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Assessment of the Suitability of Methods for Testing the Antioxidant Activity of Anti-Aging Creams

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Abstract: Anti-aging cosmetics are often sought after in order to slow down the aging process. Free radicals are one of the main causes of skin aging, and therefore antioxidants are used in anti-aging cosmetics. The aim of this study was to investigate which method is the most suitable for determining the antioxidant capacity of these products. Having samples extracted, the antioxidant capacity of the extracts obtained was determined by the following spectrophotometric methods: DPPH, Folin-Ciocalteu, FRAP, the ABTS method and the ferroion chelation method with ferrosine. The antioxidant capacity of the samples varied depending on the extract type and the method used. DPPH and ferroion chelation measurements with ferrosine were carried out in the part of the spectrum where plant pigments absorb. These pigments are often found in anti-aging products affecting these methods measurement results. The Folin-Ciocalteu method is suitable for researching the antioxidant capacity of hydrophilic extracts, but not lipophilic ones, where turbidity and the formation of a gelled ring occur. The FRAP method revealed similar results for all the samples and proved to be less sensitive than the others. The ABTS method for both types of extracts has proven to be the most suitable and sensitive method for determining the antioxidant capacity of anti-aging products.

Keywords: antioxidants; antioxidant capacity; anti-aging cosmetics; spectroscopy

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1. Introduction

The term "successful ageing" comprises being health-focused and actively participating in all areas of life. It speaks out against the traditional understanding of ageing as a peaceful and sedentary lifestyle. One of its aims is to minimise the signs of ageing. Therefore, anti-aging cosmetics containing ingredients that minimise these effects are widely used today [1]. Two types of active ingredients are used in anti-aging cosmetics: antioxidants and cell regulators. Antioxidants such as vitamins, polyphenols and flavonoids reduce collagen breakdown by reducing free radicals. Cell regulators have a direct effect on collagen metabolism and stimulate the production of collagen and elastic fiberes [2]. Hydrophilic vitamin C and lipophilic vitamin E are the gold standards in anti-aging treatment. When applied topically, vitamin C increases the amount of the messenger ribonucleic acid mRNA and stimulates the synthesis of I and III collagen types, thereby stimulating the cells to regenerate. This should improve the skin condition. Applied topically, vitamin E also acts as an inhibitor of prostaglandin and nitric oxide synthesis and prevents oedema, UV-B-induced lipid peroxidation and sunburn. It plays a role in the formation of photoadducts and immunosuppression [3,4]. Topical preparation manufacturers very often add ingredients that facilitate the penetration of the active ingredient through the stratum corneum by inducing the fluidization of the stratum corneum lipids. These ingredients increase the bioavailability and effectiveness of the product [5]. Surfactants are among the ingredients that are frequently added to topical and oral formulations in

order to improve skin penetration [6]. Nonionic surfactants, such as: polysorbate, sorbitan ester, polyoxyethylene alkyl ether, polyoxyethylene alkylphenol and poloxamers, have the lowest toxicity and cause the least skin irritation, that is why they are most frequently added to topical preparations [7]. In many cases, manufacturers use plant extracts in their creams which are supposed to act as antioxidants and exhibit antioxidant activity in combination with classical antioxidants [8]. Whether a cream has an antioxidant effect or is just a marketing trick can only be determined by examining the antioxidant capacity. Such tests are analytically demanding, as creams are complex samples consisting of several phases of hydrophilic and lipophilic antioxidants. Hydrophilic and lipophilic antioxidants are incorporated into these phases, so phase separation and the testing of antioxidant capacity in each phase is a challenge for the analyst. Having in mind the complexity of the sample, the selection of testing methods is another challenging step in determining the antioxidant activity of anti-aging products. The methods for determining the antioxidant capacity are numerous and can be divided in different ways, in vitro and in vivo, but the most commonly used are spectrophotometric methods (in vitro) [9]. Depending on the mechanism of interaction between free radicals and antioxidants, spectrophotometric methods are divided into: HAT (hydrogen atom transfer methods) such as ORAC (oxygen radical absorption capacity) and ET (electron transport reaction methods) such as DPPH (2,2-diphenyl picryl hydrazyl), ABTS (2,2′ azino-to 3-ethylbenzothiazoline-6-sulphonic acid), FRAP (Ferric Reducing Antioxidant Power) etc. [10] In order to select the appropriate method, it is necessary to consider the principle of the method as well as its advantages and disadvantages. Sometimes, it is necessary to combine several methods in order to understand the antioxidant capacity [11] in a better way.

