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A microplate adaptation of the thiobarbituric acid reactive substances assay to determine lipid peroxidation fluorometrically in small sample volumes

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Abstract: A simple, fast, reproducible and low-cost assay for thiobarbituric acid reactive substances (TBARS) has been adapted for use with a microplate spectrofluorometer. The technique allows rapid analysis of multiple samples and requires a very small sample volume (50 µl of red cell homogenates from passerine birds at protein concentrations of 3.4-8.9 mg/ml in this study), what is of special interest for biomonitoring studies working with small-sized animals from which a limited amount of sample can be obtained. The TBARS test involves the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) under heating (90°C), leading to the formation of products that can be measured fluorometrically using black 384-well plates at excitation/emission wavelength of 532/553 nm. The concentrations of peroxidized lipids in samples were determined by extrapolation from a MDA standard curve. Two different excitation/emission combinations (532/553 and 530/550 nm) were used and both pairs were suitable for this technique. Intra- and inter-plate variability was < 20% and a good linearity of the standard curve was observed (R2 > 0.99). The research use of this microplate adaptation of the TBARS assay will provide further data and understanding of lipid peroxidation reducing the limitation of small sample volume.

Keywords: lipid peroxidation; TBARS; malondialdehyde; oxidative stress; erythrocytes.

Resumen: ***Una adaptación de microplacas del ensayo de sustancias reactivas del ácido tiobarbitúrico para determinar la peroxidación lipídica fluorométricamente en pequeños volúmenes de muestra.***

El presente trabajo adapta un ensayo sencillo, rápido, reproducible y económico de sustancias reactivas al ácido tiobarbitúrico (TBARS) para su uso en espectrofluorómetro para microplacas. La técnica permite un análisis rápido de múltiples muestras y requiere un mínimo volumen de muestra (50 µl de un homogeneizado de eritrocitos de aves paseriformes a una concentración proteica de 3.4-8.9 mg/ml en este estudio), lo cual resulta de especial interés en estudios de biomonitorización que trabajan con animales de pequeño tamaño de los que se puede obtener una cantidad de muestra limitada. El ensayo TBARS consiste en la reacción del ácido tiobarbitúrico (TBA) con malondialdehído (MDA) en condiciones de calor (90°C), formando productos que pueden medirse fluorométricamente usando microplacas negras de 384 pocillos a 532/553 nm de excitación/emisión. La concentración de peróxidos lipídicos en la muestra se determinó por extrapolación de una curva de MDA. Se utilizaron dos combinaciones diferentes de excitación/emisión (532/553 and 530/550 nm) y ambas fueron apropiadas para la técnica. La variabilidad intra- e inter-placa fue < 20% y se observó una buena linealidad de la curva estándar (R2 > 0.99). El uso científico de la adaptación a microplaca del ensayo TBARS proporcionará más datos y comprensión sobre la peroxidación lipídica reduciendo la limitación que supone los pequeños volúmenes de muestra.

Palabras clave: peroxidación lipídica; TBARS; malondialdehído; estrés oxidativo; eritrocitos.

Introduction

Oxidative processes and the subsequent generation of free radicals are normal in the cellular metabolism (Finkel and Holbrook, 2000). In response to this processes, organisms are equipped with an antioxidant defense system able to inhibit the generation of reactive oxygen species (ROS) and reduce the oxidation and the consequent cellular damage (McGraw, 2011). However, different exogenous factors such as the exposure to environmental pollutants, radiation or infections can deplete the major antioxidants of cells and induce ROS generation leading to oxidative stress (imbalance between the antioxidant and pro-oxidant levels in favor of the latter; Halliwell and Gutteridge, 2007), which may cause oxidative damage to membrane lipids (Ahmad, 1995; Schwarz, 1996; Bayoumi et al., 2001; Ercal et al., 2001; García-Fernández et al., 2002; Azzam et al., 2012).

