CORSO DI LAUREA IN BIOLOGIA PER LA SOSTENIBILITÀ

METODOLOGIE BIOANALITICHE
Modulo B (6 CFU)

LEZIONE 3

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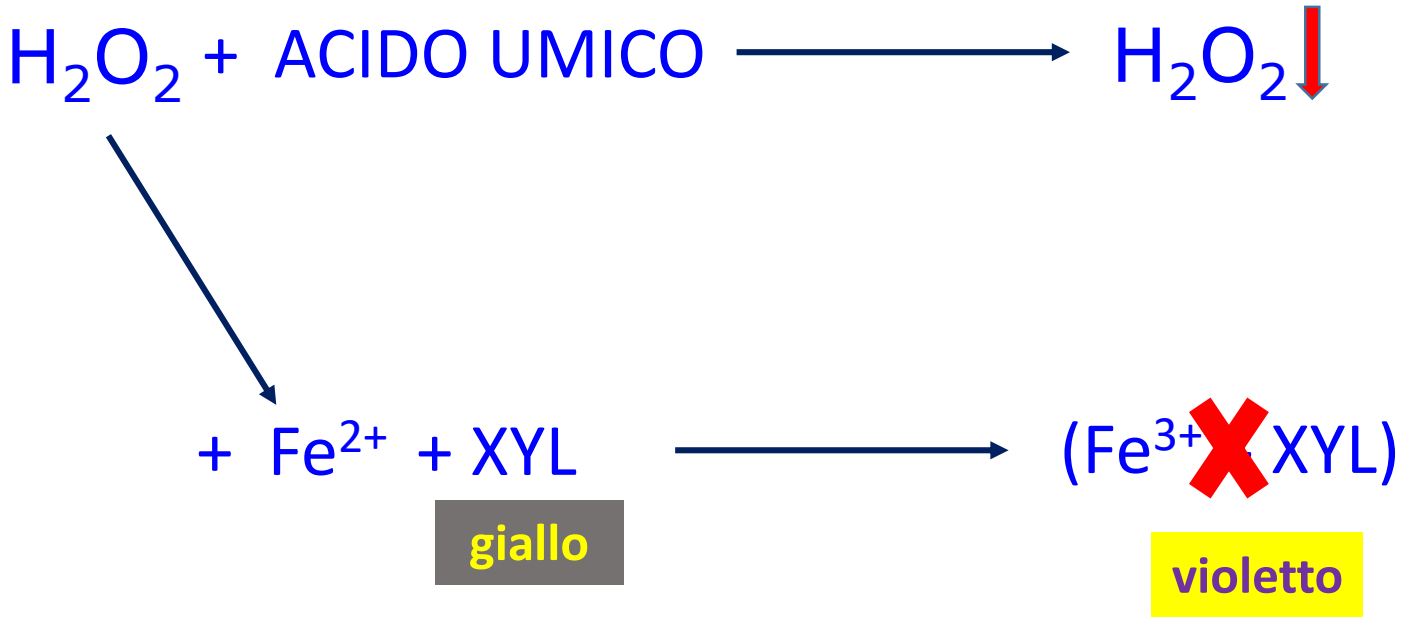
METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Un esempio pratico: studio delle proprietà antiossidanti degli acidi umici come scavengers di perossidi (ROOH)



Metodo del FOX (ferrous oxidation–xylenol orange)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- Costruzione curva di taratura dello strumento
- Confronto con la curva di taratura per valutare l'effetto del potenziale antiossidante

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- **Costruzione curva di taratura dello strumento**

	H_2O_2 25µM	H_2O_2 12,5µM	H_2O_2 10µM
A1 @593nm	2,492	1,119	0,754
A2 @593nm	2,160	1,079	0,741
Valore medio	2,326	1,099	0,748
Deviazione standard	*0,235*	*0,028*	*0,009*