2. Materials and Methods

In order to evaluate and select the most suitable analytical method for testing the antioxidant activity of anti-aging creams in the experiment, we applied most of the in vitro methods commonly used to test antioxidant capacity [9,10]. The aim was to select the most suitable in vitro method for determining the antioxidant capacity in both the hydrophilic and lipophilic part of these topical preparations. The chosen method would be used to evaluate the efficacy of this type of preparation. During the experimental work, the antioxidant capacity of hydrophilic and lipophilic extracts after extracting anti-aging cream samples was tested using the DPPH method, and the total phenol content was determined using the Folin-Ciocalteu method, FRAP method, ABTS method and ferroion complexation method with ferrosine. All the measurements were carried out spectrophotometrically and performed in triplicate. The results were statistically processed by the determination of mean value ($\overline{X}$), standard deviation (SD) and coefficient of variation (KV).

2.1. Standards and Reagents

2.1.1. Chemicals

During the experiment, the following standards and reagents were used: alpha tocopherol (Sigma, NY, USA), Trolox (Sigma, NY, USA), (Sigma, NY, USA), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, Steinheim, Germany), gallic acid (Fluka Chemika, Buchs, Switzerland), Folin-Ciocalteu reagent (Sigma-Aldrich, Steinheim, Germany), ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid), ferro (II) sulfate heptahydrate (Chemistry, Zagreb, Croatia), ferrosine (BioSystem, Barcelona, Spaine). Seven anti-aging creams available on the market in Bosnia and Herzegovina were tested.

2.1.2. Instrument

Spectrophotometric analyses were performed on a Spectronic® Genesis tm Spectrophotometer tm 2. Milton Roy Company, Ivyland, PA, USA.

2.2. Sample Preparation

2.2.1. Extraction

The samples consisted of seven different creams with declared antioxidant properties. All the samples were prepared in the same way and their preparation took three days (Scheme 1). On the first day, the triple extraction of each sample was perfomed according to the following procedure. 5 g of cream was measured on a technical balance and put into Erlenmeyer flasks with 60 mL of acetone. The reaction mixtures were covered with aluminium foil, put in an ultrasonic bath and mixed for 20 min. The mixtures were then filtrated with qualitative filter paper. The filtrates were labelled as Filtrate 1 of each cream the residues were put back into new Erlenmeyer flasks and the whole process was repeated two more times. Filtrates 1, 2 and 3 were stored in a refrigerator. The next day, Filtrates 1, 2 and 3 were once again filtrated and we obtained new filtrates labelled 1′, 2′ and 3′. On the third day, the filtrates were first thermostated to room temperature and then put into separation funnels with 30 mL of distilled water and hexane and 2.5 g of solid sodium chloride. The mixtures were shaken and left for complete separation. The lower layers represented hydrophilic parts and the upper layers represented extracts of each cream sample. The extracts obtained were used for further analysis (Scheme 1).

