

AN ABSTRACT OF THE THESIS OF

Shu Jiang for the degree of Master of Science in Food Science and Technology presented on September 16, 2014.

Title: Selective Oxidation of Biological Matrices for Subsequent Peroxidase and Peroxidase-Based Quantitative Analyses

Abstract approved:

Michael H. Penner

Hydrogen peroxide quantification is of broad interest due to the common use of hydrogen peroxide as an oxidizing agent in industrial processing and laboratory research. Hydrogen peroxide assays are also of general importance for biological studies aimed at understanding the role of *in situ* generated reactive oxygen species. In the latter scenario particularly, assays amenable to high throughput processing are needed. Peroxidase-based methods are appropriate for such applications due to the high selectivity and sensitivity of enzyme catalyzed reactions. A problem commonly encountered when using peroxidase-based methods to quantify the level of hydrogen peroxide in biological samples is assay interference due the presence of assay-modifying endogenous compounds. This type of interference has limited the applicability of peroxidase/chromophore-linked assays which are commonly used elsewhere for high throughput screening (e.g., the glucose oxidase/peroxidase assay for glucose quantification). Potential mechanisms of assay interference include enzyme inhibition/inactivation, substrate competition and product modification. In the present study we addressed the different mechanisms of interference, especially product (oxidized reporter molecule) modification, using the following system: horseradish peroxidase, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and a hydrogen peroxide-containing garlic paste extract (GPE). Methods using ABTS as an appropriate reporter molecule to circumvent the interference are based on removal

of confounding compounds, particularly referred to as natural antioxidants, prior to initiating the assay. Because confounding compounds interfere with the peroxidase-based assay by converting peroxidase-catalyzed ABTS oxidation product, $\text{ABTS}\cdot^+$ back to ABTS, prepared $\text{ABTS}\cdot^+$ was used to selectively oxidize, thus inactivate confounding compounds that would cause confounding in this assay. A calibration curve generated by using $\text{ABTS}\cdot^+$ treated GPE sample was not significantly different ($p>0.05$) from the curve obtained in the model buffer system. In contrast to a flat baseline generated by original GPE sample, the effectiveness of $\text{ABTS}\cdot^+$ treatment in hydrogen peroxide quantification in the presence of interference was proved. This assay allows one to simply determine the amount of hydrogen peroxide in a product in situ and thus avoids the need for sophisticated separation techniques. The limitation of the method is that the treatment required for removal of confounding compounds takes on the order of minutes and thus the method has the possibility of underestimating the hydrogen peroxide content in systems where such concentrations are changing on the seconds to minutes time scale.

The other focus of this project was a modified assay that eliminates a source of underestimation of peroxidase activity in plant extracts. Natural reducing agents endogenous to plant materials, such as phenolic compounds and ascorbic acid, may interfere with traditional peroxidase assays by reducing the oxidized product generated in the peroxidase reaction; in such assays the oxidized product is typically the reporter molecule that is monitored for enzyme quantification. The action of such reducing compounds results in an apparent lag in product development, which is interpreted as a lower enzyme activity. In such cases the time course of product production may appear sigmoidal. In some cases, these compounds may be sufficiently active as to completely obscure the rate of the reaction. This study describes a relatively simple way to alleviate complications from these compounds. The method is based on using ABTS as the reporter substrate. The oxidized product of the reaction is $\text{ABTS}\cdot^+$, which can be followed spectrophotometrically due to its relatively high molar absorptivity in the visible region. It is herein shown that one can selectively remove complicating

endogenous reducing compounds by treating the enzyme preparation with the oxidized product itself, ABTS^{•+}, prior to initiating the assay. This approach is highly selective for those compounds likely to interfere with peroxidase quantification via reaction product reduction. The presented method is herein shown to remove lag phases associated with different plant extracts and, thus, more accurately reflect total peroxidase activity. The improved assay is relatively simple and should be applicable to a range of biological systems.

©Copyright by Shu Jiang
September 16, 2014
All Rights Reserved

Selective Oxidation of Biological Matrices for Subsequent Peroxidase and
Peroxidase-Based Quantitative Analyses

by
Shu Jiang

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented September 16, 2014
Commencement June 2015

Master of Science thesis of Shu Jiang presented on September 16, 2014.

APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Shu Jiang, Author

ACKNOWLEDGEMENTS

I would like to express my very great appreciation to my major advisor, Dr. Michael Penner, for his patient guidance and valuable suggestions during the project work. His support and encouragement are of importance when the times got rough. My completion of this project could not have been accomplished without him. I am also thankful to my thesis committee members: Dr. Sandra Loesgen, Dr. Elizabeth Tomasino, and Dr. Theresa Filtz, my graduate council representative, for sharing their thoughts on this project.

I am particularly grateful for the assistance given by Tiraporn, who has been so kind and helpful. I would also like to thank to all my friends for their help and encouragements.

Finally, I would like to thank my dear parents for their support and encouragements. Because of them, I can have a good chance to study here and realize my dream.

TABLE OF CONTENTS

	<u>Page</u>
1 General Introduction	1
1.1 Hydrogen Peroxide	1
1.1.1 Nature of Hydrogen Peroxide	1
1.1.2 Relevance of Hydrogen Peroxide in Food System	2
1.1.3 Role of Hydrogen Peroxide in Plant Tissues	5
1.1.4 Relevance of Hydrogen Peroxide in Non-Food Biological Systems	5
1.1.5 Analytical Approaches to Quantify Hydrogen Peroxide	7
1.2 Peroxidase	10
1.2.1 Nature of Peroxidase	10
1.2.2 Relevance of Peroxidase in Food Systems	12
1.2.3 Relevance of Peroxidase in Biological Systems	14
1.2.4 Analytical Approaches to Quantification of Peroxidase	16
2 Selective Oxidation of Biological Matrices for Subsequent Peroxidase-Based Assays: Hydrogen Peroxide Quantification	20
2.1 Abstract	21
2.2 Introduction	21
2.3 Materials and Methods	24
2.4 Results and Discussion	26
3 Selective Oxidation of Enzyme Extracts for Improved Peroxidase Quantification	46
3.1 Abstract	47
3.2 Introduction	47
3.3 Materials and Methods	50
3.4 Results and Discussion	53
3.5 Conclusion	58
4 General Conclusion	68
Bibliography	70

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1.1. Structure of hydrogen peroxide	2
1.2. Reaction cycle of HRP with a reducing substrate (AH). The rate constants k_1 , k_2 and k_3 describe the rate of compound I formation, compound I and compound II reduction respectively	12
2.1. Standard curves for hydrogen peroxide determination in a model buffer system and in GPE using the HRP/ABTS system	38
2.2. A plausible mechanism for assay interference. The concentration of hydrogen peroxide in a sample is based on the amount of $\text{ABTS}^{\bullet+}$ formed following initiation of the peroxidase reaction.....	39
2.3. Experimental design of modified assay for the determination of hydrogen peroxide in biological matrices	40
2.4. Standard curve for hydrogen peroxide determination in $\text{ABTS}^{\bullet+}$ -treated GPE	41
2.5. Comparison of standard (calibration) curves for hydrogen peroxide determination in $\text{ABTS}^{\bullet+}$ -treated GPE using different concentrations of ABTS in the reaction mixture	42
2.6. Time-course of $\text{ABTS}^{\bullet+}$ reduction in GPE. The insert depicts time-courses reflecting rates of $\text{ABTS}^{\bullet+}$ reduction following the addition of sufficient $\text{ABTS}^{\bullet+}$ to the $\text{ABTS}^{\bullet+}$ -treated GPE to adjust its $\text{ABTS}^{\bullet+}$ concentration back to that amount present at zero time of the initial $\text{ABTS}^{\bullet+}$ treatment	43
2.7. Time course of hydrogen peroxide loss, measured as absorbance change due to presence of hydrogen peroxide (y-axis), in GPE	44
2.8. Comparison of standard (calibration) curves obtained by following spiking known amounts of hydrogen peroxide into $\text{ABTS}^{\bullet+}$ -treated and untreated GPE samples	45

LIST OF FIGURES (Continued)

<u>Figure</u>	<u>Page</u>
3.1. Time-courses showing product accumulation, as determined by increase in absorbance, during peroxidase activity measurements using the traditional and the modified ABTS-based peroxidase assay procedures	64
3.2. Time-course showing product accumulation, as determined by increase in absorbance, during peroxidase activity measurement of sweet yellow pepper extract using the traditional guaiacol-based assay procedure	66
3.3. Time-courses of ABTS ^{••} decay, determined as decrease in absorbance, in vegetable extracts	67

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1.1 Uses of H ₂ O ₂ in food processing	4
3.1 Comparison of peroxidase activity of sweet yellow peppers and yellow onions as determined by the traditional and the modified peroxidase assays	63

1 General Introduction

Hydrogen peroxide quantification is of great interest due to its common use as an oxidizing, bleaching, and/or antimicrobial agent in industrial processing. This project was initiated by a local food company, and the aim of this project was to determine hydrogen peroxide in food products. Hydrogen peroxide quantification in foods is needed as an excess of hydrogen peroxide causes tissue and cell damage, resulting in inflammatory disease, cardiovascular diseases and cancers. Hydrogen peroxide assays are also of general importance for biological studies aimed at understanding the role of *in situ* generated reactive oxygen species (e.g. harmful agents causing oxidative damage in pathologies, regulatory agents in a range of biological phenomena) (Murphy et al., 2011). Peroxidase-based methods have been widely applied in food and biological systems, but the encountered problem is enzyme-based assay interference, resulting from endogenous confounding compounds in the detecting system. It has also been found that these endogenous confounding compounds can interfere with assays for peroxidase quantification in biological systems. Therefore, it is necessary to pay attention to possible interference mechanisms in peroxidase and peroxidase-based hydrogen peroxide assays. Methods for peroxidase and peroxidase-based hydrogen peroxide quantification in biological systems, where endogenous confounding compounds are likely to be present need to be developed.

1.1 Hydrogen Peroxide

1.1.1 Nature of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a chemical compound consisting of two atoms of hydrogen (H) and an oxygen-oxygen single bond (O_2). The structure of H_2O_2 is illustrated in Figure 1.1.

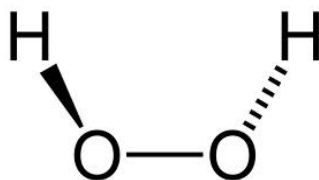


Figure 1.1 Structure of hydrogen peroxide

H₂O₂ solution occurs as a clear, colorless liquid at ambient temperature and are miscible in water. It is a strong two-electron oxidant due to its reduction potential (E_0') of 1.349 V at pH 7.0 (Wood, 1988). However, with a considerably high activation energy barrier, which causes difficulty in releasing oxidizing power, H₂O₂ is unreactive to most biological molecules, including low-molecular-weight antioxidants (Winterbourn, 2013). H₂O₂ does directly react with thiols, but the reaction with low-molecular-weight thiols and cysteine residues in most proteins is slow. However, it is much more reactive with transition metal centers (e.g., low-molecular-weight chelates, heme peroxidase, other redox-active metalloproteins such as iron or sulfur proteins), selenoproteins, and select thiol proteins. These proteins include: catalase, glutathione peroxidases, and peroxiredoxins (Winterbourn, 2013; Winterbourn, 2014).

1.1.2 Relevance of Hydrogen Peroxide in Food Systems

Exogenous sources of H₂O₂

In July of 1986, the U.S. Food and Drug Administration (FDA) approved the GRAS (Generally Recognized as Safe) status of H₂O₂ as a direct component of foods. The concentration of food grade H₂O₂ is usually between 30% and 50% (Food Chemicals Codex, 2003).

There are several uses of H₂O₂ in food processing (Table 1.1) especially for the dairy industry. H₂O₂ acts as an antimicrobial agent and is approved for preparations of milk for cheese making, modified whey products, and thermophile-free starch to extend the shelf life of food products (Food Chemicals

Codex, 2003). Instead of pasteurization, H_2O_2 has been used to remove undesired pathogenic bacteria in cheese milk, especially when certain cheeses such as Swiss are made. The use of H_2O_2 also protects some natural milk enzymes that are susceptible to heat of pasteurization. These enzymes are usually beneficial to flavor development of the cheese (Scott et al., 1998).

H_2O_2 is an oxidizing agent that can be applied to foods such as starch, and corn syrup to reduce or remove sulfur dioxide (SO_2). H_2O_2 also can bleach foods such as tripe, beef feet, herring, instant tea, colored (annatto) cheese whey and starch to improve color (Food Chemicals Codex, 2003). Jervis et al. (2013) stated that the annatto-colored cheddar whey is bleached with H_2O_2 with a maximum usage level of 0.05% or 500 ppm, and improving H_2O_2 bleaching efficacy with minimal protein damage and off-flavor development is of great importance to the dairy industry. Although H_2O_2 is GRAS, there is a maximum treatment level in foods allowed as seen in Table 1.1 (Food Chemicals Codex, 2003). Catalase is usually used to remove the residual H_2O_2 as it can convert H_2O_2 to water and oxygen. This is essential because of H_2O_2 cellular toxic effect, promoting serious clinical conditions (e.g., cancer, cardiovascular diseases and inflammatory states) (Segundo et al., 2013).

Table 1.1 Uses of H₂O₂ in food processing

Food	Maximum treatment level in food (percent)	Functional use
Milk, intended for use during the cheesemaking process as permitted in the appropriate standards of identity for cheese and related cheese products under part 133 of this chapter.	0.05	Antimicrobial agent as defined in § 170.3 (o)(2) of this chapter
Whey, during the preparation of modified whey by electrodialysis methods.	0.04	do.
Dried eggs, dried egg whites, and dried egg yolks as in §§ 160.105, 160.145, and 160.185 of this chapter.	Amount sufficient for the purpose.	Oxidizing and reducing agent as defined in § 170.3 (o)(22) of this chapter
Tripe	do	Bleaching agent.
Beef feet	Amount sufficient for the purpose. (Hydrogen peroxide may be in the form of a compound salt, sodium carbonate peroxide).	Bleaching agent.
Herring	Amount sufficient for the purpose.	do.
Wine	do	Oxidizing and reducing agent as defined in § 170.3 (o)(22) of this chapter.
Starch	0.15	Antimicrobial agent as defined in § 170.3 (o)(2) of this chapter, to produce thermophile-free starch; Remove sulfur dioxide from starch slurry following steeping and grinding operations of corn refining.
Instant tea	Amount sufficient for the purpose.	Bleaching agent.
Corn syrup	0.15	Reduce sulfur dioxide levels in the finished corn syrup.
Colored (annatto) cheese whey	0.05	Bleaching agent.
Wine vinegar	Amount sufficient for the purpose.	Remove sulfur dioxide from wine prior to fermentation to produce vinegar.
Emulsifiers containing fatty acid esters	1.25	Bleaching agent.

Endogenous sources of H₂O₂

H₂O₂ is an endogenous component found in honey, which imbues honey with antibacterial properties, making honey function as a natural antimicrobial agent. Originating from hypopharyngeal glands of honey bees, H₂O₂ is easily produced by glucose oxidase and glucose when honey is diluted. Since catalase from pollen in honey can decompose H₂O₂ to water and oxygen, the level of H₂O₂ in honey depends on relative levels of glucose oxidase and catalase. Higher H₂O₂ level are associated with higher level of glucose oxidase and lower level of catalase (Taormina et al., 2001; Manzoori et al., 2006; Franchini et al., 2008). Another endogenous source of H₂O₂ that has been reported is the polyphenol-rich beverages, such as green tea, black tea, and coffee, where polyphenols are easily oxidized by oxygen and a transition metal ion to their corresponding quinones and reactive oxygen species such as O₂⁻ and H₂O₂ are produced (Akagawa et al., 2003).

1.1.3 Role of Hydrogen Peroxide in Plant Tissues

In plant tissues, H_2O_2 plays a crucial role as a key-signaling molecule, getting involved in responses to environmental stresses, either biotic or abiotic stresses, including: pathogen attacks, extreme temperatures, excessive radiation, ozone, and wounding (Olson et al., 1993; Slesak et al., 2007). After exposure to environmental stresses, H_2O_2 concentration increases to control stress responses and physiological adjustments. The rate of H_2O_2 production and H_2O_2 levels differ for various types of stresses, and the strength and duration of stresses as well. For example, in response to pathogen infection, H_2O_2 production is a major defense against pathogens by killing pathogens directly causing a hypersensitive response, which resulting in cell death at infection sites rapidly or cross-linking of cell wall structural proteins lignification (Olson et al., 1993). This process can reinforce the cell wall, consequently blocking the spread of harmful pathogens. Apart from being a defensive signal molecule, H_2O_2 also functions as a signaling molecule in a wide range of physiological processes, such as photorespiration and photosynthesis, senescence, stomatal movement, cell cycle, and plant growth and development (Quan et al., 2008). Moreover, H_2O_2 serves as a regulator of the expression of various genes, including modulators of H_2O_2 production and those encoding antioxidant enzymes (Slesak et al., 2007).

1.1.4 Relevance of Hydrogen Peroxide in Non-Food Biological Systems

Reactive oxygen species (ROS) in health and disease

ROS are produced in biological system (e.g., superoxide anion, hydroxyl radical, peroxy radical, and H_2O_2) among which H_2O_2 is of great interest due to its high permeability within the cell and across cellular membranes (Segundo et al., 2013). In *vivo*, H_2O_2 is produced by several different ways, including the peroxisomal pathway for β -oxidation of fatty acids, various reactions catalyzed by oxidative enzymes (e.g., glucose oxidase, D-amino acid oxidase, glycollate and monoamine oxidase) and dismutation of superoxide radical ($\text{O}_2^{\bullet-}$), a free and unstable radical,

catalyzed by superoxide dismutase or spontaneously in the absence of enzymes (Gulcin, 2012; Halliwell et al., 2000). Regarded as a double-edged molecular sword, H_2O_2 displays positive and negative effects under physiological and pathological levels, respectively. Low levels of H_2O_2 (20-50 μM or below) can be useful in metabolism. For example, H_2O_2 is used by thyroid peroxidase to produce thyroid hormones. Actually H_2O_2 serves as an important redox-signaling compound to indicate oxidative stress (Halliwell et al., 2000; Gulcin, 2012). In living organisms, H_2O_2 levels are controlled by a complex web of antioxidant defenses in order to minimize oxidative damage to biomolecules (Halliwell, 2005). Therefore, it is reasonable that there needs to be balance between production of ROS, including H_2O_2 , and antioxidant defense for proper cellular metabolism. Negative effects of H_2O_2 are due to generally high levels of H_2O_2 ($\geq 50 \mu\text{M}$) that are considered as being cytotoxic to a wide range of animal, plant and bacterial cells in culture even though several factors (e.g., cell type, physiological state of the cell, length of exposure to H_2O_2 and the H_2O_2 concentration used) decide LD_{50} (median lethal dose) values and the cell death mode (Halliwell et al., 2000). Acting as a mediator, H_2O_2 disrupts cellular homeostatic mechanisms by mediating cytotoxicity through alternations in protein, lipid, and nucleic acid structure and function, so causing a variety of cellular injuries such as mutagenesis and carcinogenesis in biological systems (Takahashi et al., 1999; Tarpey et al., 2004). It was reported that H_2O_2 induced cancer in the duodenum of mouse after it is administered in the drinking water at 0.1% and 0.4% (Toyoda et al., 1982). H_2O_2 also provides mainly positive results in short-term genotoxicity tests (Abbas et al., 2010). Increased DNA strand breakage appears before detectable lipid peroxidation or oxidative protein damage when H_2O_2 is added to many mammalian cells (Halliwell, 2005). H_2O_2 has an ugly reputation due to the reactive hydroxyl radical ($\bullet\text{OH}$) generated either by exposure to ultraviolet light or rapid interaction with the reduced form of a range of transition metal ions, of which iron is the most important. Hydroxyl radicals are strong oxidants that react with almost any other molecules to produce other radicals, which results in cellular damage such as lipid peroxidation, oxidative DNA damage, and protein oxidation (Halliwell et al., 2000). Another biologically damaging effect of H_2O_2 is

due to the formation of hypochlorous acid (HOCl) from interaction between H_2O_2 and heme peroxidases (e.g., myeloperoxidase, lactoperoxidase, and eosinophil peroxidase). This oxidizing acid can cause damages to tissues during inflammation and oxidize protein (Winterbourn, 2013; Halliwell et al., 2000; Forman, 2008).

1.1.5 Analytical Approaches to Quantify Hydrogen Peroxide

H_2O_2 can be measured through several techniques (e.g., colorimetric assays, fluorometric assays, chemiluminescent assays, HPLC, electrochemical approaches) (Tarpey et al., 2004; Marquette & Blum, 2006; Takanami et al., 2009).

Colorimetric assays with tube-based or microplate-based detection techniques are usually simple, fast, economical, but less sensitive. Fluorescent detections are more sensitive than colorimetric methods, but instrumentation costs of fluorescent detection are considerably higher. HPLC, chemiluminescent and electrochemical detection are also typically sensitive, but they are complex, and require expensive equipment and operator training. These methods are usually classified into two kinds of assays, non-enzyme coupled assay and enzyme-coupled assay.

