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A Novel Combination of Methods for Evaluating the Balance Between Oxidative Stress Damage and System's Intrinsic Total Oxidative Capacity

Georgios K Efthymiadis[1]*, Alexandros Tsoupras[2] and Chryssa Anastasiadou[3]*

[1]*University of Vienna, Department of Molecular Evolution and Development, 1030, Austria*

[2]*Hephaestus, Laboratory, School of Chemistry, Faculty of Sciences, Democritus University of Thrace, St Lukas, 65404, Greece*

[3]*Fisheries Research Institute, 64007, Nea Peramos, Kavala, Greece*

***Corresponding author:** Georgios K Efthymiadis, University of Vienna, Department of Molecular Evolution and Development, 1030, Austria, georgios.efthymiadis@univie.ac.at

Chryssa Anastasiadou, Fisheries Research Institute, 64007, Nea Peramos, Kavala, Greece, anastasiadou@inale.gr

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ABSTRACT

Oxidative stress is a multifaceted process of critical importance in the development of various diseases, driven by the degeneration of cellular lipids through reactive species, primarily reactive oxygen and nitrogen species (ROS and RNS). These agents initiate a chain reaction that can spread across tissues, contributing to cellular damage. The mechanisms underlying the production of these reactive species and their role in disease progression have been extensively studied, leading to advancements in therapeutic strategies and diagnostic methods that detect end-products of lipid oxidation, such as malondialdehyde (MDA). In parallel, measuring the total antioxidant capacity (TAC) of a system provides valuable insight into its ability to counteract oxidative damage and protect cellular integrity. This review discusses the combined application of two established methods in oxidative stress research: one that quantifies cell wall damage via lipid peroxidation (TBARS) and another that assesses the antioxidant defense system via the Blue CrO5 assay. By integrating these approaches, we propose a more robust framework for evaluating oxidative stress and antioxidant capacity, offering deeper insights into the mechanisms of cellular protection and advancing our understanding of how oxidative damage can be mitigated in biological systems.

Keywords: Oxidative Stress; Total Antioxidant Capacity; MDA; Blue CrO5; TBARS

Abbreviations: MDA: Malondialdehyde; TAC: Total Antioxidant Capacity; TBARS: Thiobarbituric Acid Reactive Substances; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species

Oxidative Damage and Its Role in Cell Wall Degradation

Oxidative stress arises from an imbalance between oxidative substances and antioxidants, leading to redox disequilibrium (Preedy, et al. [1]) and subsequent cell degeneration, primarily through lipid peroxidation of the cell wall (Etsuo N, et al. [2,3]). This process is driven by free radicals-molecules with unpaired electrons in their outer shell (C Parthiban, et al. [4])- originating from diverse chemical categories. The most prominent contributors are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Xianghong Yan, et al. [5]). Among ROS, superoxide anion (O_2•-), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2) are particularly biologically significant (Y Simos, et al. [6]). These radicals are by-products of the evolutionary adaptation of oxygen as the terminal electron acceptor in the mitochondrial electron transport chain during cellular energy production (Charalambidis, et al. [7]) (Table 1).

Table 1: Various free radicals of ROS and RNS involved in oxidative processes. Free radicals are characterized by unpaired electrons, while non-radicals include stable molecules that can still participate in oxidative reactions. (Adapted from Sies, et al. 2017).

Free radicals	Non-radicals
Reactive Oxygen Species (ROS)	
Superoxide anion radical ($O_2^{-\bullet}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl radical (OH•)	Organic hydroperoxide (ROOH)
Peroxyl radical (ROO•)	Singlet oxygen ($^1O_2$)
Alkoxyl radical (RO•)	Electronically excited carbonyls (RCO)
Reactive Nitrate Species (RNS)	
Nitrogen monoxide (NO•)	Nitrite (NO_2^-)
Nitrogen dioxide ($NO_2^{\bullet}$)	Nitroxylanion (NO^-)