2.2.2. In Vitro Methods of Testing of the Antioxidant Capacity

DPPH Method

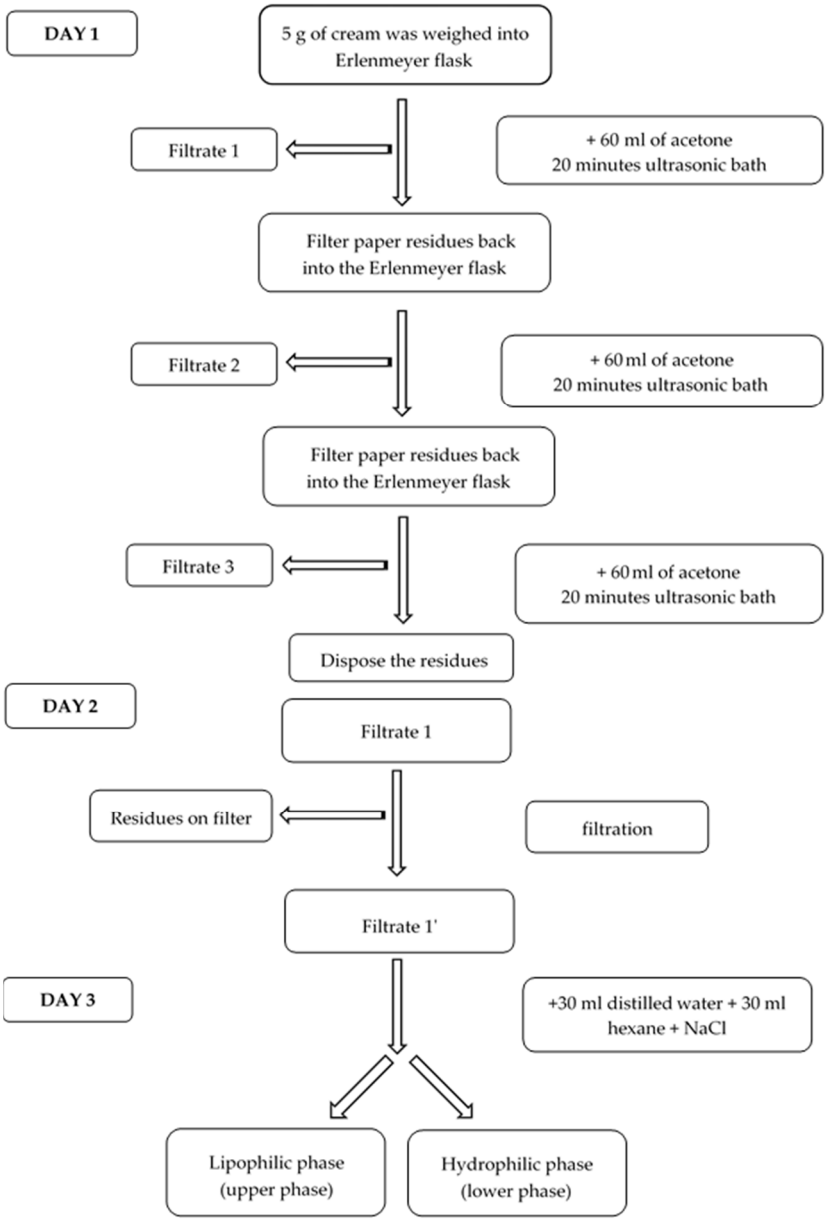
Solutions of DPPH in ethanol and methanol were prepared at a concentration of 1 mmol/L. Standard solutions were prepared. A standard solution of alpha tocopherol in hexane was prepared at a concentration of 1 mg/mL. A series of dilutions in the concentration range of 2 to 5 μg/mL was made. A standard solution of Trolox in a solution of water:acetone (1:2) with a concentration of 0.1 mg/mL was also prepared. A series of dilutions in the concentration range of 0.4 to 20 μg/mL was made of this solution. DPPH solution was added to each prepared solution, and calibration curves were created for the series dilutions of standard alpha tocopherol and Trolox solutions. The same amount of DPPH solution was added to the obtained hydrophilic and lipophilic cream extracts. The content was shaken and left in a dark place for 3 h and 30 min. The absorbances of the standards and samples were measured at 517 nm.

Folin-Ciocalteu Method

A dilution of Folin-Ciocalteu reagent (1:10), a sodium carbonate solution at a concentration of 75 g/L and standard solutions of gallic acid at a concentration of 0.5 mg/mL and alpha tocopherol in hexane at a concentration of 1 mg/mL were prepared. Series of dilutions were made. Their concentrations were 20–70 μg/mL for gallic acid and 5–25 μg/mL for alpha tocopherol. Prepared reagents were added to each prepared solution, and calibration curves were obtained for series dilutions of the standard gallic acid and alpha tocopherol solutions. Diluted Folin-Ciocalteu reagent and sodium carbonate solution were also added to the obtained hydrophilic and lipophilic cream extracts. The contents of the tubes were shaken and left in a dark place for 2 h. The absorbances of the standards and samples were measured at 765 nm.

FRAP Method

Reagents were prepared: 0.2 M phosphate buffer; 1% potassium ferricyanide (III) solution; 10% trichloroacetic acid solution; a 0.1% solution of fero (III) chloride. Standard solutions of alpha tocopherol in hexane at the concentration of 1 mg/mL and Trolox in a solution of water: acetone (1:2) at a concentration of 0.1 mg/mL were prepared. Batch dilutions were made with concentrations ranging from 2 to 5 μg/mL for alpha tocopherol and from 20 to 90 μg/mL for Trolox. Calibration curves for the standard solutions were created according to the protocol described by Oyaiza et al. [8] Samples of hydrophilic and lipophilic cream extracts were prepared according to an identical protocol. The absorbances of the standards and samples were measured at 700 nm.



Scheme 1. Sample preparation.

ABTS Method

Reagents were prepared: an ABTS solution in distilled water at a concentration of 7 mM; a solution of potassium persulfate in distilled water at a concentration of 2.45 mM; ABTS reagent and working ABTS reagent made by diluting the ABTS reagent. Standard solutions was prepared of Trolox in a solution of water:acetone (1:2) with a concentration of 0.1 mg/mL and alpha tocopherol in hexane at a concentration of 1 mg/mL. Batch dilutions were made from standard solutions to which ABTS working reagent was added. The tube contents were shaken and left at room temperature for 7 min after which the absorbances were measured. Calibration curves of the standard solutions were prepared in this way. Hydrophilic and lipophilic extracts of the prepared samples were analysed in the same way. The absorbances were measured at 734 nm.

