What are the main physicochemical factors involved in antioxidant action of phenolics to counteract lipid oxidation ?

A new approach through the Conjugated Autoxidizable Triene (CAT) assay

M. Laguerre, L. J. Lopez Giraldo, J. Lecomte, P. Villeneuve

CIRAD PERSYST, Lipotechny Laboratory, Joint Research Unit on Agropolymers & Emerging Technologies, Montpellier, France

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1) THE ISSUE OF EVALUATING ANTIOXIDANTS

1) How to evaluate their antioxidant capacity ?

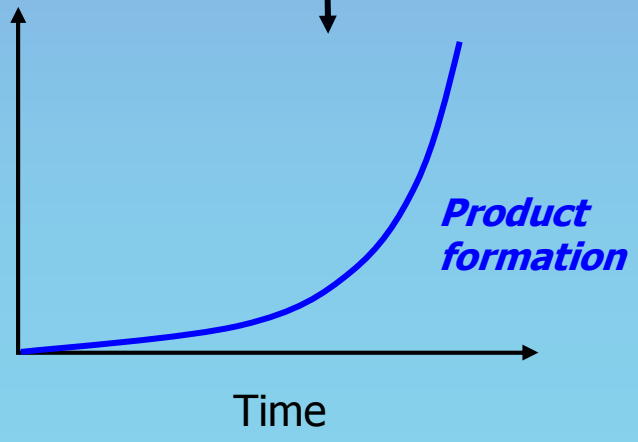
2) What are the main physicochemical factors governing it ?

Antioxidant capacity can be measured by monitoring the inhibitory effect of an antioxidant on the oxidation of a substrate

Kinetic approach 1

Spectral measurement of oxidation products formation

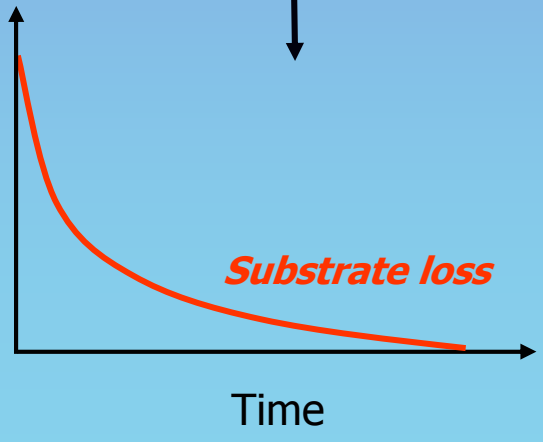
Conjugated dienes, TBARS…



Kinetic approach 2

Spectral measurement of substrate loss

ORAC, BODIPY and Crocin bleaching tests



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Kinetic approach 1

Spectral measurement of oxidation products formation

Conjugated dienes, TBARS…

Problem

1) No universal marker is currently available

Lipid oxidation leads to the formation of both primary and secondary oxidation products

It is essential to evaluate both primary and secondary products to obtain a reliable overall picture of the oxidation level

→ Very tedious

Kinetic approach 2

Spectral measurement of substrate loss

ORAC, BODIPY and Crocin bleaching tests

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Spectral measurement of substrate loss

ORAC, BODIPY and Crocin bleaching tests

Two main problems

1) The substrate must be detectable by a spectral method and thus, must exhibit specific spectrum

→ Not often the case with natural lipids *(ω9, ω6 or ω3)*

Fluorophoric or chromophoric substrates are often unrepresentative of natural oxidizable substrates

← BODIPY, Crocin, Fluorescein disodium salt

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Two main problems

2) The antioxidant is often **in excess** compared to the oxidizable substrate

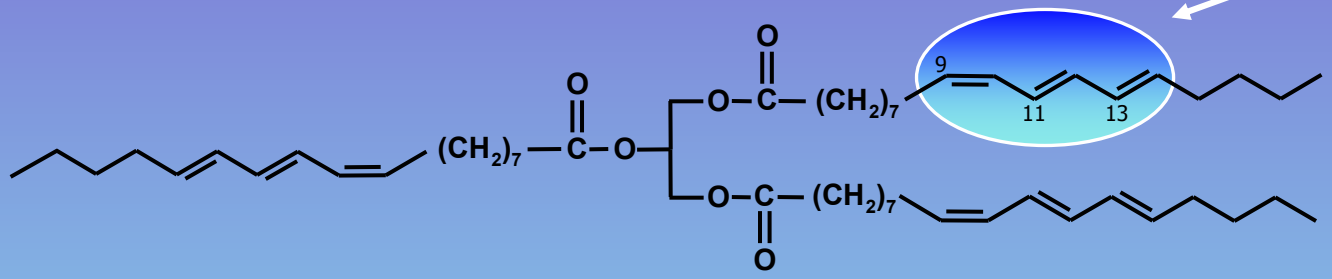
An antioxidant is *« a substance that when present at **low concentration compared to oxidizable substrate**, significantly delays or prevents oxidation of that substrate »* Halliwell and Gutteridge, 1990

2) CONJUGATED AUTOXIDIZABLE TRIENE (CAT) ASSAY

1) Commercial availability
2) Cheapness compared to other oxidizable substrates
3) High sensitivity to oxidation
4) Use of natural TAGs as oxidizable substrate

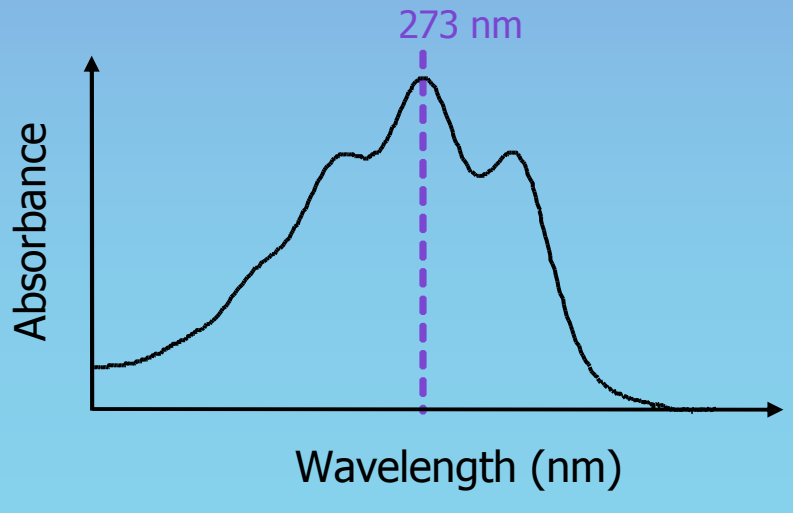
CAT assay is based on the spectral properties of **triacylglycerols (TAGs)** naturally present in Tung oil

This particular oil is composed of ~ 77 % of trieleostearin which contains **3 conjugated trienes**

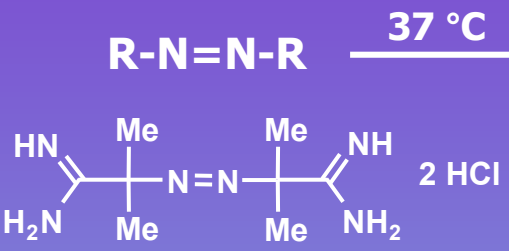


This conjugated system exhibits a strong **UV-absorption** at 273 nm

Thus allowing the monitoring of its oxidative degradation by **UV-spectrophotometry**