Non-enzyme coupled assays

Based on various chemical reactions related to H_2O_2 , numerous non-enzyme coupled assays for quantification of H_2O_2 have been investigated. Lu et al. (2009) measured H_2O_2 in apple fruit tissues by using the chemiluminescence reaction with luminal, in which contaminants were efficiently removed by PVPP. With the strong oxidizing ability of the Fenton reaction, Abbas et al. (2010) developed a more sensitive fluorometric method for the determination of H_2O_2 in milk samples. Residual H_2O_2 was quantified in dried bean curbs and disposable chopsticks by using a non-enzymatic colorimetric method based on nitrophenylboronic acids reacting with H_2O_2 chemoselectively under alkaline conditions to produce yellow nitrophenolates (Lu et al., 2011). In addition, the high-performance liquid chromatography (HPLC) method is one of the most popular H_2O_2 detection

methods in many programs. Takanami et al. (2009) described a method for separating H_2O_2 by HPLC method with the electrochemical detector (ECD). This method has been applied to an aqueous extract of cigarette smoke for H_2O_2 analysis by taking into consideration that there are many redox-active compounds exist in the cigarette smoke matrix. Steinberg (2013) developed an HPLC method for quantification of H_2O_2 in irradiated mineral and soil suspensions, which was based on the reaction between H_2O_2 and iodide in the presence of ammonium molybdate and vanillic acid, producing iodovanillic acid, which is separated and quantified by reversed-phase HPLC with UV detection at 280 nm.

Enzyme-coupled assays

Enzyme-coupled assays, generally conducted with horseradish peroxidase (HRP), are commonly used for quantification of H_2O_2 in foods or biological systems. In general enzyme-based assays, H_2O_2 is usually colorimetrically or fluorometrically detected because it is colorless. Even though H_2O_2 can be detected at 240 nm by UV spectrophotometer, the molar absorptivity of H_2O_2 is low, indicating low sensitivity. Thus an appropriate colored or fluorescent detecting compound produced by some stoichiometric reactions related to H_2O_2 needs to be used. With the necessary substrate, H_2O_2 , peroxidase assay also depends on oxidation of another certain substrate, an indicator, such as phenol red, tetramethylbenzidine, 4-aminoantipyrine/phenol, ABTS, homovanillic acid, and Amplex Red, to a colored or fluorescent product detected by a spectrophotometer or spectrofluorometer (Winterbourn, 2013). It has been demonstrated that residual H_2O_2 in noodles, fish paste, dried fish, and herring roe was detectable to 2 ppm by using spectrophotometric method in which H_2O_2 reacted with phenol, 4-aminoantipyrine, and peroxidase to formulate the stable quinoneimine dye (Ito et al., 1981). Wei et al. (2008) reported that peroxidase-like Fe_3O_4 magnetic nanoparticles catalyzed the oxidation of ABTS by H_2O_2 to its stable colored cation, $\text{ABTS}^{\bullet+}$, providing the detection of H_2O_2 . A versatile method for the differential amperometric determination of H_2O_2 in honey samples has been developed with the use of an on-line tubular reactor containing peroxidase immobilized on Amberlite IRA-743

resin (Franchini et al., 2007). Manzoori et al. (2006) presented a spectrofluorometric detection method for H_2O_2 with high sensitivity and selectivity, which was based on oxidation of homovanillic acid with H_2O_2 and peroxidase obtained from crude extract of kohlrabi (*Brassica oleracea gongylodes*). Currently, fluorimetric detection with the substrate, Amplex Red, is widely used due to its great sensitivity provided by oxidation of Amplex red (10-acetyl-3, 7-dihydroxyphenoxazine) to resorufin, a fluorescent product (Winterbourn, 2013; Brudzynski et al., 2012).

Limitations of enzyme-coupled assays

Enzyme-based methods are of great interest in H_2O_2 analysis mainly due to their high sensitivity and selectivity since peroxidase has considerably high specificity to H_2O_2 , but in non-enzyme coupled assays, some other compounds rather than H_2O_2 could participate in chemical reactions producing detecting signals. When enzymatic assays are performed using 96-well microplates or cuvettes, quantification of H_2O_2 is relatively quick and simple compared to other methods (e.g., HPLC, electrochemical detection) (Brudzynski et al., 2012). However, they have limited availability. The presence of interfering compounds (i.e., reducing agents) including thiols and ascorbate, especially in biological systems, affects enzymatic assays because the compounds can serve as substrate for HRP, competing with the oxidation of detector molecules, and resulting in an underestimation of H_2O_2 . In addition, interfering compounds reduce oxidized detector molecules directly by electron transport components, leading to loss of utility of detector compounds (Tarpey et al., 2004). For example, free radical scavengers can inhibit the response of H_2O_2 by scavenging the probe radical when extracellular H_2O_2 is detected (Winterbourn, 2013). It has been found that the oxidized indicator, 3,3', 5, 5'-tetramethylbenzidine•2HCl (TMB) interacts with electron carriers from respiring mitochondria, being reduced to its original form that does not have maximum absorbance at 465 nm (Staniek et al., 1999), leading to a failure in assessing mitochondrial H_2O_2 generation. Overestimation of H_2O_2 has been demonstrated in the presence of dietary antioxidants when low

concentrations of H_2O_2 were quantified based on resorufin, the oxidation product of Amplex Red by H_2O_2 /peroxidase. This probably can be explained by a direct interaction between dietary antioxidants and peroxidase, causing formation of observed resorufin even in the absence of H_2O_2 (Serrano et al., 2009). H_2O_2 quantification in plant and fruit tissues was inhibited by interference from other redox-active compounds, including ascorbate and phenolic compounds. One possible solution to solve this problem is the removal of interfering compounds through separation chemistry. Interfering compounds, including ascorbate and polyphenols, can be efficiently removed by ascorbate oxidase and PVPP, respectively prior to initiating the peroxidase-coupled assay (Lu et al., 2009; Veljovic-Jovanovic et al., 2002). However, this is a limited solution due to the high enzymatic and chemical specificity, which means other interfering compounds, would remain in the detection system. Also enzyme treatment is rather costly and the stability of ascorbate oxidase is low. Solid phase extractions and/or chromatography have been applied as pre-treatment steps prior to H_2O_2 detection (Ito et al., 1981; Tarvin et al., 2010). The disadvantage is that solid phase extractions are rather non-specific, while chromatography is time-consuming and difficult to incorporate into rapid automated/semi-automated analytical systems. Therefore, it is necessary to develop a simple and quick method for dealing with interfering compounds in food and biological systems.

1.2 Peroxidase

1.2.1 Nature of Peroxidase

Peroxidases, members of the oxidoreductase enzyme group, are widely distributed and play multiple physiological roles in living organisms (e.g., plants, animals, and microorganisms). Based on various source and mode of action, peroxidases are classified as three super families: plant peroxidases, animal peroxidases and catalases. Plant peroxidases can further be divided into three classes, based on structural similarities and certainly in a suspected common evolutionary origin. Class I contains mitochondrial yeast cytochrome c peroxidases, such as

chloroplast and cytosol ascorbate peroxidases from higher plants and bacterial peroxidase. Class II is composed of all secretory fungal (manganese) peroxidase. All of the secretory plant peroxidases, including horseradish peroxidase (HRP), turnip peroxidase (TuP), tomato peroxidase (TomP), belong to class III, which usually have a wide range of substrates, especially for phenolic compounds. This shows distinctive features from plant ascorbate peroxidases (class I), which use ascorbic acid as the preferential electron donor (Azevedo et al., 2003; Almagro et al., 2009). In the animal peroxidase superfamily, there are two subsequent classes: halide peroxidases (myeloperoxidase, thyroid peroxidase, lactoperoxidase) and prostaglandin synthases, such as prostaglandin H₂ synthase. Catalase catalyzes the dismutation of H₂O₂ to water and oxygen, preventing an excessive accumulation of H₂O₂. Of all the peroxidases of living organisms, plant peroxidases have been widely studied, especially class III peroxidases, in which HRP receives a special attention and is fairly popular in biotechnological applications. The general peroxidase-catalyzed reaction is a three-step cyclic process. The first step is the enzyme oxidation by H₂O₂ to generate an oxidized enzyme intermediate referred to as “compound I”. Compound I is then reduced by a hydrogen donor involving a one-electron transfer to form a second enzyme intermediate referred to as “compound II” and a radical product. The reaction cycle is completely by a second one-electron reduction step, in which compound II is reduced by a second hydrogen donor back to the native enzyme, with the formation of a second radical product. Take HRP for example, this reaction cycle is illustrated in Figure 1.2 (Regalado et al., 2004; Ngo, 2010; Azevedo et al., 2003; Veitch, 2004).

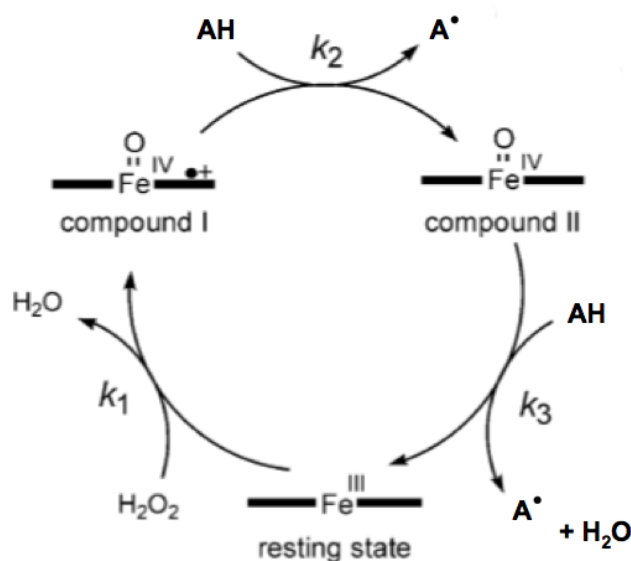


Figure 1.2. Reaction cycle of HRP with a reducing substrate (AH). The rate constants k_1 , k_2 and k_3 describe the rate of compound I formation, compound I and compound II reduction respectively.

As already mentioned, H_2O_2 is the main peroxide substrate participating in peroxidase-catalyzed reaction, but high concentrations of H_2O_2 can inhibit the enzyme. Contrary to high specificity of peroxidase for H_2O_2 , the specificity for hydrogen donor substrates is low. A variety of commonly used hydrogen donor substrates are including guaiacol, o-dianisidine, o-phenylene diamine, p-phenylene diamine, o-tolidine, ABTS, pyrogallol, 4-aminoantipyrine (Vamos-Vigyazo, 1981; Azevedo et al., 2003). How to select a specific hydrogen donor substrate depends on the characteristic of different enzyme assays.

1.2.2 Relevance of Peroxidase in Food Systems

Endogenous sources

Peroxidase is commonly found in almost all vegetables and fruits, with fruits usually having lower activities than most of the vegetables. Peroxidase is responsible for the quality of raw and processed fruits and vegetable due to its association with losses in color, flavor and nutritional values (Vamos-Vigyazo,

1981; Burnette, 1977; Serrano-Martinez et al., 2008), which makes this enzyme of great interest in food technology. In the food industry, peroxidase is generally considered as an indicator of the adequacy of vegetable blanching because of its high thermal stability and wide distribution, which indicates that other enzyme systems will not survive if peroxidase is destroyed (Vamos-Vigyazo, 1981; Regalado et al., 2004; Goncalves et al., 2010; Serrano-Martinez et al., 2008; Ali et al., 2011). However, complete inactivation of peroxidase is not necessary to prevent off-colors and off-flavors in vegetable and fruits since overblanching occurs, causing loss of quality (Burnette, 1977; Williams et al., 1986).

Lactoperoxidase (LPO), a peroxidase in milk, has an inhibitory effect on microorganisms with H_2O_2 and thiocyanate (SCN^-) due to the SCN^- peroxidation products (OSCN^-), which will kill or inhibit the growth of microorganisms but are not toxic to mammalian cells (Fox, 2003). In the presence of indigenous SCN^- and H_2O_2 produced by certain bacteria, LPO plays an important role in the quality of raw and pasteurized milk. LPO is considered as an index of super-HTST (high temperature short time) pasteurization (e.g., temperatures $> 75^\circ\text{C}$ for 15 sec) to avoid over pasteurization because high temperature such as 80°C treatment would almost completely inactivate LPO and no sufficient residual enzyme activity to exert an effect on the keeping quality of the milk (Fox, 2003; Barrett et al., 1999).

Exogenous sources

Peroxidase preparation from genetically modified *Aspergillus niger* is generally recognized as safe (GRAS) for use in cheese-whey, soy milk and cream as a bleaching agent to remove the amount of carotenoids present in final products by reducing hydrogen peroxide that is added as co-factor. Cheese-whey is also in the processing of other food products including bakery and dairy products, beverages and infant formula (GRAS notification for peroxidase). It has been reported, that in baking, peroxidase participates in catalyzing the gelation of arabinoxylans by the formation of diferulic acid linkages with H_2O_2 or crosslinking arabinoxylans to side chains of amino acids, such as tyrosine and cysteine. This makes an

improvement on the handling properties of the dough, including volume and crumb characteristics and the final bakery products (Hilhorst et al., 1999; Boeriu, 2008). Treatment of apple extracts with exogenous peroxidase and polyphenol oxidase decreased its allergenicity much faster via reduction of the IgE binding levels by Mal d 1, the major apple allergen, which is likely to be due to enzyme-catalyzed oxidation of phenolic compounds to o-quinones or other intermediates modifying the structure of the allergens and cross-linking of the proteins (Garcia et al., 2007). Therefore, the role of both endogenous and exogenous peroxidase is of great importance.

1.2.3 Relevance of Peroxidase in Biological Systems

It is well known that peroxidases play an important role in biological systems. Their physiological functions include: controlling ROS, lignin biosynthesis and degradation, protection of tissues from physically damage or infection by pathogens or insects, auxin metabolism (Mehlhorn et al., 1996; Fortea et al., 2011; Hamid et al., 2009).

Role in controlling ROS

In plant cells, ROS are produced in various metabolic pathways that are localized in organelles, such as chloroplast, mitochondria or peroxisomes. Exposure to abiotic stresses, including salt, UV, drought, heavy metals, air pollutants can increase the production of ROS. Overproduction of ROS in plants cause damage to proteins, lipids, carbohydrates and DNA, ultimately resulting in oxidative stress. However, plants have antioxidant defense systems, enzymatic and non-enzymatic systems respectively, to protect cells from oxidative damage via scavenging of ROS. Peroxidases, including ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), and glutathione peroxidase (GPX), act as enzymatic antioxidants that greatly contribute to cell protection against various stresses. H_2O_2 can be reduced or removed by APX and GPOX using different electron donors. APX uses ascorbate as a specific electron donor, while GPOX prefers aromatic electron

donors. GPX catalyzes the reduction of not only H_2O_2 , but also other hydroperoxides (ROOH) with glutathione (GSH). The activity of peroxidases is variable, depending on plant species and stresses conditions (Gill et al., 2010; Matés, 2000). However, on certain occasions, peroxidases actually produce ROS. It has been investigated that ROS were generated by extracellularly secreted peroxidase in the elicitor-treated plants. Salicylic acid (SA), aromatic monoamines (AMAs) and chitooligosaccharides (COSs) are ROS-generating peroxidase substrates because a model enzyme, HRP, catalyzed the rapid production of $\text{O}_2\bullet^-$ following the addition of SA, AMAs or COSs in the presence of dissolved oxygen and traces of H_2O_2 added to the system (Kawano, 2003). H_2O_2 also can be produced by peroxidases from a number of reducing substrates, such as cysteine, glutathione, NADPH, ascorbate, and indole-3-acetic acid (Boerjan et al., 2003). Although peroxidases may paradoxically and transitorily produce H_2O_2 or $\text{O}_2\bullet^-$, they would not be considered as a possible enzyme responsible for ROS generation because no net H_2O_2 / $\text{O}_2\bullet^-$ production indicates the balance of peroxidase/oxidase reactions in a closed system (Almagro et al., 2009).

Role in lignin biosynthesis and degradation

Peroxidases participate in lignin biosynthesis and degradation as well. Lignins are complex heteropolymers derived mainly from three hydroxycinnamyl alcohols (monolignols): p-coumaryl, coniferyl, and sinapyl alcohols (Almagro et al., 2009). Lignification is based on peroxidase-catalyzed monolignol unit oxidation with H_2O_2 to monolignol phenoxy radicals, which couple spontaneously to growing polymers, extending the complex three-dimensional lignin networks. H_2O_2 is provided by NADH oxidation catalyzed by NADH oxidases (Azevedo et al., 2003). The formation of a physical barrier of lignin limits dehydration and pathogen invasion (Passardi et al., 2004; Boerjan et al., 2003). Peroxidases involved in lignin degradation include lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). It has been well established that LiP, isolated from white-rot fungi, can effectively oxidize phenolic and non-phenolic compounds with H_2O_2 , while in reactions MnPs participate in, Mn^{2+} ,

serving as a necessary substrate, is preferentially oxidized by MnP to a strong oxidant, Mn^{3+} , which is stabilized by organic acid chelators. Chelated Mn^{3+} usually oxidizes phenolic compounds other than non-phenolic units of lignin. This makes depolymerization of lignin possible because of the formation of unstable free phenoxy-radicals that tend to disintegrate spontaneously (Hofrichter et al., 2002). With activity combination of LiP and MnP, VP naturally degrades lignin by oxidizing hydroquinone in the presence of Mn^{2+} , even though no exogenous H_2O_2 appears in the reaction (Martínez et al., 2005; Pérez et al., 2002).

Others

Encountering infection by various pathogens, plants can give multiple responses to resist these pathogens. One of responses is the formation of bioactive (anti-fungal and anti-bacterial) plant products synthesized by peroxidases-mediated reactions. For example, oat leaves, after infection with pathogenic fungi, are able to produce the phenolic phytoalexins, a well-known bioactive product to protect tissues (Almagro et al., 2009). Plant hormones, auxins play an important role in plant growth regulations and development, and as one of the most significant auxins, indole-3-acetic acid (IAA) is oxidized by peroxidases (Azevedo et al., 2003). It has been found that peroxidases also participate in ethylene biosynthesis, hormone balance, respiration control, ripening and senescence (Vamos-Vigyazo, 1981).

1.2.4 Analytical Approaches to Quantification of Peroxidase

Analytical approaches for enzymes in general

Enzymes, acting as catalysts, speed up the rate of a given reaction by lowering activation energy. Enzyme assays generally refers to enzyme activity measurements and enzyme amount/concentration measurements. The concentration of an enzyme can be measured by immunological technique [e.g., Enzyme-Linked Immunosorbent Assay (ELISA), Western Blotting]. For example,

specific antibody concentration can be quantified by coating the specific antigen of interest with the capture antibody provided with the ELISA kit (Barrette et al., 2006). Mandel et al. (2010) reported that the amount of salivary amylase was measured by immunoblotting. Enzyme activity describes the general catalytic properties of an enzyme, and can be quantified by several techniques (e.g., UV-visible spectrophotometry, fluorimetry, luminometry). Simple and practical enzyme activity assays are usually based on chromogenic or fluorogenic substrates that formulate a colored or fluorescent product upon enzyme reaction. In some cases, a detectable signal is produced through a specific reaction or chemical transformation, in which indirect indicators are used. These indicators can be as simple as a pH-indicator or as complex as a functionalized nanoparticle (Reymond et al., 2009). Enzyme activity assays are usually classified into two kinds of assays: stopped assays and continuous assays. In stopped assays, how much product has been formed or how much substrate has been used up is measured over a given time. At least two time points need to be measured to ensure the linearity of the rate of enzyme reaction through the selected period for the standard method. Contrary to a stopped assay, a continuous assay is related to a reaction process, which can be monitored as it occurs. This assay is more convenient since result is shown fast and any deviation the initial velocity displays from linearity can be observed (Scopes, 2002; Rogers et al., 2009).

Peroxidase assays in general

The determination of the peroxidase activity has been studied by using a variety of methods, including colorimetric, fluorimetric (Proctor & Chan, 1994), chemiluminescence (Katsuragi et al., 2000), or electrochemical detection (Stiene et al., 2002), among which colorimetric detection is the most popular as it is simple and economical. Quantification is based on the spectrophotometric measurement of colored products, formed from the peroxidase reducing substrates/peroxidase/H₂O₂ system, at a specific wavelength at which they have maximum absorption. Since peroxidase shows a low specificity for the reducing substrate or hydrogen donor substrate, several possible substrates are: guaiacol,

ABTS, p-phenylenediamine, o-dianisidine, 3,5,3',5'-tetramethylbenzidine, pyrogallol, or o-tolidine. In spite of undefined mixture of oxidation products and undetermined absolute molar absorptivity, guaiacol is one of the most widely used hydrogen donors for determination of peroxidase activity in vegetable and fruits, especially for checking thermal treatments during blanching process (Vidigal et al., 2010; Vamos-Vigyazo, 1981; Muftugil, 1985; Tan et al., 2014; Goncalves et al., 2010; Ali et al., 2011; Suha et al., 2013).