Method of Chelating Ferro (II) Ions with Ferrosine

Reagents were prepared: 0.25 mM ferrosine working solution and 0.1 mM ferrous (II) sulfate working solution. A standard Na_2EDTA solution at a concentration of 100 mg/L was prepared. A series of dilutions in the concentration range of 1 to 20 mg/L were prepared

from this standard solution. The calibration curve was made by adding previously prepared reagents to the prepared solutions. The tube contents were shaken and the absorbances were measured after 10 min. Hydrophilic and lipophilic cream extracts were prepared in the same way as the calibration curve for the EDTA dilution series. The absorbances were measured at 562 nm.

3. Results

3.1. Results of In Vitro Testing of the Antioxidant Capacity

3.1.1. DPPH Method

A calibration curve for Trolox and alpha-tocopherol was created to calculate the micrograms of the Trolox/alpha tocopherol equivalent per gram of sample (µg TE/g and µg AE/g) in the hydrophilic and lipophilic extracts of the samples tested. The calibration curve for Trolox was in the concentration range of 0.4–20 µg/mL, while the calibration curve for alpha-tocopherol was in the concentration range of 2–5 µg/mL. The absorbances for the hydrophilic and lipophilic extracts were measured for each sample and based on the data obtained (Tables 1 and 2), expressed as the percentage of inhibition and micrograms of Trolox equivalent per gram (µg TE/g) for the hydrophilic extracts and as the percentage of inhibition and micrograms of alpha tocopherol equivalent per gram(µg AE/g) for the lipophilic extracts (Tables 3 and 4).

Table 1. Absorbances obtained for hydrophilic extracts of cream samples (DPPH method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
BLANK	2.416	2.417	2.416	2.416	0.0007	0.03
1	1.993	1.994	1.993	1.993	0.0007	0.04
2	2.008	2.007	2.008	2.008	0.0010	0.05
3	2.108	2.106	2.106	2.016	0.0010	0.05
4	1.174	1.175	1.178	1.176	0.0026	0.22
5	1.752	1.751	1.748	1.750	0.0021	0.12
6	2.197	2.195	2.200	2.197	0.0025	0.11
7	1.889	1.900	1.899	1.899	0.0007	0.04

Table 2. Absorbances obtained for lypophilic extracts of cream samples (DPPH method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
BLANK	1.924	1.923	1.923	1.923	0.0010	0.04
1	1.002	1.003	1.002	1.002	0.0007	0.04
2	1.616	1.616	1.615	1.616	0.0070	0.35
3	1.482	1.481	1.484	1.483	0.0010	0.05
4	0.323	0.323	0.324	0.323	0.0021	0.18
5	1.553	1.553	1.554	1.553	0.0021	0.12
6	1.551	1.554	1.553	1.552	0.0025	0.11
7	0.368	0.368	0.367	0.368	0.0007	0.04

Table 3. Results expressed as percentage of inhibition and in µg Trolox equivalent (TE)/g of sample (DPPH method).

Sample	% of Inhibition	µg TE/g of Sample
1	17.51	82.31
2	16.89	79.88
3	16.56	78.56
4	51.32	215.69
5	27.57	121.98
6	9.06	49.00
7	21.81	97.66

Table 4. Results expressed as percentage of inhibition and in μg AE/g of sample (DPPH method).

Sample	% of Inhibition	μg AE/g of Sample
1	47.89	28.64
2	15.96	7.26
3	22.88	11.89
4	83.20	52.29
5	19.24	9.45
6	20.85	9.48
7	80.86	50.72

In the hydrophilic samples, the extract from sample 4 had the highest antioxidant capacity, followed by extracts 5 and 7. Slightly lower, but similar values were found in cream extracts 1, 2, and 3. The hydrophilic extract from cream 6 had the lowest antioxidant capacity. Among the lipophilic extracts, cream extracts 4 and 7 had the highest antioxidant capacity, followed by the lipophilic extract of cream 1. The antioxidant capacity values were similar among the lipophilic cream extracts 3, 6, and 5 while the extract of cream 2 had the lowest antioxidant capacity.

3.1.2. Folin-Ciocalteu Method

In this method, after the absorbances for the hydrophilic (Table 5) and lipophilic cream samples (Table 6) were obtained, the results were determined in micrograms of gallic acid equivalent per gram (μg GAE/g) of sample for the hydrophilic extracts, while for the lipophilic extracts the results for antioxidant capacity were determined in micrograms of alpha-tocopherol equivalent per gram (μg AE/g) of sample. The gallic acid calibration curve was in the range of 20–70 μg/mL, while the calibration curve for alpha-tocopherol for the Folin-Ciocalteu method was in the range of 5–30 μg/mL.

