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A COMPARISON OF ABTS AND DPPH METHODS FOR ASSESSING THE TOTAL ANTIOXIDANT CAPACITY OF HUMAN MILK

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ABSTRACT

Introduction. The Total Antioxidant Capacity (TAC) of human milk reflects the concentration and the activity of many components which prevent oxidative degradation of fats and proteins. This study compares the effectiveness of ABTS and DPPH tests with regard to the recovery, precision and sensitivity (detection and quantification limit) of (TAC) values in human milk.
Material and methods. TAC values were determined in twenty five samples of human milk obtained from healthy mothers, residents of Gdańsk, on the 14th day postpartum.
Results. The average TAC of human milk determined by ABTS assay was 19.61 ±3.311 mg TE (Trolox Equivalents)/100 cm^3, the average values obtained by the DPPH assay reached 9.95 ±4.36 mg TE/100 cm^3. For each milk sample the TAC determined by the ABTS test was significantly higher than the values produced by the DPPH test. The above findings can be attributed to the presence of substances whose spectra overlap with DPPH$^•$ spectra. ABTS test was characterised by a higher sensitivity and repeatability of the determination of TAC in human milk compared to the DPPH test.
Conclusions. Comparing the calculated values for the validation parameters of both methods and taking into account the solubility of DPPH only in polar matrices, slower reaction of selected antioxidants with DPPH radical, and the presence in human milk constituents absorbing electromagnetic radiation in the absorption of DPPH be assumed that the ABTS test is more appropriate method of determining of TAC in breast milk.

Key words: human milk, total antioxidant capacity, ABTS test, DPPH test

INTRODUCTION

Human milk is the optimal nutritional source for infants and children in the first months of life. In addition to the nutrients required for infant development, it contains immune defense and growth promoting factors [Lönnerdal 2000, Picciano 2001]. Human milk is a source of multiple components, enzymatic and non-enzymatic antioxidant constituents which prevent oxidative rancidity [Li et al. 2009].

The antioxidant enzymes found in human milk include superoxide dismutase (SOD), catalase, glutathione peroxidase [Lindmark-Mansson and Akesson 2000] and coenzyme Q10 [Compagnoni et al. 2004]. Selected non-enzymatic antioxidants can be synthesized in the body (iron-binding protein lactoferrin), while others have to be supplied with food (ascorbic acid, tocopherols, tocotrienols, carotenoids, isoflavones, selenium) [Lindmark-Mansson and Akesson 2000]. Antioxidants can scavenge radicals, hydrogen peroxide and other peroxides and prevent the formation of radicals. Other antioxidant enzymes catalyze the synthesis or the regeneration of non-enzymatic antioxidants.

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The measurement of total antioxidant capacity (TAC) is a useful tool in evaluating of the antioxidative role of the investigated compounds. The total antioxidant capacity of human milk reflects the presence and the activity of antioxidant components. The most popular methods of evaluating TAC levels in human milk are spectrophotometric ABTS, spectrophotometric DPPH$^{\bullet}$ and fluorescence ORAC (Oxygen Radical Absorbance Capacity) assays. Test results are expressed in terms of millimoles or milligrams of Trolox equivalents (synthetic vitamin E analogue) per unit volume of milk.

In the ABTS test, 2,2'-azinobis (3-ethylbenzthiazoline-6-acid) (ABTS) is converted into its radical cation (ABTS$^{\bullet+}$) by addition of sodium persulphate. This blue-green radical cation absorbs light at 734 nm. ABTS$^{\bullet+}$ is reactive towards most antioxidants. It is not affected by ionic strength, and it can be used to determine both hydrophilic and hydrophobic antioxidant capacities. During this reaction, the blue-green ABTS radical cation is converted back into its colourless neutral form. The reaction may be monitored spectrophotometrically [Aycicek et al. 2006, Cubero et al. 2009, Matos et al. 2009].

