them paramagnetic properties. Often they are highly reactive, unstable and undergoing oxidation and/or reduction reactions. They impair the structure of cell membranes, lipids, proteins and nucleic acids in the cells. As a result, they can cause damage of the vital functions of cells, tissues of the body and the whole body [1].

Living organisms developed many defense mechanisms to protect themselves against free radicals. The defense system of living organisms against free radicals can be divided into three stages: (i) preventing the generation of free radicals (*e.g.*, chelators of transition metals), (ii) removing radicals (antioxidants or scavengers of the free radicals) or (iii) repairing system of damages [2]. According to [3] antioxidant is "any substance that delays, prevents, or removes oxidative damage to a target molecule". Maintaining a balance between free radicals and antioxidants is essential to maintaining the health.

In the literature, it is observed that the increased interest in compounds (antioxidants) intended to prevent the harmful effects of free radicals on the human body. The current concern is focused on the vegetables, fruits, herbs, spices and honey [4]. There is very little information available on the antioxidative properties of food of animal origin. It should be noted here that after slaughter, a number of uncontrolled chemical reactions (acidification, oxidation, etc.) run in the meat. Next, during the meat preparation for the human consumption, changes in muscle fibers and connective tissue microstructure cause changes in the meat antioxidative properties [5].

Meat is essential for the proper functioning of the human body. Meat from the slaughtered animals contains healthy proteins and essential amino acids (needed to build tissues that the human body cannot create itself). From a nutritional point of view, meat is also a source of minerals [6]. It contains a lot of antioxidants, such as carotenoids (β-carotene, zeaxanthin, lutein), the unsaturated aliphatic compounds (characterized by orange-pink color) and vitamins A and E [7]. Vitamin E (α-tocopherol) is the most active lipophilic antioxidant in the human body. It is soluble in fats and most of the organic solvents. Carnosine is a basic dipeptide and it is also the major non-protein nitrogen-containing compound in the skeletal muscles of vertebrates [8]. It is soluble in water and can catalyze lipid

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oxidation by free radicals. Its concentration depends on age, race, gender, and color of meat. Vitamin C (ascorbic acid) is involved in the metabolism and biochemical reactions, like a synthesis of hormones or biosynthesis of collagen. It forms an oxidation-reduction buffer of blood plasma and forms the first line of defense against free radicals in the cytoplasm. Glutathione is a tripeptide consisting of glycine, cysteine, and glutamate. Inside the living organisms, it is available in several forms - reduced (GSH), oxidized (GSSG), S-nitrosoglutathione (GSNO) and the mixture of glutathione disulfide and proteins [9]. It is the most important part of the antioxidant system, maintaining the balance between free radicals and antioxidants. Heat treatment, such as cooking, significantly reduces the concentration of antioxidants [10, 11].

DPPH$^{\bullet}$ is a free radical and a scavenger for other radicals. It can be either reduced or oxidized. Reactions on the surface of the electrode in the first stage lead to the formation of an anion DPPH^{-} or cation DPPH^{+}. In the solution, it can be oxidized by strong oxidants, such as hydroxyl radicals, or reduced by antioxidants. Its reduction is, usually, based on the acceptance of a hydrogen atom. It may be a multi-stage reaction. In such case, the electron will be first accepted into the DPPH$^{\bullet}$ molecule, followed by the proton, or vice versa. These reactions strongly depend on the environment in which they run, as it was discussed in detail in the paper [12].

The DPPH$^{\bullet}$ are used as (i) an external standard of electron paramagnetic resonance (EPR) and (ii) to determine sample cleavage [13]. During reduction they change the color. They are used to the photometric or chromatographic estimation of the total antioxidant potential (TAP). Therefore, they are also used to determine total antioxidant potential (TAP) by photometric method [14, 15]. In our previous paper, we have used the TLC for the determination of the oxidized form of DPPH [16]. In this case, the TAP measure was the change of absorbance of the DPPH$^{\bullet}$ spot which is correlated to the change of the DPPH$^{\bullet}$ concentration after its reaction with antioxidants. It is worth to note that previously DPPH$^{\bullet}$ was used to visualize spots on the TLC plate (yellow spots on a purple background) [17].

Measurements of antioxidative properties are usually based on the reduction of colored DPPH$^{\bullet}$. Changes of the DPPH$^{\bullet}$ color are observed photometrically. The TAP measure is the decrease of absorption at λ = 517 nm, after the reaction between radicals and sample. These measurements are interfered by both, the tested samples and reduced DPPH.