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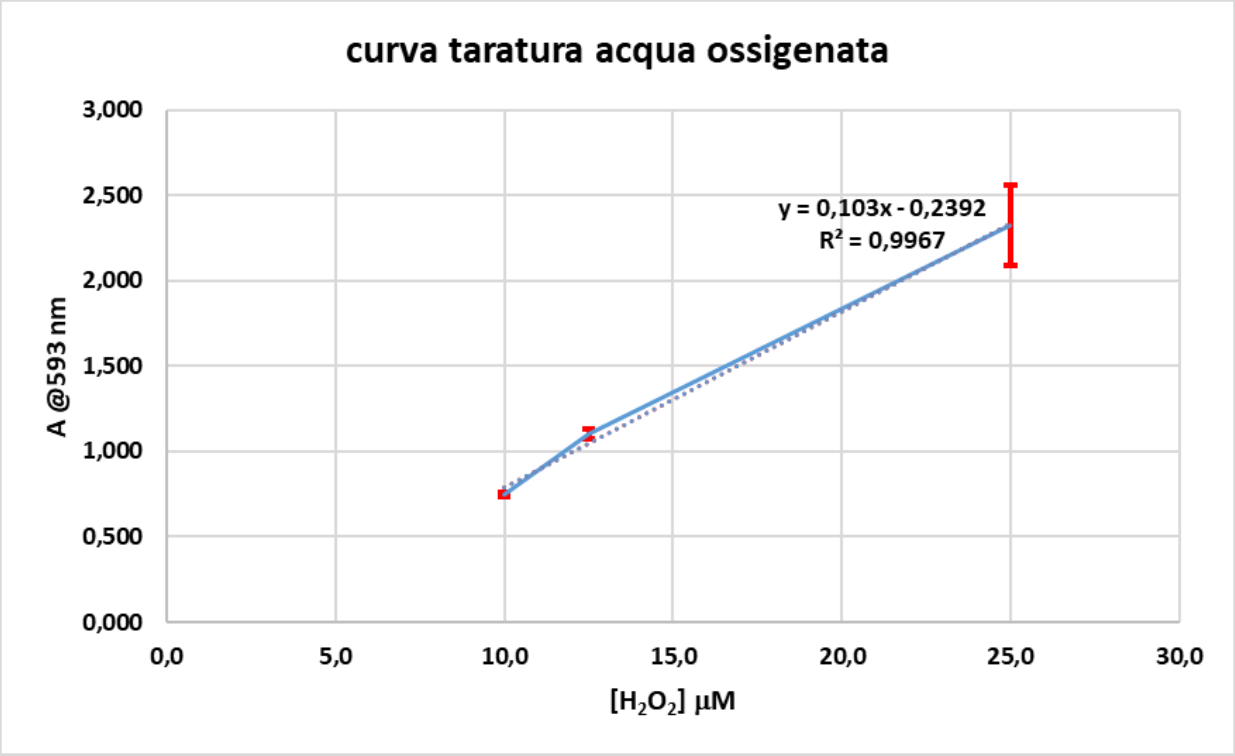
Metodo del FOX (ferrous oxidation–xylenol orange)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- **Costruzione curva di taratura dello strumento**



Metodo del FOX (ferrous oxidation–xylenol orange)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- Confronto con la curva di taratura per valutare l'effetto del potenziale antiossidante

$$y = 0{,}103x - 0{,}2392$$

A @593nm

$[H_2O_2]$ µM

Metodo del FOX (ferrous oxidation–xylenol orange)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- Confronto con la curva di taratura per valutare l'effetto del potenziale antiossidante

	H_2O_2 25µM + acido umico	H_2O_2 12,5µM + acido umico	H_2O_2 10µM + acido umico
A1 @593nm	2,118	0,951	0,641
A2 @593nm	1,836	0,917	0,630
Valore medio	1,977	0,934	0,635
Deviazione standard	*0,200*	*0,024*	*0,008*