Table 5. Absorbances obtained for hydrophilic extracts of cream samples (Folin-Ciocalteu method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
1	0.028	0.029	0.029	0.029	0.0007	2.41
2	0.072	0.073	0.073	0.073	0.0007	0.96
3	0.233	0.233	0.234	0.233	0.0007	0.30
4	0.357	0.356	0.357	0.357	0.0007	0.20
5	0.178	0.178	0.177	0.178	0.0007	0.39
6	0.024	0.023	0.023	0.023	0.0007	3.04
7	0.172	0.173	0.172	0.172	0.0007	0.41

Table 6. Absorbances obtained for lypophilic extracts of cream samples (Folin-Ciocalteu method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
1	0.239	0.242	0.242	0.141	0.0017	0.70
2	0.479	0.479	0.478	0.179	0.0007	0.15
3	0.119	0.117	0.119	0.119	0.0014	1.18
4	0.305	0.304	0.304	0.304	0.0007	0.23
5	0.071	0.070	0.070	0.070	0.0007	1.00
6	0.128	0.128	0.127	0.128	0.0007	0.101
7	0.131	0.131	0.130	0.131	0.0007	0.53

Based on the obtained absorbances of the hydrophilic and lipophilic extracts, the micrograms of gallic acid and alpha-tocopherol equivalents per gram of sample (μg TE/g and μg AE/g) were calculated for each extract. The results are shown in Tables 7 and 8.

Table 7. Results expressed in μg gallic acid equivalent per gram (GAE)/g of sample (Folin-Ciocalteu method).

Sample	μg GAE/g of Sample
1	32.89
2	83.87
3	268.91
4	412.39
5	205.27
6	25.92
7	198.56

Table 8. Results expressed in μg AE/g of sample (Folin-Ciocalteu method).

Sample	μg AE/g of Sample
1	137.46
2	201.71
3	96.88
4	413.07
5	17.41
6	115.96
7	120.56

The results obtained indicate that the hydrophilic extract from cream 4 had the highest antioxidant capacity. The results for extracts from creams 3, 5 and 7 were similar. followed by the extracts from creams 2 and 1 and cream 6 the lowest antioxidant capacity. In the lipophilic extracts the highest antioxidant capacity was in lipophilic cream extract 4, followed by cream extracts 3, 5, 7 and 2. The values for extracts 1 and 6 were similar and these samples showed the lowest antioxidant capacity according to the Folin-Ciocalteu method.

3.1.3. FRAP Method

The results for the antioxidant capacity of the creams tested using the FRAP method are expressed as micrograms of Trolox equivalent per milligrame of sample for the hydrophilic extracts and as micrograms of equivalent of alpha-tocopherol per milligrame of sample for the lipophilic extracts. A calibration curve for Trolox in the concentration range of 20–90 μg/mL and a calibration curve for alpha-tocopherol in the concentration range of 10 to 80 μg/mL were constructed. The absorbances for the hydrophilic (Table 9) and lipophilic extracts of the cream samples (Table 10) were obtained.

The results are expressed in micrograms of Trolox/alpha tocopherol equivalents per gram of sample (μg TE/g and μg AE/g) for each extract as shown in Tables 11 and 12.

Table 9. Absorbances obtained for hydrophilic extracts of cream samples (FRAP method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
1	0.191	0.192	0.192	0.192	0.0007	0.36
2	0.104	0.101	0.101	0.102	0.0016	1.57
3	0.155	0.154	0.154	0.154	0.0007	0.45
4	0.195	0.194	0.195	0.195	0.0007	0.36
5	0.138	0.139	0.139	0.139	0.0007	0.50
6	0.080	0.081	0.081	0.081	0.0007	0.86
7	0.455	0.455	0.455	0.455	0	0

Table 10. Absorbances obtained for lypophilic extracts of cream samples (FRAP method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
1	0.199	0.199	0.198	0.199	0.0007	0.35
2	0.176	0.175	0.175	0.175	0.0007	0.40
3	0.165	0.165	0.165	0.165	0	0
4	0.181	0.183	0.183	0.183	0.0014	0.76
5	0.205	0.206	0.206	0.206	0.0007	0.34
6	0.137	0.136	0.136	0.136	0.0007	0.51
7	0.194	0.195	0.194	0.194	0.0007	0.36

Table 11. Results expressed in µg TE/g of sample (FRAP method).

Sample	µg TE/g of Sample
1	220.19
2	90.07
3	165.25
4	224.53
5	143.57
6	59.71
7	600.43

Table 12. Results expressed in µg AE/g of sample (FRAP method).