The aim of this study was to (i) develop an assay for determining total antioxidant potential [4, 17, 18] related to the DPPH$^{\bullet}$ and (ii) evaluation of antioxidant properties of different kinds of meat. This includes (i) the separation of different forms of DPPH$^{\bullet}$ using TLC; (ii) examine the possibility of determining the TAP by TLC (the TAP measure was the reduction in the absorption at λ = 517 nm after the DPPH$^{\bullet}$ reaction with the sample) and (iii) the use of the developed method for the determination of TAPs of meat products.

The aim of this study was to improve the assay for TAP [4, 16, 18, 19] related to the DPPH$^{\bullet}$. Conditions for the separation of various forms of DPPH were developed. The effect of mixtures of various solvents and the values of their Hildebrand coefficients on the selectivity of the chromatographic system was investigated. The elaborated method was practically used to evaluate the antioxidant properties of various meat species.

2 Materials and methods

2.1 Instrumentation

Samples were prepared using ultrasonic bath UM-0,5 (Unitra-Unima, Olsztyn, Poland), and centrifuge MPW-251 (MPW Med. Instruments, Warsaw, Poland). Measurements were performed using the DS-II-5x10 or DS-II-20x20 (Chromdes, Lublin, Poland) TLC chambers and Helios Epsilon spectrophotometer (Thermo Spectronic, Rochester, NY, USA). Samples were separated on 5 cm x 10 cm commercial glass plates precoated with 0.25 mm layers of silica gel 60 or silica gel 60 F254 and RP-18W modified silica gel coated with fluorescent indicator F254 plates (Merck, Darmstadt, Germany). The chromatograms were scanned using HP Scanjet G210, with software Scion Image and ImageJ [16].

2.2 Reagents

Gallic acid, 96.6% ethanol, 2-propanol, disodium hydrogen phosphate, dihydrogen sodium phosphate (Chempur, Piekary Śląskie, Poland), HCl, NaOH (POCh, Gliwice, Poland), acetone, n-hexane, methanol, DPPH - 2,2-diphenyl-1-picrylhydrazyl, acetonitrile (Sigma-Aldrich, St. Louis, MO, USA), acetone (StanLab, Lublin, Poland) were of analytical-reagent grade and were used without further purification. Water was three times distilled in a

quartz apparatus. Solvents were filtered through a (0.22 μm) membrane filter (Millipore, Bedford, USA).

2.3 Samples

The research material consisted of two types of poultry (turkey and chicken fillets), beef (sirloin, veal) and pork (neck, shoulder) meat sample and smoked salmon meat. The meat was bought from the butcher shop at Siedlce (Poland).

2.4 Raw samples preparation

Extracts of the meat at a concentration of 0.5 g/mL were prepared by dissolving 2 g of thawed, minced meat (60 seconds milling) in 4 mL of solvent (methanol, ethanol, hexane, acetone, acetonitrile, water or 0.01 M HCl, pH 2). The meat extracts were placed in an ultrasonic bath, filtered and centrifuged (3 min; 18000 r/min). Such prepared test material was stored tightly in aluminum foil in the refrigerator at a temperature of +5°C.

2.5 TAP measurements

The sample solution (200 mL) was mixed with 100 mL of 1 mM DPPH• in methanol. The mixture was shaken vigorously and left for 5 min at the room temperature in the dark. It turned out that absorbance decreased for five minutes reaching an asymptote. For chromatographic separation 5 μL volume of the sample solutions were applied to the plates as spots with a syringe (Hamilton, Bonaduz, Switzerland). The normal (NP) and reversed (RP-18W) plates were developed after saturation of the tanks with mobile phase vapor for 0.5 h. A TAP of meat samples was determined by measuring the intensity of the purple spots of DPPH•, which turns to the light yellow of DPPH-H. After separation, the plates were dried at ambient temperature for 0.5 h and the surface of each plate was scanned in 8-bit resolution RGB color mode, in the direction of chromatogram development, by means of an HP Scanjet G2410 scanner equipped with Windows-compatible Scion Image software.