$$y = 0{,}103x - 0{,}2392$$

$[H_2O_2]$ µM

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

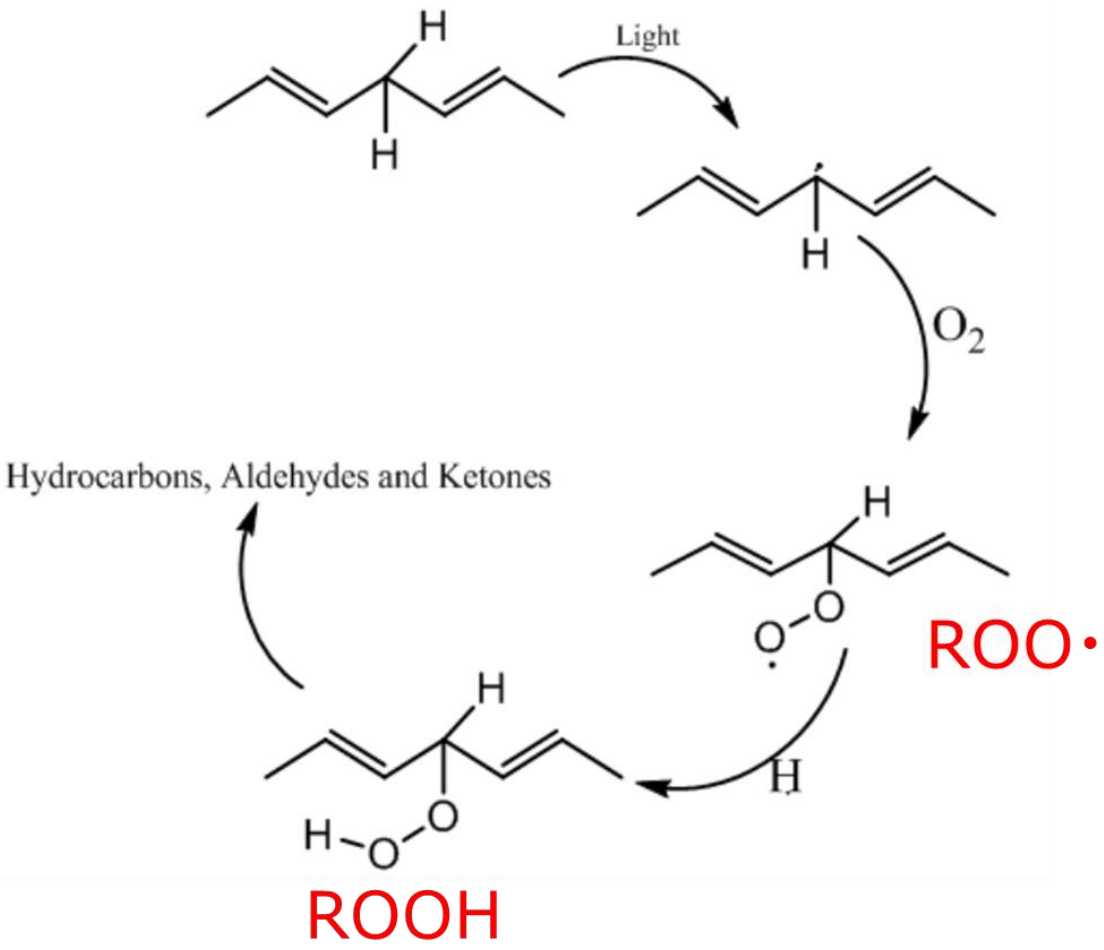
Ulteriori applicazioni: determinazione di perossidi (ROOH) in campioni biologici

$$ROOH + Fe^{2+} + XYL \longrightarrow (Fe^{3+} - XYL)$$

giallo

violetto

- **ROS: origine dei perossidi lipidici**



METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Altre applicazioni: determinazione di perossidi (ROOH) in campioni biologici

248 *J. Agric. Food Chem.* **2002**, *50*, 248–254

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Using a Modified Ferrous Oxidation–Xylenol Orange (FOX) Assay for Detection of Lipid Hydroperoxides in Plant Tissue

JOHN M. DELONG,*,† ROBERT K. PRANGE,† D. MARK HODGES,† CHARLES F. FORNEY,† M. CONNY BISHOP,† AND MICHAEL QUILLIAM‡

Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, 32 Main Street, Kentville, Nova Scotia B4N 1J5, Canada, and Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia B3L 3V2, Canada