Lipids are essential to maintain the structure of cell membranes and control the function of cells, and they are the primary targets of the attack by ROS (Yin et al., 2011). The process in which oxidants attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs), is called lipid peroxidation (Ayala et al., 2014). This process results in a wide variety of oxidation products, the main primary products being the lipid hydroperoxides (LOOH), and two secondary products extensively studied the aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (see references in Ayala et al., 2014). Malondialdehyde and thiobarbituric acid reactive substances (TBARS) have been widely used as standard biomarkers of lipid peroxidation for many years because of its reaction with thiobarbituric acid (TBA) and its simplicity and low cost (Ayala et al., 2014; Niki, 2014). The TBARS test involves the reaction of MDA with TBA under acidic condition and heating, leading to the formation of pink-colored and fluorescent products that can be measured by colorimetric and fluorometric methods. Although originally it was accepted that the TBARS assay measured MDA, it is not exclusively measuring MDA, but also other aldehydes and decomposition products from hydroperoxides. However, even though there remains a controversy regarding the specificity of TBARS and artefactual production during analytical processes, it still remains among the most popular and commonly applied assays to determine lipid peroxidation (Niki, 2014).

In the field of toxicology, numerous studies have observed an increase in TBARS values as a response to pollutant-related oxidative stress in different organisms (e.g. Howlett and Avery, 1997; Oakes and Van Der Kraak, 2003; Stepić et al., 2012; Espín et al., 2014a; Osičková et al., 2014). Particularly in avian ecotoxicology, the number of studies evaluating the effects of pollutants such as metals on oxidative stress biomarkers (including TBARS) has significantly increased in the last years (Mateo and Hoffman, 2001; Mateo et al., 2003; Koivula and Eeva, 2010; Martínez-Haro et al., 2011; López-Antia et al., 2013; Espín et al., 2014a; 2014b; Ortiz-Santaliestra et al., 2015; Espín et al., 2016a), and results suggest that TBARS is a convenient, simple and low-cost method that may function as a useful biomarker of pollutant-induced lipid peroxidation in birds. When working with wild species and particularly with small animals (e.g. passerine birds), only minimal volumes of sample are available for the analysis of a battery of biomarkers. Therefore, adaptations of techniques to minimize the sample volume are needed in order to be able to evaluate those biomarkers in a wide range of species (Koivula et al., 2011; Espín et al., 2016b). The main aim of this study is to describe a microplate TBARS assay to determine lipid peroxidation in small sample

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volumes using red blood cells (RBC) from nestlings of a passerine bird species (great tit, *Parus major*). For this purpose, we developed an adaptation of the TBARS technique described by Alonso-Álvarez et al. (2008) following the technique by Aust (1985) with different modifications to minimize the sample volume and by using fluorometry. The principle of the assay is based on the fact that different tissues contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, and their concentrations increase due to oxidative stress (Alonso-Álvarez et al., 2008).

Reagent preparation

The sodium chloride (NaCl, 0.9%) was prepared by dissolving 0.9 g NaCl (27810.295, PROLABO, VWR Chemicals™) in 100 ml milliQ-water. For TBARS reagent preparation (15% trichloroacetic acid, TCA; 0.25 N hydrochloric acid, HCl; 0.375% 2-thiobarbituric acid, TBA), 7.5 g TCA (1.00807.0100, EMSURE, Merck™), 0.1875 g TBA (T-5500, Sigma™) and 1.035 ml HCl (37%; 30721, Riedel-de Haën™) were dissolved in 50 ml milliQ-water. The butylated hydroxytoluene (BHT, 2%) was prepared by dissolving 0.2 g BHT (B-1378, Sigma™) in 10 ml ethanol (99.5%, ALTIA Oyj™). Finally, the stock malonaldehyde solution (MDA, 417 µM) for the standard curve was prepared by dissolving 17.25µl MDA (malonaldehyde bis (dimethyl acetal) or 1,1,3,3-Tetramethoxypropan 99%; 10,838-3, Aldrich™) in 250 ml ethanol. All the reagents were stored at 4ºC.

Method procedure

Sample collection

This method is described for bird erythrocytes, but it can be applicable to other biological sample types. Blood samples from great tit nestlings (14 days old) were collected during the breeding season 2015 in southwestern Finland. Blood samples (approximately 75 µl) were collected by venipuncture of the brachial vein with a needle and using sodium-heparinized microhematocrit capillary tubes (80 iu/ml, Marienfeld™). Tubes were centrifuged in the field (4400 g, 5 min) and RBCs were split in 200-µl microcentrifuge tubes and kept in liquid nitrogen and then conserved at −80°C in the laboratory. A total of 100 RBC samples were used in this study.