As a preliminary study, a method of separation of reduced and oxidized forms of DPPH was elaborated. The influence of the chromatographic conditions for separation (chamber saturated or unsaturated, normal (NP) or reversed (RP-18W) phase systems, the effect of temperature, mobile phase composition, and multiple development techniques) were investigated [16]. The developed method was used to determine the TAPs of meats. Unless it will be specified otherwise the measurements were performed on the NP or RP-18W plates at 20°C, in a saturated chamber, using the mobile phase hexane/acetone (4:1, *v/v*). A solution of DPPH• in methanol was used as a control sample. The chromatograms were scanned at a resolution of 300 dpi, 24 million colors mode and stored in TIFF formats.

The results are expressed as gallic acid equivalent (GAE) [mg of gallic acid on g of sample]:

$$TAP[GAE] = \frac{\alpha_{GA}}{\alpha_i}$$

where: α_{GA} and α_i denote slopes of the calibration curves of gallic acid or investigated sample, respectively.

2.6 Statistical analysis

The measurements of the antioxidant capacity were repeated three times for each sample and the results were averaged and expressed relative to the average result for the control samples containing no analyte. The significance of differences between means was determined by Student's t-test at $p < 0.05$. In all cases, the percent relative standard deviation (RSD) did not exceed 10%.

3 Results and discussion

At the beginning, conditions for the chromatographic separation of various forms of DPPH were developed. The dependence of the inverse of retardation factor, R_f, of DPPH• on the inverse of the acetone concentration is shown in Figure 1 and the dependence of the retardation factor on the Hildenbrand parameter in Figure 2. In the first case, a linear correlation, in the second one the inverse proportionality was found. The dependence of the ratio (as well as the difference) of the retardation factors of DPPH• and DPPH-H on the Hildebrand parameter is presented in Figure 3. In both, normal and reversed phase, no elution of DPPH• was observed in hexane. From the other side, the maximal elution (no retention) ($R_f \approx 1$) was observed in the pure polar solvents (methanol, acetonitrile, acetone, dichloromethane or 2-propanol). Water could not be used as the mobile phase due to the insolubility of the DPPH•. An exemplary TLC plate is shown in Figure 4.

The antioxidant properties (TAP) of meat are caused by the presence of various compounds in the sample, i.e., enzymatic antioxidants as well as small-molecule ones,

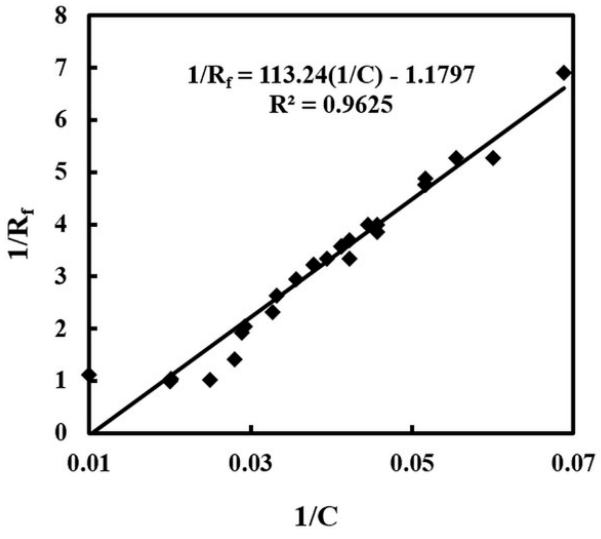

Figure 1: Dependence of the inverse of retardation factor of DPPH•, $1/R_f$, on the inverse of the concentration of acetone, 1/C. Chromatographic conditions: NP plate, temp. +20°C, saturated chamber.

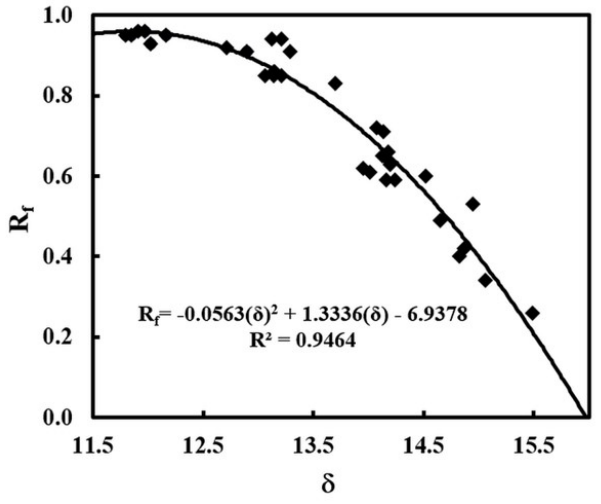

Figure 2: Dependence of the retardation factor, R_f, on the Hildenbrand parameter, δ. Tested mixtures - methanol/water, acetonitrile/water, acetonitrile/methanol, hexane/acetone, hexane/acetone/ethanol. Chromatographic conditions: RP-18W plate, temp. +20°C, saturated chamber.