Sample	µg AE/g of Sample
1	244.47
2	213.83
3	201.06
4	224.04
5	253.40
6	164.04
7	238.06

Looking at the results shown in Table 11. it appears that the highest values of antioxidant capacity were obtained with the hydrophilic sample of cream 7, while the results for the samples of the cream 4, 1, 3 and 5 were similar. The FRAP method showed the lowest antioxidant capacity for samples 2 and 6. The lipophilic extracts showed similar antioxidant capacities (Table 12). There was a deviation in the cream sample 6 where the antioxidant capacity was the lowest.

3.1.4. ABTS Method

The results for the antioxidant capacity of the samples tested using the ABTS method are expressed as percentage of inhibition and micrograms of Trolox and alpha-tocopherol equivalents per gram of sample (µg TE/g and µg AE/g) for each extract. The calibration curve for Trolox was in the range of 0.005–5.6 µg/mL, while for alpha tocopherol, the calibration curve concentrations were in the range of 0.125–2 µg/mL. The absorbances for the hydrophilic and lipophilic extracts of the cream samples are shown in Tables 13 and 14.

The results showed that in the hydrophilic sample, sample 4 had the highest value of antioxidant capacity and sample 6, the lowest. In the lipophilic sample, sample 4 also had the highest value and sample 6 had the lowest.

The values of the percentage of inhibition µg TE/g and µg AE/g (Tables 15 and 16) were determined based on the measured absorbances of the tested hydrophilic and lipophilic samples.

Table 13. Absorbances obtained for hydrophilic extracts of cream samples (ABTS method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
BLANK	0.320	0.320	0.322	0.320	0.0007	0.22
1	0.209	0.212	0.210	0.209	0.0020	0.96
2	0.203	0.203	0.202	0.203	0.0007	0.34
3	0.035	0.036	0.034	0.035	0.0010	2.86
4	0.003	0.003	0.003	0.003	0	0
5	0.079	0.079	0.078	0.079	0.0007	0.50
6	0.250	0.249	0.247	0.249	0.0016	0.89
7	0.123	0.121	0.125	0.123	0.0020	1.62

Table 14. Absorbances obtained for lipophilic extracts of cream samples (ABTS method).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
BLANK	0.526	0.525	0.527	0.526	0.0010	0.19
1	0.232	0.231	0.232	0.232	0.0007	0.30
2	0.188	0.190	0.188	0.188	0.0014	0.74
3	0.493	0.493	0.494	0.493	0.0007	0.14
4	0.099	0.101	0.100	0.100	0.0010	1.00
5	0.141	0.139	0.139	0.140	0.0007	0.50
6	0.522	0.521	0.522	0.522	0.0007	0.13
7	0.350	0.351	0.350	0.350	0.0007	0.20

Table 15. Results expressed as percentage of inhibition and in µg TE/g of sample (ABTS method).

Sample	% of Inhibition	µg TE/g of Sample
1	34.69	29.76
2	36.56	30.87
3	89.06	66.36
4	99.06	73.12
5	75.31	57.06
6	22.19	21.15
7	61.56	47.77

Table 16. Results expressed as percentage of inhibition and in µg AE/g of sample (ABTS method).

Sample	% of Inhibition	µg AE/g of Sample
1	55.89	19.73
2	64.26	23.04
3	6.08	0.07
4	80.99	79.67
5	73.38	26.66
6	0.76	0.02
7	33.46	10.84

3.1.5. Ferroion Chelation Method with Ferrosine

The method of the chelation of ferroions with ferrosine is one of the methods used to determine antioxidant capacity. The antioxidant capacity was expressed as milligrams of EDTA equivalent per gram of sample. By this method, it was possible to test the antioxidant capacity of the hydrophilic samples only, while the antioxidant capacity of the lipophilic samples could not be determined. An EDTA calibration curve was constructed in the concentration range of 1–20 mg/mL.

The absorbances for the hydrophilic samples were obtained (Table 17) and the milligrams of EDTA/gram of sample was calculated (Table 18).

Table 17. Absorbances obtained for hydrophilic extracts of cream samples (ferroion chelation method with ferrosine).

SAMPLE	A1	A2	A3	$\bar{X}$	SD	CV (%)
BLANK	0.603	0.603	0.602	0.603	0.0007	0.12
1	0.460	0.461	0.461	0.461	0.0007	0.15
2	0.456	0.455	0.455	0.455	0.0007	0.15
3	0.478	0.479	0.479	0.479	0.0007	0.14
4	0.460	0.460	0.460	0.460	0	0
5	0.482	0.483	0.483	0.483	0.0007	0.14
6	0.482	0.480	0.480	0.481	0.0010	0.21
7	0.093	0.093	0.092	0.093	0.0007	0.75

Table 18. Results expressed in mg EDTA/g of sample (ferroion chelation method with ferrosine).