Limitations of general peroxidase assays

Although peroxidase assay is relatively easy to operate, a problem encountered in detecting system, particularly in crude peroxidase extract is interference due to the presence of endogenous compounds. In the early 1980's, Osborne et al. (1984) concluded that the guaiacol assay, as well as other biochemical assays based on other substrates, including ABTS, o-dianisidine, and o- and p-phenylenediamine used for determination of peroxidase activity, are hindered by an endogenous factor found in organs including liver and kidney of animals. This was named as peroxidase interfering activity (PIA). It has been reported that lag phases in horseradish peroxidase catalyzed-oxidation of guaiacol by H_2O_2 were observed in *Sedum* and *Pelargonium* extracts, which was due to inhibition of endogenous ascorbic acid and phenolic compounds respectively (Castillo et al., 1984). This inhibition was also shown in a simple ascorbic acid assay based on the lag produced in peroxidase/ H_2O_2 with homovanillic acid or guaiacol applied to crude biological extracts (Celardin et al., 1982). Therefore, measurements of peroxidase activity obtained from crude extracts become controversial since various compounds present in biological samples can hinder this assay, leading to erroneous results.

However, current researches have given much less attention to the problem caused by interfering compounds in peroxidase/ H_2O_2 system. Usually researchers only pointed out that peroxidase activity was calculated from the initial linear portion of a curve by plotting absorbance versus time (Ali et al., 2011; Igual et al., 2013;

Yu et al., 2010). This may be used because peroxidase activity is still detectable even in the presence of lag time, and it seems unnecessary to focus on the lag produced in the reaction system. It is unsurprising that latent peroxidase activity would make people conclude that no activity was observed in samples during a short detecting time. Hence, testing the presence of interfering substances is of great importance and it is crucial to find solutions to determine valid peroxidase activity. There is no doubt that sample purification is one way to remove interfering compounds while retaining peroxidase (Osborne et al., 1984). Interfering substances causing a lag were removed from orange juice serum by dialysis or by chromatography on Sephadex-G25 (Bruemmer et al., 1976). Pre-incubation of Sedum enzyme extract with ascorbate oxidase eliminated the lag phase completely due to ascorbic acid, the main reducing substance responsible for the lag. Insoluble polyvinyl-pyrrolidone (PVP) is useful for separating interfering substances, especially for phenolic inhibitors (Castillo et al., 1984). However, specific instruments needed for purification may not be commonly found in every laboratory and some substances used for removing interfering compounds are considerably specific with limited availability. Therefore, a valid, simple and quick assay for measuring peroxidase activity in the presence of interfering compounds needs to be developed. The improvement in efficiency and accuracy of peroxidase activity measurements will help to get a better understanding of peroxidases assays.

2 Selective Oxidation of Biological Matrices for Subsequent Peroxidase-Based Assays: Hydrogen Peroxide Quantification

Shu Jiang & Michael H. Penner
Department of Food Science and Technology
Oregon State University
Corvallis, OR 97331-6602

2.1 Abstract

Hydrogen peroxide quantification is of broad interest due to the common use of hydrogen peroxide as an oxidizing agent in industrial processing and laboratory research. Hydrogen peroxide assays are also of general importance for biological studies aimed at understanding the role of *in situ* generated reactive oxygen species. In the latter scenario particularly, assays amenable to high throughput processing are needed. Enzyme-based methods are appropriate for such applications. A problem commonly encountered when using enzyme-based methods to quantify the level of hydrogen peroxide in biological samples is assay interference due the presence of assay-modifying endogenous compounds. This type of interference has limited the applicability of peroxidase/chromophore-linked assays which are commonly used elsewhere for high throughput screening (e.g., the glucose oxidase/peroxidase assay for glucose quantification). Potential mechanisms of assay interference include enzyme inhibition/inactivation, substrate competition and product modification. In the present study we addressed the different mechanisms of interference using the following system: horseradish peroxidase, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and a hydrogen peroxide-containing garlic extract. Included experiments evaluated the nature of assay-interference in this system, which is overwhelmingly due to reduction of generated ABTS-radicals. Methods to circumvent the interference are presented; the methods are based on removal of interfering hydrogen donors prior to initiating the assay. The results from this work are expected to be generally applicable to other peroxidase-based assays for the quantification of hydrogen peroxide in complex biological systems.

2.2 Introduction

Hydrogen peroxide is a reactive oxygen species of general relevance to many biochemical systems. It is present naturally in biological systems at relatively low amounts where, depending on the circumstances, it is associated with either

beneficial or detrimental consequences (Segundo et al., 2013; Winterbourn, 2013). It is widely used in industrial processing due to its oxidizing, bleaching, and/or antimicrobial properties. Examples of its use in bio-material processing can be found in the pulp (Hage & Lienke, 2006), textile (Hage & Lienke, 2006), food (Jervis et al., 2013), dental (Westbroek et al., 2007), cosmetic (Chiu et al., 2011), and forensic (Barni et al., 2007) industries. The quantification of hydrogen peroxide in bio-based systems is thus relevant to a wide range of applications, from fundamental questions of cellular metabolism to applied aspects of industrial bio-processing. It is generally recognized that many of the current methods used for the quantification of hydrogen peroxide are of questionable applicability when applied to biological matrices due to the confounding nature of components endogenous to such systems (Murphy et al., 2011). Advances in approaches toward hydrogen peroxide quantification in biological systems are thus needed to extend the applicability of existing methods.

There are several approaches to the quantification of hydrogen peroxide. The “best” method for any particular application will be based on the typical factors: sensitivity, detection limit, nature of interfering compounds, equipment required, etc. A common approach to the quantification of hydrogen peroxide in biological systems is based on hydrogen peroxide-consuming peroxidase-catalyzed reactions that change the optical properties of a reporter molecule; the reporter molecule is typically the reducing substrate in the peroxidase-catalyzed reaction. Such assays include those based on spectrophotometric (Rhee et al., 2010), fluorimetric (Winterbourn, 2014), and chemiluminescent (Marquette & Blum, 2006) techniques. A situation that often limits the interpretation of data obtained with such methods is the presence of confounding compounds that alter the stoichiometry of the reaction, *i.e.*, alter the number of moles of reporter molecules oxidized per mole hydrogen peroxide consumed (Wardman, 2007). Separation steps are sometimes included in assays to circumvent this problem, including the use of solid phase extractions and/or chromatography prior to hydrogen peroxide quantification (Tarvin et al., 2010). The former approach is typically rather non-specific and the latter requires relatively sophisticated equipment, is time

consuming, and is difficult to incorporate into rapid automated/semi-automated analytical systems. An alternative to separating the confounding compounds from hydrogen peroxide prior to its quantification is to inactivate the confounding compounds *in situ*.

The objective of this study was to evaluate the potential of using a heretofore unexplored *in situ* method to deal with endogenous components capable of confounding peroxidase-based assays for hydrogen peroxide quantification in biological matrices. The presented method is based on the selective oxidation of confounding compounds prior to initiating the peroxidase-catalyzed reaction. The term “selective” in this sense indicates that the pre-quantification oxidative treatment effects only those compounds likely to reduce the reporter molecule used for hydrogen peroxide quantification. The appropriate oxidizing agent for such a treatment is the reporter molecule itself, thus insuring maximum selectivity. This approach successfully inactivates those confounding compounds that react with the reporter molecule and thus significantly decrease molar signal yields. Confounding compounds that work via this mechanism include a wide range of reductants/antioxidants present in biological systems (Tarpey et al., 2004). The reporter molecule used in this study was 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS). When ABTS is oxidized by hydrogen peroxide via the peroxidase-catalyzed reaction it yields the corresponding cation radical ($\text{ABTS}^{\bullet+}$); $\text{ABTS}^{\bullet+}$ is the reporter molecule in the assay due to its relatively high molar absorptivity in the visible region (Prior et al., 2005). The selective oxidizing agent used in the treatment phase is thus $\text{ABTS}^{\bullet+}$. The bio-based hydrogen peroxide-containing matrix used to test the validity of the proposed approach was a garlic (*Allium sativum*) extract representative of that used in the food processing industry. The primary outcome of the study is knowledge of the potential for using the proposed approach of selective oxidation to extend the applicability of peroxidase-based methods for the quantification of hydrogen peroxide in bio-based materials.

2.3 Materials and Methods

Reagents.

Hydrogen peroxide (30 wt.%, ACS reagent grade), horseradish peroxidase (HRP, type II, 150-250 units/mg solid), ABTS [2,2'-Azino-Bis(3-ethylbenzo Thiazoline-6-Sulfonic acid) diammonium salt], monobasic sodium phosphate and potassium persulfate were purchased from Sigma-Aldrich, USA

Analyte-Containing Sample.

A food-grade commercial garlic paste preparation was used as the bio-based sample matrix for all analyses. According to the manufacturer, the paste was a blend of dehydrated garlic, palm oil, soy lecithin, tocopherol and ascorbyl palmitate. In processing, the paste is treated with hydrogen peroxide and heated to reduce the microbial load. The sample is herein referred to as "garlic paste."

Sample Extract.

Five grams garlic paste were mixed with 15 ml 0.1M sodium phosphate (pH 6.0) in a 20 ml scintillation vial and vortexed for approximately 3 minutes. Two ml aliquots of the resulting suspension were then added to a series of micro-centrifuge tubes and subsequently centrifuged at 10,000 rpm for 10 minutes. Sample-containing tubes were then incubated in an ice-bath for approximately 10 minutes to solidify lipid components. The resulting tubes contained a pellet at the bottom, an aqueous liquid phase, and a solidified-lipid phase at the top. A needle/syringe was then used to quantitatively remove the liquid phase from each centrifuge tube. The liquid phase was then extracted with hexane (1:1, v/v) and filtered using Milipore Swinnex syringe glass fiber filters. The resulting clear solution is herein referred to as "garlic paste extract" (GPE).

Preparation of ABTS radical cation solution.

ABTS radical cation ($\text{ABTS}^{\bullet+}$) containing solutions were prepared as described by Huang *et al.* (2005) by incubating an aqueous 7mM ABTS, 2.45mM potassium persulfate, solution overnight (12-16 hours) in the dark, at room temperature. The solution was then made 100 mM (sodium) phosphate, pH 6.0, prior to being used in subsequent experiments.

Standard method for hydrogen peroxide quantification (peroxidase-ABTS assay).

In a typical assay with hydrogen peroxide in non-interfering buffer systems, 0.2 ml of color-forming reagent (0.86mM ABTS, 0.1M sodium phosphate, pH 6.0) was added to 2ml of H_2O_2 -containing sample. The signal producing reaction was then initiated by adding 100 μl HRP (100 $\mu\text{g/ml}$); the absorbance was read after mixing (~ 30 seconds, at which time color development was stable) at 734 nm. Enzyme solutions were kept on ice until initiation of the reaction. All reactions were done at ambient temperature.

Modified peroxidase-ABTS assay incorporating $\text{ABTS}^{\bullet+}$ -treatment.

In a typical assay, to a given amount of H_2O_2 -containing sample (typically from 20 – 200 μl) is added sufficient aqueous $\text{ABTS}^{\bullet+}$ solution (prepared as described above) such that following the treatment period the absorbance is in the range .1 to .3 (the extent of the treatment period is dependent on sample, see “Results and Discussion”). Peroxidase was then added to the treated samples for hydrogen peroxide quantification as described for the standard method above.

Time course of hydrogen peroxide degradation in GPE.

Hydrogen peroxide-free GPE was spiked with known amounts of hydrogen peroxide to initiate the reaction. At selected times $\text{ABTS}^{\bullet+}$ -containing solution was added to the reaction mixture to treat the sample for subsequent hydrogen

peroxide quantification. Peroxidase was then added to the treated samples and the assay completed as described for the standard method above. The hydrogen peroxide content of the GPE at the initiation of the experiment was taken as the amount measured for a sample to which a known amount of hydrogen peroxide was added to the ABTS \bullet^+ -pretreated GPE sample and immediately assayed.

2.4 Results and Discussion

A common problem associated with peroxidase-based assays for hydrogen peroxide quantification is illustrated in Figure 2.1. The figure shows a representative standard curve for the quantification of hydrogen peroxide using the HRP/ABTS assay in a model buffer system. The depicted sensitivity and linearity are typical of many such assays when applied to model systems. The second curve of Figure 2.1, which reflects the complete absence of signal, covers the same amounts of hydrogen peroxide within a bio-based matrix (a garlic paste extract, GPE). The latter assay was initiated by the addition of hydrogen peroxide to GPE containing all of the relevant HRP/ABTS assay components. A plausible explanation for the absence of signal when applying the assay to the GPE matrix is that components endogenous to GPE confound the assay by reacting with the product/reporter molecule (ABTS \bullet^+) generated in the peroxidase-catalyzed reaction. This rationale is consistent with HRP-catalyzed ABTS oxidation by hydrogen peroxide being rapid relative to the reactivity of hydrogen peroxide with other components typical of biological systems (Winterbourn, 2013). It is also consistent with the documented reactivity of ABTS \bullet^+ in biological matrixes, including garlic preparations (Leelarungrayub et al., 2006). The likelihood of product modification was verified in the present case in a series of experiments in which ABTS \bullet^+ was prepared in a buffer system and subsequently added to GPE; in all cases there was a time-dependent decrease in the absorbance attributed to ABTS \bullet^+ . In further experiments fleeting color development was observed in the initial seconds following the addition of relatively high amounts of hydrogen peroxide to GPE samples containing ample quantities of ABTS and HRP, indicating HRP-catalyzed ABTS \bullet^+ production had occurred. A plausible

mechanism for assay interference that is consistent with these observations is presented in Figure 2.2.

In this scenario, ABTS is oxidized to $\text{ABTS}^{\bullet+}$ in the process of peroxidase-catalyzed hydrogen peroxide reduction. This reaction alone should result in color/signal formation. However, as depicted in Figure 2.2, $\text{ABTS}^{\bullet+}$ is reduced back to ABTS by confounding compounds. The net result is diminished $\text{ABTS}^{\bullet+}$ accumulation and corresponding underestimates of hydrogen peroxide concentration. This type of interference is expected from compounds that show antioxidant activity (Prior et al., 2005). Indeed, a decrease in signal due to $\text{ABTS}^{\bullet+}$ reduction is the basis of the Trolox equivalent antioxidant capacity (TEAC) assay, a widely used assay for quantifying antioxidant activity in foods (Zulueta et al., 2009). The above interference scenario is not limited to peroxidase-based assays which use ABTS as the reducing substrate/reporter molecule. The problem of endogenous components confounding peroxidase-based assays that incorporate a range of different chromophores, fluorophores, and chemiluminescent compounds as reporter molecules is known (Staniek et al., 1999). In most instances, this complication is considered an inherent limitation of directly applying peroxidase-based assays to biological samples without prior analyte separation.

In the present work the cyclic nature of the $\text{ABTS}/\text{ABTS}^{\bullet+}$ redox reaction is used to circumvent the problem of reporter molecule reduction by endogenous confounding compounds. This is possible because $\text{ABTS}^{\bullet+}$ is a relatively stable radical with sufficient lifetime to allow it to be used as a selective oxidizing agent prior to ABTS being used as the reporter molecule for hydrogen peroxide quantification. The pertinent reactions along with a schematic illustrating the nature of the overall assay, including the pre-quantification $\text{ABTS}^{\bullet+}$ treatment, are shown in Figure 2.3. The schematic depicts the decrease in signal as the sample is first treated with $\text{ABTS}^{\bullet+}$, during which time endogenous interfering compounds are oxidized as they reduce the added $\text{ABTS}^{\bullet+}$ to ABTS. Once this treatment has subsided, HRP is added to the sample mixture to initiate the hydrogen peroxide

specific conversion of ABTS back to $\text{ABTS}^{\bullet+}$. The analytical signal attributed to hydrogen peroxide is thus the difference in the absorbance at the end of the pre-quantification $\text{ABTS}^{\bullet+}$ treatment and the absorbance obtained following HRP addition (which corresponds to newly generated $\text{ABTS}^{\bullet+}$).

Potassium persulfate was found to be a convenient reagent for preparing appropriate $\text{ABTS}^{\bullet+}$ solutions (Re et al., 1999). Such solutions must contain sufficient $\text{ABTS}^{\bullet+}$ to account for all confounding compounds in the sample. This can be assured by verifying that the signal of the treated sample does not decrease to the point obtained in the absence of $\text{ABTS}^{\bullet+}$. Having excess $\text{ABTS}^{\bullet+}$ in the HRP-phase of the assay is not a problem in this analytical scheme since the analyte signal is taken as the difference in absorbance before and after the addition of HRP (extremely high $\text{ABTS}^{\bullet+}$ levels should be avoided in order to keep the baseline absorbance within a reasonable range). A second consideration with respect to $\text{ABTS}/\text{ABTS}^{\bullet+}$ concentrations is that the concentration of ABTS, during the HRP-reaction phase, must be sufficient to account for all of the hydrogen peroxide in the system. This is confirmed by verifying that supplemental ABTS does not result in an increase in absorbance. Lastly, it is imperative that excess ABTS be included during the initial formation of $\text{ABTS}^{\bullet+}$ since the presence of potassium persulfate in the analyte-containing reaction mixture would be problematic. The stoichiometric ratio for the reaction of ABTS with persulfate is 2:1 (ABTS:persulfate) (Venkatasubramanian et al., 1989; Henriquez et al., 2002).

The inclusion of an $\text{ABTS}^{\bullet+}$ -treatment to deal with confounding compounds in peroxidase-based assays is particularly appealing due to its simplicity and because it specifically targets those compounds likely to pose a problem in the subsequent assays. Importantly, as used herein, ABTS and $\text{ABTS}^{\bullet+}$ do not react with hydrogen peroxide in the absence of HRP and, at least with respect to the systems dealt with here, neither do the $\text{ABTS}^{\bullet+}$ -oxidized confounding compounds generated during the $\text{ABTS}^{\bullet+}$ -treatment. Hence, the $\text{ABTS}^{\bullet+}$ -treatment per se does

not interfere with the subsequent HRP-based hydrogen peroxide quantification; it simply alleviates compounds likely to confound the assay.

The analytical approach described herein is applicable when using reporter molecules which when oxidized have sufficient lifetime to be used in the pre-quantification treatment phase of the assay. ABTS is particularly well suited for this approach due to the relative stability of its cation radical. Many of the traditional reducing substrates used for monitoring peroxidatic reactions, such as guaiacol (Doerge et al., 1997) and the benzidine derivatives (Joseph et al., 1982), are not appropriate for this approach due to the instability of the radical formed as a result of hydrogen peroxide reduction. Assays based on the latter types of reporter molecules are typically described as follows (Adak et al., 1996):



The problem with such reporter molecules is that the polymerized product is stable and, thus, cannot be used in a treatment step to alleviate confounding compounds. Keep in mind that confounders are capable of reducing the initially formed radicals prior to their polymerization, thereby decreasing the analytical signal, just as is observed with the ABTS substrate. The key point with respect to doing a pre-quantification treatment is that ABTS is rather unique in its suitability for this assay approach.

The time course of $\text{ABTS}\cdot^+$ reduction during the pre-quantification treatment step is expected to be sample specific. It is preferable for the rate of $\text{ABTS}\cdot^+$ reduction to be negligible by the end of the treatment step such that changes in absorbance due to $\text{ABTS}\cdot^+$ reduction are insignificant when considered in relation to the amount of $\text{ABTS}\cdot^+$ generated during the peroxidase-dependent hydrogen peroxide reduction. The HRP-catalyzed reaction upon which hydrogen peroxide quantification is based is relatively fast; in the present experiments the HRP-

catalyzed reaction was complete in the time samples had been mixed and readied for absorbance readings (always < 30 seconds). Hence, relatively slow rates of ABTS \bullet^+ reduction during the HRP-treatment step are manageable (see example below). Rates of ABTS \bullet^+ reduction by many compounds common to biological materials have been documented. Compounds such as phenolics are relatively slow to reduce ABTS \bullet^+ whereas rates of reduction by others, such as ascorbic acid, are nearly instantaneous (Walker & Everette, 2009; Tian & Schaich, 2013). ABTS \bullet^+ also undergoes a slow disproportionation reaction (Childs & Bardsley, 1975), but this reaction is not significant when considering the timeframe of this assay. A method for dealing with slow rates of ABTS \bullet^+ reduction is presented in the application below.

The complexity of using peroxidase-based assays for the quantification of hydrogen peroxide in biological matrixes is illustrated in the following example in which the HRP/ABTS assay is used to assess the reactivity of hydrogen peroxide in a garlic paste matrix representative of those available in the food industry. Recall that ABTS \bullet^+ production could not be observed in the HRP/ABTS-containing GPE spiked with hydrogen peroxide (the relevant calibration curve of Figure 2.1 was essentially a flat baseline). The absence of an absorbance change due to ABTS \bullet^+ generation in the GPE to which hydrogen peroxide, ABTS and HRP were added was attributed to the rapid reduction of ABTS \bullet^+ by endogenous components. To circumvent this problem GPE was treated with ABTS \bullet^+ , as proposed above, to selectively oxidize the sample's confounding compounds prior to the initiating the hydrogen peroxide quantification reaction. A calibration curve representative of those prepared with the ABTS \bullet^+ -treated GPE is given in Figure 2.4; this curve is analogous to the calibration curves of Figure 2.1. The most obvious result is that ABTS \bullet^+ was generated in the ABTS \bullet^+ -treated GPE in proportion to the amount of hydrogen peroxide present. A statistical comparison of the slopes of the calibration curves made in the two matrixes, i.e. the buffer system and the ABTS \bullet^+ -treated GPE, indicates the calibration sensitivity (slope) of the assay is not significantly different ($p > 0.05$) when quantifying hydrogen peroxide in the model matrix versus the ABTS \bullet^+ -treated GPE matrix. These

results demonstrate the effectiveness of selectively oxidizing endogenous confounding compounds prior to running the HRP-based quantification assay.