The ferrous oxidation−xylenol orange (FOX) assay was adapted for quantifying lipid hydroperoxides (LOOHs) in plant extracts. Excised pieces of several fruit and vegetable species were exposed to 83 kJ m^{-2} day^{-1} of biologically effective ultraviolet B irradiance (UV-B_{BE}) for 10−12 days to induce cellular oxidation. The LOOH and thiobarbituric acid reactive substance (TBARS) concentrations of these plant tissues were assessed with the FOX and iodometric assays for the former and a modified TBARS assay for the latter. There was generally good agreement between the FOX and iodometric methods both prior to and following the UV exposure. However, the iodometric assay appeared to have some difficulty in consistently quantifying lower LOOH levels (<11 μM), whereas the FOX assay measured LOOH concentrations as low as 5 μM. All tissues exhibited UV-induced increases in TBARS, indicating a marked degree of cellular oxidation in the exposed tissue segments. Compared with the iodometric assay, the FOX method consistently generated less variable LOOH values. The presence of authentic linoleic acid−OOHs in spiked avocado and spinach samples (11 μM) was identified with liquid chromatography−mass spectrometry techniques, which validated corresponding FOX assay results. The FOX method is inexpensive, is not sensitive to ambient O_2 or light levels, and can rapidly generate LOOH measurements. The physiological value of the FOX assay resides in its ability to measure initial rather than more advanced fatty acid oxidation; hence, early membrane-associated stress events in plant tissue can be detected.

KEYWORDS: Fatty acid oxidation; ferrous oxidation−xylenol orange (FOX) assay; lipid hydroperoxides; oxidative stress

FONTE: De Long et al. 2002 (materiale integrativo file: ANTIOX_ROOH_De Long_2002

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INTRODUCTION

Fatty acid oxidation has been implicated in normal and stress-induced plant metabolism (*1*−*4*), in plant senescence mechanisms (*5*, *6*), in the etiology of various animal-based diseases such as atherosclerosis (*7*), coronary heart disease (*8*), and cancer (*9*, *10*), and in inflammation disorders such as arthritis (*11*) and asthma (*12*).

Membrane fatty acids are susceptible to oxidative degradation under a variety of cellular stress conditions often resulting in the eventual breakdown of membrane structure and function (*13*, *14*). As polyunsaturated fatty acids (PUFA) undergo nonenzymic peroxidative reactions or enzyme-catalyzed oxidation, molecular (O_2) or singlet ($^1O_2$) oxygen is incorporated into the hydrocarbon skeleton, resulting in the formation of lipid hydroperoxides (LOOHs) (*15*−*17*).

Hence, the formation of hydroperoxide moieties is an initial event in the oxidative degradation of fatty acid molecules. Hydroperoxides can then undergo various decomposition pathways often involving transition metals (notably Fe^{2+}) or enzyme-mediated reactions that yield various products such as alkenals, hydroxyalkenals, alkanes, and jasmonic acid (*2*, *18*).

Lipid hydroperoxides have been measured by using a variety of techniques including high-performance liquid chromatography (HPLC) (*19*), gas chromatography (GC) (*20*), electrospray mass spectrometry (*21*), iodide oxidation (*22*), heme degradation of peroxides (*23*), cylco-oxygenase activation (*24*), and microperoxidase−luminol chemiluminescence (*25*). These methods are time-consuming or costly or require strict control of ambient oxygen levels. In recent years, another method has been developed that is based upon the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions by LOOHs with the subsequent binding of the Fe^{3+} ion to the ferric-sensitive dye xylenol orange (*26*−*28*). Known as the ferrous oxidation−xylenol orange (FOX) assay (versions I and II), the technique is sensitive (nanomole to micromole levels of LOOHs), inexpensive, and not affected by ambient oxygen concentrations. As version II permits the quantification of low concentrations of LOOHs in the presence

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FONTE: De Long et al. 2002 (materiale integrativo file: ANTIOX_ROOH_De Long_2002

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Table 4. LOOHs and TBARS in Various Plant Tissues Prior to and Following 10–12 Days of Continuous UV-B_{BE} Exposure[a]

tissue	UV-B exposure (days)	FOX[b]	iodometric[b]	TBARS[c]
avocado	0	20 ± 1 a[d]	17 ± 3 a	7 ± 1
	11	268 ± 55 a	241 ± 57 b	33 ± 3
pear	0	21 ± 4 a	47 ± 26 a	0
	12	190 ± 14 a	189 ± 26 a	120 ± 7
potato	0	0 a	0 a	0.7 ± 0.3
	10	9 ± 1 a	17 ± 3 a	10 ± 1
red cabbage	0	0 a	0 a	6 ± 3
	12	5.2 ± 0.9 a	0 b	29 ± 3
red pepper	0	0 a	0 a	45 ± 3
	12	28 ± 6 a	11 ± 10 b	74 ± 5
spinach	0	199 ± 5 a	110 ± 12 b	27 ± 2
	10	384 ± 12 a	341 ± 51 a	234 ± 5