Sample and MDA standard curve preparation

RBC were homogenized in 0.9% NaCl to maximize the volume and to get protein concentrations between 3.4 and 8.9 mg/ml, working on ice to avoid oxidation. The protein concentration (mg/ml) was measured using the Pierce™ BCA Protein Assay Kit from ThermoFisher Scientific, Waltham, Massachusetts, USA. In brief, the BCA reaction mix is made according to the kit instructions (50:1, BCA Reagent A:B). A serial dilution of bovine serum albumin (BSA, 10 mg/ml) is used as protein standard. One µl of each BSA standard dilution, control (salmon liver) or sample is pipetted in a transparent 384-well plate in triplicate. Then, 50 µl of BCA reaction mix are added to each well with a multichannel pipette and briefly mixed using a plate shaker. Then the plate is incubated at 37 ºC for 30 min. Finally, the protein concentration is measured spectrophotometrically at an absorbance of 562 nm.

Fifty µl of each homogenate was split in a 1.5-ml microcentrifuge tube for TBARS assay, and the remaining was divided into different microcentrifuge tubes for other oxidative stress measurements. For 5 of the samples, 250 µl of homogenate was divided in 5 tubes (50 µl per tube) in order to use them as controls in the different plates and evaluate the inter-assay precision. All measurements (standards, controls and samples) were done in triplicate in each plate to evaluate the intra-assay variability.

Seven standard dilutions of MDA (from 0 to 0.5 nmol/ml) were prepared using the stock MDA solution (417 µM) and milliQ-water according to the instructions provided in Table 1. Firstly, 100 µl from the stock MDA solution were dissolved in 900 µl of millliQ-water to prepare solution C, 100 µl from solution C were dissolved in 900 µl of milliQ-water to prepare solution B, and 149.9 µl from solution B were dissolved in 850.1 µl of milliQ-water to prepare solution A. The standard point number 7 was prepared by dissolving 800 µl from solution A in 200 µl of millliQ-water, the standard point number 6 was prepared by dissolving 500 µl from standard 7 in 500 µl of milliQ-water, etc., following the process shown in Table 1.

TBARS assay description

This method is described to work in sets of 19 different unknown samples, 5 control samples and a standard curve of 7 points. Therefore, 95 unknown samples were analyzed in 5 different assays, and the other 5 unknown samples were used as control samples in all the assays. Several sets can be done the same day, and a standard curve should always be included in each assay in order to calculate the final MDA concentration.

Before starting the assay, a set of 1.5-ml microcentrifuge tubes containing 500 µl of water is prepared (31 tubes in total, one per standard point, sample and control). Tubes are labelled with the standard number or sample identification code and a glass insert (with conical base and plastic bottom spring, 6 x 29 mm) is introduced inside each tube (the water will facilitate the heat transfer to the sample that will be inside the glass insert). The standard curve and a 1:100 mix of BHT 2% and TBARS reagent (a mix of 40 µl BHT and 4 ml TBARS reagent will be needed for each set of 19 samples, 5 controls and the standard curve) are prepared daily. All reagents except samples, controls and standards must be equilibrated to room temperature before beginning the assay.

A diagram summarizing the assay protocol is shown in Figure 1.

Table 1: *Standard curve preparation (7 points) for a microplate adaptation of the TBARS assay.*

MDA concentration (nmol/ml)	0	0.03125	0.0625	0.09375	0.125	0.25	0.5	0.625	4.17	41.7	417
Standard number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	A	B	C	Stock
MilliQ-water (µl)	1000	500	500	977.52	500	500	200	850.1	900	900	
MDA (µl)	0	500 from 3	500 from 5	22,48 from B	500 from 6	500 from 7	800 from A	149.9 from B	100 from C	100 from Stock	

MDA: malondialdehyde

Table reading: 100 µl from the stock MDA solution were dissolved in 900 µl of millliQ-water to prepare solution C, 100 µl from solution C were dissolved in 900 µl of milliQ-water to prepare solution B, and 149.9 µl from solution B were dissolved in 850.1 µl of milliQ-water to prepare solution A. The standard points (1-7) were prepared by dissolving X µl from solution X in X µl of millliQ-water as shown in the table.