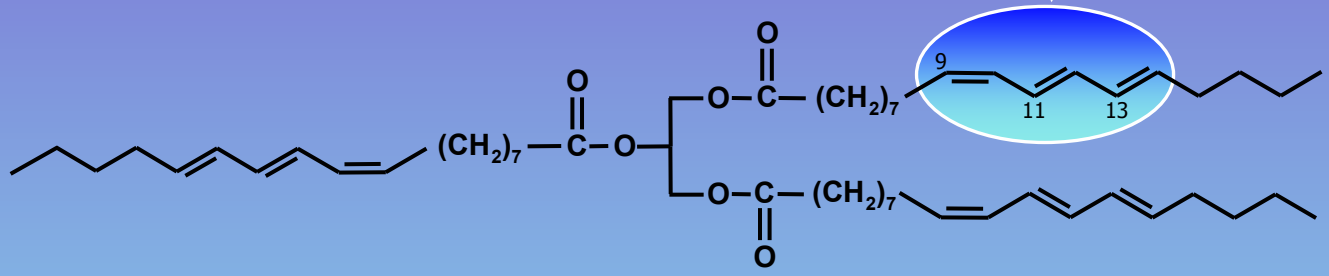


2) CONJUGATED AUTOXIDIZABLE TRIENE (CAT) ASSAY

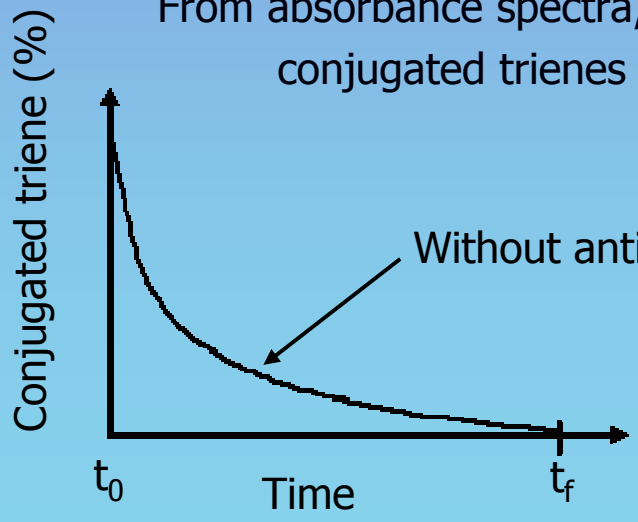


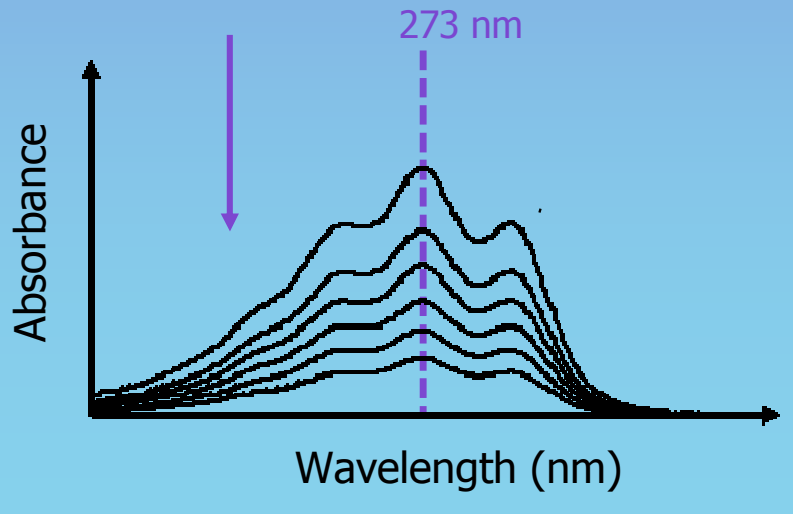
Oxidizing species: hydrophilic azo-initiator AAPH

Peroxyradicals will then destroy the conjugated triene, leading to a **decrease in absorbance**



From absorbance spectra, the kinetic loss of conjugated trienes is then established





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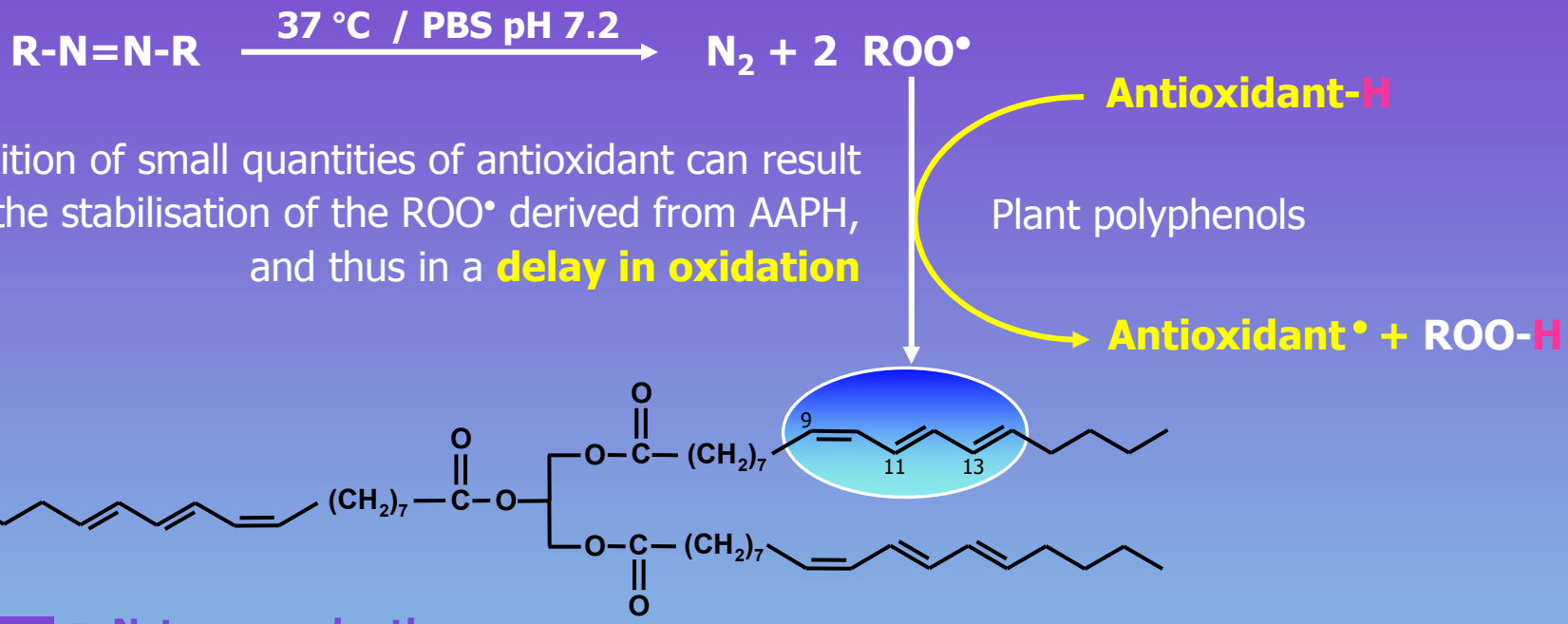
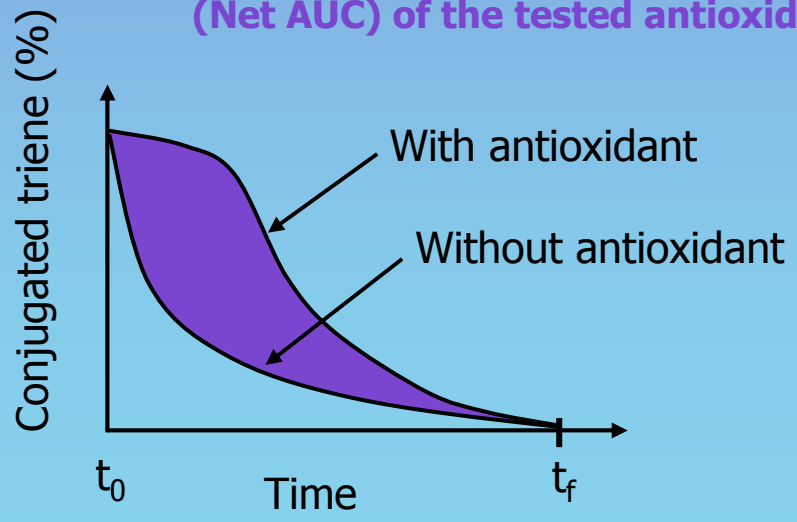


Addition of small quantities of antioxidant can result in the stabilisation of the ROO• derived from AAPH, and thus in a **delay in oxidation**