Sample	mg EDTA/g of Sample
1	18.80
2	22.97
3	6.51
4	19.54
5	3.77
6	5.14
7	271.2

The results obtained by this method show that the hydrophilic sample 7 had the highest value of antioxidant capacity. The results for the other samples were similar and much lower than those for sample 7.

4. Discussion

In order to investigate whether the cosmetic products on the market actually have antioxidant activity as declared, their antioxidant capacity was tested using selected analytical methods. The antioxidant capacity was examined in hydrophilic and lipophilic extracts of the products tested. As the anti-aging creams themselves contain antioxidants soluble in water and lipids phase separation was necessary in order to gain a clear insight into the antioxidant capacity of the samples tested. As they were very complex samples, the extraction process itself had to be optimised beforehand and adapted to this type of sample. The methods used to test the antioxidant capacity were spectrophotometric based on the ET mechanism. Based on the results obtained, the antioxidant activity of the extracts was compared with the corresponding standards and the results are presented as their equivalents. As a review of the literature did not reveal similar determination in the individual phases of a cosmetic product. the suitability of the test methods for determining the antioxidant capacity was estimated.

The DPPH method only took into consideration values obtained for the first hydrophilic and lipophilic extracts. This is because only the first extracts had absorbance values lower than the positive blank, which means that the substances responsible for the antioxidant activity are extracted immediately at the beginning of the extraction. The results show that the lipophilic extracts had higher equivalents per gram of sample and therefore, a stronger antioxidant activity, which is expected since tocopherol and other fat-soluble antioxidants in the composition of the cream are at higher percentages than hydrophilic antioxidants. The absorbance values for extracts 2 and 3 were much higher if compared to the corresponding positive blanks. This means that these extracts contain components that interfere with the measurement by absorbing at the same wavelength or forming complexes with DPPH and preventing its reduction. It is known that plant pigments interfere with the DPPH method because they absorb visible light in the same region in which the measurement is carried out [12–14]. Creams are extremely complex

matrices wich can be seen on the compositions indicated on the packaging. Each of the creams analysed contains extracts of different plant species that have been added with the aim of improving the antioxidant activity.

These extracts contain plant pigments that are both water-soluble and fat-soluble and are distributed in the appropriate phases by extraction (water: acetone or hexane). Therefore, their influence can be found in both types of extracts. Since the identification and determination of the contents of plant pigments in the extracts examined did not take place, their presence in the first extracts and their effects on the results obtained cannot be excluded with certainty. For more precise results, it would be necessary to repeat the measurements with techniques such as electronic paramagnetic resonance, which can almost eliminate the effect of plant pigments on the measurements [15,16]. The most significant interferences in the results of DPPH tests are caused by plant pigments and therefore the focus was on them.

The results obtained with the Folin-Ciocalteu method were similar to those obtained using the DPPH method. Antioxidant activity was detected in the initial extracts and the values were higher in the lipophilic extracts. Folin-Ciocalteu reagent itself is sensitive not only to the presence of phenols, but also to the presence of other reducing substances (vitamin C and other antioxidants), so it is expected for the first extracts to have higher absorbance values [17]. The obtained absorbances for the first extracts are generally low and indicate low antioxidant activity. Absorbance, though low, was observed in both the second and third extracts and most likely caused by the presence of polyphenols from the plant extracts. Since the presence of antioxidants was only detected in the first extracts using these two methods, for practical reasons further measurements were carried out only in the first extracts.

The FRAP method showed that the antioxidant capacity of the samples to reduce the ferro (III) ion to the ferro (II) ion was very low for both the hydrophilic and lipophilic extracts for approximately all the samples and both types of extracts. This is due to factors such as the reactivity of ferro ions, which is lower compared to more sensitive DPPH and ABTS radicals. Other reasons may be an acidic medium that reduces the reactivity of some compounds, particularly thiols, and a possible chelation of ferro ions produced in reaction with some antioxidants [18,19] and with other compounds found in creams. The influence of plant pigments on the results can be excluded because the measurement was carried out in the part of the spectrum in which the pigments do not absorb. Based on all this, more sensitive methods should be chosen to test the antioxidant capacity of these samples.

The results for both types of extracts obtained using the ABTS method show that the lipophilic extracts had a higher antioxidant capacity. Therefore, it was necessary to modify the method as the discoloration of the samples occurred almost immediately and it was impossible to measure the absorbances after 10 min. ABTS is fast, simple, sensitive and suitable for the determination of the antioxidant activity in both lipophilic and hydrophilic extracts [20]. Therefore, measurements of this type of samples in both types of extracts could be performed quickly and with distinction between individual preparations.