DPPH$^{\bullet}$ (2,2-diphenyl-1-picryhydrazyl) is one of the few stable organic nitrogen radicals. A DPPH$^{\bullet}$ solution in ethanol has an intensive deep purple colour with a strong VIS absorption at 515 nm. When it reacted with an antioxidant, the DPPH$^{\bullet}$ radical is converted into DPPH, and its colour changed from purple to yellow. The antioxidant effect may be easily evaluated by observing the decrease in VIS absorption at 515 nm [Li et al. 2009, Thaipong et al. 2006].

In the ORAC method, a peroxyl radical is generated by thermal decomposition using 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH). Peroxyl radicals decrease the fluorescence of the secondary reagent (B- or R-phycoerythrin PE). The decrease constant for the PE fluorescence decay profile is used to determine the antioxidant capacity of the added sample [Alberti-Fidanza et al. 2002, Li at el. 2009, Sáenz et al. 2009].

The objective of this study was to compare the effectiveness of determination of breast milk TAC levels by the most popular spectrophotometric tests: ABTS and DPPH. Both methods were validated statistically, and their effectiveness was compared with regard to the recovery, precision, detection and quantification of TAC levels in human milk.

MATERIAL AND METHODS

Ethics. All the procedures of this study were approved by the Local Ethics Committee of the Medical University of Gdańsk. The subjects gave their informed consent before the start of any procedure.

Breast milk samples. Twenty five samples of human milk were collected from healthy mothers, residents of Gdańsk, on the 14th day postpartum. The mothers, aged 21-35, received a normal diet without pharmacological treatment. Milk samples were collected by the mothers in sterile plastic containers before the first morning feeding (between 6 and 8 a.m.). The samples were freeze stored at home (–18°C) delivered to laboratory on ice within six hours after collection, and immediately frozen at –80°C until analysis.

Analytical procedure

Determination of TAC levels in human milk by the ABTS method. The TAC of human milk was determined by the modified method proposed by Turoli et al. [2004]. The stock solution of the ABTS radical was prepared by dissolving 38.4 mg of 2,2'-azinobis (3-ethylbenzthiazoline-6-acid) (ABTS) in 10 cm^3 of a sodium persulphate solution (2.45 mM), and the mixture was dark stored for 12 hours. The working solution was obtained by diluting the stock solution of the ABTS radical cation with methanol to obtain an absorbance of 0.7 ±0.005 at 734 nm.

Human milk samples (0.01 cm^3) were reacted with 1 cm^3 of the ABTS$^{\bullet+}$ working solution for 10 minutes at room temperature. After *centrifuging* (5 min, 8000 rpm) absorbance was measured at 734 nm against the reference sample (methanol). The antioxidant capacity of the human milk samples was expressed in milligrams of the Trolox Equivalent TE per 100 cm^3 of milk on the basis of the calibration curve and percentage recovery.

Determination of TAC levels in human milk by the DPPH method. The analytical procedure was performed with the use of modified methods proposed by Thaipong et al. [2006]. The stock solution was prepared by dissolving 10 mg of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical solution in 10 cm^3 methanol. The working solution was obtained by diluting of the stock solution of the DPPH radical with methanol to produce an absorbance of 1.0 ±0.005 at 515 nm.

Human milk samples (0.02 cm^3) were reacted with 1 cm^3 of the DPPH working solution and 0.5 cm^3 of chloroform for 15 minutes at room temperature. After *centrifuging* (5 min at 8000 rpm), absorbance was measured at 515 nm against the reference sample (methanol:chloroform, 2:1 (v:v)). The results calculated on the basis of calibration curve and the recovery percentage were expressed in mg TE in 100 cm^3 of human milk.

Validation and linearity

The methods were validated for recovery (internal standard method), repeatability (RSD relative standard deviation for 6 independent analyte measurements in 3 different matrices), detection (Limit of Detection, LOD, and Method Detection Limit, MDL) and limit of quantification (LOQ). LOD was calculated in view of the standard deviation of signals and the slope of the calibration curve where LOQ is three times more of the LOD [Konieczka et al. 2004]. Calibration curves were plotted using standard solutions of Trolox [97%, Sigma]. The linearity of the analytical methods for fresh methanolic solution of Trolox were confirmed by determining of the statistical significance of calibration curve coefficients (> 0.994). The calibration curve for ABTS test was linear within the concentration range of 0.129-3.842 μg TE/cm^3 (r = 0.996), while the calibration curve for DPPH test was linear in the concentration range of 0.228 to 7.957 μg TE/cm^3 (r = 0.994).