FONTE: De Long et al. 2002 (materiale integrativo file: ANTIOX_ROOH_De Long_2002

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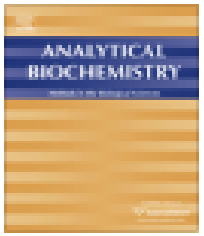

Review

Determination of hydroperoxides in foods and biological samples by the ferrous oxidation–xylenol orange method: A review of the factors that influence the method's performance

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FONTE: Bou et al. 2008 (materiale integrativo file: ANTIOX_ROOH_Bou_2008

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Altre applicazioni: determinazione di perossidi (ROOH) in campioni biologici

The oxidation of lipids yields both primary and secondary oxidation compounds that produce undesirable biological effects [1–3] and includes loss of nutritional value and sensory problems in foods [4,5]. Primary oxidation products include lipid hydroperoxides (HP)[1] which can further decompose into secondary oxidation products and/or react with other compounds present in the food or biological material [6–8].

The measurement of HP, in connection with free radicals and other reactive oxygen species, has been used as indication of oxidative stress in biological samples [7,9–13] and associated with the pathogenesis of several diseases such as atherosclerosis [14,15], cancer [16,17], and neurodegenerative diseases [18–20]. Unfortunately, the majority of analyses to assess lipid oxidation in biological and food samples determine only secondary oxidation products, whereas the determination of HP could give an early and more accurate indication of the oxidative status. Hence, a proper assessment of the degree of oxidation in any kind of sample should be accomplished by the appropriate selection of the methods that include the determination of both primary oxidation products and their decomposition products [8,21,22].

However, the determination of lipid HP is quite challenging because many different kinds of HP are produced from lipid oxidation and HP are reactive compounds that rapidly react and decompose even at moderate temperatures [22]. Thus, a great variety of methods have been proposed to assess lipid HP in biological samples and foods [8,21–24]. Among them, chromatographic (GC and HPLC), spectroscopic (NMR and ESR), and enzymatic methods showed high sensitivity, selectivity, and reproducibility [21,24–30] but their application to routine analysis is compromised and not all laboratories have the necessary instrumentation. More simple methods that measure HP by titration and colorimetric methods based on iodide or iron oxidation are also available [31–33]. However, a simple routine method should be reproducible and sensitive also. In this frame, and based on the oxidizing properties of HP, the ferrous oxidation–xylenol orange (FOX) method is of interest.

Briefly, the FOX method is based on the ability of HP to convert ferrous ions into ferric ions which subsequently form a complex with xylenol orange (XO) that is determined through spectrophotometry. This method offers the possibilities to determine the total HP content rapidly and with low cost and to assess the susceptibility to oxidation [34]. Moreover, the FOX method has been reported to be simple, sensitive, and selective for the total amount of HP, with a good precision and potentially useful in a variety of matrices [34–36]. Despite that, many variables have been reported also to influence the performance of this method [37–39]. This paper reviews the major advantages and drawbacks of the FOX method, its applicability in different matrices, and the factors that influence its specificity, selectivity, and sensitivity.

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[1] *Abbreviations used:* FOX, ferrous oxidation-xylenol orange; HP, hydroperoxide; GC, gas chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ESR, electron spin resonance; XO, xylenol orange; FTIR, Fourier transform infrared; TPP, Triphenylphosphine; BHT, butylated hydroxytoluene; LDL, low-density lipoprotein.