The data of Figure 2.1 and Figure 2.4 also address the extent to which the ABTS serves as the sole reducing substrate in the hydrogen peroxide consuming reaction. The relevance of this question is due to the non-specificity of the peroxidase enzyme. It is possible that endogenous components may compete with ABTS as a substrate for this reaction, thus lowering the molar absorbance change for the assay. The similarity of the molar absorbance changes in the model and GPE matrixes, as indicated by the similarity in slopes of the calibration curves in the two systems, suggest that competing substrates in GPE are not an issue in the current experimental design. A further experiment comparing molar absorbance yields, also depicted in Figure 2.5 as calibration curves, from the assay run at different ABTS concentrations support this interpretation; this rationale is based on the assumption that increasing the concentration of ABTS in the reaction mixture would increase molar absorbance yields if there were significant competition with a fixed amount of alternative substrates.

A representative time-course of $\text{ABTS}^{\bullet+}$ reduction following its addition to GPE is depicted in Figure 2.6. This type of data provides information important when considering pre-quantification $\text{ABTS}^{\bullet+}$ -treatment times. The kinetics of $\text{ABTS}^{\bullet+}$ reduction in such systems will be dependent on the nature and concentration of reactive endogenous constituents (Tian & Schaich, 2013; Walker & Everette, 2009). The relatively slow reduction of $\text{ABTS}^{\bullet+}$ in GPE is consistent with that reported for garlic-based preparations treated with $\text{ABTS}^{\bullet+}$ as a means of assessing antioxidant capacity (Leelarungrayub *et al.*, 2006). The insert to Figure 2.6 shows that the rate of decrease in absorbance, attributable to continued $\text{ABTS}^{\bullet+}$ reduction, following $\text{ABTS}^{\bullet+}$ treatments of 15, 30, 60, 120 and 180 minutes was .006, .004, .003, .002, and .002 absorbance units per minute, respectively. These values provide a guide for calculating the extent to which confounding compounds remaining in samples following $\text{ABTS}^{\bullet+}$ treatments are likely to affect absorbance changes during the HRP-catalyzed hydrogen peroxide

quantification reaction. The values are thus to be considered in the context of the hydrogen peroxide-quantifying reaction being complete in approximately 30 seconds. These relatively small adjustments can be accounted for, if necessary, when calculating absorbance changes for hydrogen peroxide-quantification.

The time required for the pre-quantification ABTS^{•+} treatment will largely dictate the extent to which the assay can be applied to kinetic studies. Incorporating the treatment into peroxidase-based assays is straightforward for applications with static systems where the concentration of hydrogen peroxide remains essentially constant over the time required for the assay; recall that the time required for the assay is largely dictated by the time chosen for the ABTS^{•+} treatment phase of the assay. In the present study ABTS^{•+} treatments ranged from 15 to 180 minutes. A concern when working with relatively unstable systems is that hydrogen peroxide concentrations could change during the ABTS^{•+} treatment itself. The extent to which this may occur is dependent on the kinetics of the systems. As discussed above, hydrogen peroxide does not react with ABTS in the absence of HRP, so the loss of hydrogen peroxide referred to in this context is due to reaction of hydrogen peroxide with non-ABTS endogenous components. The ideal treatment for such unstable systems would terminate all hydrogen peroxide consuming/producing reactions instantaneously while also removing those compounds that may inhibit the subsequent peroxidase-based assay. The extent to which this ideal was approached in the present work with the GPE matrix is illustrated in Figure 2.7 (time-course “b”). The time course depicts hydrogen peroxide loss in GPE to which ABTS^{•+} and hydrogen peroxide were added simultaneously. Hence, the data describes the maximum amount of hydrogen peroxide loss that may occur in the GPE matrix during the ABTS^{•+} treatment. It is a maximum value because the reagents were added to fresh GPE (“fresh” is used here to emphasize that the GPE had no previous exposure to hydrogen peroxide or ABTS^{•+}). For the present system, this maximum was .044 μ mole. The data show that the decrease in hydrogen peroxide occurred within 15 minutes following the addition of hydrogen peroxide and ABTS^{•+} to GPE; after 15 minutes the concentration of hydrogen peroxide remained constant. The absolute amount of

hydrogen peroxide consumed during the initial treatment period was constant over the different hydrogen peroxide concentrations tested. This is evident when comparing the calibration curves of Figure 2.8 which are parallel to one another but offset to the extent that hydrogen peroxide was consumed during the treatment period. It is clear that there exist endogenous components in the fresh GPE that react with hydrogen peroxide at a rate which is competitive with its rate of reaction with $\text{ABTS}^{\bullet+}$. The notion of $\text{ABTS}^{\bullet+}$ and hydrogen peroxide reacting with the same endogenous components is reasonable based on their both being relatively strong oxidizing agents. In follow-up experiments it was shown that $\text{ABTS}^{\bullet+}$ treatment of fresh GPE for 1 hour or more oxidized GPE to the extent that added hydrogen peroxide was stable over the timeframe of these experiments (i.e., up to 3 hours, data not show). The time allotted for the $\text{ABTS}^{\bullet+}$ treatment does not appear critical, provided it is greater than the established minimum of 15 minutes for GPE (see above). This statement is based on experiments where GPEs containing fixed amounts of hydrogen peroxide, arrived at by allowing GPE/hydrogen peroxide mixtures to react for a period time, were treated/terminated for different times with $\text{ABTS}^{\bullet+}$ prior to hydrogen peroxide quantification. In all such experiments the amount of hydrogen peroxide in samples treated with $\text{ABTS}^{\bullet+}$ for different times, ranging from 15 minutes to 1 hour, were not significantly different.

The data of Figure 2.7 (curve “a”) illustrate the application of the proposed method for determining the stability of hydrogen peroxide in GPE. The time-course of curve “a” depicts changes in hydrogen peroxide content with time following addition of a known amount of hydrogen peroxide to GPE; curve “b”, as discussed above, depicts changes in hydrogen peroxide content with time following simultaneous addition of hydrogen peroxide and $\text{ABTS}^{\bullet+}$ to GPE (curve “b” was discussed previously with respect to termination of the reaction). Note that the early data points of the time-course correspond to shorter times than required for the $\text{ABTS}^{\bullet+}$ treatment. As noted previously, this is not a problem provided hydrogen peroxide concentrations do not change during the $\text{ABTS}^{\bullet+}$ treatment. Curve “b” of Figure 2.7 shows that for this system anyway, this ideal is

approached but not obtained. That is because there is a relatively small decline in hydrogen peroxide content in the presence of ABTS•⁺. The decline was complete within the first 15 minutes following simultaneous addition of hydrogen peroxide and ABTS•⁺ to fresh GPE. The nature of the hydrogen peroxide consuming reaction remains to be determined. With respect to data interpretation, it means the early time points making up the time course of curve “a” (Figure 2.7) may somewhat underrepresent the actual amount of hydrogen peroxide remaining in the reaction mixture.

The aim of the presented study was to develop a strategy that would allow the direct use of peroxidase-based assays for the quantification of hydrogen peroxide in biological matrixes. Such applications of peroxidase-based assays are often not feasible due to the presence of endogenous confounding compounds that reduce the oxidized reporter molecule generated by the peroxidase reaction. It is here shown that this problem can be circumvented by treatment of the biological matrix with the oxidized reporter molecule itself prior to initiating the peroxidase reaction. The advantages and limitations of this approach have been presented through a series of experiments evaluating the stability of hydrogen peroxide in a garlic-based product representative of those in the food industry. The primary advantage of the presented assay is that it allows one to directly quantify hydrogen peroxide in complex biological matrixes and thus avoids the need for additional separation techniques. A possible limitation of the method is that the treatment required for removal of confounding compounds takes on the order of minutes and thus the method has the possibility of underestimating the hydrogen peroxide content in systems where such concentrations are changing on the seconds-to-minutes time scale. Experiments presented herein provide an example of the extent to which this limitation may be a factor. The approach described herein of selectively oxidizing confounding compounds with ABTS•⁺ prior to using ABTS/peroxidase-based methods for analyte quantification should be generally applicable. This applicability is expected to include peroxidase-based coupled enzyme assays for a range of analytes in different bio-based systems.

References

- Adak, S., Mazumder, A. & Banerjee, R.K. (1996) "Probing the active site residues in aromatic donor oxidation in horseradish peroxidase: involvement of an arginine and a tyrosine residue in aromatic donor binding" *Bipchem. J.* 314, 985-991
- Barni, F., Lewis, S.W., Berti, A., Miskelly, G.M. & Lago, G. (2007) "Forensic application of the luminol reaction as a presumptive test for latent blood detection" *Talanta* 72, 896-913
- Childs, R.E. & Bardsley, W.G. (1975) "The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromagen" *Biochem. J.* 145, 93-103
- Chiu, M.-H., Kumar, A.S., Sornambikai, S., Chen, P.-Y., Shih, Y. & Zen, J.-M. (2011) "Cosmetic hydrogen peroxide detection using nano bismuth species deposited built-in three-in-one screen-printed silver electrode" *Int. J. Electrochem. Sci.* 6, 2352-2365
- Doerge, D.R., Divi, R.L. & Churchwell, M.I. (1997) "Identification of the colored guaiacol oxidation products produced by peroxidases" *Anal. Biochem.* 250, 10-17
- Grieshaber, D.; MacKenzie, R.; Vörös, J.; Reimhult, E. (2008) "Electrochemical biosensors – sensor principles and architectures" *Sensors* 8, 1400-1458
- Hage, R. & Lienke, A. (2006) "Applications of transition-metal catalysts to textile and wood-pulp bleaching" *Angew. Chem. Int. Ed.* 45, 206-222
- Henriquez, C., Aliaga, C. & Lissi, E. (2002) "Formation and decay of the ABTS derived radical cation: a comparison of different preparation procedures" *International Journal of Chemical Kinetics* 34, 659-665
- Huang, D., Ou, B. & Prior, R.L. (2005) "The chemistry behind antioxidant capacity assays" *J. Agric. Food Chem.* 53, 1841-1856
- Jervis, S.M. & Drake, M. (2013) "The impact of iron on the bleaching efficacy of hydrogen peroxide in liquid whey systems" *Journal of Food Science* 78, R129-R137
- Joseph, P.D., Eling, T. & Mason, R.P. (1982) "The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine" *The Journal of Biological Chemistry* 257, 3669-3675
- Leelarungrayub, N., Rattanapanone, V., Chanarat, N. & Gebicki, J.M. (2006) "Quantitative evaluation of the antioxidant properties of garlic and shallot preparations" *Nutrition* 22, 266-274

- Marquette, C.A. & Blum, L.J. (2006) "Applications of the luminol chemiluminescent reaction in analytical chemistry" *Anal. Bioanal. Chem.* 385, 546-554
- Murphy, M.P., Holmgren, A., Larsson, N.-G., Halliwell, B., Chang, C.J., Kalyanaraman, B., Rhee, S.G., Thornalley, P.J., Partridge, L., Gems, D., Nyström, T., Belousov, V., Schumacker, P.T. & Winterbourn, C.C. (2011) "Unraveling the biological roles of reactive oxygen species" *Cell Metab* 13, 361-366
- Prior, R.L., Wu, X. & Schaich, K. (2005) "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements" *J. Agric. Food Chem.* 53, 4290-4302
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999) "Antioxidant activity applying an improved ABTS radical cation decolorization assay" *Free Radical Biology & Medicine* 26, 1231-1237
- Rhee, S.G., Chang, T.-S., Jeong, W. & Kang, D. (2010) "Methods for detection and measurement of hydrogen peroxide inside and outside of cells" *Mol. Cells* 29, 539-549
- Segunda, M.A., Castro, C., Magalhães, L.M. & Reis, S. (2013) "Determination of scavenging capacity against hydrogen peroxide: recent trends on chemical methods" in *Hydrogen Peroxide Detection, Applications and Health Implications* (G. Aguilar & R. A. Guzman, Editors), Novo Science Pub Inc., 203-218
- Staniek, K. & Nohl, H. (1999) "H₂O₂ detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system" *Biochimica et Biophysica Acta* 1413, 70-80
- Tarpey, M.M., Wink, D.A., & Grisham, M.B. (2004) "Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations." *Am J Physiol Regul Integr Comp Physiol.* 286, R431-R444
- Tarvin, M., McCord, B., Mount, K., Sherlach, K. & Miller, M.L. (2010) "Optimization of two methods for the analysis of hydrogen peroxide: high performance liquid chromatography with fluorescence detection and high performance liquid chromatography with electrochemical detection in direct current mode" *J. Chromatogr. A* 1217, 7564-7572
- Tian, X. & Schaich, K.M. (2013) "Effects of molecular structure on kinetics and dynamics of the Trolox equivalent antioxidant capacity assay with ABTS^{•+}" *J. Agric. Food Chem.* 61, 5511-5519
- Venkatasubremanian, L. & Maruthamuthu, P. (1989) "Kinetics and mechanism of formation and decay of 2,2'-Azinobis- (3-Ethylbenzothiazole-6-Sulphonate) radical cation in aqueous solution by inorganic peroxides" *International Journal of Chemical Kinetics* 21, 399-421

- Walker, R.B. & Everette, J.D. (2009) "Comparative reaction rates of various antioxidants with ABTS radical cation" *J. Agric. Food Chem.* 57, 1156-1161
- Wardman, P. (2007) "Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects" *Free Rad. Biol. Med* 43, 995-1022
- Westbroek, P., Hakuzimana, J., Gasana, E., Lombaert, P. & Kiekens, P. (2007) "Voltammetric detection of hydrogen peroxide in teeth whitening gels" *Sensors and Actuators B* 124, 317-322
- Winterbourn, C.C. (2013) "The biological chemistry of hydrogen peroxide" *Meth. Enzymol.* 528, 3-25
- Winterbourn, C.C. (2014) "The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells" *Biochim Biophys Acta* 1840, 730-738
- Zulueta, A., Esteve, M.J. & Frígola, A. (2009) "ORAC and TEAC assays comparison to measure the antioxidant capacity of food products" *Food Chemistry* 114, 310-316

Figures

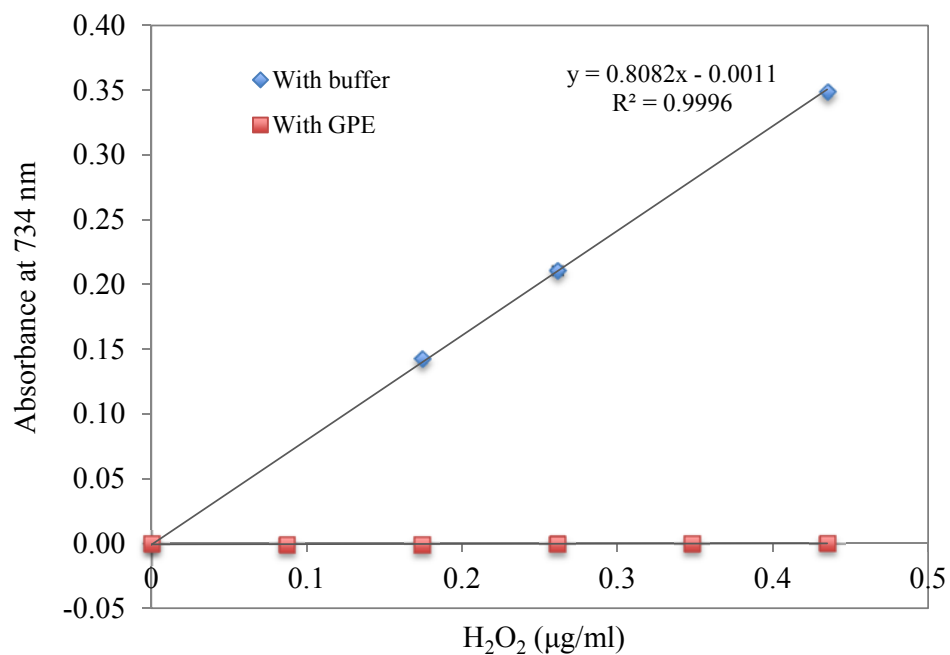


Figure 2.1. Standard curves for hydrogen peroxide determination in a model buffer system and in GPE using the HRP/ABTS system. Final values are means \pm standard deviation from triplicate measurements. Points without visible error bars have standard deviations smaller than the data points.

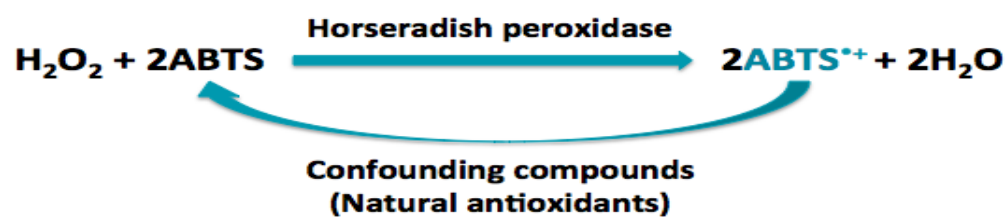


Figure 2.2. A plausible mechanism for assay interference. The concentration of hydrogen peroxide in a sample is based on the amount of $\text{ABTS}^{\bullet+}$ formed following initiation of the peroxidase reaction.

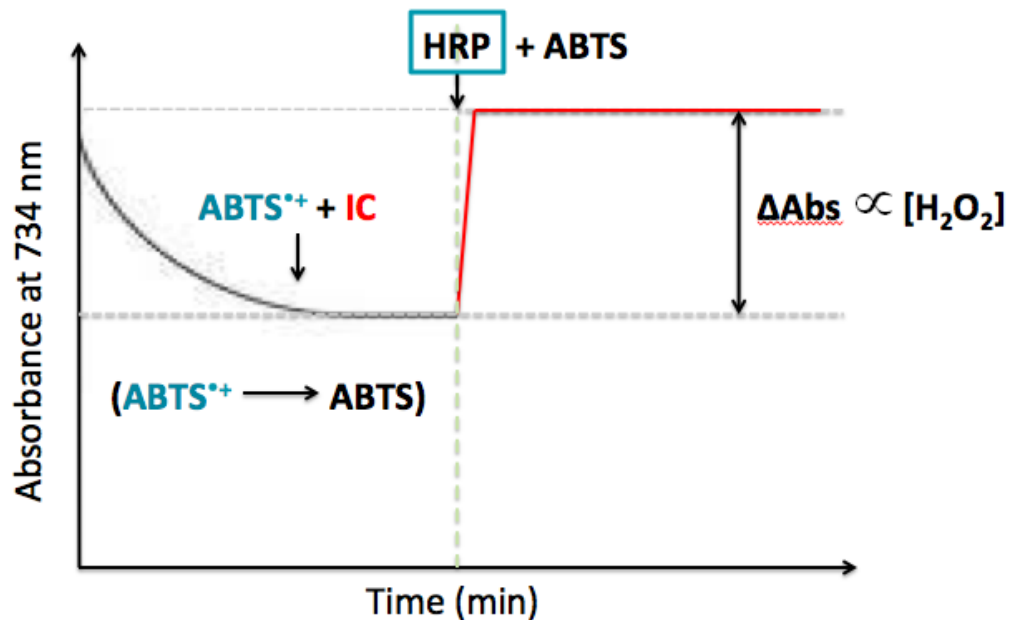


Figure 2.3. Experimental design of modified assay for the determination of hydrogen peroxide in biological matrices. Initially, an aliquot of ABTS and ABTS^{•+}-containing solution is added to the hydrogen peroxide containing sample. This step allows confounding compounds to react with ABTS^{•+}. Horseradish peroxidase is then added to catalyze for the formation of ABTS^{•+} in proportion to the amount of hydrogen peroxide in the reaction mixture. Hydrogen peroxide is quantified based on the change in absorbance before and after the addition of peroxidase.