soluble in water or in lipids. Therefore, it is impossible to obtain complete information about the sample antioxidant activity just using one extraction solvent. The TAP value depends on extraction conditions and type of meat. The TAP values of various meats after extraction using different solvents are presented in Figure 5, as a function of the time of extraction are presented in Figure 6.

The retardation factor, R_f, depends on the mobile phase, especially on the used solvent. If, as a mobile phase the mixture of two solvents is used, then R_f depends on their relative concentrations. It was found that there is a linear correlation between $1/R_f$ of DPPH• and inverse of the concentration of less polar solvent in the mobile phase, as it is presented for the normal phase system in Figure 1. However, concentration as the measure of the mobile phase polarity can be easily used only in binary systems. When the mobile phase consists of more than two components or if we want to compare the different phases then it should be used an universal parameter describing the polarity of the mobile phase. We found that the retention of DPPH• was strongly dependent on the δ - Hildebrand polarity parameter (Figure 2). In the reversed phase system (e.g. using the RP-18W plates) Rf increased with the decrease of the δ parameter. As the mobile phases the mixtures of methanol/water, acetonitrile/water, acetonitrile/methanol, hexane/acetone and hexane/acetone/ethanol were investigated. It turned out that, in this case, the Hildebrand parameter is a good measure of the polarity of the solvent mixtures as well as retardation factor. The best separation was obtained at the average values of the Hildebrand coefficients (Figure 3).

For the binary mixtures, such as methanol/water (5 to 20% of water) a slight decrease of the retardation factor (from 0.9 to 0.67) was observed with the increase of water concentration. It means that the retardation factor decreases with the increase of the concentration of more polar solvent. The addition of water caused a slight separation of the two forms of DPPH from each other. However, the better separation was achieved using a mixture of hexane and acetone. Spots on the plates RP-18W are narrower and less pronounced than on NP ones (Figure 4). In addition, to the spots originating from the oxidized and reduced forms of DPPH there are other spots (pink and brown). Multiple developing does not improve the separation.

The TAP values depend on the type of meat and its preparation (extraction conditions). Among them, the samples extracted in methanol (Figure 5), are characterized by the highest total antioxidant activities. They are in the range of 52.43 GAE for smoked salmon and 2.57 GAE obtained for pork. It was found that the concentration of polyphenols is not correlated with the TAP^{DPPH} values of meat extracted in methanol. The TAP values of water extracts ranged from 31.24 GAE for chicken fillet to 23.52 GAE of turkey fillet.

It turned out that the results obtained with TLC are, on average, 1.8 times higher than those obtained with the classical photometric technique. For example, the