Production of Free Radicals: Pathways and Implications

Free radicals are generated through multifaceted processes involving both exogenous and endogenous factors. For instance, Fenton-type reactions produce highly reactive hydroxyl radicals (•OH), which inflict substantial damage on biological systems (Winterbourn, et al. [8]):

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + HO\bullet + OH^- \quad (1)$$

Endogenously, free radicals often originate from immune cells such as phagocytes (macrophages, monocytes, and neutrophils) as part of the body's defense mechanisms. During immune responses, these cells release reactive species to combat invading pathogens (Rosen, et al. [9]). Various studies so far have demonstrated that specific ROS, including superoxide anion radicals (O_2•–) and singlet oxygen ($^1O_2$), play a critical role in bacterial destruction during phagocytosis. Following this process, enzymatic systems neutralize many of these reactive products, though secondary metabolites may form as a result of metabolic activity (Di Meo, et al. [10,11]).

$$NADPH + O_2 \xrightarrow{NOX2} NADP + NADP + O^-_2 + H^+ \quad (2)$$

$$O^-_2 + OH \rightarrow OH^- + {}^1O_2 \quad (3)$$

Furthermore, exogenous sources, like the rising temperatures and elevated atmospheric CO_2 directly influence free radical production and magnify oxidative stress. These changes can disrupt cellular functions, impair growth and reproduction, and increase mortality rates (Feidantsis, et al. [12]; Sies et al 2017).

A System's Intrinsic Ability to Inhibit the Free Radicals Contains Various Mechanisms

The intrinsic ability of a system to counteract free radicals relies on a variety of antioxidant mechanisms. The concept of Total Antioxidant Capacity (TAC) encompasses the combined activity of both enzymatic and non-enzymatic antioxidants (Chodakowska, et al. [13,14]). TAC serves as a key metric, providing a broad yet precise assessment of a system's overall defensive capability to mitigate oxidative damage and protect cellular macromolecules from oxidative factors. The dynamic interplay between endogenous and exogenous oxidants and their corresponding counteractive mechanisms has garnered significant attention across disciplines, from biochemistry to health sciences. Within a biological cell the most common antioxidant enzymes that simulate antioxidant defense are the Superoxide of dismutase responsible for catalyzing harmful ROS to their less reactive species (4), Catalase which breaks down hydrogen peroxide to water and oxygen (5) and Glutathione peroxidase, which reduces lipid peroxides and minimizes lipid deterioration (6) (Sáez, et al. [15,16]):

$$SOD: \cdot O_{2^-} + 2H + \rightarrow H_2O_2 \quad (4)$$

$$CAT: 2H_2O_2 \rightarrow 2H_2O + O_2 \quad (5)$$

$$GPx: 2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (6)$$

However, to gain a comprehensive understanding of antioxidant defense, it is crucial to have a method that accurately captures the synergistic action of not only the enzymatic related mechanisms, but also the non-enzymatic ones, like the antioxidant mechanistic benefits of ascorbic acid and α-tocopherol, just to name a few. This can provide a broader perspective on the system's overall ability to combat oxidative stress, beyond focusing on individual components or specific phases.

A Combined Approach for a Holistic Evaluation of a System's Oxidative Stress Damage and Resilience

Research on oxidant regulation offers a wide array of opportunities, with projects spanning environmental, health, and food science domains. Previous endeavors (Feidantsis, et al. [12]) emphasize the growing impact of oxidative damage on marine organisms, driven by abiotic factors, which can increase free radical production, while others highlight the pathogenic role of oxidative stress-induced cellular damage in diseases such as Alzheimer's, skin disorders, and cancer (Charalambidis, et al. [7]; Nakei & Tsuruta 2017). As mentioned before, traditional methods for oxidative stress assessment often focus on identifying specific by-products of lipid peroxidation. Techniques such as the widely used TBARS (Thiobarbituric Acid Reactive Substances) assay, exemplify such an approach. Even though these approaches provide valuable insights, they predominantly address oxidative damage retrospectively, with limited emphasis on evaluating the total intrinsic antioxidant defenses of the organism. To address this gap, we propose an innovative framework that integrates two complementary perspectives:

1) Assessing cellular damage from oxidative stress, focusing on lipid peroxidation in cell membranes, and

2) Quantifying TAC to measure the system's ability to neutralize free radicals. Together, these methods provide a comprehensive evaluation of oxidative stress and resilience, offering critical insights into the balance between oxidative damage and antioxidant defense. This holistic perspective facilitates targeted strategies in environmental conservation, disease management, and food quality improvement.

TBARS Assay: Capturing Oxidative Stress Damage Through Lipid Peroxidation

During lipid oxidation of polyunsaturated fatty acids (PUFAs) by free radicals, various by-products are generated, with malondialdehyde (MDA) being a widely recognized biomolecular marker over the years (Pryor WA, et al. [17]). MDA forms from the breakdown of lipids containing three or more double bonds, a process catalyzed by cyclooxygenase (Gaetano, et al. [18]). As a reactive aldehyde, it is part of a broader group of reactive electrophile species that induce cellular toxic stress by forming covalent bonds with proteins (Farmer, et al. [19]). The TBARS (Thiobarbituric Acid Reactive Substances) method, first introduced by Buege and Aust in 1978, is a well-established assay for measuring lipid peroxidation. It detects MDA, through its reaction with 2-thiobarbituric acid (TBA) as depicted in Figure 1 and can be easily detected with spectrophotometric means. However, TBARS results can be influenced by potential biases, including MDA production through auto-oxidation during assay conditions and cross-reactivity with non-MDA substrates or other aldehydes (Oakes, et al. [20,21]) Despite these limitations, the TBARS method remains a valuable supplementary approach in projects assessing oxidative stress. The continuous application of the TBARS assay is demonstrated by its utility in recent endeavors, particularly in biomedical studies of oxidative stress-related diseases as well as about environmental toxicology (Figure 1).

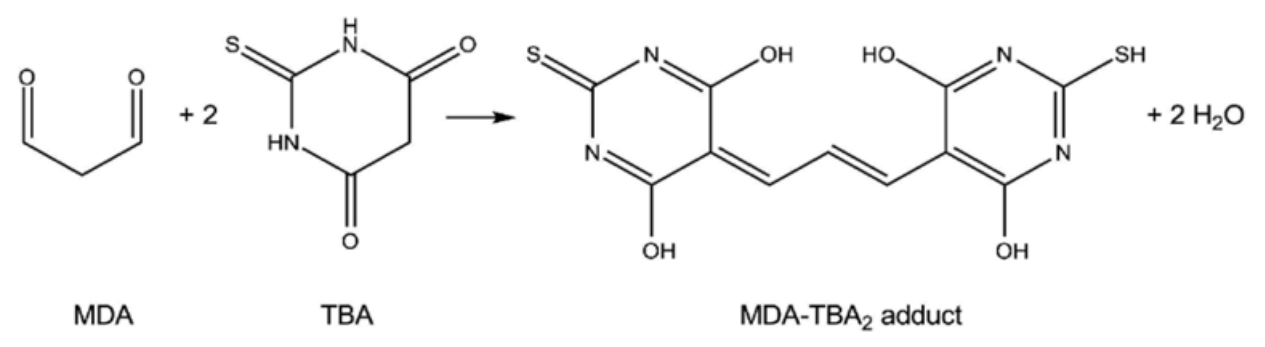

Figure 1: Malondialdehyde reacts with two molecules of 2-thiobarbituric acid to form a characteristic chromophore, as indicators of lipid peroxidation (adapted from Weitner, et al. [29]).