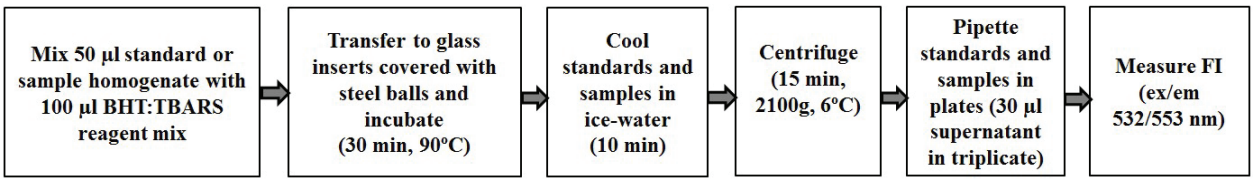

Figure 1 Diagram summarizing the microplate TBARS assay protocol.

Table 2: *Validation parameters (i.e. intra/inter-assay coefficient of variability and linearity of the standard curve) for a microplate adaptation of the TBARS assay.*

Excitation/emission wavelength	Sample type	Intra-assay CV (%)[a]	Inter-assay CV (%, global)[b]	Inter-assay CV (%, different days)[c]	Inter-assay CV (%, different researchers)[d]	Linearity (R^2)[e]
532/553 nm	Control	2.73 (1.78-4.02)	15.95 (11.66-19.36)	14.38 (13.34-16.05)	5.89 (1.67-8.59)	-
	Standard curve	4.13 (2.70-5.35)	12.03 (3.34-27.03)	4.85 (0.53-16.19)	8.40 (0.76-26.00)	0.995 (0.991-0.999)
530/550 nm	Control	2.66 (1.86-3.15)	15.38 (10.94-20.24)	13.99 (10.80-17.04)	4.98 (0.55-7.99)	-
	Standard curve	4.13 (3.29-5.44)	11.03 (1.31-22.51)	3.87 (0.06-15.04)	7.30 (0.72-21.65)	0.994 (0.991-0.998)

[a] *Intra-assay precision reflects variability among triplicates within the same assay run (same microplate) (mean, min and max CV for 5 control samples or 7 standard points in the 5 plates)*
[b] *Inter-assay precision (global) reflects variability among microplates for the same sample/standard (mean, min and max CV for 5 control samples or 7 standard points)*
[c] *Inter-assay precision (different days) reflects variability with time (mean, min and max CV for 5 control samples or 7 standard points)*
[d] *Inter-assay precision (different researchers) reflects variability among researchers (mean, min and max CV for 5 control samples or 7 standard points)*
[e] *Linearity calculated using 7 different standard points from 0 to 0.5 nmol MDA/ml (mean, min and max R^2 for 5 standard curves)*

The tubes with 50 µl of the RBC homogenates and 7 tubes with 50 µl of each standard point are kept on ice and mixed with 100 µl of the mix TBARS reagent plus BHT. The whole mix is transferred to glass inserts kept inside appropriately labelled 1.5-ml microcentrifuge tubes with 500 µl of water, and then warmed for 30 min at 90ºC in a thermoblock. During the incubation in the thermoblock, keep the microcentrifuge tubes open and place stainless steel balls (6 mm) covering all the glass inserts. The steel balls prevent the sample evaporation but allow the escape of excess gas. After the incubation, the steel balls are removed, the tubes are closed carefully with the inserts inside, and the samples are cooled in ice-water for 10 min to stop the reaction. The tubes are centrifuged for 15 min at 6ºC and 2100 g. Subsequently, the 7 standard points, samples and controls are pipetted in the microplate in triplicate (a total of 93 wells are used). A volume of 30 µl of supernatant in triplicate (30 µl per well) is pipetted in black 384-well plates (OptiPlate, PerkinElmer), keeping the plate on ice while pipetting. There is no specific pattern for using the wells on the microplate and it is not necessary to use all the wells on the microplate at one time. Supernatant has to be taken with caution while pipetting it in the microplate to avoid the pellet-supernatant mixture after centrifugation. Glass inserts with conical base and plastic bottom spring (6 x 29 mm) are recommended since the conical base will help to keep the pellet at the bottom of the insert after centrifugation. Some trials were done using glass inserts with flat base and there were pellet-supernatant mixture problems. It is also possible to filter the sample before pipetting. However, part of the sample can be lost during this process and it should be done carefully in order to have enough volume for the triplicates (90 µl in total).

