

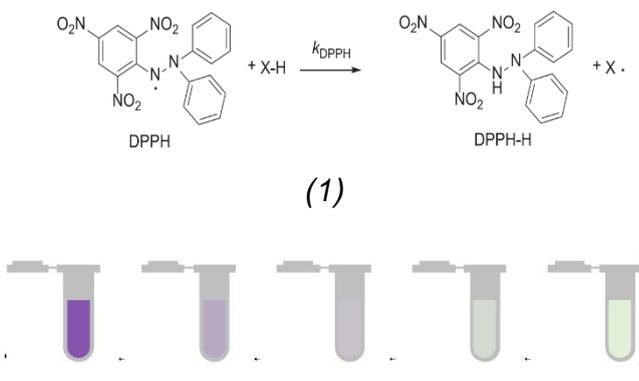
A new procedure for the Antioxidant capacity dpph assessment in small samples

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Graphical Abstract	Abstract.
 <p style="text-align: center;">(1)</p>	<p>The antioxidant capacity of a fluid consists in its capacity to prevent or slow down oxidation. It can be measured by several biochemical methods, one of them is the colorimetric reaction of 2-2-diphenyl-1-picrylhydrazyl (DPPH), developed by Brand-Williams in 1995. In order to apply this method to very small and diverse samples, we adapted the method. The use of a nanodrop for the colorimetric assessment allows a 20 fold reduction of the required sample volume, and a dpph sonication prior to use, and an extra centrifugation prior to the measurement allowed us to assess the antioxidant capacity of very reduced samples of breast milk and colostrum, and open the possibility for further analysis impossible until now, such as tears. For industrial purposes, it will allow a significant reduction of the volume used for the antioxidant capacity determination.</p>

Introduction (optional)

The antioxidant capacity of a substance is its ability to prevent or slow down oxidation. It is a reaction of a transfer of electrons. The molecule catching electrons is referred to as the oxidative agent and the molecule giving electrons as the reducing agent. Oxidative stress is toxic for the living cells, and it can be limited by different pathways: the inhibition of enzymes responsible of the production of oxidative species, the synthesis, activation or stabilization of antioxidant enzymes, and finally by a direct scavenging of radical species.(1)

In living organisms, especially in association to the respiration process, free radicals are commonly produced. Antioxidants are naturally present to prevent damages, and therefore the antioxidant capacity of a substance is a key element to evaluate. Several methods can be employed to assess total antioxidant capacity in a liquid, one of the most reliable and frequently used is based on a colorimetric reaction involving 2,2-diphenyl-1-picrylhydrazyl (DPPH) (2).

Brand & Williams developed in 1995 (3) the method of assessment of antioxidant capacity based on the color change of DPPH according to its oxidation status. The oxidized DPPH is a radical, with dark purple color and in contact with antioxidant species, reduced in contact with antioxidant species, turning to yellow-green color. The amount of oxidized DPPH can be quantified at 517nm spectrophotometrically. Using a known dose of antioxidant molecule, such as Trolox, a standard curve of antioxidant can be obtained. The antioxidant capacity of any sample can be assessed measuring the oxidized DPPH, and the result is expressed in Trolox equivalents. This method has been adapted by Martinez et al. in 2005 in order to assess antioxidant capacity in plasma samples (4).

Despite this modification, we encountered difficulties to proceed to this measurement on small samples of breast milk. The obstacles we have met using this method are (i) the important volume of sample required and (ii) the interference of molecules of fat present in some biological fluids (breast milk). We therefore adapted the original method in order to perform antioxidant capacity assessment on our samples. Here is presented the adapted protocol, and an example of results obtained.

Materials and Methods (optional)

Table 1. Trolox standard curve		
Trolox (μM)	Methanol (μL)	Trolox mother solution (μM)
800	0	1000
700	125	875
600	250	750
500	375	625
400	500	500
300	625	375
200	750	250
100	875	125
75	917	83
50	937	63
25	969	31
10	987	13
1	999	1
0	1000	0

Table 1: Standard curve concentrations of Trolox

Protocol

1. Label 1,5ml Eppendorf from 0 to 800 μM as indicated in table 1, and 0,5ml Eppendorf for the DPPH reaction (14 for the standard curve + the number of samples)
2. Prepare 200ml of 80% methanol

3. dissolve 10mg Trolox in 50ml methanol 80%
4. In each tube of the standard curve, add Trolox and methanol following the proportions indicated in table 1
5. Vortex pulse each tube
6. Dissolve 3,9mg dpph in 100ml methanol 80% and sonicate 10minutes
7. Pipet 10µL of sample or standard + 290µL of dpph in the labelled 0,5ml ependorfs
8. Vortex pulse each tube
9. Incubate the standard curve and the samples 30 min at room temperature in the dark
10. Centrifuge the samples 10 min at 5000G in order to prevent artefacts due to the nature of the sample
11. Measure the absorbance with a nanodrop at 517 nanometer : 2,5µl in the reader.
12. A curve is obtained from the standard Trolox solution absorbance, and the equation of this curve is used to obtain the value of antioxidant capacity of each sample, expressed in Trolox equivalent.