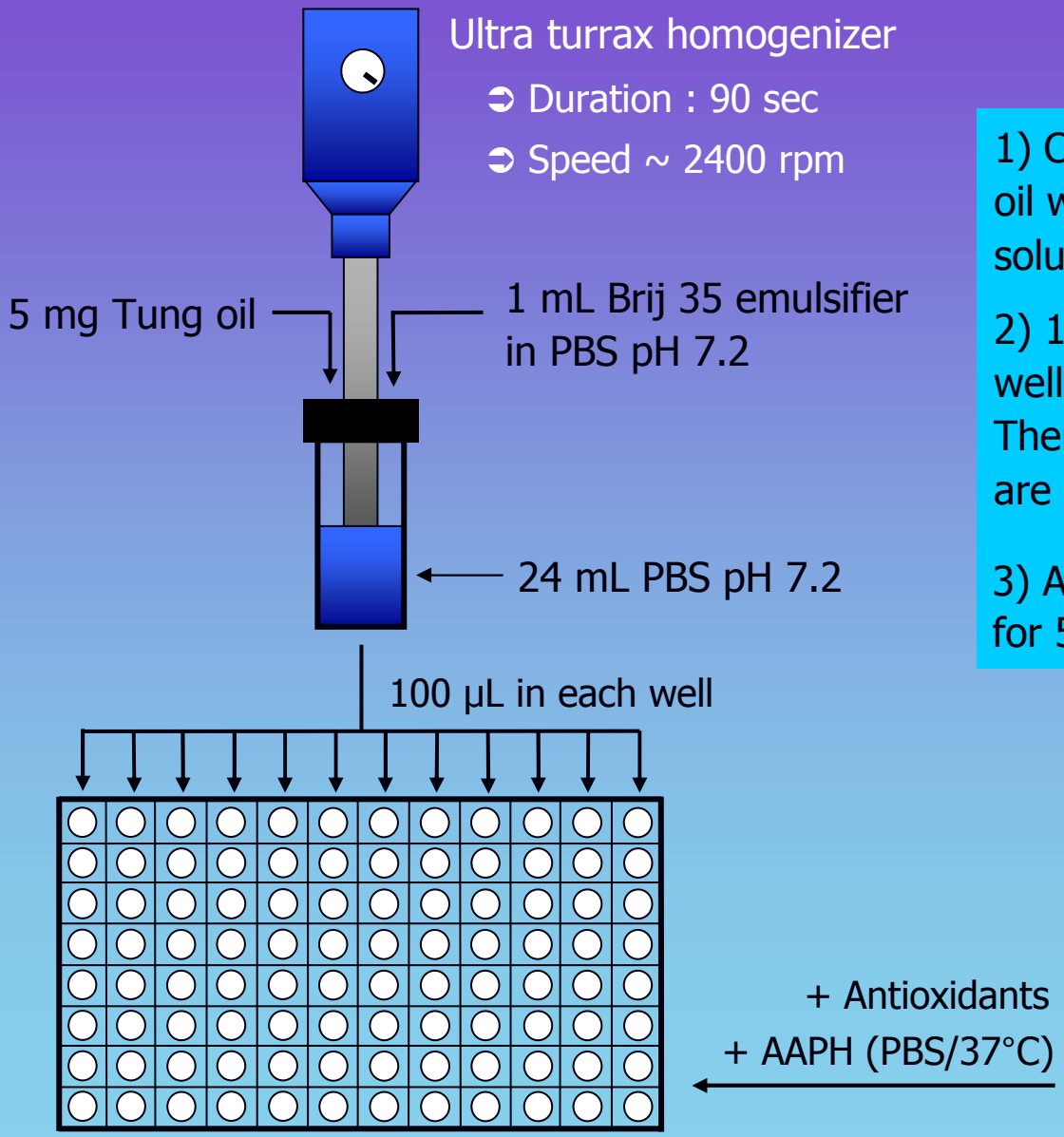
= **Net area under the curve (Net AUC) of the tested antioxidant**



Extraction of quantitative information from the data can be namely performed by using a classical area under the curve calculation

CAT Value = (Net AUC $_{Sample}$ / Net AUC $_{Trolox}$) x (moles of Trolox/moles of sample)

3) EXPERIMENTAL PROTOCOL



CAT in 3 steps

1) CAT assay is performed mixing Tung oil with Brij emulsifier in phosphate buffer solution with an Ultra Turrax homogenizer

2) 100 µL of this are dispensed into each well of microplate.
Then, various antioxidant concentrations are added, followed by addition of AAPH

3) Abs at 273 nm is monitored each min for 5 hours with a microplate reader

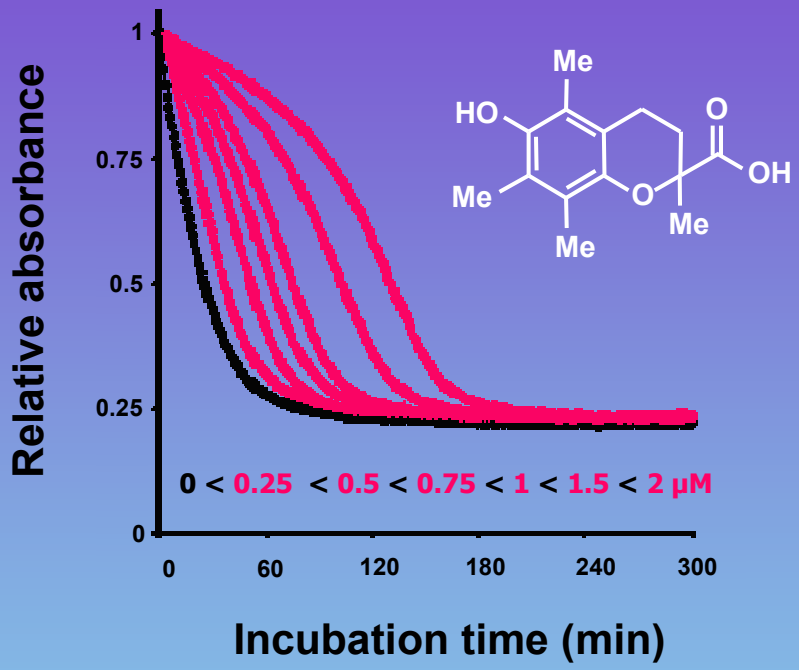
Antioxidant is ~ 100-times less concentrated than Tung oil

↓

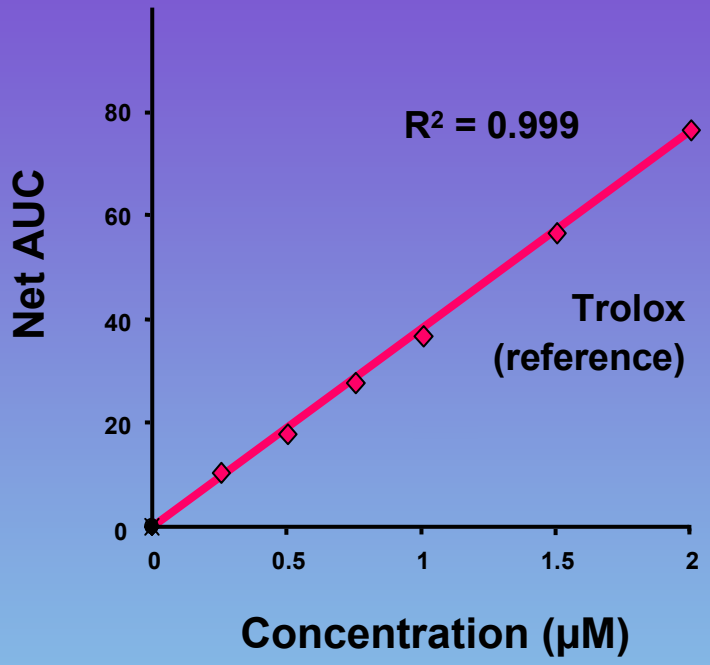
More biologically relevant than ORAC assay