Finally, the fluorescence intensity (FI) is measured at an excitation/emission wavelength of 532/553 and 530/550 nm with the microplate spectrofluorometer (EnSpire 2300 Multilabel Reader, PerkinElmer™).

Calculations

After calculating the mean fluorescence for triplicate measurements of each standard, control and sample, the coefficient of variability (CV) for triplicates is determined as follows (equation 1):

$$CV\ (\%) = (SD/M) \times 100 \qquad (1)$$

where SD is the standard deviation and M is the arithmetic mean value for the repeated measurements. If a high dispersion of triplicates is observed (CV values > 20%), this may be due to pipetting errors or presence of bubbles in the well. A meticulous pipetting is recommended to prevent sample splash from the wells and the plate can be carefully tapped with the fingers to remove bubbles before the FI measurement.

The fluorescence values of each standard are plotted as a function of the MDA concentration by linear regression analysis [y = (slope) x + y-intercept]. The concentrations of peroxidized lipids in samples and controls are determined by extrapolation from the MDA standard curve from each assay. This way, we will obtain the nmol of MDA per ml of homogenate (equation 2).

$$MDA\ (nmol/ml\ homogenate) = [(FI - y\text{-intercept}) / slope] \qquad (2)$$

If MDA is not detected in the samples, this may be due to a low MDA concentration or the sample being too diluted, thus a lower RBC dilution in order to have a more concentrated homogenate may help to detect MDA.

As explained before, the total protein concentrations were analyzed in the same homogenates and were expressed as mg per ml of homogenate. The final MDA concentration can be expressed in relation to the mg of protein in RBC homogenates (nmol of MDA per mg of protein). Alternatively, the final MDA concentration can be expressed in relation to the amount or volume of the original tissue (e.g. nmol of MDA per mg of RBC, nmol of MDA per ml of blood). In this case, the sample values must be corrected for any dilutions performed during sample preparation, and the original sample amount/volume must be recorded.

Fluorescence measurement (excitation and emission wavelengths)

In fluorometric determination of TBARS, discrepant data for excitation and emission wavelengths have been reported in literature (Yin, 1995). Although TBARS assay can be measured by colorimetric methods (absorbance at 535 nm), higher volumes of sample are needed to obtain reliable data. However, fluorometric assays may be more sensitive and, therefore, more suitable for small amount of sample and samples containing low lipid peroxidation products (Yagi, 1976; Jo and Ahn, 1998). According to Yagi (1976), the excitation/emission maxima of the MDA-TBA reaction product were observed at 532/553 nm, while Yin (1995) found an excitation/emission maxima of the MDA-TBA product at 536/549 nm. The latter recommends excitation/emission wavelengths as close as possible to these values in order to obtain the greatest sensitivity.

In the present study, the fluorescence excitation/emission spectrum was studied on a microplate spectrofluorometer (EnSpire 2300 Multilabel Reader, PerkinElmer™) by scanning wavelengths of an excitation light while a wavelength in the emission detector was fixed and vice versa, and the highest fluorescence intensity was observed at an excitation wavelength of 530-535 nm and an emission wavelength of 550-555 nm. Therefore, the fluorometric measurement at excitation/emission wavelengths of 532/553 nm reported by Yagi (1976) was used. This was possible because of the ability of our instrument to select any specific wavelength; however, some instruments do not have this flexibility. Therefore, all the plates were also measured at 530/550 nm according to other methods described in the literature. The excitation/emission wavelength of 536/549 nm could not be tested since a minimum distance of 20 nm between excitation and emission wavelengths is needed in our instrument. Figure 2 shows the mean standard curves plotting fluorescence and MDA concentration (nmol/ml) obtained at 532/553 nm and 530/550 nm. Measurements at excitation/emission wavelengths of 532/553 nm and 530/550 nm provided very similar intra- and inter-assay CV and fluorescence intensity for control samples and standards, and the linearity of the standard curve showed R^2 values > 0.99 (Table 2), thus both excitation/emission pairs seem to be suitable for this technique.