The Fox Method: Principle, advantages, and disadvantages

Gupta [40] first described the FOX method for the analysis of hydrogen peroxide in irradiated solutions. Later, Jiang et al. [41]

FONTE: Bou et al. 2008 (materiale integrativo file: ANTIOX_ROOH_Bou_2008

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Altre applicazioni: determinazione di perossidi (ROOH) in campioni biologici

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Hydroperoxide formation in different lean meats

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ABSTRACT

Peroxide is one of the compounds that are indicated to be toxic in the human digestion system. Lean fresh meat samples were collected from beef, lamb, pork and chicken to investigate their hydroperoxide formation potential. Total peroxides of fresh comminuted raw meat were determined by analysing protein-bound peroxides and hydroperoxide compounds in water–methanol and chloroform extracted phases. The amount of total peroxides was ranked as: beef > pork > lamb > chicken. Hydroperoxide formation was examined at different pH values and at different incubation times, using beef and chicken samples. All peroxides were transient, with a maximum value after 2–4 h of incubation at 37 °C. When pH fell from 7 to 1.5, the different peroxides fell by 10–20%. Non-polar peroxide formation could largely (70%) be described by variation in fatty acid composition and hemin content of the meat, while protein-bound peroxide variation was less explained by these variables. Liposome addition increased (40%) the amount of protein-bound peroxides.

FONTE: Yi et al. 2013 (materiale integrativo file: ANTIOX_ROOH_Yi_2013

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del FOX (ferrous oxidation–xylenol orange)

Altre applicazioni: determinazione di perossidi (ROOH) in campioni biologici

1. Introduction

Meat consumption from some land-based animals has come under attack due to unclear status regarding many diseases. Colon cancer is among these diseases, and it is one of the major causes of death in western countries (Sesink, Termont, Kleibeuker, & Van der Meer, 1999). It has been recognised that many genetic factors are involved as determinants of colorectal cancer (Fearon & Jones, 1992), but environmental factors have appeared to contribute to the incidences of colon cancer (MacLennan, 1997). The World Cancer Research Fund panel has judged that the evidence of red meat and processed meat being a cause of colon cancer is convincing (WCRF, 2007), and a western style diet with a high red meat consumption is suggested as a risk factor for colon cancer (Sesink et al., 1999). Increased consumption of meat can be due to improved efficiency in agriculture, which has then created sufficient amounts of relatively cheap meat products. Animal breeding has so far given most priority to rapid animal growth and cost-effective feeds. But meat should also have a good oxidative and microbial shelf life. Sufficient oxidative stabilization is paramount for meat flavour. A present understatement is that oxidised food can be consumed as long as the microbiology and sensory quality are acceptable to consumers. Compounds that could increase the genetic instability of colon cells and the appearance of cancer have received much attention (Ferguson, 2010). Lipid or lipid-derived peroxides are a major source of dietary pro-oxidants speculated to be of toxicological importance (Halliwell & Chirico, 1993).

An *in vitro* study on intake of fat and derived peroxides has identified this as one of many important factors in colon cancer (Angeli et al. 2011). Lipid peroxides are set with an acceptable upper level of 5–10 mmol/kg in oil or fat (Sattar & Deman, 1976). Peroxide limits are normally not defined for products other than oil/fats. However, it is more common to eat larger amounts of lean meat than of pure oil/fats in a meal. Heated turkey meat has been reported to have 1 mmol of lipid hydroperoxide/kg wet weight (Kuffa, Priesbe, Krueger, Reed, & Richards, 2009). This suggests a high peroxide value in the endogenous lipids (~100 mmol/kg lipid). In addition, proteins may also carry peroxides equal to 3–22 mmol/kg of protein (Salminen and Heinonen, 2008). Proteins damaged by free radicals in the presence of oxygen can yield relatively long-lived protein peroxides (Davies, Fu, & Dean, 1995; Gebicki & Gebicki, 1993), which have been shown to readily degrade to free radicals upon reaction with iron (II) complex. It is therefore necessary to include them in an assay for hydroperoxide measurements, in particularly in lean meat where the lipid content is low relative to the protein content.