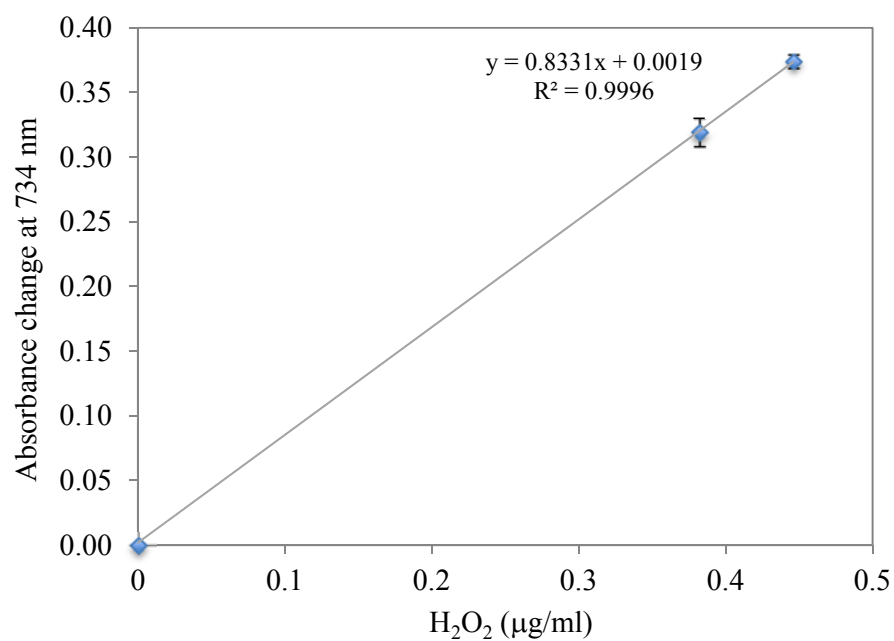


Figure 2.4. Standard curve for hydrogen peroxide determination in ABTS^{•+}-treated GPE. Final values are means \pm standard deviation from triplicates.

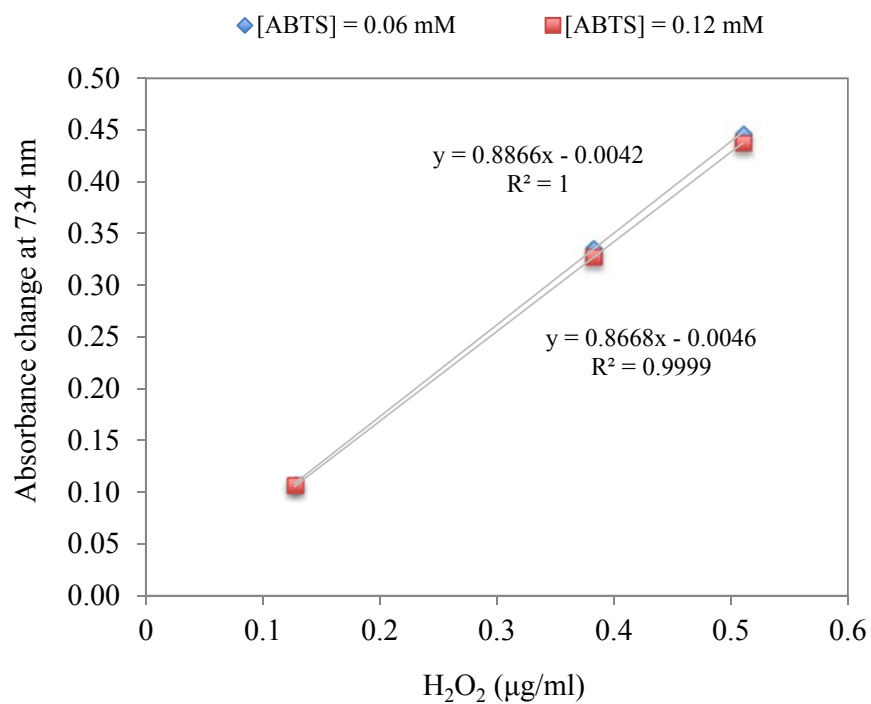


Figure 2.5. Comparison of standard (calibration) curves for hydrogen peroxide determination in $ABTS\bullet^+$ -treated GPE using different concentrations of ABTS in the reaction mixture. Final values are means \pm standard deviation from triplicates.

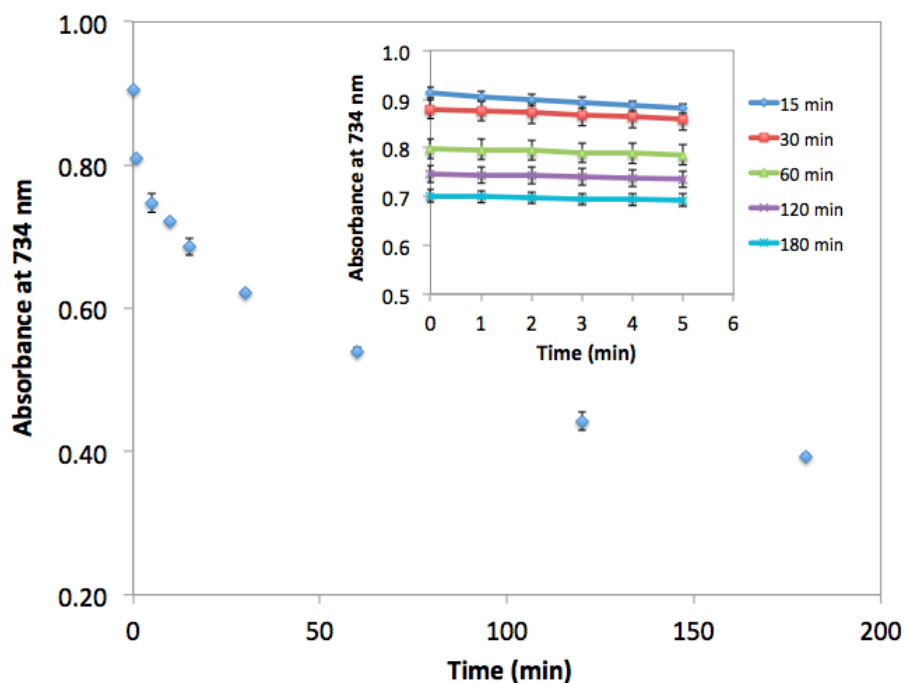


Figure 2.6. Time-course of ABTS \bullet^+ reduction in GPE. The insert depicts time-courses reflecting rates of ABTS \bullet^+ reduction following the addition of sufficient ABTS \bullet^+ to the ABTS \bullet^+ -treated GPE to adjust its ABTS \bullet^+ concentration back to that amount present at zero time of the initial ABTS \bullet^+ treatment. The initial ABTS \bullet^+ -treatment was done for different times prior to the addition of the supplemental ABTS \bullet^+ (initial ABTS \bullet^+ treatment times, prior to adding supplemental ABTS, are indicated to the right of insert). Rates of ABTS \bullet^+ reduction ($\Delta\text{Abs}/\text{min}$), as depicted in the insert, were 0.006, 0.004, 0.003, 0.002, and 0.002 after 15, 30, 60, 120, and 180 min ABTS \bullet^+ treatments, respectively. Data points represent means \pm standard deviation from triplicate samples.

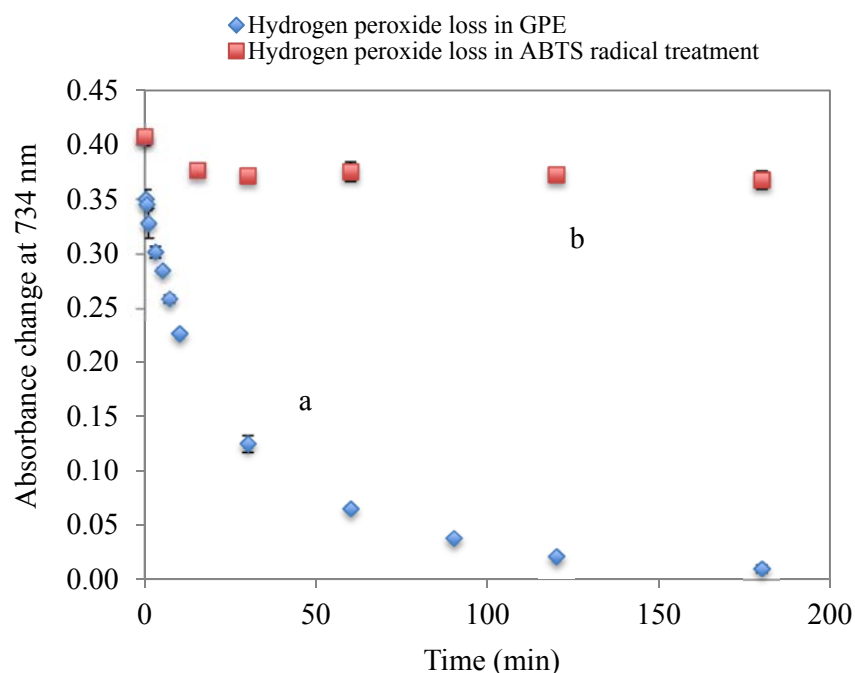


Figure 2.7. Time course of hydrogen peroxide loss, measured as absorbance change due to presence of hydrogen peroxide (y-axis), in GPE. Curve “a” was generated by adding hydrogen peroxide to GPE, allowing it to react for the specified time (as specified on x-axis) before adding ABTS^{•+} to eliminate confounding compounds and terminate hydrogen peroxide reduction. Peroxidase was subsequently added to the ABTS^{•+}-containing sample for hydrogen peroxide quantification. Curve “b” was obtained by simultaneously adding hydrogen peroxide and ABTS^{•+} to GPE and allowing the mixture to react for the specified times (as specified on x-axis) prior to quantification of hydrogen peroxide by the addition of peroxidase. Data points are means \pm standard deviation from triplicate measurements.

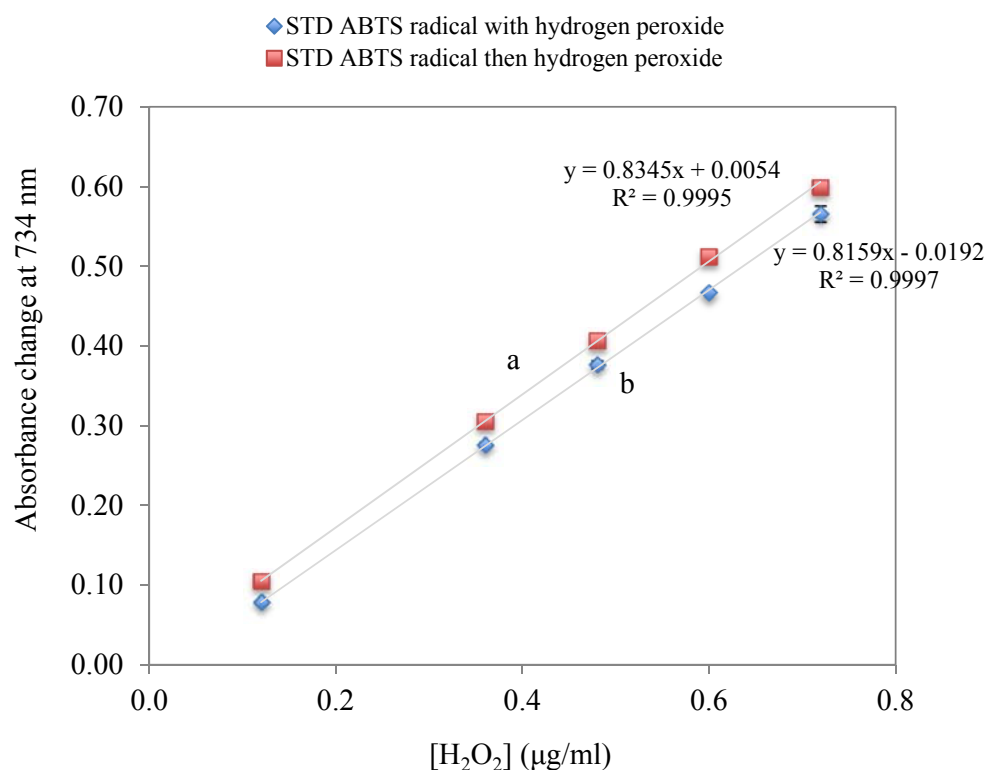


Figure 2.8. Comparison of standard (calibration) curves obtained following spiking known amounts of hydrogen peroxide into ABTS^{•+}-treated and untreated GPE samples. Curve “a” was obtained by measuring hydrogen peroxide levels following its addition to 3 hr ABTS^{•+}-treated GPE (as in Figure 2.4); Curve “b” was obtained by measuring hydrogen peroxide produced following the addition of hydrogen peroxide and ABTS^{•+} simultaneously to untreated GPE, waiting 15 minutes, and then adding HRP for hydrogen peroxide quantification. Data points are means \pm standard deviation from triplicates.

3 Selective Oxidation of Enzyme Extracts for Improved Peroxidase Quantification

Shu Jiang and Michael H. Penner
Department of Food Science and Technology
Oregon State University
Corvallis, OR 97331-6602

3.1 Abstract

A modified assay that eliminates a source of underestimation of peroxidase activity in plant extracts has been developed. Natural components endogenous to plant materials, such as phenolics and ascorbic acid, may interfere with traditional peroxidase assays by reducing the oxidized product generated as a result of the peroxidase-catalyzed reaction; the problem in such cases is that the oxidized product is typically the reporter molecule that is monitored for enzyme quantification. The reduction of the reporter molecule results in an apparent lag in product accumulation, which is observed as an artificially low enzyme activity. The artificially low activity is thus a consequence of the rate of product accumulation being lower than the rate of product production. This behavior may be reflected in the time course of product accumulation being sigmoidal. This paper describes a relatively simple way to alleviate complications arising from the presence of compounds that confound the peroxidase assay without the need to fractionate the enzyme-containing sample. The method is based on using ABTS as the reporter (reducing) substrate. The oxidized product of the reaction is $\text{ABTS}^{\bullet+}$, which can be followed spectrophotometrically due to its relatively high molar absorptivity at 734 nm. It is herein shown that one can selectively inactivate complicating endogenous confounding compounds by treating the enzyme preparation with the oxidized product itself, $\text{ABTS}^{\bullet+}$, prior to initiating the assay. This approach is expected to be selective for those compounds likely to interfere with peroxidase quantification via the reductive mechanism described above. The presented method is herein shown to alleviate complications associated with lag phases typical of plant extracts and, thus, to more accurately reflect total peroxidase activity. The presented assay is relatively simple and should be applicable to a range of biological systems.

3.2 Introduction

Peroxidase enzymes are common in plants where they appear to be involved in a wide range of physiological functions, including hydrogen peroxide metabolism,

the formation of lignin and suberin, cross-linking of cell wall components, and plant defense mechanisms (Almagro et al., 2009; Hiraga et al., 2001; Mehlhorn et al., 1996; Fortea et al., 2011; Hamid & Rehman, 2009). Along with their importance for plant vitality, peroxidases also impact the consumer acceptability of plant-based foods through their role in fruit and vegetable coloration/discoloration (Adams & Brown, 2007), flavor development (Burnette, 1977), nutritional properties (Vamos-Vigyazo, 1981) and texture (Manu & Rao, 2011). Furthermore, peroxidases are used as indicators of the adequacy of vegetable blanching due to their high thermal stability and wide distribution (Serrano-Martinez et al., 2008; Goncalves et al., 2010; Ali et al., 2011). Selected plant peroxidases, particularly horseradish peroxidase (HRP), are widely used in biotechnological applications, including the decolorization of synthetic dyes, chemical syntheses, bioremediation, biosensors, and a range of analytical applications (Regalado et al., 2004; Azevedo et al., 2003; Ngo, 2010). Current understanding of the role of peroxidases in each of the aforementioned areas is not complete. It is anticipated that improvements in methods for the quantification of peroxidase activity *in situ* will further such understanding.

A variety of methods exist for the determination of peroxidase activity, including those based on colorimetric (Xianyu et al., 2013, Fortea et al., 2011), fluorometric (Acharya et al., 2013), chemiluminescent (Katsuragi et al., 2000), and electrochemical (Stiene & Bilitewski, 2002) detection. Among these, colorimetric detection is most commonly used as it is simple and economical (Dosoretz & Ward, 2006). Colorimetric quantification is based on the formation of visible light-absorbing products as a result of the peroxidase-catalyzed reaction between a “reporter molecule” and hydrogen peroxide. The reporter molecule is the hydrogen/electron donating (reducing) substrate which, after its oxidation, absorbs light in the visible region. Various reducing substrates, including guaiacol, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), *p*-phenylenediamine, tetramethylbenzidine (TMB), and *o*-dianisidine, have been used as reporter molecules. The flexibility in choosing a reporter molecule is due to the rather broad specificity of these peroxidases. A compendium focusing on the

quantification of horseradish peroxidase lists over 200 substrates for peroxidase activity determinations (Silaghi-Dumitrescu, 2010). As may be expected, commercially available kits for the quantification of peroxidase activity offer a range of reducing substrates. A common concern with many of the substrates used for colorimetric-based assays, especially when applied directly to biological matrixes, is interference due to the presence of confounding compounds endogenous to living tissues. An important class of such compounds are those that react with the assay's reporter molecule, *i.e.* they react with the oxidized product derived from the reducing substrate as a result of the peroxidase-catalyzed reaction (Castillo et al., 1984; Osborne & Metzler, 1984). The net effect of these confounding compounds is a reduction in the observed rate of product accumulation. In such cases the actual rate of product production is less than the rate of product accumulation, leading to underestimates of enzyme activity. To avoid such complications one can separate the confounding compounds from the enzyme prior to quantifying enzyme activity. Approaches toward this end may include a range of separation techniques previously used in peroxidase quantification and/or purification studies (Mall et al., 2013; Valetti & Picó, 2013; Motamed et al., 2009; Fraguas et al., 2004; Magri et al., 2003; Regaldo et al., 1996; Castillo et al., 1984; Osborne & Metzler, 1984). Disadvantages associated with methods aimed at fractionating out confounding compounds include the need for relatively costly specialized equipment (*e.g.*, chromatographs), difficulty in identifying optimum fractionation parameters, difficulty in estimating the extent of confounding compound removal, the time required for such separations, and/or inherent limitations in the extent to which one can minimize changes in the enzyme's environment if interested in *in situ* activity.

The goal of the work presented in this paper was to develop a colorimetric method for the quantification of peroxidase activity that accounts for confounding compounds typical of biological matrices without requiring a fractionation step. Confounding compounds widely present in biological matrices, *e.g.* plant extracts, include a range of natural antioxidants that are capable of reducing the reporter molecules generated during peroxidase quantification. The outcome of this study

is an improved method based on the use of ABTS as the reducing substrate and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($\text{ABTS}^{\bullet+}$) as the corresponding reporter molecule. $\text{ABTS}^{\bullet+}$ is generated as a result of the peroxidase-catalyzed reaction between ABTS and hydrogen peroxide. The presented assay effectively eliminates problems associated with confounding compounds inherent in peroxidase-containing samples by selectively oxidizing these compounds prior to peroxidase quantification. Selective oxidation is achieved by treating samples with $\text{ABTS}^{\bullet+}$. During this treatment $\text{ABTS}^{\bullet+}$ is reduced to ABTS. The ABTS generated in this way later serves in the peroxidase-quantification reaction as the reducing substrate, where the rate at which ABTS is converted to $\text{ABTS}^{\bullet+}$ reflects the sample's peroxidase activity. The presented method improves the accuracy of peroxidase activity measurements by eliminating lag phases associated with the presence of confounding compounds. The method retains the simplicity of the traditional ABTS-based colorimetric assay upon which it is based (Childs & Bardsley, 1975).

3.3 Materials and Methods

Reagents.

Hydrogen peroxide (30 wt.%, ACS reagent grade), horseradish peroxidase (HRP, type II, 150-250 units/mg solid), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], guaiacol, potassium persulfate and monobasic sodium phosphate were purchased from Sigma-Aldrich, USA.

Sample.

Sweet yellow peppers (*Capsicum annuum*) and yellow onions were purchased from local markets.

Preparation of Enzyme Extracts.

Vegetables were washed and rinsed with distilled water, cut into small pieces and weighed. Approximately 100 g of cut vegetable weighed to the nearest 0.1 g was homogenized for 30 seconds in 100 mM sodium phosphate buffer (pH 6.0) such that the vegetable:buffer ratio was 1:2 (by weight). The resulting homogenate was filtered through two layers of cheesecloth and then Whatman #1 filter paper. The resulting filtrate from sweet yellow peppers was subsequently filtered through 0.45 μm PTFE syringe-type filters; the resulting filtrate from yellow onions was filtered through 1.2 μm syringe glass fiber filters (Millipore Swinnex). Clear filtrates resulting from these processes were kept in an ice-bath until assayed for peroxidase activity. Enzyme extracts prepared in this way from sweet yellow peppers and yellow onions are hereafter referred to as sweet yellow pepper extract (SYPE) and yellow onion extract (YOE), respectively.

Preparation of ABTS radical cations.

ABTS radical cation ($\text{ABTS}^{\bullet+}$) containing solutions were prepared as described by Huang *et al.* (2005) by incubating an aqueous 7mM ABTS, 2.45mM potassium persulfate, solution overnight (12-16 hours) in the dark, at room temperature. The resulting solution is then made 100 mM sodium phosphate, pH 6.0 prior to being used in subsequent experiments.

Traditional peroxidase assay using ABTS.