4) TROLOX : HYDROPHILIC ANALOGUE OF α-TOCOPHEROL

A) Antioxidant capacity of Trolox



B) Net antioxidant area *vs.* concentration

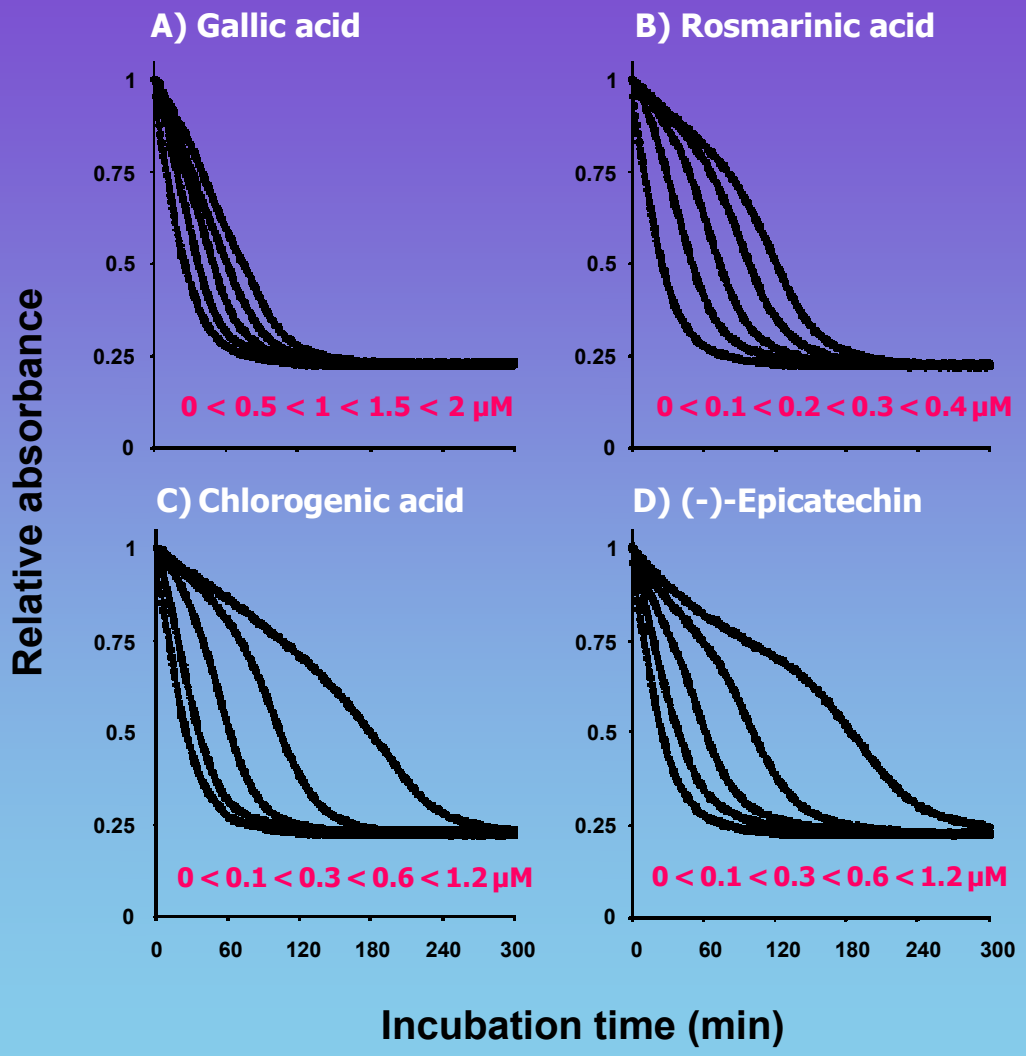


Addition of increasing quantities of Trolox

Observations — ***Trolox delays oxidation in a dose-dependent manner***

* Laguerre M, Lopez Giraldo LJ, Lecomte J, Baréa B, Cambon E, Tchobo PF, Barouh N, Villeneuve P. **Conjugated Autoxidizable Triene (CAT) assay : a novel spectrophotometric determination of antioxidant capacity using triacylglycerol as ultraviolet probe.** *Submitted to Analytical Biochemistry.*

5) ANTIOXIDANT CAPACITY OF VARIOUS PHENOLIC COMPOUNDS



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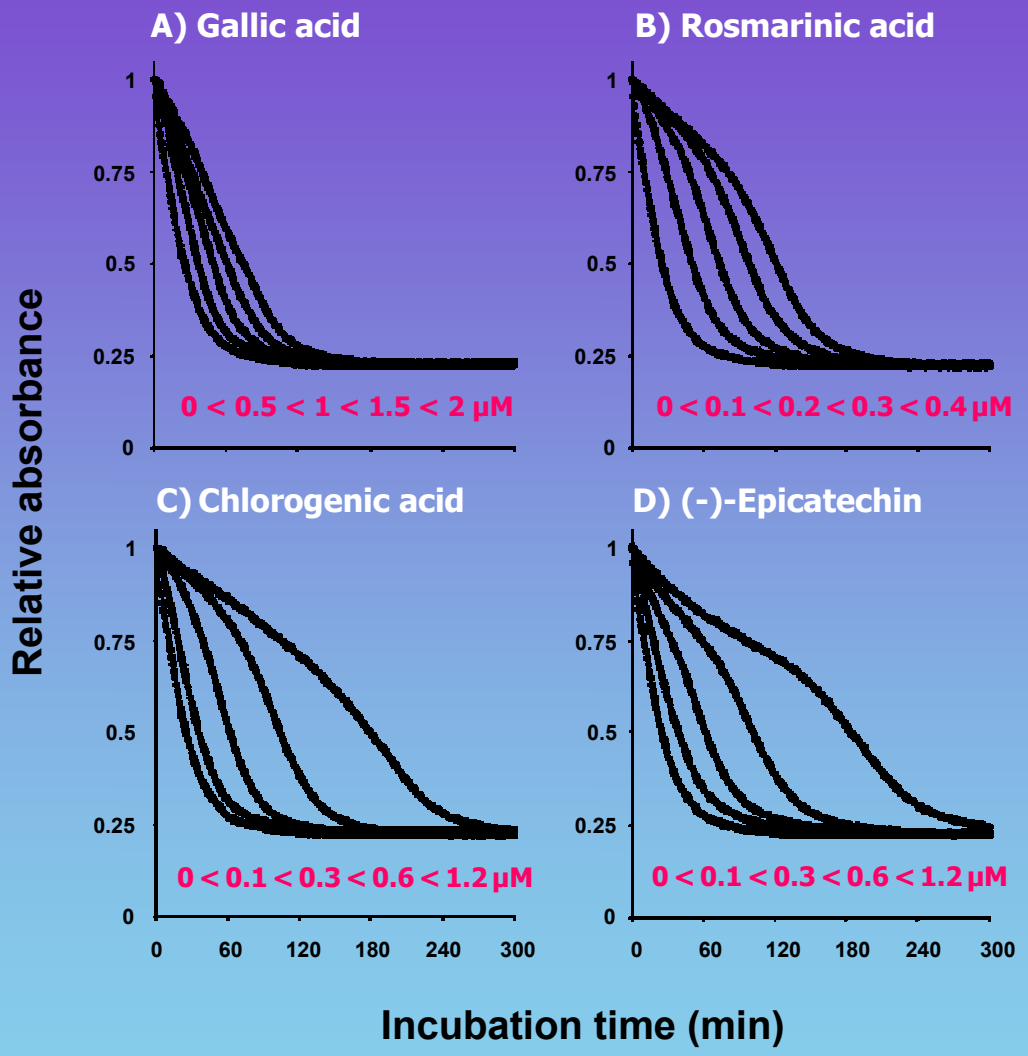
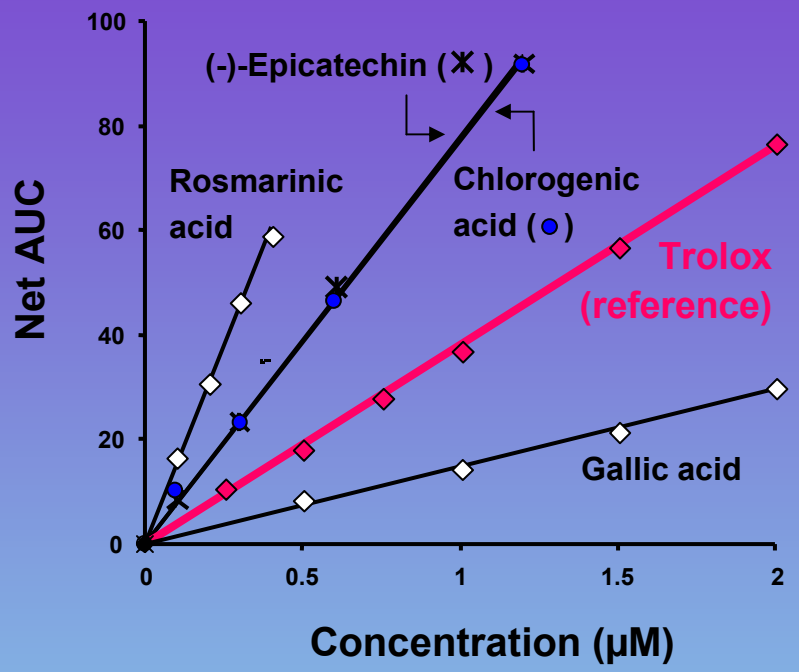




Similar to Trolox, an AUC calculation shows that all tested antioxidants acted in a dose-dependent manner, which enables their CAT value (in Trolox equivalent) to be calculated

↓

CAT Value = slope ratio between tested molecule and Trolox

5) ANTIOXIDANT CAPACITY OF VARIOUS PHENOLIC COMPOUNDS

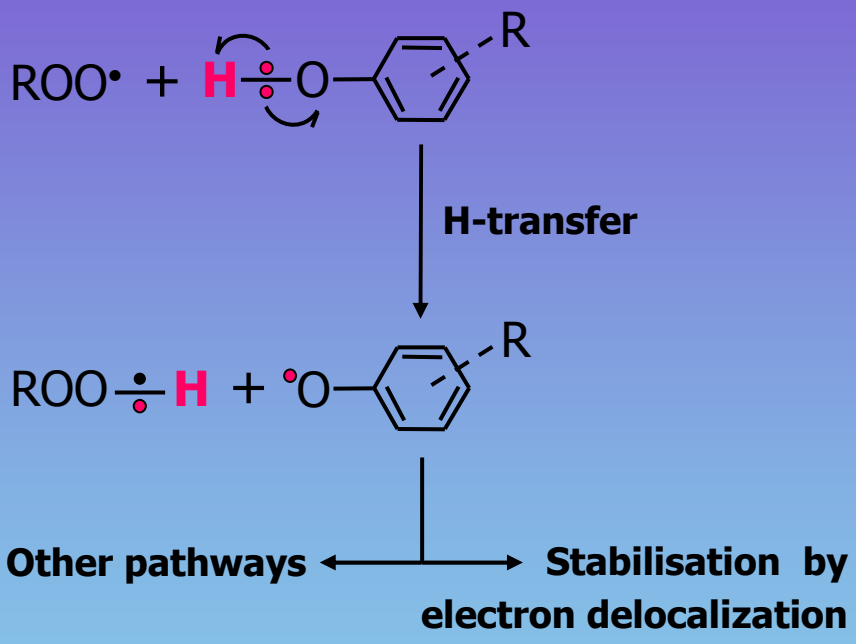
A : Antioxidant effectiveness order of 10 phenolic compounds