With sufficient amounts of efficient antioxidants, meat should be a homoeostatic system which remains reduced or without oxidised compounds and reactive components. The aim of this study was: (1) to set up a new model system for measuring total hydroperoxide values of lean meat and the reactivity of lean meat towards liposomes, (2) to discover if the lipid peroxides were always dominant over the protein-bound peroxides, (3) to investigate whether the peroxides were stable when incubated

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FONTE: Yi et al. 2013 (materiale integrativo file: ANTIOX_ROOH_Yi_2013

- **Saggi di attività antiossidante**

Saggi ET (electron transfer): basati sul trasferimento di elettroni

Saggi HAT (hydrogen atom transfer): basati sul trasferimento di atomi di idrogeno

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

- **Saggi HAT (hydrogen atom transfer): meccanismo di azione dell'antiossidante (AH/ArOH)**

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

Con questo tipo di Saggi si dosano antiossidanti che agiscono da scavengers di radicali donando un atomo di idrogeno

- **Saggi HAT (hydrogen atom transfer)**

L'antiossidante reagisce con il probe colorato donando ad esso un atomo di idrogeno: tale reazione causa la variazione di colore

Saggi spettrofotometrici basati sulla variazione di colore del probe ad opera dell'antiossidante: metodi con i radicali stabili DPPH e ABTS

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del radicale stabile DPPH (2,2-diphenyl-1-picrylhydrazyl)

Messa a punto dell'esperimento:

1. Preparazione delle soluzioni
2. Valutazione delle quantità da dosare
3. Elaborazione dei dati sperimentali

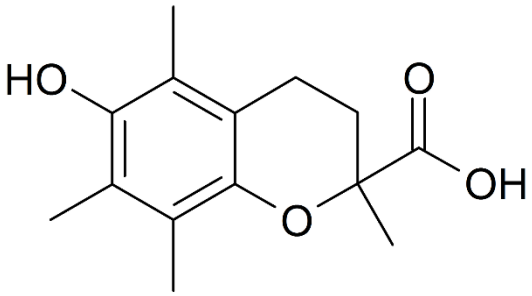
METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del radicale stabile DPPH (2,2-diphenyl-1-picrylhydrazyl)

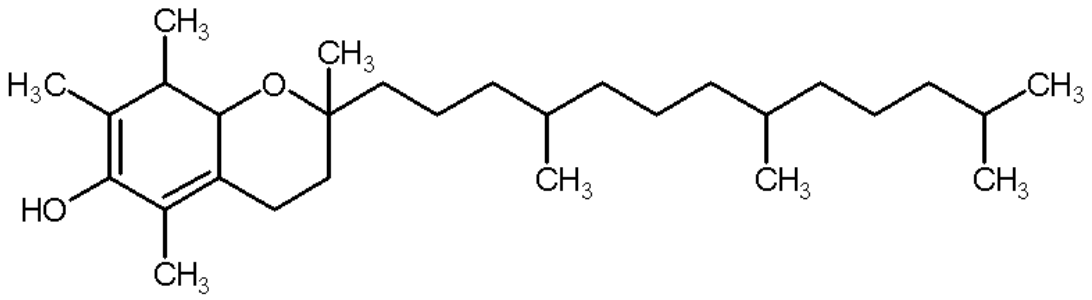
Messa a punto dell'esperimento:

1. Preparazione delle soluzioni

- radicale DPPH: soluzione metanolica con concentrazione: 3 mg/25 ml
- il potenziale antiossidante viene espresso come capacità Trolox equivalenti (TEAC)
- Trolox: acido 6-idrossi-2,5,7,8-tetrametilcroman-2-carbossilico, un derivato idrosolubile della vitamina E