Aliquots of SYPE (20 μl) or YOE (100 μl) were added to 2 ml of color-forming reagent (0.9 mM ABTS, 0.15 mM H_2O_2 , 0.1M sodium phosphate, pH 6.0) to initiate the reaction. Assays were performed at ambient temperature (20-22°C). The increase in absorbance at 734 nm resulting from peroxidase-catalyzed $\text{ABTS}^{\bullet+}$ production was monitored for 30 minutes. Initial velocities were calculated from the linear portion of the reaction time course having the highest positive slope. One unit of peroxidase activity, based on initial velocity

determinations, is defined as that amount of enzyme that catalyzes the production of 1 $\mu\text{mole ABTS}^{\bullet+}$ per minute under the defined conditions. The absorptivity of $\text{ABTS}^{\bullet+}$ was taken as $15,000 \text{ M}^{-1}\text{cm}^{-1}$ (Re et al., 1999).

Traditional peroxidase assay using guaiacol.

A 20 μl aliquot of SYPE was added to 2.18 ml of color-forming reagent (0.04% (v/v) guaiacol, 0.135 mM H_2O_2 , 0.1M sodium phosphate, pH 6.0). The increase in absorbance at 470 nm resulting from peroxidase-catalyzed guaiacol oxidation was monitored for 30 minutes. Assays were performed at ambient temperature (20-22°C). Initial velocities were calculated from the linear portion of the reaction time course having the highest positive slope. One unit of peroxidase activity is defined as that amount of enzyme that catalyzes the reaction at a rate corresponding to an absorbance change of 0.1 unit per minute under the defined conditions.

Modified peroxidase assay using ABTS.

Aliquots of SYPE (20 μl) or YOE (100 μl) were selectively oxidized by mixing with 2 ml of an appropriately diluted $\text{ABTS}/\text{ABTS}^{\bullet+}$ solution, prepared as described below, and allowed to react for 2 minutes (SYPE) or 30 minutes (YOE) in the dark at 0°C. Peroxidase quantification was then initiated by adding 0.2 ml of color-forming reagent (9 mM ABTS, 1.5 mM H_2O_2 , 0.1M sodium phosphate, pH 6.0) to the extract containing solution. Absorbance changes due to peroxidase-catalyzed ABTS oxidation were monitored at 734 nm. One unit of peroxidase activity in the modified assay is defined as in the “Traditional peroxidase assay using ABTS” (above).

Time course of $\text{ABTS}^{\bullet+}$ reduction in SYPE and YOE.

Aliquots of SYPE or YOE, ranging from 20 to 100 μl , were added to 1.98 ml $\text{ABTS}^{\bullet+}$ solution having an initial absorbance at 734 nm of between 0.9 and 1.0.

The decrease in absorbance at 734 nm resulting from $\text{ABTS}^{\bullet+}$ reduction was monitored spectrophotometrically for 30 minutes. For reference purposes, controls were included which monitored changes in the absorbance of $\text{ABTS}^{\bullet+}$ solutions to which aliquots of buffer were added.

3.4 Results and Discussion

Typical time courses depicting product accumulation in the traditional and the modified ABTS-based peroxidase assays applied to SYPE and YOE are shown in Figure 3.1. The time-courses obtained using the traditional assay reflect a lengthy lag period prior to accumulation of product. The lag period can be explained by the presence of confounding compounds common to vegetable extracts that react with the chromophoric oxidized reporter molecule ($\text{ABTS}^{\bullet+}$) generated in the peroxidase-catalyzed reaction. This type of interference can be described as follows:



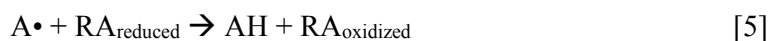
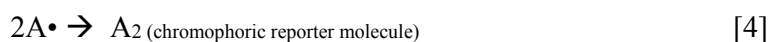
RA = reducing agent, defined as any compound that reduces $\text{ABTS}^{\bullet+}$ at a rate which is significant in reference to the measurement of peroxidase.

If the rate of reaction [2] is significant with respect to the rate of reaction [1], then the peroxidase activity of the reaction mixture will be underestimated, at least until RA is depleted to the extent that reaction [2] is insignificant. The underestimate of enzyme activity may appear as a complete lack of peroxidase activity if the rate of [2] is much greater than that of reaction [1], such that $\text{ABTS}^{\bullet+}$ is reduced immediately upon being formed. Or it may reflect a fractional underestimate of peroxidase activity if rates of reactions [1] and [2] are similar. One would expect to see an increase in peroxidase activity with time as RA is depleted, provided there is an excess of added substrates (ABTS and H_2O_2) in the reaction mixture.

The time-courses depicted in Figure 3.1 which were obtained with the proposed modified assay do not show a lag prior to product accumulation. This is a result of incorporating into the assay a sample treatment step, prior to peroxidase quantification, to selectively oxidize those compounds likely to reduce $\text{ABTS}^{\bullet+}$. The selective oxidation is done by adding the oxidized reporter molecule, $\text{ABTS}^{\bullet+}$, to the reaction mixture prior to adding the substrates for peroxidase quantification (*i.e.*, ABTS and H_2O_2). During the pre-quantification period, when the sample is exposed to $\text{ABTS}^{\bullet+}$, those components most reactive with $\text{ABTS}^{\bullet+}$ are oxidized. The treatment is selective in that it specifically targets those compounds that modify the oxidized reporter molecule, as expected since it is the oxidized reporter molecule itself that is used as the oxidizing agent. The time allotted for the pre-quantification treatment is somewhat flexible; it is dependent on the nature and amount of confounding compounds in the sample and the sample's peroxidase activity. These are major factors dictating the relative importance of reaction [2] in peroxidase quantification. The outcome of incorporating the $\text{ABTS}^{\bullet+}$ treatment in the peroxidase assay is a decrease in the error associated with reaction [2] (as depicted in the curves of Figure 3.1 obtained using the modified assay).

The modified assay addresses a problem that has been noted by many researchers (e.g., Fortea et al., 2011; Reszka & Britigan, 2007; Osborne & Metzler, 1984; Celardin et al., 1982; Bruemmer et al., 1976; Castillo et al., 1984). Compounds likely to interfere with the traditional ABTS-based peroxidase assay include a wide range of natural antioxidants. This is not surprising since $\text{ABTS}^{\bullet+}$ reduction is the basis of the Trolox equivalent antioxidant capacity (TEAC) assay, which is widely used for determining the antioxidant capacity of biological materials (Huang et al., 2005). Interestingly, the lag phase in the peroxidase/ H_2O_2 /ABTS system, as depicted in the time-courses obtained using the traditional peroxidase assay, has been suggested as an indicator of the total antioxidant capacity in foods (Arnao et al., 1996). The premise being the length of the lag phase is dictated by the amount of antioxidant in the matrix.

The type of assay interference described by equation [2] is not limited to the ABTS substrate. It is likely to occur with many of the common chromophoric reducing substrates used for peroxidase quantification. This is because the initial reporter molecule-product resulting from the peroxidase-catalyzed reaction is a radical. In many cases it is assumed that these radicals polymerizes to make the final chromophoric reporter molecule. Inhibition of the peroxidase assay by this type of generic mechanism may be described follows:



RA = “reducing agent”, here defined as any compound that reduces the initial radical product formed in the peroxidase reaction.

This reaction scheme shows how confounding compounds reacting with the initial product of the peroxidase reaction, $\text{A}\bullet$, will prevent the formation of A_2 . This in turn results in an underestimation of peroxidase activity because A_2 is the chromophoric reporter molecule upon which peroxidase quantification is based. A classic reducing substrate used in many peroxidase quantification assays is guaiacol. Assays using guaiacol are susceptible to the type of inhibition described by the above equations. In Figure 3.2 we demonstrate the lag observed when measuring peroxidase activity in SYPE using guaiacol as the reducing substrate. The observed lag is again indicative of the presence of confounding compounds in SYPE. Such lags in peroxidase assays have been noted indirectly before with respect to the behavior of antioxidants (Sanchez et al., 1997; Arnao et al., 1996; Takahama & Oniki, 1997; Demirevska-Kepova & Bakardjieva, 1976). Unfortunately, in contrast to the ABTS substrate, the primary product of guaiacol oxidation, the guaiacol radical, is not sufficiently stable to enable using it in a pre-quantification treatment as proposed for $\text{ABTS}\bullet^+$ (*i.e.*, the guaiacol radical rapidly dimerizes; see Doerge et al., 1997). $\text{ABTS}\bullet^+$ is somewhat unique in this respect since it is relatively stable in common buffer systems, thus allowing it to be

prepared and used for the selective oxidation of the peroxidase-containing matrix as described herein.

Investigators using traditional peroxidase assays sometimes report that their initial velocity values, *i.e.* the values used for calculating enzyme activities, were derived from the first linear portion of the time-courses of product accumulation. One assumes the phrase “first linear portion” is not referring to that associated with the lag, but rather the first linear portion that reflects maximum enzyme activity. Hence, the question arises as to whether there is a consequence of measuring enzyme activity in this way. The data of Table 3.1 demonstrate that there is a consequence of measuring enzyme activity following the lag phase. The peroxidase activity was approximately 2-fold higher when determined using the proposed modified assay compared to that obtained using the traditional assay. The slower rate of reaction coming out of the lag phase may be rationalized by at least four mechanisms: (1) slower reacting ABTS \bullet^+ -reducing compounds remain in the reaction mixture following the lag phase when ABTS \bullet^+ begins to accumulate, thus the rate of accumulation of ABTS \bullet^+ is decreased to some extent due to a lingering but significant rate of ABTS \bullet^+ reduction, (2) sufficient hydrogen peroxide is consumed during the lag phase such that the post-lag phase reaction rate reflects the lower hydrogen peroxide concentration (note that in the simplified scheme above the concentration of ABTS would not change during the lag phase since the absence of reporter molecule accumulation is due to ABTS \bullet^+ produced as a result of the peroxidase/hydrogen peroxide/ABTS reaction being reduced back to ABTS), (3) ABTS \bullet^+ -oxidizable competing substrates remain in the reaction mixture following the lag phase when ABTS \bullet^+ begins to accumulate, thus the rate of accumulation of ABTS \bullet^+ is slowed due to the enzyme's interaction with non-chromophoric competing substrates, and (4) the enzyme is inactivated over the course of the lag phase (*e.g.*, suicide inhibition; Valderrama et al., 2002). It is important to recognize that the successful application of the proposed ABTS \bullet^+ treatment prior to peroxidase quantification should alleviate the above complications.

The key point in considering activities obtained using the traditional versus the modified assay is that the higher activity is expected to more accurately reflect the true peroxidase activity of an enzyme preparation. This reasoning is based on the premise that ABTS^{•+} activation of peroxidase activity per se is unlikely. The effect of ABTS^{•+} on horseradish peroxidase (HRP) was directly assessed in this study by doing two minute exposures of HRP to ABTS^{•+}, these treatments are analogous to those used in the SYPE experiments. The results showed no demonstrable change in HRP activity due to ABTS^{•+} treatments. Assuming this result is applicable to other plant peroxidases, it can be concluded that the higher activity reflected in the modified assay is solely a consequence of removing confounding compounds.

An important parameter with respect to the application of the modified assay is the time required for the pre-peroxidase quantification ABTS^{•+} treatment. The time required for such treatments will be sample specific. The data of Figure 3.3 summarize the rate of ABTS^{•+} reduction by SYPE and YOE. The differences in rates are striking. It is well known that antioxidants differ greatly in the rate with which they react with ABTS (Walker & Everette, 2009; Henriquez et al., 2004). Compounds such as ascorbic acid and tocopherol derivatives react with ABTS^{•+} relatively fast; those such as phenolics react much slower. The composition of the two enzyme preparations used in this work are consistent with the observed time-courses depicting ABTS^{•+} reduction. Ascorbic acid is the main antioxidant in SYPE (Serrano-Martinez et al., 2008) and thus the majority of its confounding components react relatively fast with ABTS^{•+} (Tian et al., 2013). In contrast, slower reacting flavonoids (e.g., quercetin, kaempferol) are the predominant antioxidants in YOE (Takahama, 2004; Lanzotti, 2006). Once the kinetics of ABTS^{•+} reduction are established for a given enzyme preparation, then one can choose an appropriate treatment time. An appropriate ABTS^{•+} treatment time need not remove all confounding compounds, but the rate of ABTS^{•+} reduction at the end of the treatment should be negligible relative to the rate of ABTS^{•+} formation due to the peroxidase quantifying reaction.

3.5 Conclusion

An important complication associated with the measurement of peroxidase activity in plant extracts has been addressed. The complication stems from the fact that plant extracts, like most biological matrixes, often contain confounding compounds that react with reporter molecules generated in typical peroxidase assays. These confounding reactions can lead to false underestimates of enzyme activity. This paper introduces an approach to circumvent this problem. The assay uses ABTS as the reducing substrate in conjunction with hydrogen peroxide. A key piece of the modified assay is the inclusion of a selective oxidizing step, using $\text{ABTS}^{\bullet+}$ as the oxidizing agent, prior to peroxidase quantification. This step is selective in that it specifically removes those confounding compounds that interfere with the assay. The modified assay improves the accuracy of enzyme activity determinations while maintaining much of the simplicity of traditional peroxidase assays. The modified assay is better suited for assessing relative activities when working with peroxidases in different biological matrices, such as may occur when applying peroxidase-based technologies in different environments or when fractionating peroxidase-containing preparations. This is because in such cases each sample/preparation/environment will have its own confounding compound profile, thus inhibiting the traditional assay to its own unique extent. This is not an issue with the modified assay since such compounds are inactivated prior to peroxidase quantification. The modified peroxidase assay is expected to be applicable to the wide range of natural and manipulated biological matrixes in which confounding compounds are likely to impact peroxidase measurements.

References

- Acharya, A.P., Nafisi, P.M., Gardner, A., MacKay, J.L., Kundu, K., Kumar, S., & Murthy, N. (2013) "A fluorexcent peroxidase probe increases the sensitivity of commercial ELISAs by two orders of magnitude" *Chem. Commun.* 49, 10379-10381
- Adams, J.B. & Brown, H.M. (2007) "Discoloration in raw and processed fruits and vegetables" *Crit. Rev. Food Sci. Nutr.* 47, 319-333

- Ali, G., Russly, A.R., Jamilah, B., Azizah, O. & Mandana, B. (2011) "Effect of heat and thermosonication on kinetics of peroxidase inactivation and vitamin C degradation in seedless guava (*Psidium guajava* L.)" *International Food Research Journal* 18, 1289-1294
- Almagro, L., Gomez Ros, L.V., Belchi-Navarro, S., Bru, R., Ros Barcelo, A. & Pedreno, M.A. (2009) "Class III peroxidases in plant defence reactions" *Journal of Experimental Botany* 60, 377-390
- Arnao, M.B., Cano, A., Hernandez-Ruiz, J., Garcia-Canovas, F. & Acosta, M. (1996) "Inhibition by L-ascorbic acid and other antioxidants of the 2,2'-Azino-Bis(3-ethylbenzo Thiazoline-6-Sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods" *Analytical Biochemistry* 236, 255-261
- Azevedo, A.M., Martins, V.C., Prazeres, D.M.F., Vojinović, V., Cabral, J.M.S. & Fonseca, L.P. (2003) "Horseradish peroxidase: a valuable tool in biotechnology" *Biotechnology Annual Review* 9, 199-247
- Bruemmer, J.H., Roe, B. & Bowen, E.R. (1976) "Peroxidase reactions and orange juice quality" *Journal of Food Science* 41, 186-189
- Burnette, F.S. (1977) "Peroxidase and its relationship to food flavor and quality: a review" *J. Food Sci.* 42, 1-6
- Castillo, F.J., Celardin, F. & Greppin, H. (1984) "Peroxidase assay in plants: interference by ascorbic acid and endogenous inhibitors in *Sedum* and *Pelargonium* enzyme extracts" *Plant Growth Regulation* 2, 69-75
- Celardin, F., Castillo, F.J. & Greppin, H. (1982) "Simple kinetic determination of trace amounts of ascorbic acid in plant extracts" *Journal of Biochemical and Biophysical Methods* 6, 89-93
- Childs, R.E. & Bardsley, W.G. (1975) "The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromogen" *Biochem J.* 145, 93-103
- Demirevska-Kepova, K.N. & Bakardjieva, N.T. (1976) "Study of competition between guaiacol and ascorbic acid in peroxidase reaction and on ion effect by means of absorption spectra" *Can. J. Bot.* Vol. 54, 90-99
- Doerge, D.R., Divi, R.L. & Churchwell, M.I. (1997) "Identification of the colored guaiacol oxidation product produced by peroxidases" *Analytical Biochemistry* 250, 10-17

- Dosoretz, C.G. & Ward, G. (2006) "Peroxidases" in *Enzyme Technology* (A. Pandey, C. Webb, C.R. Soccol and C. Larroche, Edts.) Springer Asiatech Publ., Inc., New Delhi, 399-432
- Fortea, M.I., Lopez-Miranda, S., Serrano-Martinez, A., Hernandez-Sanchez, P., Zafrilla, M.P., Martinez-Cacha, A. & Nunez-Delicado, E. (2011) "Kinetic characterization and thermal inactivation study of red alga (*Mastocarpus Stellatus*) peroxidase" *Food Chemistry* 127, 1091-1096
- Fragus, L.F., Batista-Viera, F., & Carlsson, J. (2004) "Preparation of high-density concanavalin A adsorbent and its use for rapid, high-yield purification of peroxidase from horseradish roots" *J. Chromatogr. B* 803, 237-241
- Goncalves, E.M., Pinheiro, J., Abreu, M., Brandao, T.R.S. & Silva, C.L.M. (2010) "Carrot (*Daucus carota L.*) peroxidase inactivation, phenolic content and physical changes kinetics due to blanching" *Journal of Food Engineering* 97, 574-581
- Hamid, M. & Rehman, K. (2009) "Potential applications of peroxidases" *Food Chemistry* 115, 1177-1186
- Henriquez, C., Aliaga, C. & Lissi, E. (2004) "Kinetics profiles in the reaction of ABTS derived radicals with simple phenols and polyphenols" *Journal of the Chilean Chemistry Society* 49, 65-67
- Hiraga, S., Sasaki, K., Ito, H. Ohashi, Y. & Matsui, H. (2001) "A large family of class III plant peroxidases" *Plant Cell Physiol.* 42, 462-468
- Huang, D., Ou, B. & Prior, R.L. (2005) "The chemistry behind antioxidant capacity assays" *J. Agric. Food Chem.* 53, 1841-1856
- Katsuragi, H., Takahashi, K., Suzuki, H. & Maeda, M. (2000) "Chemiluminescent measurement of peroxidase activity and its application using a lucigenin CT-complex" *Luminescence* 15, 1-7
- Lanzotti, V. (2006) "The analysis of onion and garlic" *Journal of Chromatography A* 1112, 3-22
- Magri, M.L., Cabrera, R.B., Miranda, M.V., Fernández-Lahore, H.M. & Cascone, O. (2003) "Performance of an aqueous two-phase-based countercurrent chromatographic system for horseradish peroxidase purification" *J. Sep. Sci.* 26, 1701-1706
- Mall, R., Naik, G., Mina, U. & Mishra, S.K. (2013) "Purification and characterization of a thermostable soluble peroxidase from *Citrus medica* leaf" *Prep. Biochem. Biotechnol.* 43, 137-151
- Manu, B.T. & Rao, U.J.S.P. (2011) "Role of peroxidase and H₂O₂ in cross-linking of gluten proteins" *J. Food Biochem.* 35, 1695-1702

- Mehlhorn, H., Lelandais, M., Korth, H.G. & Foyer, C.H. (1996) "Ascorbate is the natural substrate for plant peroxidases" *FEBS Letters* 378, 203-206
- Motamed, S., Ghaemmaghami, F. & Alemzadeh, I. (2009) "Turnip (*Brassica rapa*) peroxidase: purification and characterization" *Ind. Eng. Chem. Res.* 48, 10614-10618
- Ngo, T.T. (2010) "Peroxidase in Chemical and Biochemical Analysis" *Analytical Letters* 43, 1572-87
- Osborne, J.C. & Metzler, M. (1984) "Peroxidase interfering activity, an endogenous factor able to hinder the biochemical determination of peroxidase" *Analytical Letters* 17, 2035-2046
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999) "Antioxidant activity applying an improved ABTS radical cation decolorization assay" *Free Radical Biology & Medicine* 26, 1231-1237
- Regalado, C., Asenjo, J.A. & Pyle, D.L. (1996) "Studies on the purification of peroxidase from horseradish roots using reverse micelles" *Enz. Microb. Technol.* 18, 332-339
- Regalado, C., Garcia-Almendarez, B.E., & Duarte-Vazquez, M.A. (2004) "Biotechnological applications of peroxidases" *Phytochemistry Reviews* 3, 243-256
- Reszka, K.J. & Britigan, B.E. (2007) "Doxorubicin inhibits oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by a lactoperoxidase/H₂O₂ system by reacting with ABTS-derived radical" *Archives of Biochemistry and Biophysics* 466, 164-171
- Sanchez, M., Queijeiro, E., Revilla, G. & Zarra, I. (1997) "Changes in ascorbic acid levels in apoplastic fluid during growth of pine hypocotyls. Effect on peroxidase activities associated with cell walls" *Physiologia Plantarum* 101, 815-820
- Serrano-Martinez, A., Fortea, M.I., Amor, F.M. del & Nunez-Delicado, E. (2008) "Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase" *Food Chemistry* 107, 193-199
- Silaghi-Dumitrescu, R. (2010) "Assays for peroxidase activity: the HRP case" *Studia Universitatis Babes-Bolyai. Chemia* 55, 207-222
- Stiene, M. & Bilitewski, U. (2002) "Electrochemical characterization of screen-printed carbonaceous electrodes for the determination of peroxidase activity in novel screen-printed flow-through modules" *Analytical and Bioanalytical Chemistry* 372, 240-47