ANTIOXIDANT CAPACITY (− → +)

Compound	CAT Value (TEq[a])
Gallic acid	0.43 ± 0.05
Ferulic acid	1.05 ± 0.04
Protocatechuic acid	1.66 ± 0.02
Chlorogenic acid	2.21 ± 0.14
Myricetin	2.35 ± 0.08
(-)-Epicatechin	2.39 ± 0.03
Caffeic acid	2.91 ± 0.07
Catechin	3.00 ± 0.08
Quercetin	3.18 ± 0.15
Rosmarinic acid	4.21 ± 0.77

[a] Mole of Trolox/mole of compound

B) Antioxidant mechanism by H-donation



Question ***What are the main physicochemical factors involved in this antioxidant action ?***

According to the structural aspect of phenolic compounds, it is logic to consider that their antioxidant capacity is linked to the **phenolic hydroxyl(s)**, especially their **H-donation** ability

6) PHENOLIC HYDROXYL NUMBER : NO SIGNIFICANT EFFECT

Table : Antioxidant effectiveness order of 10 phenolic compounds

Compound	CAT Value (TEq[a])	Phenolic OH
Gallic acid	0.43 ± 0.05	3
Ferulic acid	1.05 ± 0.04	1
Protocatechuic acid	1.66 ± 0.02	2
Chlorogenic acid	2.21 ± 0.14	2
Myricetin	2.35 ± 0.08	5
(-)-Epicatechin	2.39 ± 0.03	4
Caffeic acid	2.91 ± 0.07	2
Catechin	3.00 ± 0.08	4
Quercetin	3.18 ± 0.15	4
Rosmarinic acid	4.21 ± 0.77	4

ANTIOXIDANT CAPACITY (− → +)

[a] Mole of Trolox/mole of compound

F = 2.4 ; p-value = 0.2 → **No significant effect**

Observation

There is no significant effect of the phenolic hydroxyl number on the CAT value

If the **NUMBER** can not only explain the CAT value, one must take into account the **POSITION** of the phenolic hydroxyls

7) CATECHOL VS. PYROGALLOL : A STRONG DIFFERENCE !

Table : Antioxidant effectiveness order of 10 phenolic compounds

Compound	CAT Value (TEq[a])
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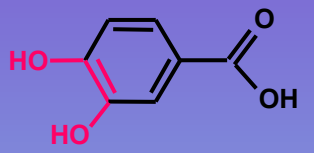
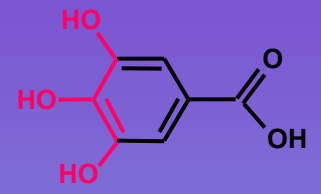
ANTIOXIDANT CAPACITY



Catecholic moiety



Pyrogallolic moiety

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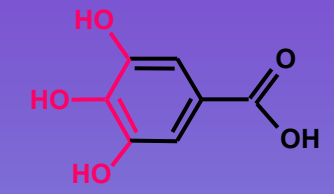
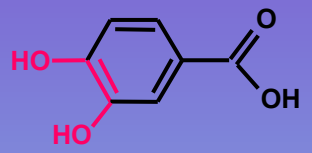
Catechin	3.00 ± 0.08
Quercetin	3.18 ± 0.15
Rosmarinic acid	4.21 ± 0.77

[a] Mole of Trolox/mole of compound

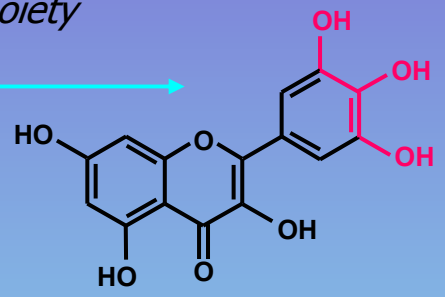
ANTIOXIDANT CAPACITY (− → +)



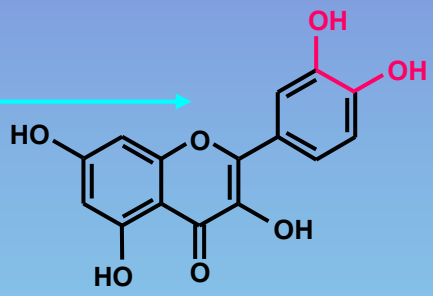
Pyrogallolic moiety



Catecholic moiety



Pyrogallolic moiety



Catecholic moiety

Observation — ***3 adjacent hydroxyls give lower CAT value than 2 adjacent hydroxyls***

- Number of **PHENOLIC HYDROXYLS** is senseless when taken independently to the position
- Number of **CATECHOLIC MOIETIES** seems to be better correlated with the CAT value

8) CATECHOL STRUCTURE NUMBER : SIGNIFICANT EFFECT

Table : Antioxidant effectiveness order of 10 phenolic compounds

Compound	CAT Value (TEq[a])	Catechol
Gallic acid	0.43 ± 0.05	0
Ferulic acid	1.05 ± 0.04	0
Protocatechuic acid	1.66 ± 0.02	1
Chlorogenic acid	2.21 ± 0.14	1
Myricetin	2.35 ± 0.08	0
(-)-Epicatechin	2.39 ± 0.03	1
Caffeic acid	2.91 ± 0.07	1
Catechin	3.00 ± 0.08	1
Quercetin	3.18 ± 0.15	1
Rosmarinic acid	4.21 ± 0.77	2

ANTIOXIDANT CAPACITY (− → +)

[a] Mole of Trolox/mole of compound

F = 15.6 ; p-value = 0.004 → **Significant effect <0.05**

Question

Is catechol number the only factor involved in the antioxidant action ?

8) CATECHOL STRUCTURE NUMBER : SIGNIFICANT EFFECT

Table : Antioxidant effectiveness order of 10 phenolic compounds

Compound	CAT Value (TEq[a])	Catechol
Gallic acid	0.43 ± 0.05	0
Ferulic acid	1.05 ± 0.04	0
Protocatechuic acid	1.66 ± 0.02	1
Chlorogenic acid	2.21 ± 0.14	1
Myricetin	2.35 ± 0.08	0
(-)-Epicatechin	2.39 ± 0.03	1
Caffeic acid	2.91 ± 0.07	1
Catechin	3.00 ± 0.08	1
Quercetin	3.18 ± 0.15	1
Rosmarinic acid	4.21 ± 0.77	2

ANTIOXIDANT CAPACITY (− → +)

6 antioxidants exhibiting very different CAT value with the same number of catechol

[a] Mole of Trolox/mole of compound

$F = 15.6$; p-value = 0.004 → **Significant effect <0.05**

Question — ***Is catechol number the only factor involved in the antioxidant action ?***

Besides the **NUMBER** AND the **POSITION** of phenolic hydroxyl, there are other involved factors

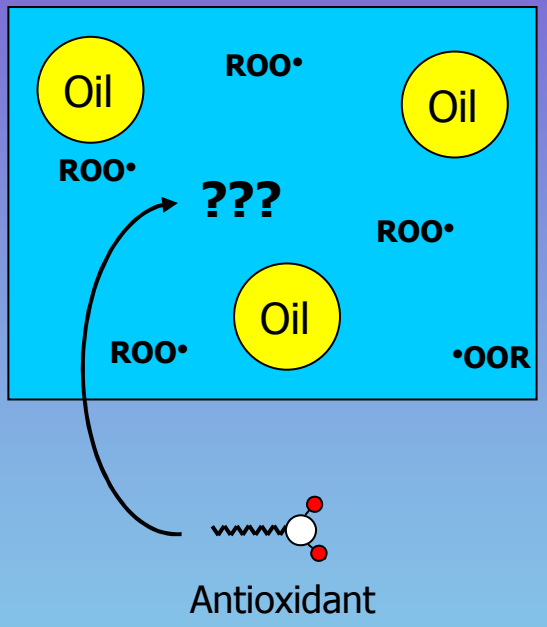
8) PARTITION BETWEEN OIL AND WATER : CHLOROGENATE MODEL

Table : Antioxidant effectiveness order of 10 phenolic compounds

Compound	CAT Value (TEq[a])	Catechol
Gallic acid	0.43 ± 0.05	0
Ferulic acid	1.05 ± 0.04	0
Protocatechuic acid	1.66 ± 0.02	1
Chlorogenic acid	2.21 ± 0.14	1
Myricetin	2.35 ± 0.08	0
(-)-Epicatechin	2.39 ± 0.03	1
Caffeic acid	2.91 ± 0.07	1
Catechin	3.00 ± 0.08	1
Quercetin	3.18 ± 0.15	1
Rosmarinic acid	4.21 ± 0.77	2