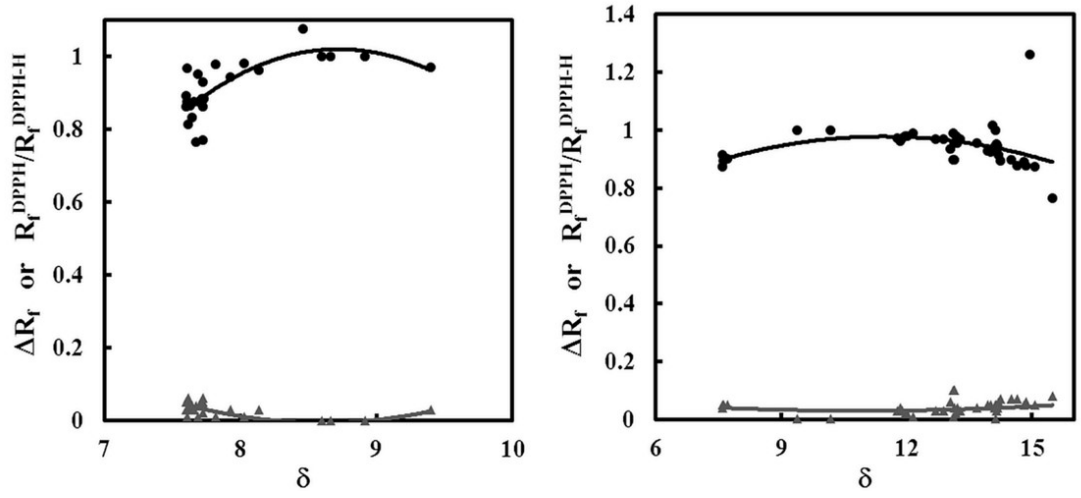

Figure 3: Dependence of the ratio (●, black line) and the difference (▲, gray line) of $R_f^{DPPH\bullet}$ and $R_f^{DPPH\text{-}H}$ on the Hildebrand parameter, δ. Chromatographic conditions: NP plate (left picture), RP (right), temp. +20°C, saturated chamber.

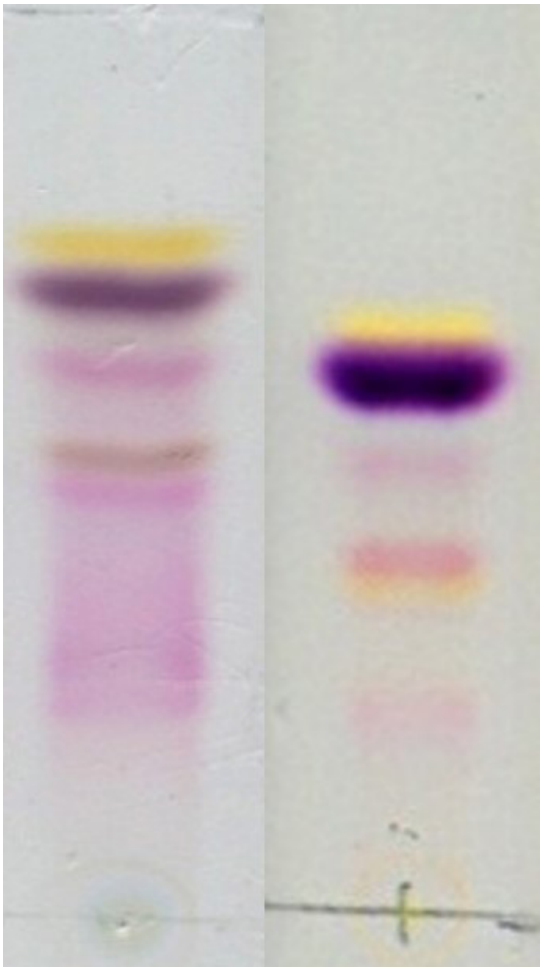

Figure 4: Separation of DPPH• (lower, purple spot) from DPPH-H (upper, yellow spot). Chromatographic conditions: plate – RP-18W (left), NP (right), temp. +20°C; mobile phase - hexane/acetone (20:80% *v/v*); chamber – saturated.

TAP [GAE] values for chicken fillet were equal 21,43 and 11,56 using TLC and photometric methods, respectively. The corresponding results for the pork neck were 12.62 and 6.96, respectively. This is due to the fact that TAP is directly proportional to the difference in spot surface area of DPPH• recorded without or with the sample. Smaller disturbances caused by the sample and the reduced form of the radicals increase the TAP values. This proves that the use of TLC is more advantageous than the photometric method.

For various meats (Figure 6A) as well as for various solvents (Figure 6B) it was found that the TAPs go through a maximum in a function of time of extraction. Initially (approx. until 5 min) with the increase of extraction time it was observed an increase of the TAP values (Figure 6). Extraction time increased the amount of leached compounds. However, further prolongation of the extraction decreases TAPs due to the oxidation of antioxidants, for example by oxygen. It turned out that similar dependencies were observed for different types of meat (Figure 6A) and various solvents used for extraction (Figure 6B). At the optimal time of extraction (5 min), the use of methanol as a solvent is preferred. It is worth to note that the antioxidative activity of meat depends also on the chemical reactions during processing and storage of meat, as well as the additives used. They cause the formation of MRPs (Maillard Reaction Products), that is the products of Maillard reaction - the reaction between amino acids and sugars. Approximately in 5 min, there is an increase antioxidant activity of certain proteins due to changes occurring in their second, third and fourth-row structure [20, 21]. Too slow freezing/thawing of meat may cause