The chelation capacity for heavy metals is also one of the ways in which antioxidant activity can be expressed. Heavy metals such as iron and copper are known to stimulate oxidative processes and contribute to the formation of free radicals. Antioxidants prevent these processes and contribute to the protection of the organism by binding metals at physiologically acceptable levels. The chelatione capacity for metals is expressed as the chelatione capacity for ferro ions with ferrosine. Measurements could only be made for the hydrophilic extracts. The results were low with the exception of those for the cream sample 7. For the lipophilic extracts, the positive blank containing hexane alone had a much lower absorbance than the lipophilic samples, which is paradoxical considering that antioxidants present in the lipophilic phase should reduce the amount of iron available for complexation with ferrosine. A possible reason for this phenomenon in the samples may be the presence of plant pigments that absorb in the same region in which the measurement was carried out; these pigments are also present in the hydrophilic phase, so it can be said that they also

had an effect on the absorbance values of the hydrophilic extracts. In the case of a positive blank of the lipophilic extract, the results show that the absorbance value of the magenta complex was significantly lower compared to the positive blank of the hydrophilic extract. The reason for this phenomenon may be the interference in hexane, such as of anions and cations, described in the research of Stookey et al. [21].

The level of the antioxidant capacity of the tested cosmetic products taken from the market varied depending on the type of tested extract and the type of method used for testing. Based on the results obtained by the DPPH method, it was found that the results were influenced by the presence of plant pigments, which can be found very often in this type of topical preparations, so this method did not prove to be sufficiently selective. The results obtained by the Follin-Ciocalteu method were similar to those with the DPPH method, but the disadvantage of this method was that the measurement was affected by the presence of polyphenolic plant components extracted together with the antioxidants from the creams. Regarding the results obtained by the FRAP method used on both the lipophilic and hydrophilic extracts of the tested creams, it can be seen that the results were almost the same and that there were no difference. This was attributed to factors such as the reactivity of the ferric ion itself which is lower compared that of the more sensitive DPPH radicals, so this method did not prove to be sufficiently sensitive for testing the antioxidant activity of topical preparations in vitro. The ABTS method proved to be fast, simple, sensitive and furthermore suitable for determination in both lipophilic and hydrophilic media, compared to other in vitro methods. The results of this method were not affected by the presence of plant pigments, so it showed sufficient selectivity and sensitivity compared to the others. The only disadvantage of this method is that the measurement must be performed within a time period of 10 min. By using the method of the chelation of ferrous ions with ferrozine, it was possible to determine the antioxidant capacity of the hydrophilic extracts, however the results could not be evaluated for the lipophilic extracts. The same as with the DPPH method, the measurement was affected by the presence of plant pigments, so this method was not sufficiently selective as well as DPPH. A combination of in vitro and in vivo methods would provide the best insight into the antioxidant capacity of these topical preparations

5. Conclusions

The antioxidant capacity of the tested anti-aging creams varies depending on the type of extract and the method used for its determination. Although the DPPH method is applicable to both types of extracts, it is not the most suitable method for determining the antioxidant capacity in anti-aging creams. The reason for this is that the measurements for this method are carried out in the part of the spectrum in which the plant pigments commonly contained in these creams absorb. The Folin-Ciocalteu method is suitable for testing the antioxidant capacity of hydrophilic extracts, while lipophilic extracts should be avoided due to the occurrence of turbidity and the formation of a gelled ring. FRAP provided similar results for all the samples and proved to be a less sensitive method compared to the other methods, mainly due to the lower reactivity of ferro ions compared to the more sensitive DPPH and ABTS radicals. The ferroion chelation method with ferrosine, similar to the DPPH method, takes measurements in the part of the spectrum where plant pigments interfere, making this method unsuitable for determining the antioxidant capacity in anti-aging creams. Furthermore, it was not possible to determine the antioxidant capacity of the lipophilic cream extracts using this method. As the antioxidant content in these creams is low, more sensitive methods are better suited for determining the antioxidant capacity in these samples. The ABTS method for both types of extracts has proven to be the most suitable method for determining the antioxidant capacity.

The advantage of in vitro methods for testing antioxidant activity is reflected by the fact that they can be used to separately determine the antioxidant capacity in hydrophilic and lipophilic cream extracts, which is not possible with in vivo methods. The disadvantage of these in vitro methods is that they cannot fully predict how these topical products will

exhibit antioxidant activity in a living organism, as well as that the results are affected by the complexity of the matrix. From all of the tested methods, only the ABTS method proved to be suitable for the in vitro testing of the antioxidant activity of this type of topical preparation. The method was sufficiently sensitive, selective, rapid and furthermore adequate for the in vitro estimation of the efficacy of this type of product in a control analytical laboratory. However. a combination of the in vitro method and the in vivo method would provide the best insight into the antioxidant activity of these products, which could be the next aim in researching the antioxidant capacity of anti-aging creams in the future.

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