[a] Mole of Trolox/mole of compound

ANTIOXIDANT CAPACITY (− → +)

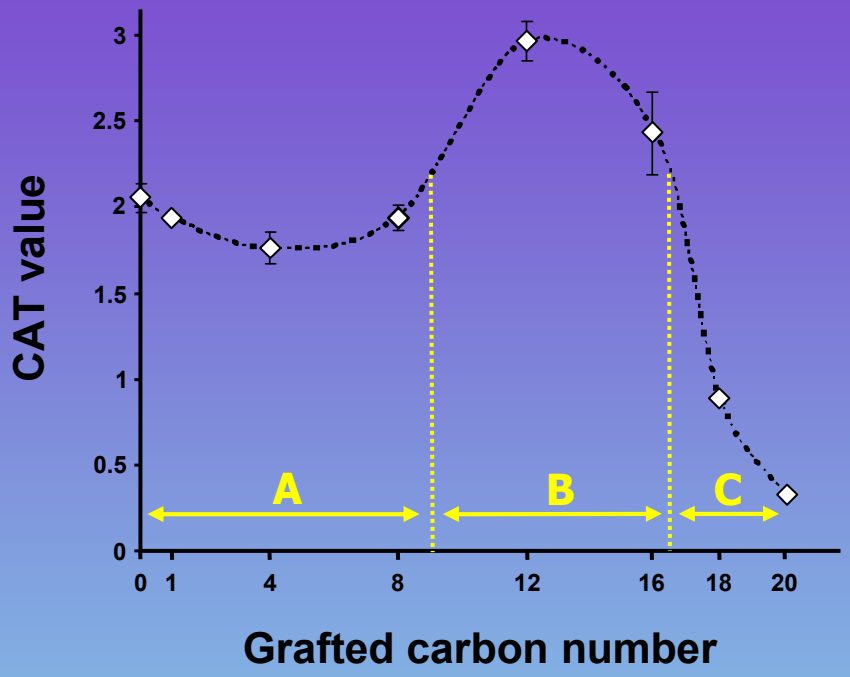


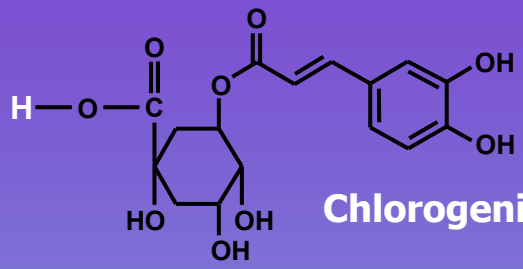
CAT assay involves a biphasic medium between oil and water

Antioxidant PARTITION between oil and water is of prime importance in the CAT value determinism

ANTIOXIDANT MODEL :
Chlorogenic acid
(Intermediate CAT value)

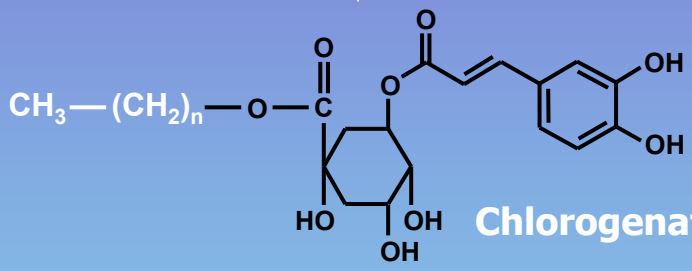
9) HYDROPHOBICITY : A STRONG NON-LINEAR EFFECT





Chlorogenic acid

Grafting of various carbon chain lenghts *

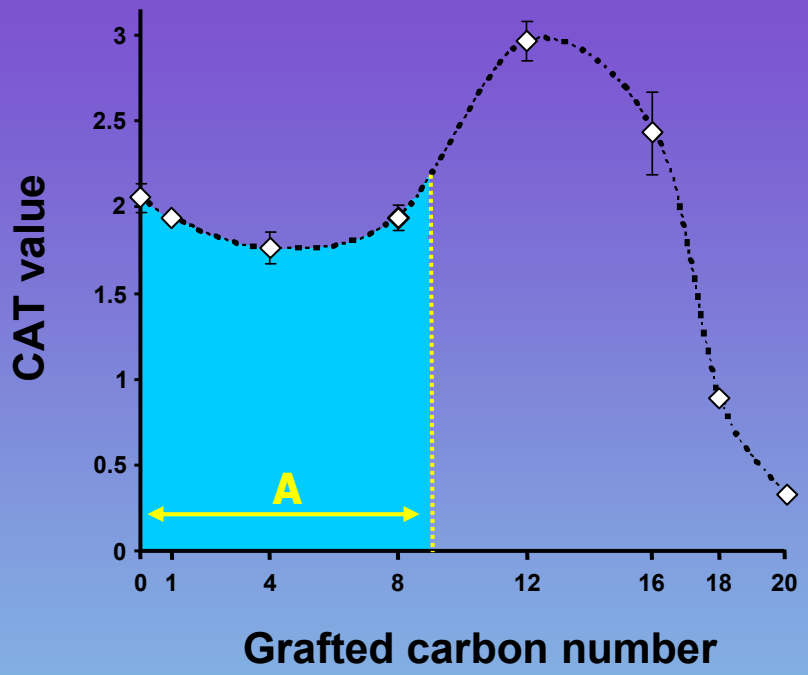


Chlorogenate esters

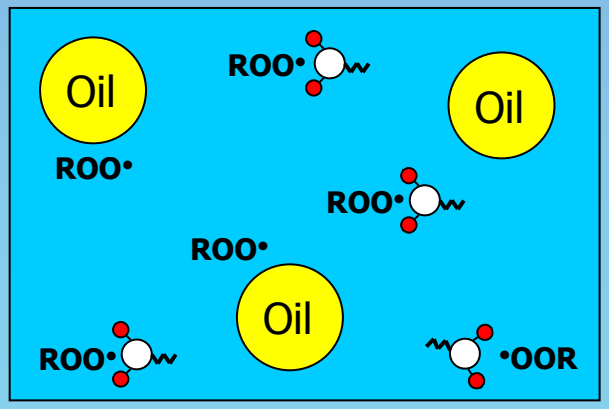
- Methyl chlorogenate (C1)	- Dodecyl chlorogenate (C12)
- Butyl chlorogenate (C4)	- Hexadecyl chlorogenate (C16)
- Octyl chlorogenate (C8)	- Octadecyl chlorogenate (C18)
	- Eicosyl chlorogenate (C20)

* Lopez Giraldo LJ, Laguerre M, Lecomte J, Figueroa-Espinoza MC, Barouh N, Baréa B, Villeneuve P. **Lipase-catalysed synthesis of chlorogenate fatty esters in solvent-free medium.** *Enzyme & Microbial Technology* ***2007***, *41:721-26.*

10) UNTIL 8 CARBONS : NO IMPORTANT EFFECT ON THE CAT VALUE



A) 0 < Carbon number < 8



<u>Hypothesis :</u>

The corresponding esters remain in the **aqueous phase**, while oxidation occurs at the **oil-in-water interphase**

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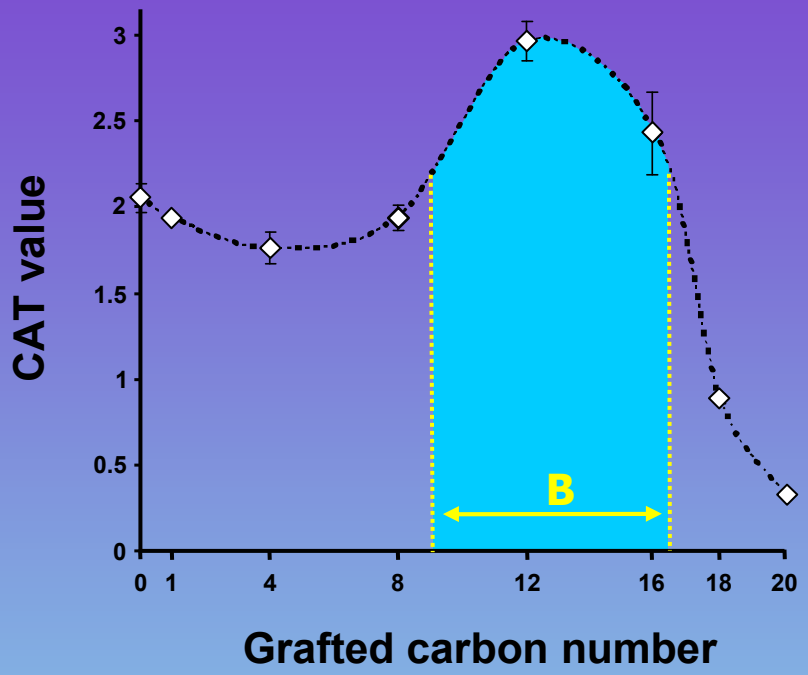
The protective effect of chlorogenate esters is not optimal, because of their bad location towards oxidizable substrate