Domanskyi, et al. [22] highlight in their recent work, how TBARS can detect oxidative stress in neurodegenerative diseases, as reactive oxygen and nitrogen species can contribute to neurodegeneration, while Fernandez, et al. [23] employed it in clinical research, to monitor oxidative stress biomarkers in patients with metabolic disorders and depression. Finally, TBARS has provided key insights in how the impact of seasonal and environmental changes on oxidative stress of commercially important invertebrates reveal seasonal shifts in metabolic rates and antioxidant defenses, underlining the vulnerability of marine species to habitat alteration due to climate change (Feidantsis, et al. [24]).

Blue CrO_5 Assay: A Robust Method to Quantify a System's TAC

The Blue CrO_5 assay, developed by Charalampidis, et al. [7], provides a robust method for quantifying Total Antioxidant Capacity (TAC), yet it has not been fully recognized for its potential. The method utilizes chromium peroxide (CrO_5), a potent oxidant, as the core reactive agent. The concentration of the deep cyan blue CrO_5 can be accurately measured spectrophotometrically, offering a reliable means to assess oxidative stress and antioxidant defense. The total reaction is described by the following equation:

$$(NH_4)_2Cr_2O_7 + 4H_2O_2 + 2H^+ \rightarrow 2Cr(O_2)_2 + H_2O + 4H_2O + \text{ammonium salt} \quad (7)$$

Chromium peroxide is formed under acidic conditions facilitated by the addition of sulfuric acid (H_2SO_4). The acid provides the necessary environment for the oxidative reaction with ammonium dichromate ($(NH_4)_2Cr_2O_7$) to form ammonium sulfate ($(NH_4)_2SO_4$). In the presence of isoamyl alcohol or propylene carbonate a bi-phasic solution is created (Figure 2), wherein CrO_5 dissolves into the organic phase. Briefly, the assay involves two key measurements:

1) The sample is introduced into the solution and its absorbance is recorded and
2) A defined quantity of H_2O_2 is added to simulate oxidative stress, initiating the formation of CrO_5 as indicated from its characteristic cyan blue color.

This oxidation process simultaneously activates the sample's antioxidant defense mechanisms. The difference in absorbance before and after the addition of H_2O_2 ($\Delta A = A_2 - A_1$) quantifies the TAC of the sample. A higher ΔA value indicates a greater antioxidant capacity, reflecting the system's ability to neutralize free radicals. The intensity of the blue coloration reflects the presence of peroxides, indicating the oxidant capacity of the sample. The inhibition of color formation represents the sample's ability to mitigate oxidative reactions, quantifying its antioxidant defenses.