Statistical analysis

The results were presented as arithmetic means and their standard deviations. The applied methods were evaluated statistically with the use of STATISTICA 8.0 (StatSoft®, Poland) software. Normal distribution of continuous variables was tested with the Kolmogorov-Smirnov test. The differences in TAC levels of human milk measured by ABTS and DPPH assays were estimated with statistical significance defined as $p < 0.05$. Additionally Pearson's linear coefficient of correlation was calculated between the TAC levels in human milk (mg TE in 100 cm^3) determined by ABTS and DPPH methods.

RESULTS

The analytical methods applied in the experiment produced highly satisfactory analyte recovery results, in excess of 95% (Table 1). The average TE recovery was 95.94% in the spectrophotometric ABTS method and 96.93% in the DPPH test.

Table 1. Recovery of TE from human milk

Added TE μg	ABTS method		DPPH method	
	determined TE μg	recovery %	determined TE μg	recovery %
0.0	0.766		0.692	
0.485	1.151	92.01	1.109	94.22
0.970	1.678	96.66	1.640	98.68
1.455	2.202	99.14	2.102	97.90
Average		95.94		96.93

The values of validation parameters reported for both analytical methods are presented in Table 2. Both ABTS and DPPH were marked by satisfactory repeatability with RSD of 2.01% and 5.66%, respectively. While both investigated methods showed satisfactory repeatability, the ABTS technique was characterized by higher levels of analyte detection and quantification. The measurement of TAC values by the ABTS

Table 2. Validation parameters associated with each analytical method

Test	Range of linearity μg TE/cm^3	LOD μg TE/cm^3	LOQ μg TE/cm^3	MDL mg TE/100 cm^3 of milk	RSD
ABTS test	0.129-3.842	0.043	0.129	1.303	0.0201
DPPH test	0.228-7.657	0.076	0.228	1.733	0.0566

assay revealed the presence of antioxidant substances with activity equivalent to 1.3 mg Trolox in 100 cm^3 of human breast milk. In the DPPH test, MDL (Method Detection Limit) values were higher, and they were equivalent to 1.73 mg of Trolox per 100 cm^3 of milk (Table 2).

Table 3. TAC of human breast milk determined by spectrophotometric ABTS and DPPH methods

Number of sample	ABTS test	DPPH test
	TAC ±SD, mg TE/100 cm^3 human milk	
1	23.41 ±0.47	12.58 ±0.26
2	18.16 ±0.36	13.65 ±0.28
3	20.17 ±0.41	14.02 ±0.29
4	17.66 ±0.36	12.46 ±0.25
5	17.95 ±0.36	3.87 ±0.08
6	18.37 ±0.37	2.45 ±0.05
7	25.93 ±0.52	5.86 ±0.12
8	17.45 ±0.35	12.81 ±0.26
9	21.48 ±0.43	17.66 ±0.36
10	17.78 ±0.36	13.48 ±0.27
11	16.52 ±0.33	13.13 ±0.26
12	13.75 ±0.28	7.46 ±0.15
13	21.94 ±0.44	5.77 ±0.23
14	17.53 ±0.35	9.75 ±0.20
15	23.32 ±0.47	6.12 ±0.12
16	17.45 ±0.35	10.74 ±0.22
17	17.36 ±0.35	3.73 ±0.07
18	19.84 ±0.40	14.25 ±0.29
19	22.36 ±0.45	14.36 ±0.29
20	25.30 ±0.51	6.02 ±0.12
21	20.30 ±0.41	7.14 ±0.15
22	16.94 ±0.34	12.27 ±0.25
23	13.33 ±0.27	12.92 ±0.26
24	22.44 ±0.45	13.01 ±0.06
25	23.41 ±0.47	8.55 ±0.17

Values presented as mean ±standard deviation; TE – Trolox equivalent.