11) TWELVE CARBONS : AN OPTIMAL CAT VALUE



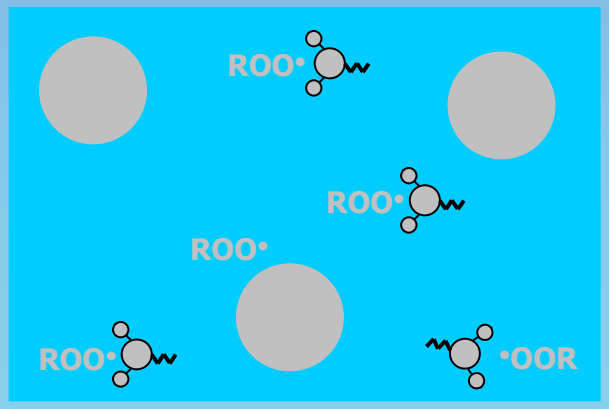
Hypothesis :

Dodecyl chain (C12) provides nearer location to oil droplet, where the oxidation occurs, than other alkyl chains

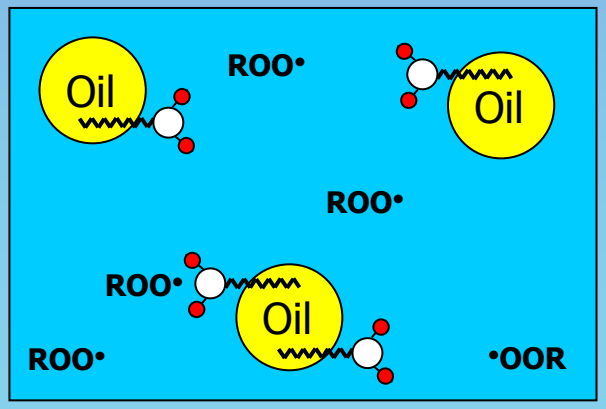
« In many biological systems, molecules show maximum membrane activity if they possess an alkyl chain length of approximately 12 carbons »

Walters *et al., Int. J. Cosmet. Sci.1993*

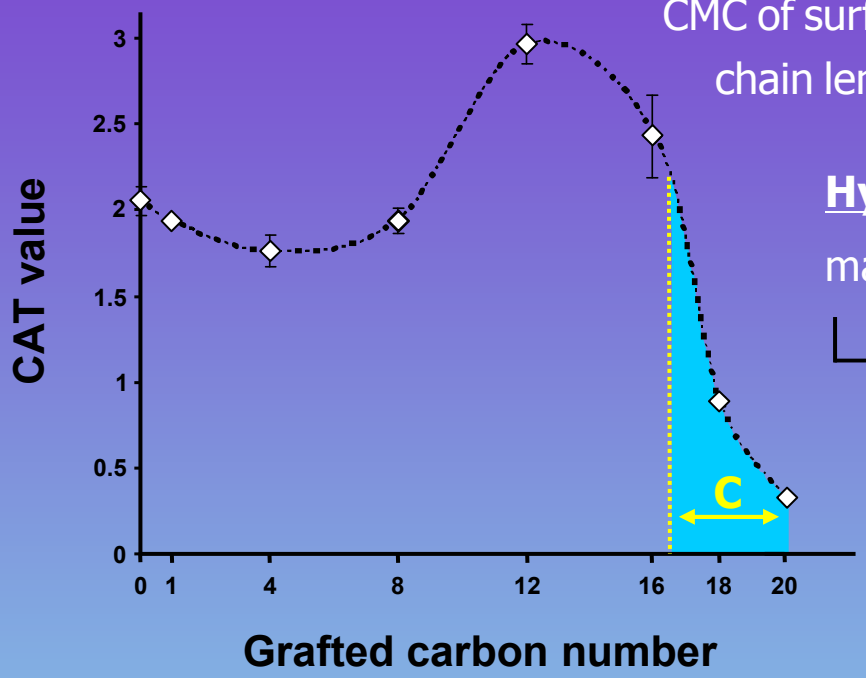
A) 0 < Carbon number < 8



B) Carbon number = 12



12) ABOVE ~ 16 CARBONS : CAT VALUE COLLAPSE



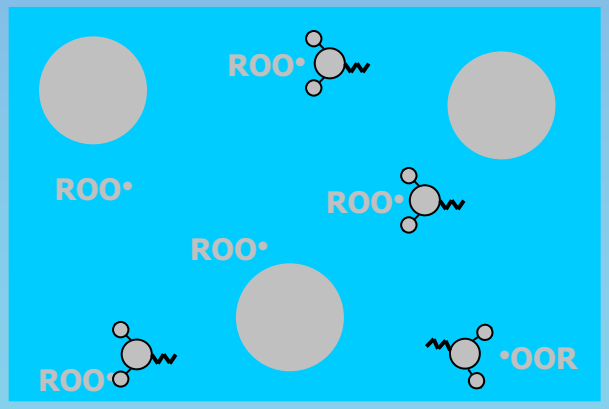
CMC of surface-active antioxidant decreases as the hydrocarbon chain length increases *(Yuji et al., J. Agric. Food Chem. 2007)*

Hypothesis : Above 16 carbons, chlorogenate esters mainly exist as micelles

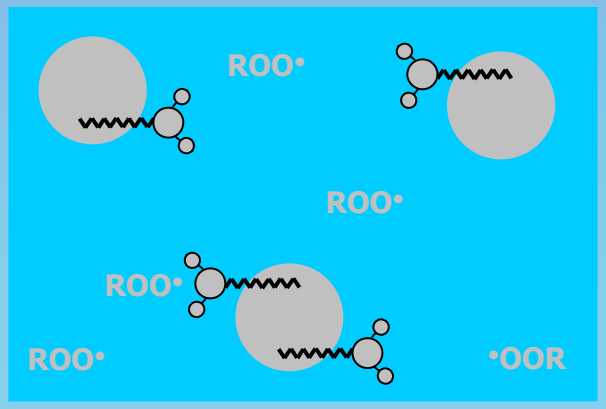
↳ 3 majors drawbacks to counteract lipid oxidation