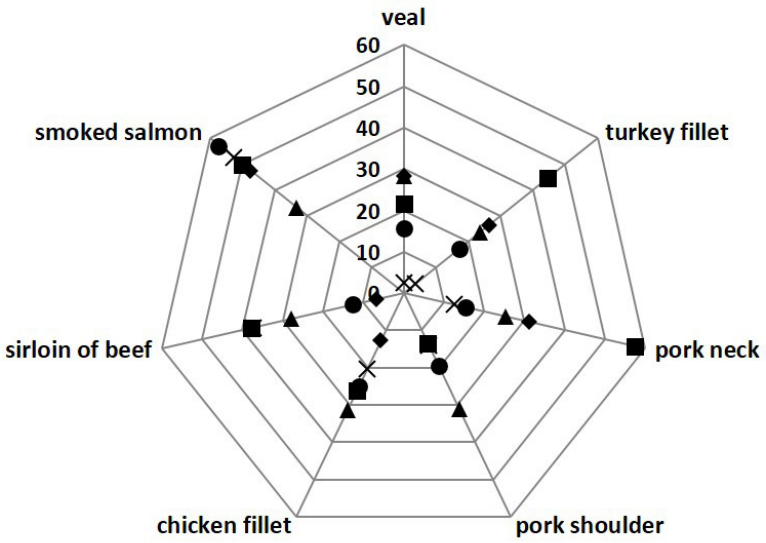

Figure 5: TAP values (GAE) of various meats (500 mg/mL) after extraction using different solvents (**x** - methanol, ▲ - water, ■ - buffer pH 2, ♦ - hexane, ● - acetone).

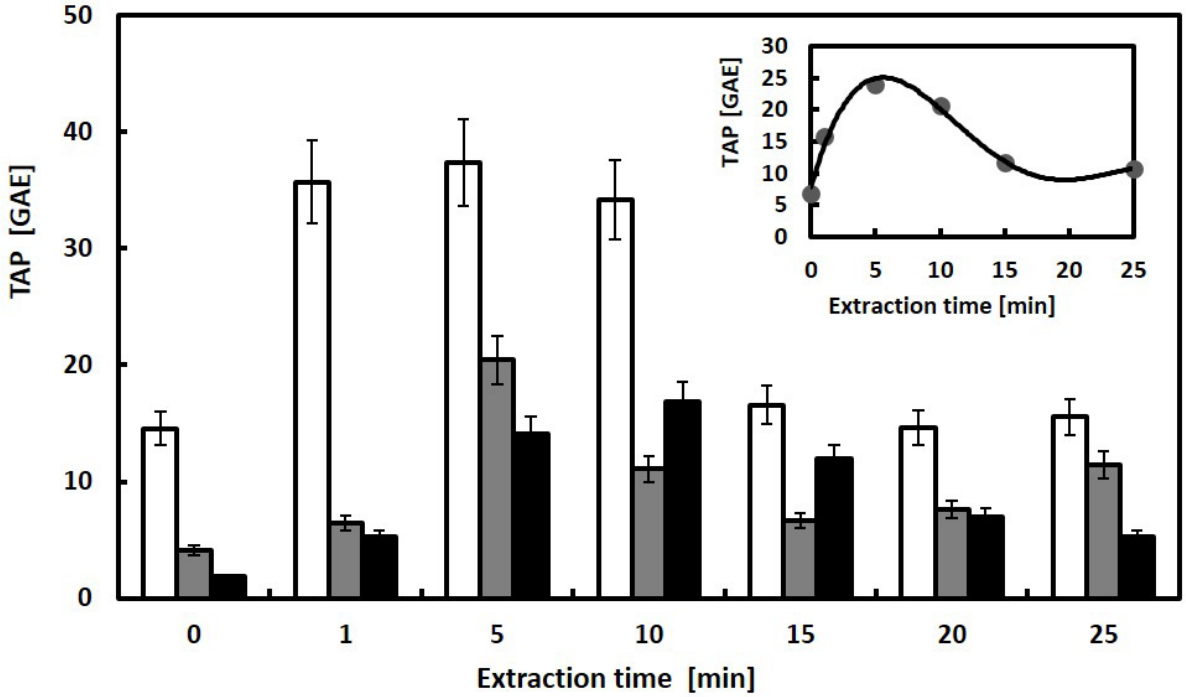

Figure 6A: The dependence of TAP values on the time of extraction in methanol. Meat samples (500 mg/mL): sirloin of beef (white), chicken fillet (gray), pork shoulder (black).