ABTS and DPPH assays were used to determine TAC levels in 25 human milk samples collected on 14th day of lactation. TAC levels were determined in the range of 13.33 to 25.93 mg TE per 100 cm^3 human milk by the ABTS method and 2.45 to 17.66 mg TE per 100 cm^3 by the DPPH test (Table 3). TAC values produced by the DPPH assay were characterised by significantly higher variation than ABTS test, and the reported variance was not symmetric. The following average TAC levels were determined in human breast milk sampled on lactation day 14: 19.61 mg TE/100 cm^3 with standard deviation 3.311 mg TE/100 cm^3 in the ABTS test, and 9.95 mg TE/100 cm^3 with SD = 4.359 mg TE/100 cm^3 in the DPPH assay.

DISCUSSION

The results of our study are similar to other published data regarding TAC levels of human milk measured by ABTS. Matos et al. [2009] in mature milk of Portuguese women observed between 6.0 and 14.8 mg TE/100 cm^3, VanderJagta et al. [2001] from 7.51 to 102.7 mg TE/100 cm^3 in milk of Nigerian women. TAC levels determined by ORAC [Alberti-Fidanza et al. 2002] and by FRAP (Ferric Reducing Antioxidant Power) method [Zarban et al. 2009] also gave consistent results – average 25.30 ±9.27 mg TE per 100 cm^3 and 21.61 ±10.71 mg TE per 100 cm^3 respectively. Three times higher concentration of TE (ABTS assay) as presented in above mentioned papers was found in milk of women in Italy (about 78 mg/100 cm^3) [Turoli et al. 2004].

The statistical test revealed significant differences between the results of the deployed analytical tests ($p < 0.05$). TAC levels determined by the ABTS method were significantly higher than those reported in the DPPH assay. The correlation between TAC values in human milk samples determined by ABTS and DPPH tests is illustrated in the diagram (Fig. 1).

The above diagram shows an absence of a linear correlation between the TAC values of milk samples measured by both techniques (Pearson's correlation coefficient $r = 0.1789$). The above suggests that human milk contains ingredients (antioxidants) which have

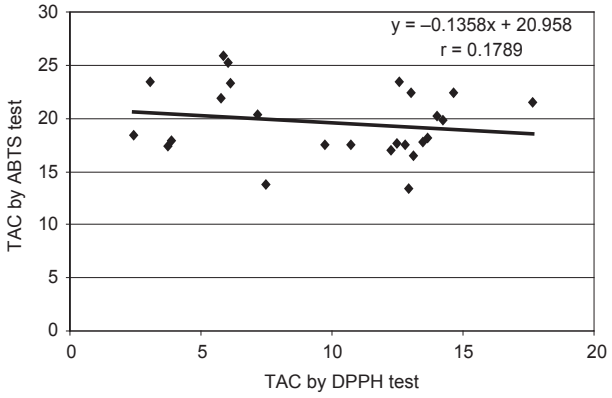

Fig. 1. Correlation between TAC levels in human milk (mg TE in 100 cm^3) determined by ABTS and DPPH methods

a reducing effect on only one of the colour reactants. ABTS and DPPH assays are usually classified as SET (Single Electron Transfer) reactions. These radical indicators may be neutralized by direct reduction via electron transfer or by radical quenching via hydrogen atom transfer [Prior et al. 2005]. In general, SET-based assays measure antioxidant reductive capacity.

ABTS radical cation ($ABTS^{\bullet+}$) is reactive towards most antioxidants, and it is soluble in both aqueous and organic solvents. The $ABTS^{\bullet+}$ method is a useful tool in determining the antioxidant activity of both lipophilic (e.g. alpha-tocopherol or beta-carotene) and hydrophilic antioxidants in various matrices (body fluids, food extracts, etc.) [Cano et al. 2000]. $ABTS^{\bullet+}$ react rapidly with antioxidants, and they can be applied over a wide pH range. Selected substances, including most phenolic compounds, reduce $ABTS^{\bullet+}$ if its redox potential is lower than that of ABTS (0.68 V).

The DPPH assay is based mainly on the electron transfer reaction, while hydrogen – atom abstraction is a marginal reaction pathway [Prior et al. 2005]. The interactions between antioxidants and $DPPH^{\bullet}$ are also determined by the antioxidant's structural conformation. Some compounds react very rapidly with $DPPH^{\bullet}$, and they reduce the number of $DPPH^{\bullet}$ molecules corresponding to the number of available hydroxyl groups.