(1) BAD LOCATION
(2) WEAK MOBILITY
(3) CLUSTER EFFECT

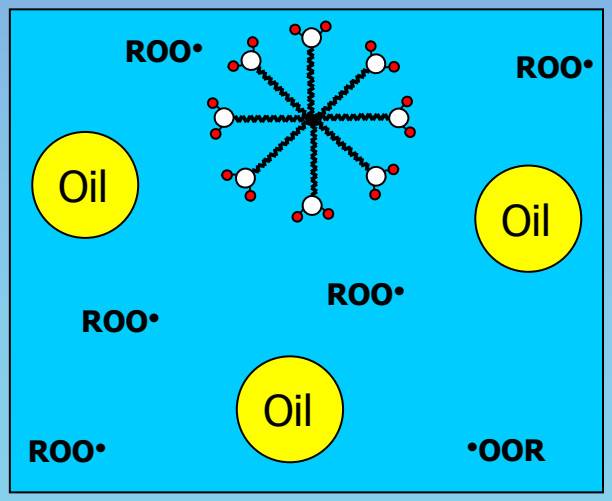
A) 0 < Carbon number < 8



B) Carbon number = 12



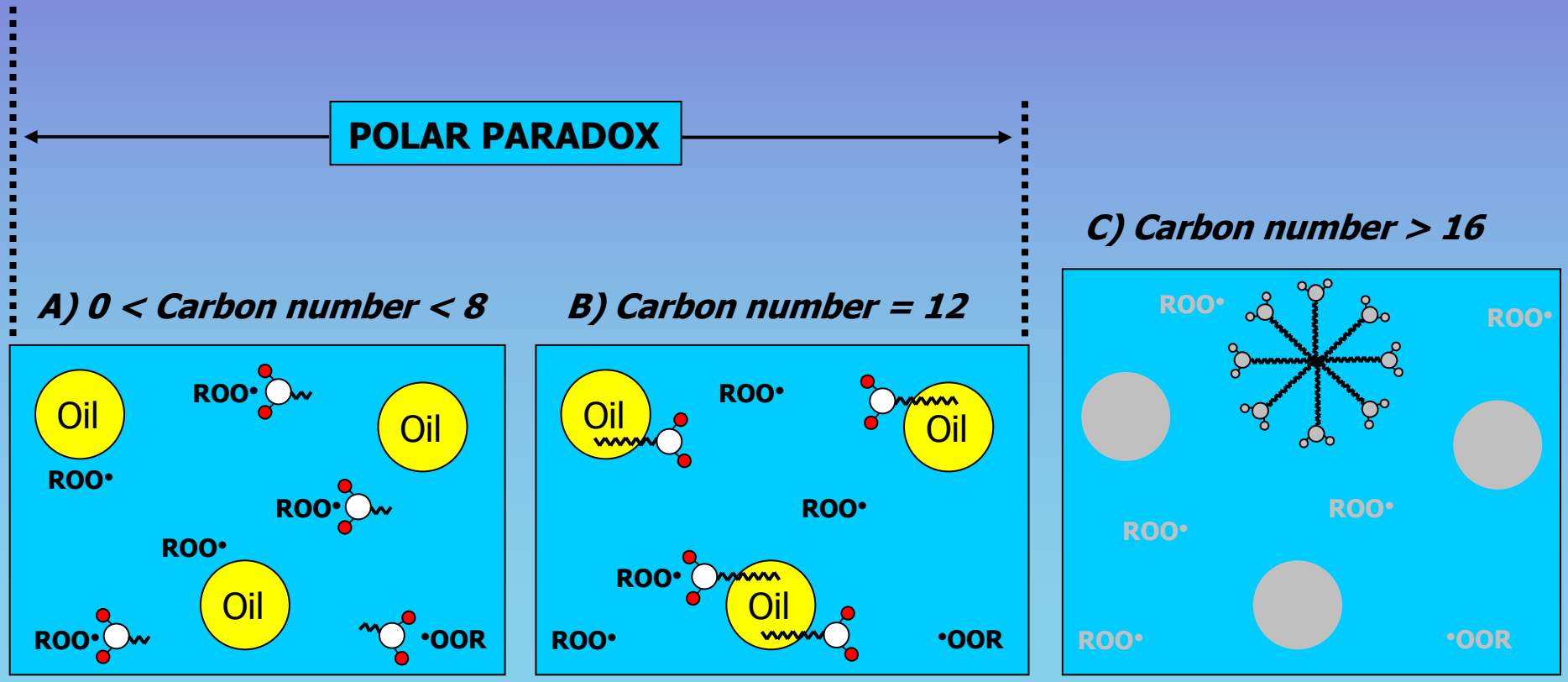
C) Carbon number > 16



13) UNTIL 12 CARBONS: PARTIAL AGREEMENT WITH POLAR PARADOX

Polar paradox *(Porter et al., J. Agric. Food Chem. 1989)* **:**

Apolar antioxidants are more active in emulsified medium than their polar homologues

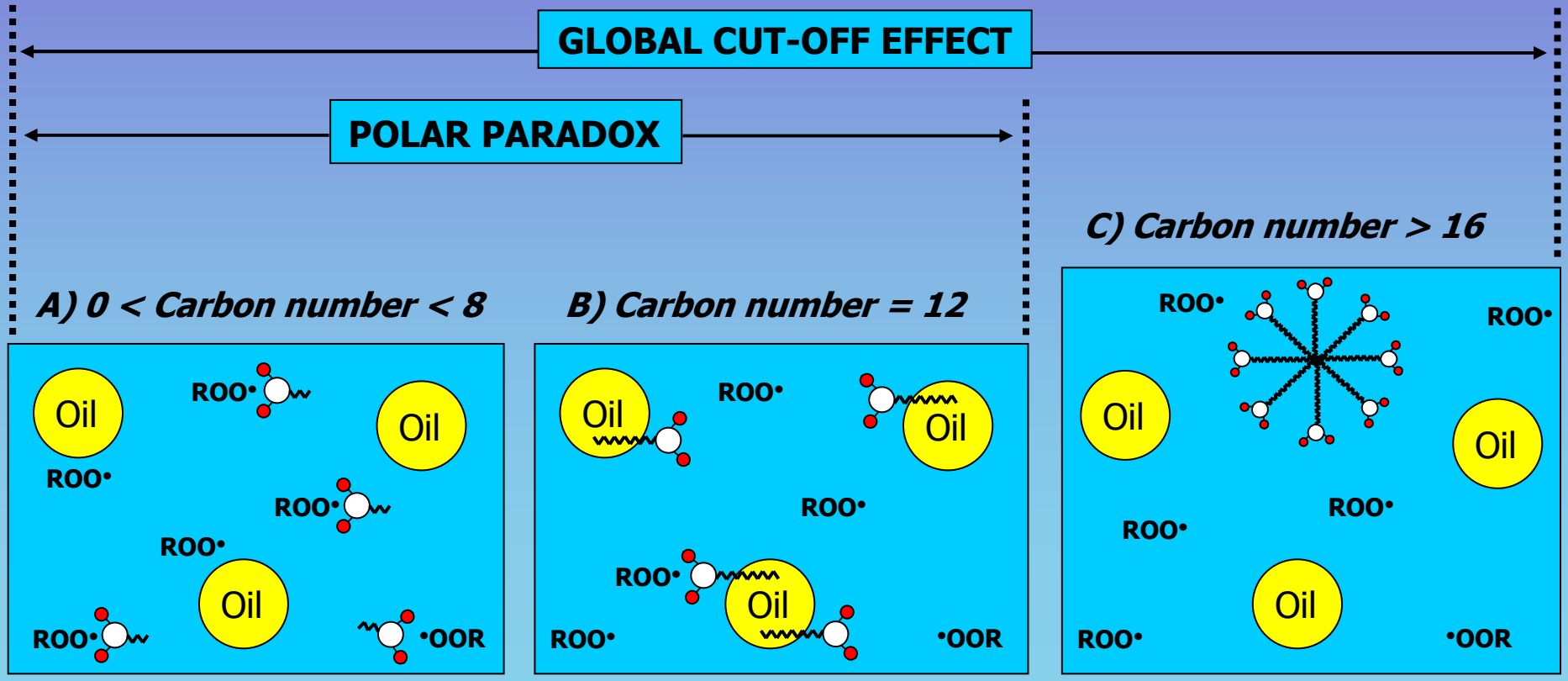


14) ABOVE 16 CARBONS : A STRONG DIFFERENCE

When the antioxidant hydrophobicity increases, the Polar paradox does not take into account the progressive predominance of the **antioxidant's accumulation in the water phase** by a micellization process, compared to its **anchorage in the oil-in-water interphase**

Beyond the Polar paradox

The Polar paradox appears to be a « **PARTICULAR CASE** » of a far more global « **CUT-OFF EFFECT** » observed here



15) HOW TO EVALUATE THE ANTIOXIDANT CAPACITY OF PHENOLICS ?

→ By means of multiple methods, in a resolutely **multidimensional approach**

Frankel and Meyer, *J. Sci. Food Chem. 2000 ;* Laguerre *et al., Prog. Lipid Res. 2007*

→ These assays must be performed in experimental conditions as near as possible the **natural conditions** (t°C, oxidizable substrate, antioxidant concentration range...).

In this way, as lipid are prime oxidative target in food and biological samples, our test seems to be well adapted to the antioxidant evaluation in the specific **lipid oxidation context**

CAT ASSAY IN FIVE ADVANTAGES

1. **Representative :** TAGs substrate possesses a triglyceridic skeleton
2. **Realistic :** Excess of substrate compared to antioxidant (physiologically relevant)
3. **Easy-to-use :** Commercially available cheap substrate / Use of a spectrophotometer
4. **High-throughput :** Adapted to a microplate reader (96-well microplate)
5. **Versatile :** Possibility to assess both hydrophilic and lipophilic molecules

16) WHAT ARE THE MAIN FACTORS GOVERNING IT ?

Table : Antioxidant effectiveness order of 17 phenolic compounds

Compound	CAT Value (TEq[a])
Eicosyl chlorogenate (C20)	0.32 ± 0.02
Gallic acid	0.43 ± 0.05
Octadecyl chlorogenate (C18)	0.88 ± 0.04
Ferulic acid	1.05 ± 0.04
Protocatechuic acid	1.66 ± 0.02
Butyl chlorogenate (C4)	1.76 ± 0.08
Methyl chlorogenate (C1)	1.94 ± 0.02
Octyl chlorogenate (C8)	1.94 ± 0.10
Chlorogenic acid (C0)	2.21 ± 0.14
Myricetin	2.35 ± 0.08
(-)-Epicatechin	2.39 ± 0.03
Hexadecyl chlorogenate (C16)	2.43 ± 0.26
Caffeic acid	2.91 ± 0.07
Dodecyl chlorogenate (C12)	2.97 ± 0.13
Catechin	3.00 ± 0.08
Quercetin	3.18 ± 0.15
Rosmarinic acid	4.21 ± 0.77

[a] Mole of Trolox/mole of compound

ANTIOXIDANT CAPACITY

Ability of phenolic compound to donate a H atom to a free radical is at once modulated by:

1. **the number of phenolic hydroxyls**
2. **their position on the aromatic ring(s)**
3. **the location and the mobility of the phenolic compound towards the oxidizable substrate**

FUTURE PROSPECTS

Further investigations must be done to determine the weight to be allocated to each factor

THANK YOU FOR YOUR ATTENTION

(AND SORRY FOR MY TERRIBLE FRENCH ACCENT)