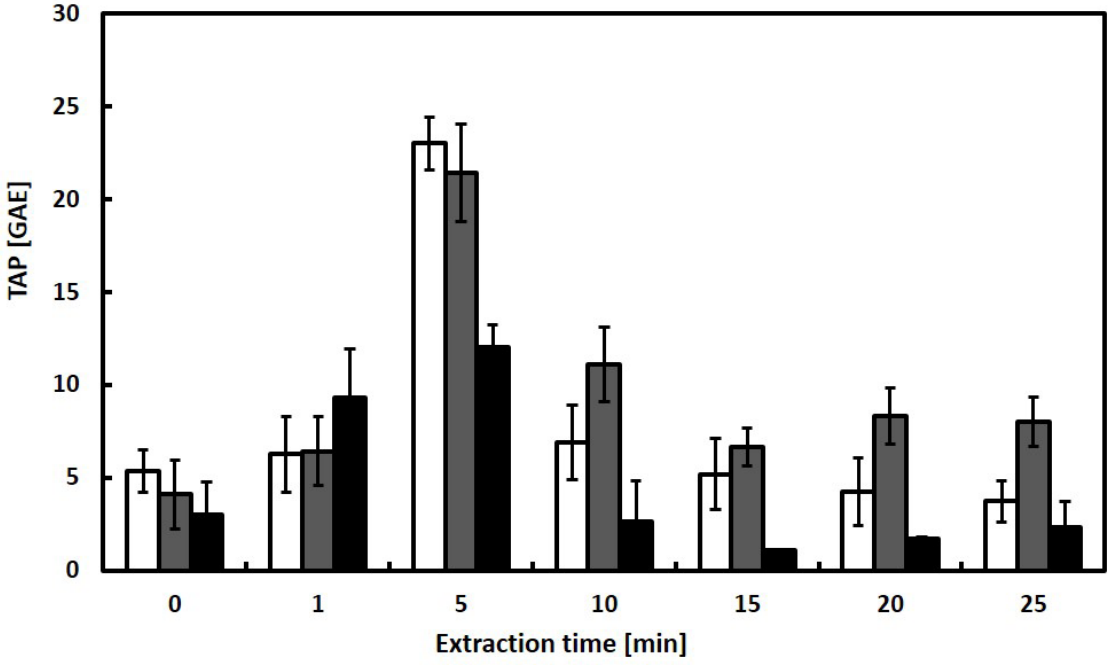

Figure 6B: The dependence of TAP of chicken fillet (500 mg/mL) on time of extraction in acetone (white), methanol (gray) and hexane (black).

water leakage, together with the dissolved antioxidants. The antioxidant activity of lean meat (veal or poultry) is relatively weak. Pork is the delicate bright red color meat, the intergrown with fat, forming a soft consistency [22]. It is a good source of selenium. Fat contains approx. 50% of unsaturated fatty acids, polyunsaturated from 7 - 14%, and monounsaturated fatty acids 41 - 52%. The most valuable meat, with an average calorific value, is the beef [23]. Fish is a source of vitamins, minerals, and proteins [24]. The content of antioxidants of extracts of different kinds of meat is depending on the solvent used for extraction (Figure 5). The highest TAP was observed for salmon fish extracted in acetone (57.16 GAE) and buffer pH 2 (49.94 GAE).

4 Conclusions

It was found that TLC can be used for the separation of different forms of DPPH$^{\bullet}$ as well as for the measurements of TAP values related to the DPPH$^{\bullet}$. The elaborated method was used to test the antioxidant properties of meat. Although meat is not characterized by the strongest antioxidant properties, it is consumed in much larger quantities by the average person than, for example, herbs. The highest antioxidant activity was observed for smoked salmon fish extracted in acetone (probably this is due to the fact that it contains the liquid smoke [25]) and for pork neck extracted in buffer pH 2, the lowest for veal and turkey fillet extracted in methanol. It turned out that the TAPs obtained with TLC are higher than those obtained using photometric method. This means that the separation of the DPPH$^{\bullet}$ from its derivatives and sample components improves the selectivity of the TAP measurement.

Conflict of interest: Authors declare no conflict of interest.

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