- Takahama, U. (2004) "Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase: Physiological significance of the oxidation reactions" *Phytochemistry Reviews* 3, 207-219
- Takahama, U. & Oniki, T. (1997) "A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells" *Physiologia Plantarum* 101, 845-852
- Tian, X. & Schaich, K.M. (2013) "Effects of molecular structure on kinetics and dynamics of the trolox equivalent antioxidant capacity assay with ABTS^{•+}" *J. Agric. Food Chem.* 61, 5511-5519
- Valderrama, B. & Ayala, M. (2002) "Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes" *Chemistry & Biology* 9, 555-565
- Valetti, N.W. & Picó, G. (2013) "A friendly method for *Raphanus sativus* L (wild radish) peroxidase purification by polyelectrolyte precipitation" *Sep. Purif. Technol.* 119, 1-6
- Vamos-Vigyazo, L. (1981) "Polyphenol oxidase and peroxidase in fruits and vegetables" *Critical reviews in food science and nutrition* 15,49-127
- Walker, R.B. & Everette, J.D. (2009) "Comparative reaction rates of various antioxidants with ABTS radical cation" *J. Agric. Food Chem.* 57, 1156-1161
- Xianyu, Y., Zhu, K., Chen, W., Wang, X., Zhao, H., Sun, J., Wang, Z. & Jiang, X. (2013) "Enzymatic assay for Cu(II) with horseradish peroxidase and its application in colorimetric logic gate" *Anal. Chem.* 85, 7029-2032

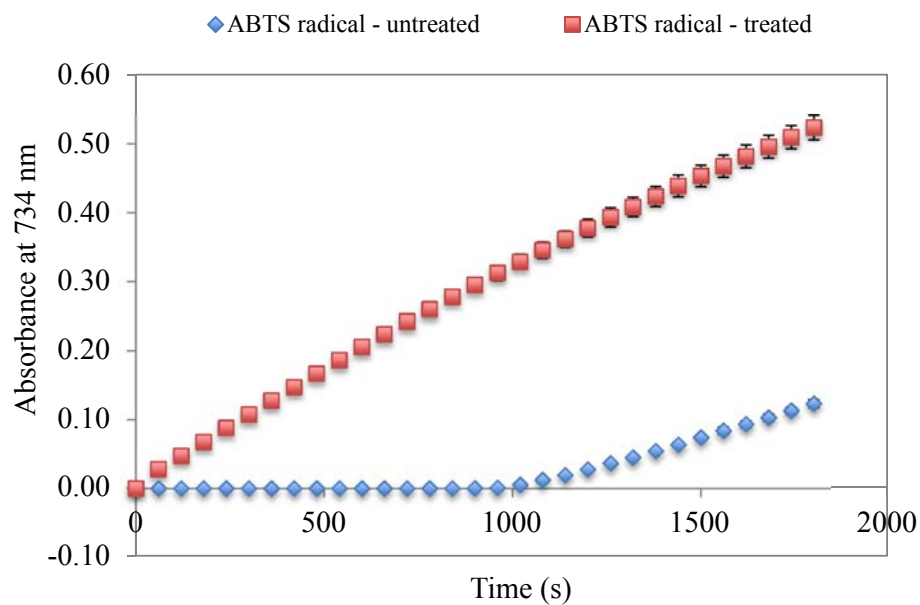
Tables

Table 3.1. Comparison of peroxidase activity of sweet yellow peppers and yellow onions as determined by the traditional and the modified peroxidase assays.

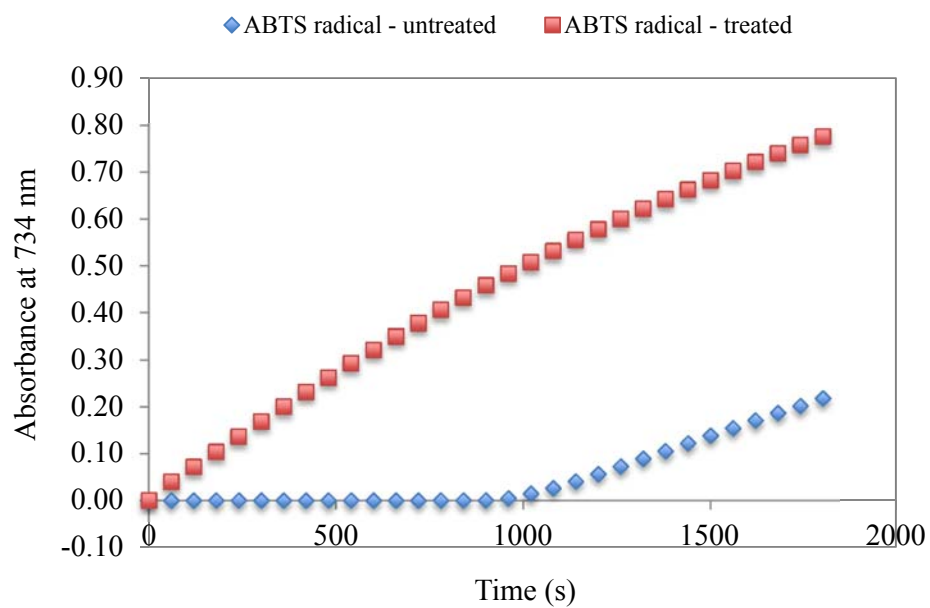
	Peroxidase activity (U/ml)	
	ABTS ^{•+} - untreated	ABTS ^{•+} - treated
Pepper #1	0.067 ± 0.002	0.136 ± 0.005
Pepper #2	0.115 ± 0.002	0.228 ± 0.004
Pepper #3	0.090 ± 0.012	0.156 ± 0.004
Onion #1	0.011 ± 0.001	0.020 ± 0.002
Onion #2	ND	0.008 ± 0.002

Values are means of three replicates ± standard deviation. ND: Not detectable

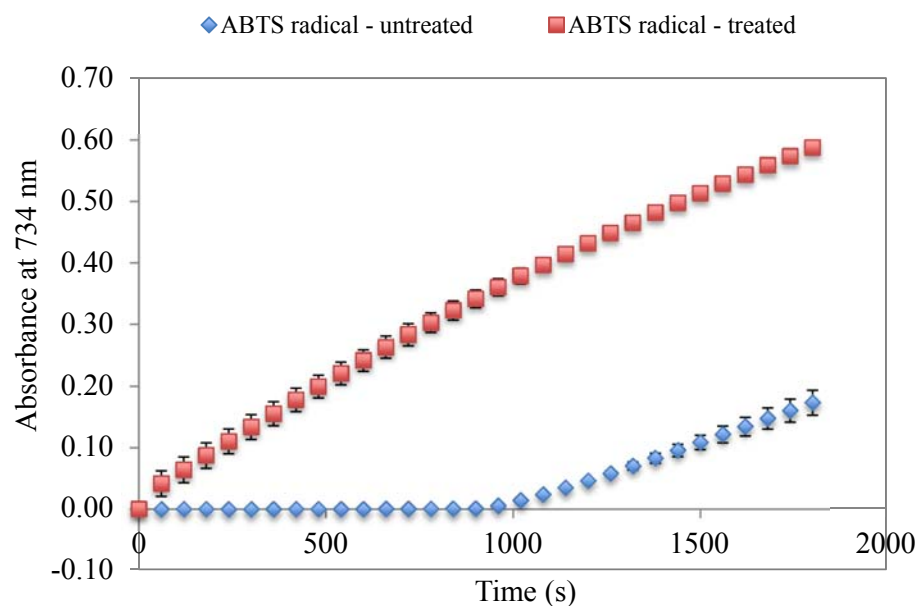
Figures



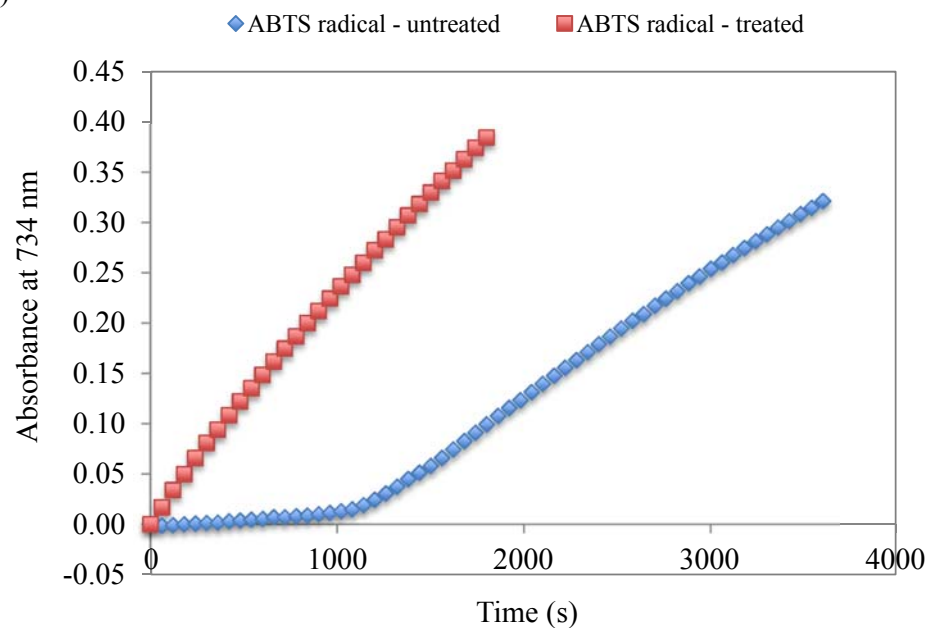
(A)



(B)



(C)



(D)

Figure 3.1. Time-courses showing product accumulation, as determined by increase in absorbance, during peroxidase activity measurements using the traditional and the modified ABTS-based peroxidase assay procedures. Time-courses depicted in (A), (B) and (C) are from assaying peroxidase activity in different sweet yellow pepper extracts; time-courses depicted in (D) are from assaying peroxidase activity in a yellow onion extract.

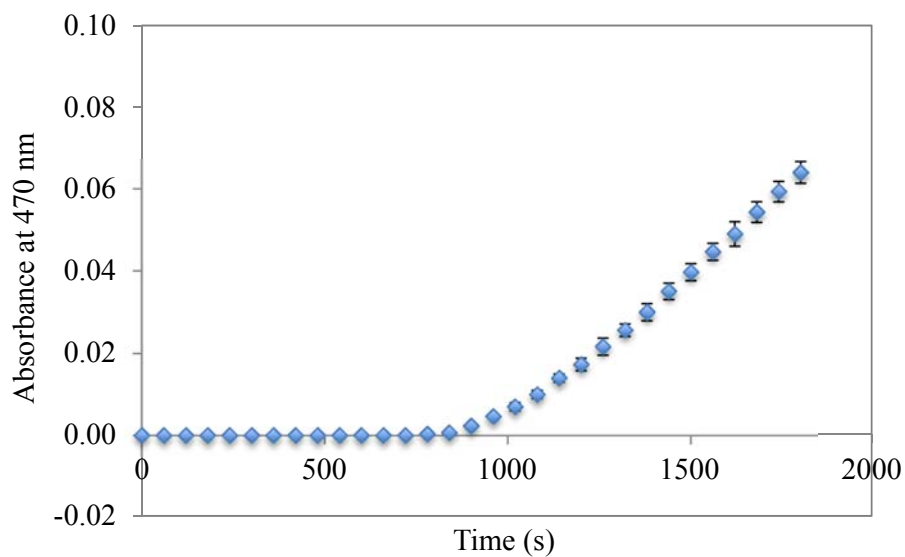


Figure 3.2. Time-course showing product accumulation, as determined by increase in absorbance, during peroxidase activity measurement of sweet yellow pepper extract using the traditional guaiacol-based assay procedure. Data points represent means \pm SD for triplicate assays.

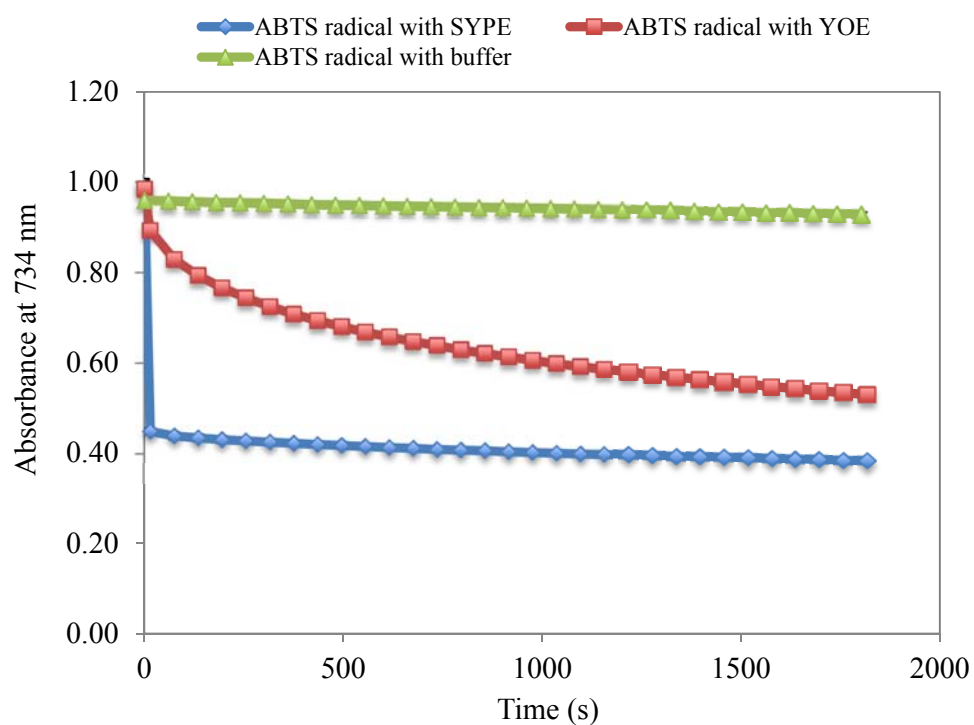


Figure 3.3. Time-courses of $\text{ABTS}^{\bullet+}$ loss, depicted as decrease in absorbance, in vegetable extracts. \blacklozenge , $\text{ABTS}^{\bullet+}$ in sweet yellow pepper extract; \blacksquare , $\text{ABTS}^{\bullet+}$ in yellow onion extract; \blacktriangle , $\text{ABTS}^{\bullet+}$ in extracting buffer (0.1 M sodium phosphate, pH 6.0).

4 General Conclusion

There are many peroxidase/reporter molecule assay systems for the quantification of hydrogen peroxide. A limitation for many of these assays, when applied to complex biological systems, is interference due to the reaction of endogenous components with the oxidized/radical form of the reporter molecule generated in the hydrogen peroxide/reporter/peroxidase reaction. These interfering reactions can modify the signal yield per mole hydrogen peroxide to an extent that is unacceptable for quantitative purposes. In this study we have presented an approach to circumvent this problem. The approach is based on treating the sample with the oxidized reporter molecule prior to initiating the peroxidase-catalyzed reaction. This “treatment” step selectively oxidizes the confounding compounds, thus effectively removes these compounds prior to initiating the hydrogen peroxide-dependent reaction, allows one to simply determine the amount of hydrogen peroxide in a product in situ and thus avoids the need for sophisticated separation techniques. The approach is demonstrated herein using an ABTS/HRP assay system. The limitation of the method is that the treatment required for removal of confounding compounds takes on the order of minutes and thus the method has the possibility of underestimating the hydrogen peroxide content in systems where such concentrations are changing on the seconds to minutes time scale. The approach is not expected to be applicable to all peroxidase/reporter molecule combinations, but it is expected to greatly widen the applicability of these useful enzyme-based assay systems in many situations. A similar problem encountered for peroxidase activity measurements that natural reducing agents endogenous to plant materials, such as phenolic compounds and ascorbic acid, may interfere with traditional peroxidase assays by reducing the oxidized product generated in the peroxidase reaction, resulting in an apparent lag in product development and underestimation of peroxidase activity. Based on highly selective removal of complicating endogenous reducing compounds likely to interfere with peroxidase quantification via reaction product reduction, oxidized reporter molecule, ABTS^{•+}, is used to treat the enzyme preparation prior to initiating the assay. This approach effectively eliminates lag phases associated

with different plant extracts and, thus, more accurately reflect total peroxidase activity. The improved assay is relatively simple and should be applicable to a range of biological systems.

Bibliography

- Abbas, M.E., Luo, W., Zhu, L., Zou, J. & Tang, H. (2010) "Fluorometric determination of hydrogen peroxide in milk by using a Fenton reaction system" *Food Chemistry* 120, 327-331
- Acharya, A.P., Nafisi, P.M., Gardner, A., MacKay, J.L., Kundu, K., Kumar, S., & Murthy, N. (2013) "A fluorexcent peroxidase probe increases the sensitivity of commercial ELISAs by two orders of magnitude" *Chem. Commun.* 49, 10379-10381
- Adak, S., Mazumder, A. & Banerjee, R.K. (1996) "Probing the active site residues in aromatic donor oxidation in horseradish peroxidase: involvement of an arginine and a tyrosine residue in aromatic donor binding" *Bipchem. J.* 314, 985-991
- Adams, J.B. & Brown, H.M. (2007) "Discoloration in raw and processed fruits and vegetables" *Crit. Rev. Food Sci. Nutr.* 47, 319-333
- Akagawa, M., Shigemitsu, T. & Suyama, K. (2003) "Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under *quasi*-physiological conditions" *Biosci. Biotechnol. Biochem.* 67, 2632-2640
- Ali, G., Russly, A.R., Jamilah, B., Azizah, O. & Mandana, B. (2011) "Effect of heat and thermosonication on kinetics of peroxidase inactivation and vitamin C degradation in seedless guava (*Psidium guajava* L.)" *International Food Research Journal* 18, 1289-1294
- Almagro, L., Gomez Ros, L.V., Belchi-Navarro, S., Bru, R., Ros Barcelo, A. & Pedreno, M.A. (2009) "Class III peroxidases in plant defence reactions" *Journal of Experimental Botany* 60, 377-390
- Arnao, M.B., Cano, A., Hernandez-Ruiz, J., Garcia-Canovas, F. & Acosta, M. (1996) "Inhibition by L-ascorbic acid and other antioxidants of the 2,2'-Azino-Bis(3-ethylbenzo Thiazoline-6-Sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods" *Analytical Biochemistry* 236, 255-261
- Azevedo, A.M., Martins, V.C., Prazeres, D.M.F., Vojinović, V., Cabral, J.M.S., & Fonseca, L.P. (2003) "Horseradish peroxidase: a valuable tool in biotechnology" *Biotechnology Annual Review* 9, 199-247
- Barni, F., Lewis, S.W., Berti, A., Miskelly, G.M. & Lago, G. (2007) "Forensic application of the luminol reaction as a presumptive test for latent blood detection" *Talanta* 72, 896-913

- Barrett, N.E., Grandison, A.S. & Lewis, M.J. (1999) "Contribution of lactoperoxidase system to the keeping quality of pasteurized milk" *Journal of Dairy Research* 66, 73-80
- Barrette, R.W., Urbonas, J. & Silbart, L.K. (2006) "Quantifying specific antibody concentrations by enzyme-linked immunosorbent assay using slope correction" *Clinical and Vaccine Immunology* 13, 802-805
- Boeriu, C. G. (2008) "Peroxidases in food industry: crosslinking of proteins and polysaccharides to impart novel functional properties" *Romanian Biotechnological Letters* 13, 81-86
- Boerjan, W., Ralph, J. & Baucher, M. (2003) "Lignin biosynthesis" *Annual Review of Plant Biology* 54, 519-546
- Bruemmer, J.H., Roe, B. & Bowen, E.R. (1976) "Peroxidase reactions and orange juice quality" *Journal of Food Science* 41, 186-189
- Brudzynski, K., Abubaker, K. & Miotto, D. (2012) "Unraveling a mechanism of honey antibacterial action: polyphenol/H₂O₂-induced oxidative effect on bacterial cell growth and on DNA degradation" *Food Chemistry* 133, 329-336
- Brudzynski, K. & Lannigan, R. (2012) "Mechanism of honey bacteriostatic action against MRSA and VRE involves hydroxyl radicals generated from honey's hydrogen peroxide" *Frontiers in Microbiology* 3, 1-8
- Burnette, F.S. (1977) "Peroxidase and its relationship to food flavor and quality: a review" *Journal of Food Science* 42, 1-6
- Castillo, F.J., Celardin, F. & Greppin, H. (1984) "Peroxidase assay in plants: interference by ascorbic acid and endogenous inhibitors in *Sedum* and *Pelargonium* enzyme extracts" *Plant Growth Regulation* 2, 69-75
- Celardin, F., Castillo, F.J. & Greppin, H. (1982) "Simple kinetic determination of trace amounts of ascorbic acid in plant extracts" *Journal of Biochemical and Biophysical Methods* 6, 89-93
- Childs, R.E. & Bardsley, W.G. (1975) "The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromagen" *Biochem. J.* 145, 93-103
- Chiu, M.-H., Kumar, A.S., Sornambikai, S., Chen, P.-Y., Shih, Y. & Zen, J.-M. (2011) "Cosmetic hydrogen peroxide detection using nano bismuth species deposited built-in three-in-one screen-printed silver electrode" *Int. J. Electrochem. Sci.* 6, 2352-2365