Results and Discussion (optional)

Measuring antioxidant capacity in breast milk using DPPH reaction presented two issues: An important volume of samples required for the test, and the artefacts produced by the complex composition of certain samples.

Reading about DPPH reaction and experiment, and performing different tests, we developed an adapted protocol which is adapted for small volumes of diverse body fluids.

To begin, fluid samples from living beings are complex, with lipids and proteins in suspension, susceptible to interfere with the colorimetric measurement. By performing a supplementary centrifugation at the end of the colorimetric reaction (5000G 10min), at a speed allowing separation of the components, a clean measurement is obtained.

Moreover, the 200µl volume required for the assessment in the previous method limits the number of experiment that can be done on one sample, and sometimes makes it impossible, especially in the colostrum samples. The use of the nanodrop device and the adaptation of the volume of reactants allows a determination with a sample volume of only 10µl.

Finally, the sonication of the DPPH solution is not specified in most of the protocols, nevertheless we could observe that it is necessary to obtain an homogeneous solution, therefore we want to insist on the importance of this step to obtain reliable results.

Using the protocol described earlier, we determined antioxidant capacity in 10 breast milk samples (table 2). In order to assess the reliability of the determination in a diluted sample, we diluted all sample in a ½ ratio with the solvent (methanol 80%).The measurements were performed with a nanodrop (a) and with a classic spectrophotometer (b).

First, we observed that the measurement of the antioxidant capacity of a diluted sample does not permit to calculate the value of the original sample, we therefore recommend not to proceed to dilution. In the case of very small volume of sample, the experiment can be done with our method, as the determination with the Nanodrop allows an assessment of antioxidant capacity equivalent to the one done with a classic spectrophotometer.

Table 2. Antioxidant capacity assessment with two different espectrofotometer devices.

N°	Nanodrop determination (a)				Classic spectrophotometer determination (b)			
	Without dilution		diluted		Without dilution		diluted	
	abs(517)	Trolox equivalent	abs(517)	Trolox equivalent	abs(517)	Trolox equivalent	abs(517)	Trolox equivalent

1	0,2	105,7	0,5	44,7	0,2	126,0	0,5	46,8
2	0,4	73,7	0,7	30,4	0,4	71,1	0,7	25,9
3	0,5	57,7	0,6	32,6	0,5	58,5	0,6	32,8
4	0,2	112,2	0,8	14,0	0,2	114,9	0,8	12,4
5	0,3	79,9	0,9	11,4	0,4	72,2	0,8	11,2
6	0,0	230,7	0,8	14,0	0,1	179,1	0,7	15,9
7	0,1	214,7	0,8	14,9	0,1	202,3	0,7	21,8
8	0,1	190,7	0,8	18,4	0,2	147,7	0,8	15,1
9	0,4	75,7	0,8	14,0	0,5	56,8	0,7	16,3
10	0,4	71,7	0,9	7,4	0,5	58,1	0,8	10,4

Table 2: Antioxidant capacity of 10 breast milk samples. Determination with nanodrop (a) and classic spectrophotometer (b) of DPPH absorbance and Trolox equivalent.

Moreover, the present method also allows antioxidant capacity determination in other body fluids, as referenced in table 3. The last centrifugation prior to measurement allows to limit the interferences due to the nature of the samples.

Sample	Trolox equivalents average	std desv	Type error %
Culture medium	175,2 ±	12,8	7.30
Urine	1120,2 ±	59,4	5.30
Breast milk	457,6 ±	33,1	7.23
Serum	405,9 ±	29,6	7.29
Saliva	271,9 ±	12,2	4.48

The applicability to very diverse and small samples opens the potential application of DPPH based antioxidant capacity measurement with a type error between 4-8 %. For instance, saliva of very young children, tears, colostrum, or other body fluids available only in reduced quantity could now be analyzed. The reduced volume required also allows to perform replicated, and also other tests. This advantage can also benefit the industry, with the assessment of the antioxidant capacity of dairy products, or of food additives (5) using reduced volumes.

Conclusions (*optional*)

We present an adaptation of the method of Brand-Williams (1995) based on the colorimetric assessment of the DPPH oxidative reaction in order to quantify the antioxidant capacity of a liquid. Our proposal consist on the use of a NanoDrop for colorimetric assessment, reducing 20 times the volume necessary for the test. Plus, we underline the interest of adding a centrifugation prior to the measurement, in order to prevent artefacts caused by the nature of several body fluids.

References (*mandatory*)

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