Trolox



Vitamina E

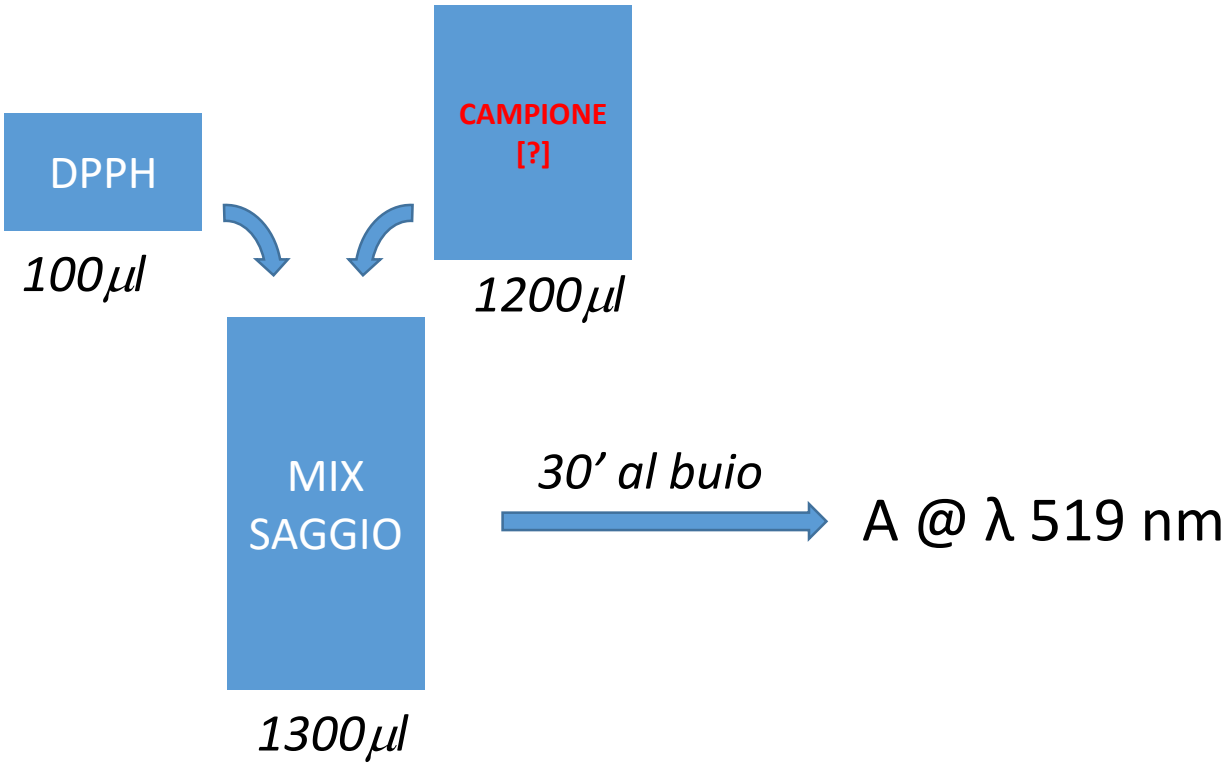
METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del radicale stabile DPPH (2,2-diphenyl-1-picrylhydrazyl)

Messa a punto dell'esperimento:

1. Preparazione delle soluzioni

- radicale DPPH: soluzione metanolica con concentrazione: 3 mg/25 ml
- Campione: soluzione acquosa da miscelare con la soluzione di DPPH



METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del radicale stabile DPPH (2,2-diphenyl-1-picrylhydrazyl)

Messa a punto dell'esperimento:

2. Valutazione delle quantità da dosare

Concentrazione	A @ 519nm				$A_S/A_0 \times 100$	% inibizione
	Valore 1	Valore 2	MEDIA	DV STD		
CONTROLLO (A_0)	0,371	0,374	0,373	0,002	100,0	
Campione (A_S)	Valore 1	Valore 2	MEDIA	DV STD		
5,0 µg/ml	0,149	0,152	0,151	0,002	40,0	60,000
2,0 µg/ml	0,278	0,281	0,280	0,002	74,2	25,772
1,5 µg/ml	0,301	0,303	0,302	0,001	79,5	20,537
1,0 µg/ml	0,329	0,330	0,330	0,001	86,8	13,154

- A_0 è l'assorbanza del radicale DPPH e A_S è l'assorbanza dopo l'addizione del campione o dello standard.

METODOLOGIE PER IL DOSAGGIO DI SOSTANZE ANTIOSSIDANTI

Metodo del radicale stabile DPPH (2,2-diphenyl-1-picrylhydrazyl)

Messa a punto dell'esperimento:

3. Elaborazione dei dati sperimentali

- Il potenziale antiossidante viene espresso come capacità Trolox equivalenti (TEAC)
- Si effettua il confronto con una opportuna curva di calibrazione
- Il potere antiossidante viene misurato come valore percentuale d'inibizione, secondo la formula:

$$\text{Inibizione } \% = (1 - A_S/A_0) \times 100$$

- dove A_0 è l'assorbanza del radicale DPPH e A_S è l'assorbanza dopo l'addizione del campione o dello standard.