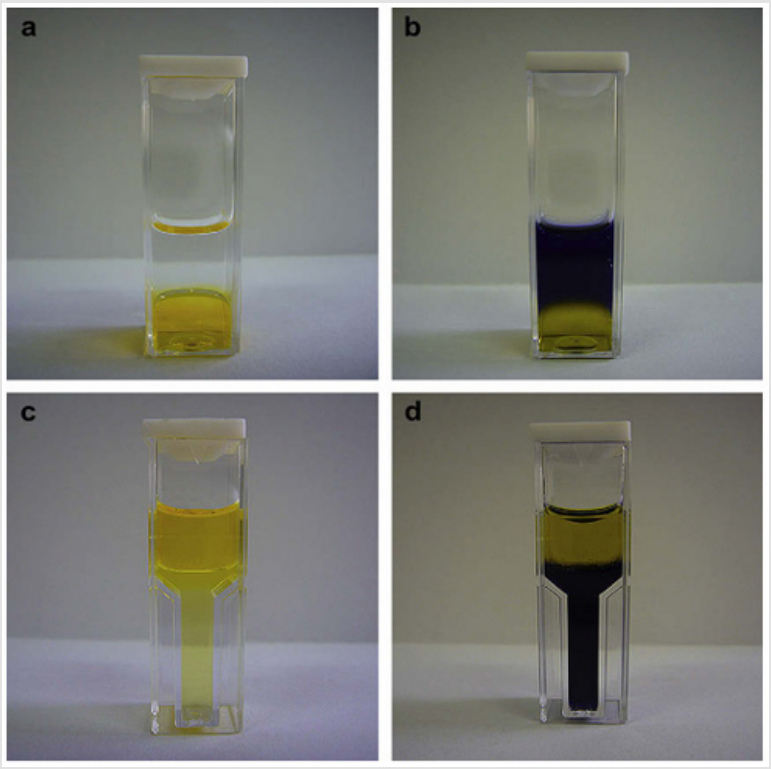

Figure 2:
a) Colorless organic phase (isoamyl alcohol) and yellow aqueous phase (ammonium dichromate) before H_2O_2 addition.
b) Blue organic phase (chromium peroxide) and yellow aqueous phase after H_2O_2 addition.
c) Yellow aqueous phase (ammonium dichromate) and colorless organic phase (propylene carbonate) before H_2O_2 addition.
d) Yellow aqueous phase (ammonium dichromate) and blue organic phase after H_2O_2 addition (Adapted from Charalambidis, et al. [7]).

Applications and Limitations of the Blue CrO_5 Assay in Antioxidant Research

The use of Blue CrO_5 assay has been proved in a variety of research areas, emphasizing its versatility. In food science Simos, et al. [25] examined the antioxidant properties of milk from different mammals, demonstrating its applicability on dietary sources while Efthymiadis et al 2024 (accepted – under publication) highlighted the course of change of TAC values and how it aligns with the external enzymatic browning in two commercial important shrimp species. Furthermore, the impact of oxidative stress on the severity of knee osteoarthritis, and antioxidant defense evaluation in biological samples, providing insights into its potential applications in clinical diagnostics and therapeutic research (Oikonomidis, et al. [26]). While these examples support the assay's utility in diverse contexts, few challenges remain. For example, the assay's results can be influenced by interfering compounds in the sample, such as substances that may react with the chromium reagent, leading to misinterpretation of antioxidant capacity. Also, like many spectrophotometric assays, there is a weak correlation between *in vitro* antioxidant capacities measured by this assay and their biological relevance *in vivo*.

Future Perspectives

While specialized assay kits allow the measurement of specific antioxidant enzymes or individual non- enzymatic antioxidant molecules such as α-tocopherol, ascorbic acid, these assays are often more expensive, require complex reagents, and cannot fully capture the overall oxidative stress and antioxidant defense balance in a system, limiting their ability to provide a comprehensive assessment. Furthermore, previous efforts have also focused on combining different methods for evaluating antioxidant defense along with lipid peroxidation, aiming to enhance the robustness of results (Sadder, et al. [27,28]). Traditional TAC evaluation methods, such as spectrophotometric assays (DPPH, ABTS, FRAP), have been effectively integrated with lipid peroxidation detection methods, including TBARS and protein carbonyl assays, which rely on by-product detection. This cross-method approach has shown promise in providing a more comprehensive analysis of oxidative stress and antioxidant mechanisms. In this short review, the proposed integration of TBARS and Blue CrO_5 assays has the potential to consist of a novel multi-method approach, offering a more comprehensive evaluation of both oxidative damage and antioxidative defense mechanisms, enhancing the depth of analysis. Both the TBARS and Blue CrO_5 assays offer practical, cost-effective methods for assessing oxidative stress and antioxidant capacity.

These techniques can be performed using standard laboratory equipment, including a simple spectrophotometer, requiring available and inexpensive reagents. In both assays, the sample preparation process is straightforward, typically involving treatment with organic solvents (commonly at a ratio of 1:10), making them accessible even for basic laboratory setups. The simplicity, reproducibility, and sensitivity of both methods make them ideal for large-scale or routine assessments, offering significant advantages over more expensive and complex assays. Thus, the proposed synergistic combination of TBARS and Blue CrO_5, could improve the accuracy of oxidative stress evaluation along with the antioxidant profile estimation, contributing to more effective interventions in areas such as environmental conservation, disease prevention, and food quality enhancement [29].

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