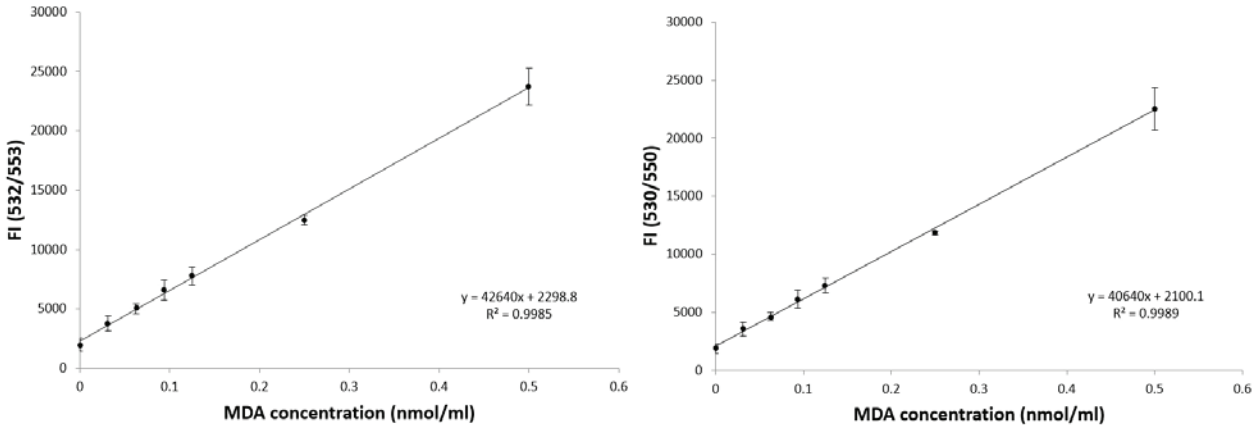

Figure 2: Standard curves plotting fluorescence intensity (FI) and MDA concentration (nmol/ml) at 532/553 nm and 530/550 nm of excitation/emission wavelengths. Each standard point corresponds to the mean value ± SD of 5 different curves (in each curve all the standard points were measured in triplicate).

Method validation

The precision of an assay can be described using repeatability and reproducibility tests. Repeatability is used to prove the ability to provide similar results when the measurement is repeated in the same sample under the same operating conditions and by the same operator. It is also called intra-assay precision. Reproducibility expresses the ability to provide similar results when the technique is repeated in the same sample but by different operators or different laboratories. The effect of random events on the precision of the assay can be also tested, and a typical variation to be studied is the inter-assay precision in different days. The precision of the analytical procedure is usually expressed as the CV of a series of measurements. Repeatability and reproducibility acceptance criterion was set at CV ≤ 20%. To validate the repeatability and reproducibility of the method, standards, samples and aliquots of a subset of 5 different samples (control samples) were analyzed in the 5 assays developed by 2 different researchers (S.E. and P.S-V.) in 2 different days. All measurements (standards, samples and controls) in each plate were done in triplicates to evaluate the intra-assay precision, reflecting the variability among triplicate determinations within the same assay run. The intra-assay CV was < 10% for the 100 samples/controls and the standards analyzed. The inter-assay variation when comparing assays done at different days was < 20% for both the standard curve and the control samples, and the variation when the standards and control samples were analyzed by different researchers was < 10% (Table 2). These results indicate that the method can be considered acceptable for the analysis of TBARS.

The linearity of the standard curve is evaluated to determine the proportionality between the concentration of MDA in the standard points and the FI. In the present study, the linearity was calculated using 7 different standard points (from 0 to 0.5 nmol MDA/ml, Table 1). Each standard point was analyzed in triplicate and a different standard curve was analyzed in each assay. Linear regression of data to a calibration curve was performed, and the linearity was accepted when $R^2 > 0.95$. A good linearity was found in all the assays, since R^2 was above 0.99 (Table 2).

Conclusiones

The microplate assay herein described is a simple, fast, reproducible and economical fluorometric method for TBARS determination in small sample volumes. Only 50 µl of diluted RBC homogenates are needed, what is of special interest for biomonitoring studies working with animals of small size from which a limited amount of sample can be obtained non-destructively. In addition, multiple samples may be analyzed simultaneously. This TBARS microplate assay format may be easily adapted to measure TBARS in different sample types and species using the appropriate concentrations in the standard curve. The research use of this microplate adaptation of the TBARS assay will provide further data and understanding of lipid peroxidation in different organisms reducing the limitation of small sample volumes.

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Declaration of interest statement

The authors declare that they have no conflict of interest.

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