- Demirevska-Kepova, K.N. & Bakardjieva, N.T. (1976) "Study of competition between guaiacol and ascorbic acid in peroxidase reaction and on ion effect by means of absorption spectra" *Can. J. Bot.* Vol. 54, 90-99
- Doerge, D.R., Divi, R.L. & Churchwell, M.I. (1997) "Identification of the colored guaiacol oxidation products produced by peroxidases" *Anal. Biochem.* 250, 10-17
- Dosoretz, C.G. & Ward, G. (2006) "Peroxidases" in *Enzyme Technology* (A. Pandey, C. Webb, C.R. Soccol and C. Larroche, Edts.) Springer Asiatech Publ., Inc., New Delhi, 399-432
- Forman, H.J. (2008) "Hydrogen peroxide: the good, the bad, and the ugly" in *Oxidants in Biology* (G. Valacchi & P.A. Davis, Editors), Springer Netherlands, 1-17
- Fortea, M.I., Lopez-Miranda, S., Serrano-Martinez, A., Hernandez-Sanchez, P., Zafrilla, M.P., Martinez-Cacha, A. & Nunez-Delicado, E. (2011) "Kinetic characterization and thermal inactivation study of red alga (*Mastocarpus Stellatus*) peroxidase" *Food Chemistry* 127, 1091-1096
- Fox, P.F. (2003) "Significance of indigenous enzymes in milk and dairy products" in *Handbook of food enzymology* (J.R. Whitaker, A.G.J. Voragen & D.W.S. Wong, Editors), Marcel Dekker, Inc., 255-277
- Fragus, L.F., Batista-Viera, F., & Carlsson, J. (2004) "Preparation of high-density concanavalin A adsorbent and its use for rapid, high-yield purification of peroxidase from horseradish roots" *J. Chromatogr. B* 803, 237-241
- Franchini, R.A.A., Souza, C.F.D., Colombara, R., Matos, M.A.C., & Matos, R.C. (2007) "Rapid determination of hydrogen peroxide using peroxidase immobilized on Amberlite IRA-743 and minerals in honey" *J. Agric. Food Chem.* 55, 6885-6890
- Franchini, R.A.A., Matos, M.A.C., Colombara, R. & Matos, R.C. (2008) "Differential amperometric determination of hydrogen peroxide in honeys using flow-injection analysis with enzymatic reactor" *Talanta* 75, 301-306
- Garcia, A., Wichers, J.H. & Wichers, H.J. (2007) "Decrease of the IgE-binding by Mal d 1, the major apple allergen, by means of polyphenol oxidase and peroxidase treatments" *Food Chemistry* 103, 94-100
- Gill, S.S. & Tuteja, N. (2010) "Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants" *Plant Physiology and Biochemistry* 48, 909-930
- Goncalves, E.M., Pinheiro, J., Abreu, M., Brandao, T.R.S. & Silva, C.L.M. (2010) "Carrot (*Daucus carota* L.) peroxidase inactivation, phenolic content and physical changes kinetics due to blanching" *Journal of Food Engineering* 97, 574-581

Grieshaber, D.; MacKenzie, R.; Vörös, J.; Reimhult, E. (2008) “Electrochemical biosensors – sensor principles and architectures” *Sensors* 8, 1400-1458

Gulcin, I. (2012) “Antioxidant activity of food constituents: an overview” *Arch Toxicol.* 86, 345-391

Hage, R. & Lienke, A. (2006) “Applications of transition-metal catalysts to textile and wood-pulp bleaching” *Angew. Chem. Int. Ed.* 45, 206-222

Halliwell, B., Clement, M.V. & Long, L.H. (2000) “Minireview-Hydrogen peroxide in the human body” *FEBS Letters* 486, 10-13

Halliwell, B. (2005) “Free radicals and other reactive species in disease” *Encyclopedia of Life Sciences*

Hamid, M. & Rehman, K. (2009) “Potential applications of peroxidases” *Food Chemistry* 115, 1177-1186

Henriquez, C., Aliaga, C. & Lissi, E. (2002) “Formation and decay of the ABTS derived radical cation: a comparison of different preparation procedures” *International Journal of Chemical Kinetics* 34, 659-665

Henriquez, C., Aliaga, C. & Lissi, E. (2004) “Kinetics profiles in the reaction of ABTS derived radicals with simple phenols and polyphenols” *Journal of the Chilean Chemistry Society* 49, 65-67

Hilhorst, R., Dunnewind, B., Orsel, R., Stegeman, P., Vliet, T. van, Gruppen, H. & Schols, H. A. (1999) “Baking performance, rheology, and chemical composition of wheat dough and gluten affected by xylanase and oxidative enzymes” *Journal of Food Science* 64, 808–13

Hiraga, S., Sasaki, K., Ito, H. Ohashi, Y. & Matsui, H. (2001) “A large family of class III plant peroxidases” *Plant Cell Physiol.* 42, 462-468

Hofrichter, M. (2002) “Review: lignin conversion by manganese peroxidase (MnP)” *Enzyme and Microbial Technology* 30, 454-466

Huang, D., Ou, B. & Prior, R.L. (2005) “The chemistry behind antioxidant capacity assays” *J. Agric. Food Chem.* 53, 1841-1856

Igual, M., Sampedro, F., Martinez-Navarrete, N. & Fan, X. (2013) “Combined osmodehydration and high pressure processing on the enzyme stability and antioxidant capacity of a grapefruit jam” *Journal of Food Engineering* 114, 514-521

Ito, Y., Tonogai, Y., Suzuki, H., Ogawa, S., Yokoyama, T., Hashizume, T., Santo, H., Tanaka, K., Nishigaki, K., & Iwaida, M. (1981) "Improved 4-aminoantipyrine colorimetry for detection of residual hydrogen peroxide in noodles, fish paste, dried fish, and herring roe" *J. Assoc. Off. Anal. Chem.* 64, 6

Jervis, S.M. & Drake, M. (2013) "The impact of iron on the bleaching efficacy of hydrogen peroxide in liquid whey systems" *Journal of Food Science* 78, R129–R137

Joseph, P.D., Eling, T. & Mason, R.P. (1982) "The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine" *The Journal of Biological Chemistry* 257, 3669-3675

Katsuragi, H., Takahashi, K., Suzuki, H. & Maeda, M. (2000) "Chemiluminescent measurement of peroxidase activity and its application using a lucigenin CT-complex" *Luminescence* 15, 1-7

Kawano, T. (2003) "Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction" *Plant Cell Reports* 21, 829–837

Lanzotti, V. (2006) "The analysis of onion and garlic" *Journal of Chromatography A* 1112, 3-22

Leelarungrayub, N., Rattanapanone, V., Chanarat, N. & Gebicki, J.M. (2006) "Quantitative evaluation of the antioxidant properties of garlic and shallot preparations" *Nutrition* 22, 266-274

Lu, C., Lin, C., Chang, C., Wu, S. & Lo, L. (2011) "Nitrophenylboronic acids as highly chemoselective probes to detect hydrogen peroxide in foods and agricultural products" *J. Agric. Food Chem.* 59, 11403-11406

Lu, S., Song, J., & Campbell-Palmer, L. (2009) "A modified chemiluminescence method for hydrogen peroxide determination in apple fruit tissues" *Scientia Horticulturae* 120, 336-341

Magri, M.L., Cabrera, R.B., Miranda, M.V., Fernández-Lahore, H.M. & Cascone, O. (2003) "Performance of an aqueous two-phase-based countercurrent chromatographic system for horseradish peroxidase purification" *J. Sep. Sci.* 26, 1701-1706

Mall, R., Naik, G., Mina, U. & Mishra, S.K. (2013) "Purification and characterization of a thermostable soluble peroxidase from *Citrus medica* leaf" *Prep. Biochem. Biotechnol.* 43, 137-151

Mandel, A.L., Peyrot des Gachons, C., Plank, K.L., Alarcon, S. & Breslin, P.A.S. (2010) "Individual differences in *AMY1* gene copy number, salivary α -amylase levels, and the perception of oral starch" *PLoS One* 5, e13352

- Manu, B.T. & Rao, U.J.S.P. (2011) "Role of peroxidase and H₂O₂ in cross-linking of gluten proteins" *J. Food Biochem.* 35, 1695-1702
- Manzoori, J.L., Amjadi, M., & Orooji, M. (2006) "Application of crude extract of kohlrabi (*Brassica oleracea gongylodes*) as a rich source of peroxidase in the spectrofluorometric determination of hydrogen peroxide in honey samples" *Analytical Sciences* 22, 1201-1206
- Marquette, C.A. & Blum, L.J. (2006) "Applications of the luminol chemiluminescent reaction in analytical chemistry" *Anal. Bioanal. Chem.* 385, 546-554
- Martínez, Á.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A. & del Río, J.C. (2005) "Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin" *International Microbiology* 8, 195–204
- Matés, J.M. (2000) "Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology" *Toxicology* 153, 83–104
- Mehlhorn, H., Lelandais, M., Korth, H.G. & Foyer, C.H. (1996) "Ascorbate is the natural substrate for plant peroxidases" *FEBS Letters* 378, 203-206
- Motamed, S., Ghaemmaghami, F. & Alemzadeh, I. (2009) "Turnip (*Brassica rapa*) peroxidase: purification and characterization" *Ind. Eng. Chem. Res.* 48, 10614-10618
- Muftugil, N. (1985) "The peroxidase enzyme activity of some vegetables and its resistance to heat" *Journal of the Science of Food and Agriculture* 36, 877-880
- Murphy, M.P., Holmgren, A., Larsson, N.-G., Halliwell, B., Chang, C.J., Kalyanaraman, B., Rhee, S.G., Thornalley, P.J., Partridge, L., Gems, D., Nyström, T., Belousov, V., Schumacker, P.T. & Winterbourn, C.C. (2011) "Unraveling the biological roles of reactive oxygen species" *Cell Metab* 13, 361-366
- National Research Council (2003) *Food Chemicals Codex: Fifth Edition*. Washington, DC: The National Academies Press
- Ngo, T.T. (2010) "Peroxidase in Chemical and Biochemical Analysis" *Analytical Letters* 43, 1572–87
- Olson, P.D. & Varner, J.E. (1993) "Hydrogen peroxide and lignification" *The Plant Journal* 4, 887-892
- Osborne, J.C. & Metzler, M. (1984) "Peroxidase interfering activity, an endogenous factor able to hinder the biochemical determination of peroxidase" *Analytical Letters* 17, 2035-2046

- Passardi, F., Penel, C. & Dunand, C. (2004) "Performing the paradoxical: how plant peroxidases modify the cell wall" *Trends in Plant Science* 9, 534–540
- Pérez, J., Muñoz-Dorado, J., Rubia, T. de la & Martínez, J. (2002) "Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview" *International Microbiology* 5, 53–63
- Prior, R.L., Wu, X. & Schaich, K. (2005) "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements" *J. Agric. Food Chem.* 53, 4290-4302
- Proctor, G.B. & Chan, K. (1994) "A fluorometric assay of peroxidase activity utilizing 2',7'-dichlorofluorescein with thiocyanate: application to the study of salivary salivary secretion" *Journal of Biochem. Biophys Methods* 28, 329-336
- Quan, L., Zhang, B., Shi, W. & Li, H. (2008) "Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network" *Journal of Integrative Plant Biology* 50, 2–18
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999) "Antioxidant activity applying an improved ABTS radical cation decolorization assay" *Free Radical Biology & Medicine* 26, 1231-1237
- Regalado, C., Asenjo, J.A. & Pyle, D.L. (1996) "Studies on the purification of peroxidase from horseradish roots using reverse micelles" *Enz. Microb. Technol.* 18, 332-339
- Regalado, C., Garcia-Almendarez, B.E., & Duarte-Vazquez, M.A. (2004) "Biotechnological applications of peroxidases" *Phytochemistry Reviews* 3, 243-256
- Reymond, J.L., Fluxà, V.S. & Maillard, N. (2009) "Enzyme assays" *Chem. Commun.* 34-46
- Rhee, S.G., Chang, T.-S., Jeong, W. & Kang, D. (2010) "Methods for detection and measurement of hydrogen peroxide inside and outside of cells" *Mol. Cells* 29, 539-549
- Rogers, A. & Gibon, Y. (2009) "Enzyme kinetics: theory and practice" in *Plant Metabolic Networks* (J. Schwender, Editor) Springer, Berlin (in press)
- Sanchez, M., Queijeiro, E., Revilla, G. & Zarra, I. (1997) "Changes in ascorbic acid levels in apoplastic fluid during growth of pine hypocotyls. Effect on peroxidase activities associated with cell walls" *Physiologia Plantarum* 101, 815-820

Scopes, R.K. (2002) "Enzyme activity and assays" In: Encyclopedia of life sciences, Macmillan Publishers Ltd, Nature Publishing Group

Scott, R., Robinson, R.K. & Wilbey, R.A. (1998) "Cheesemaking Practice (3rd edition)" Gaithersburg, Maryland: Aspen Publishers Inc.

Segundo, M.A., Castro, C., Magalhães, L.M. & Reis, S. (2013) "Determination of scavenging capacity against hydrogen peroxide: recent trends on chemical methods" in *Hydrogen Peroxide Detection, Applications and Health Implications* (G. Aguilar & R. A. Guzman, Editors), Novo Science Pub Inc., 203-218

Serrano, J., Jové, M., Boada, J., Bellmunt, M.J., Pamplona, R. & Portero-Otín, M. (2009) "Dietary antioxidants interfere with Amplex Red-coupled-fluorescence assays" *Biochemical and Biophysical Research Communications* 388, 443–49

Serrano-Martinez, A., Fortea, M.I., Amor, F.M. del & Nunez-Delicado, E. (2008) "Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase" *Food Chemistry* 107, 193-199

Silaghi-Dumitrescu, R. (2010) "Assays for peroxidase activity: the HRP case" *Studia Universitatis Babes-Bolyai. Chemia* 55, 207-222

Slesak, I., Libik, M., Karpinska, B., Karpinski, S. & Miszalski, Z. (2007) "The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses" *Acta Biochimica Polonica* 54, 39-50

Staniek, K. & Nohl, H. (1999) "H₂O₂ detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system" *Biochimica et Biophysica Acta* 1413, 70-80

Steinberg, S.M. (2013) "High-performance liquid chromatography method for determination of hydrogen peroxide in aqueous solution and application to simulated Martian soil and related materials" *Environ Monit Assess.* 185, 3749-3757

Stiene, M. & Bilitewski, U. (2002) "Electrochemical characterization of screen-printed carbonaceous electrodes for the determination of peroxidase activity in novel screen-printed flow-through modules" *Analytical and Bioanalytical Chemistry* 372, 240–47

Suha, O.A., Babiker, E.M. & Babiker, E.E. (2013) "Thermostability at different pH levels of peroxidase extracted from four vegetables" *International Food Research Journal* 20, 715-719

Takahama, U. (2004) "Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase: Physiological significance of the oxidation reactions" *Phytochemistry Reviews* 3, 207-219

Takahama, U. & Oniki, T. (1997) "A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells" *Physiologia Plantarum* 101, 845-852

Takahashi, A., Hashimoto, K., Kumazawa, S., & Nakayama, T. (1999) "Determination of hydrogen peroxide by high-performance liquid chromatography with a cation-exchange resin gel column and electrochemical detector" *Analytical Sciences* 15, 481-483

Takanami, Y., Moriyama, T., Kosaka, Y. & Nakayama, T. (2009) "Analysis of hydrogen peroxide in an aqueous extract of cigarette smoke and effect of pH on the yield" *Biosci. Biotechnol. Biochem.* 73, 2222-2225

Tan, T., Cheng, L., Bhat, R., Rusul, G. & Easa, A.M. (2014) "Composition, physicochemical properties and thermal inactivation kinetics of polyphenol oxidase and peroxidase from coconut (*Cocos nucifera*) water obtained from immature, mature and overly-mature coconut" *Food Chemistry* 142, 121-128

Taormina, P.J., Niemira, B.A. & Beuchat, L.R. (2001) "Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power" *International Journal of Food Microbiology* 69, 217-225

Tarpey, M.M., Wink, D.A., & Grisham, M.B. (2004) "Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations." *Am J Physiol Regul Integr Comp Physiol.* 286, R431-R444

Tarvin, M., McCord, B., Mount, K., Sherlach, K. & Miller, M.L. (2010) "Optimization of two methods for the analysis of hydrogen peroxide: high performance liquid chromatography with fluorescence detection and high performance liquid chromatography with electrochemical detection in direct current mode" *J. Chromatogr. A* 1217, 7564-7572

Tian, X. & Schaich, K.M. (2013) "Effects of molecular structure on kinetics and dynamics of the Trolox equivalent antioxidant capacity assay with ABTS^{•+}" *J. Agric. Food Chem.* 61, 5511-5519

Toyoda, M.T., Ito, Y., Iwaida, M. & Fujii, M. (1982) "Rapid procedure for the determination of minute quantities of residual hydrogen peroxide in food by using a sensitive oxygen electrode" *J. Agric. Food Chem.* 30, 346-349

Valderrama, B. & Ayala, M. (2002) "Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes" *Chemistry & Biology* 9, 555-565

Valetti, N.W. & Picó, G. (2013) "A friendly method for *Raphanus sativus* L (wild radish) peroxidase purification by polyelectrolyte precipitation" *Sep. Purif. Technol.* 119, 1-6

- Vamos-Vigyazo, L. (1981) "Polyphenol oxidase and peroxidase in fruits and vegetables" *Critical reviews in food science and nutrition* 15,49-127
- Veitch, N.C. (2004) "Horseradish peroxidase: a modern view of a classic enzyme" *Phytochem* 65, 249-259
- Veitch, N.C. (2004) "Structural determinants of plant peroxidase function" *Phytochemistry Reviews* 3, 3-18
- Veljovic-Jovanovic, S., Noctor, G. & Foyer, C.H. (2002) "Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate" *Plant Physiology and Biochemistry* 40, 501-507
- Venkatasubremanian, L. & Maruthamuthu, P. (1989) "Kinetics and mechanism of formation and decay of 2,2'-Azinobis- (3-Ethylbenzothiazole-6-Sulphonate) radical cation in aqueous solution by inorganic peroxides" *International Journal of Chemical Kinetics* 21, 399-421
- Vidigal, Susana S. M. P., Tóth, I.V. & Rangel, António O. S. S. (2010) "Sequential injection lab-on-valve system for the determination of the activity of peroxidase in vegetables" *Journal of Agricultural and Food Chemistry* 58, 2071-2075
- Walker, R.B. & Everette, J.D. (2009) "Comparative reaction rates of various antioxidants with ABTS radical cation" *J. Agric. Food Chem.* 57, 1156-1161
- Wardman, P. (2007) "Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects" *Free Rad. Biol. Med* 43, 995-1022
- Wei, H. & Wang, E. (2008) "Fe₃O₄ magnetic nanoparticles as peroxidase mimetics and their applications in H₂O₂ and glucose detection" *Anal. Chem.* 80, 2250-2254
- Westbroek, P., Hakuzimana, J., Gasana, E., Lombaert, P. & Kiekens, P. (2007) "Voltammetric detection of hydrogen peroxide in teeth whitening gels" *Sensors and Actuators B* 124, 317-322
- Williams, D.C., Lim, M.H., Chen, A.O., Pangborn, R.M. & Whitaker, J.R. (1986) "Blanching of vegetables for freezing-which indicator enzyme to choose" *Food Technology* 40, 130-140
- Winterbourn, C.C. (2013) "The biological chemistry of hydrogen peroxide" *Meth Enz.* 528, 3-25

- Winterbourn, C.C. (2014) "The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells" *Biochim Biophys Acta* 1840, 730-738
- Wood, P.M. (1988) "The potential diagram for oxygen at pH 7" *Biochemical Journal* 253, 287-289
- Xianyu, Y., Zhu, K., Chen, W., Wang, X., Zhao, H., Sun, J., Wang, Z. & Jiang, X. (2013) "Enzymatic assay for Cu(II) with horseradish peroxidase and its application in colorimetric logic gate" *Anal. Chem.* 85, 7029-2032
- Yu, B., Jin, Z., Deng, L., Xu, X., He, L., Wang, J., Tian, Y. & Chen, H. (2010) "Kinetic study of thermal inactivation of potato peroxidase during high-temperature short-time processing" *Journal of Food Science and Technology* 47, 67-72
- Zulueta, A., Esteve, M.J. & Frígola, A. (2009) "ORAC and TEAC assays comparison to measure the antioxidant capacity of food products" *Food Chemistry* 114, 310-316