Nevertheless, this mechanism seems to be more complex and the observed reactions are slower in most antioxidants [Brand-Williams et al. 1995]. The DPPH test does not support the determination of TAC compounds whose spectra overlap with $DPPH^{\bullet}$ spectra, in particular carotenoids [Prior et al. 2005]. In the DPPH test, samples containing carotenoids show absorbance levels at 515 nm that exceed the actual amount of DPPH radicals. Carotenoids are non-enzymatic antioxidants, and their content in mature human milk may be as high as 0.20 μM/dm^3 [Gossage et al. 2002].

The DPPH method yielded lower TAC values than the ABTS assay, probably because the DPPH method has more limitations. As shown above DPPH method is characterized by a lower sensitivity than ABTS assay. The reaction of $DPPH^{\bullet}$ with most antioxidants is slower than in the case of $ABTS^{\bullet+}$. Moreover, DPPH dissolves only in polar matrices and human milk contains components whose spectra overlap with $DPPH^{\bullet}$ spectra what can significantly distort the spectrophotometric measurement. All these arguments suggest that the DPPH method is not suitable for the determination of TAC values in human milk.

CONCLUSIONS

The reported values of validation parameters indicate that both analytical methods are suitable for the determination of TAC in human milk, but the ABTS test is marked by higher repeatability, detectability and sensibility.

The presence of compounds that absorb light at 515 nm (such as carotenoids) in the sample can result in a significant falsification of TAC values measured by the DPPH test.

The ABTS test supports the quantification of a larger range of antioxidants.

The limitations of DPPH support the choice of ABTS as the preferred method for the determination of TAC values in human milk.

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PORÓWNANIE METODY ABTS ORAZ DPPH SŁUŻĄCYCH DO OKREŚLENIA CAŁKOWITEJ ZDOLNOŚCI PRZECIWUTLENIAJĄCEJ MLEKA LUDZKIEGO

STRESZCZENIE

Wstęp. Całkowitą zdolność przeciwutleniająca (TAC) mleka ludzkiego odzwierciedla zawartość i aktywność w mleku składników, które zapobiegają degradacji tłuszczów i białek na drodze utlenienia. Celem badania było porównuje testu ABTS i DPPH w odniesieniu do odzysku, precyzji i czułości (granica wykrywalności i oznaczalności) obu metod służących do oznaczania wartości TAC mleka ludzkiego.

Materiał i metody. Wartości TAC zostały określone dla dwudziestu pięciu próbek mleka uzyskanych od zdrowych matek, mieszkanek Gdańska, w 14 dobie po porodzie.

Wyniki. Średnia wartość TAC mleka ludzkiego oznaczona testem ABTS wynosiła 19,61 ±3,311 mg TE (równoważniki Trolox)/100 cm^3, średnia wartość uzyskana testem DPPH wynosiła 9,95 ±4,36 mg TE/100 cm^3. Dla każdej badanej próbki mleka kobiecego poziom TAC określony metodą ABTS był wyższy niż wartości oznaczone testem DPPH co ma prawdopodobnie związek z obecnością w mleku ludzkim substancji, których

widma pokrywają się z widmem rodnika DPPH. Test ABTS charakteryzował się większa czułością i powtarzalnością oznaczania TAC mleka ludzkiego w porównaniu do testu DPPH.
Wnioski. Porównując obliczone wartości parametrów walidacji obu metod oraz biorąc pod uwagę rozpuszczalność rodnika DPPH tylko w rozpuszczalnikach polarnych, wolniejszy przebieg reakcje przeciwutleniaczy z rodnikiem DPPH oraz obecność w mleku ludzkim składników absorbujących promieniowanie elektromagnetyczne w zakresie absorpcji DPPH należy przyjąć, że test ABTS jest metodą właściwszą oznaczania TAC mleka ludzkiego.

Słowa kluczowe: mleko ludzkie, całkowita zdolność utleniająca, test ABTS, test DPPH

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