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# **Origin of the Variability of the Antioxidant Activity Determination of Food Material**

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Additional information is available at the end of the chapter

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## **1. Introduction**

With the development of functional foods having beneficial effects for the health, the interest of scientists, consumers and industrialists in raw materials rich in antioxidants has increased considerably over last few years. Moreover, consumers require more accurate information on the composition of the food which they eat. Natural antioxidants such as phenolic compounds have been reported to possess beneficial bioactivities due to their capacity to act as antioxidant; anticarcinogenic, antibacterial, antimutagenic, anti-inflammatory, and antiallergic. These activities contribute to bringing a feeling of well-being to consumers. In fact, it was observed that the consumption of foods rich in antioxidants leads to the decrease of some diseases such as diabetes, cancer, cardiovascular or neuronal diseases [1]. To evaluate the antioxidant activity of raw materials several methods were investigated. These methods go through two steps. The first one is the extraction of the antioxidant compounds from the matrix of raw material and the second one consists in the determination of the antioxidant activity. For each step several alternatives are described in the bibliography. The data obtained showed a large variability depending on the method used. This renders the choice of an appropriate method a very sensitive task. The objective of this chapter is to make a critical study of the various methods of evaluation of the antioxidant activity of food raw materials. For this, in the first part, we will underline the different sources of variability from several examples of food raw materials. Then in the second part the different methods used for the extraction of antioxidants will be detailed. The third part will bring a complete view of the existing methods to measure antioxidant activity. The last part will deal with a general discussion on the meaning of the different values of antioxidant activity.

## 2. Identification of variability factors on the measurement of antioxidant activity

The scientific community is facing an accumulation of data on antioxidant activity. These data indicated a great variability depending on the method used, which does not render their comparison easy. To understand such variability, we analyzed several cases to highlight the factors that could be at the origin of the variability observed.

### Case 1: Investigation of the antioxidant activity of pure components

The antioxidant activity obtained by different methods (ABTS, DPPH and ORAC) of 25 phenols was compared. The results obtained are summarized in table 1.

		ABTS	DPPH	ORAC
Flavonols	Q	1.8	0.9	4.2
	K	0.5	0.8	6.2
	M	1.5	1.8	3.6
	Q-R	0.6	1.0	4.6
	K-G	0.2	0.0	6.6
	M-R	6.6	2.0	6.0
Anthocyanins	C	2.0	0.5	4.4
	D	2.3	1.5	3.8
	C-G	1.9	0.6	7.3
	C-R	1.7	0.8	5.5
	C-GA	2.3	0.5	5.8
	D-G	3.5	1.4	5.9
Flavanones	HE NA	0.5	0.0	4.5
		0.0	0.0	5.6
Flavan-3-ols	CA	1.1	0.8	7.9
	ECa	1.3	1.0	5.1
	EGCa	1.2	1.5	3.1
	GCa	4.9	8.5	8.3
	EGCaG	2.0	3.7	3.4
Phenolic acids	GA CA EA	2.1	0.9	1.0
		1.3	0.9	5.3
		0.7	0.8	2.9

With: AA (ascorbic acid); C (cyanidin); C-G (cyanidin-3-O-glucoside); C-Ga (cyanidin-3-O-galactoside); C-R (cyanidin-3-O-rutinoside); Ca (catechin); CA (chlorogenic acid); D (delphinidin); D-G (delphinidin-3-O-glucoside); EA (ellagic acid); ECa (epicatechin); EGCa (epigallocatechin); EGCaG (epigallocatechin gallate); GA (gallic acid); GCa (gallocatechin); Gl (reduced glutathione); HE (hesperidin); K (kaempferol); K-G(kaempferol-3-O-glucoside); M (myricetin); M-R (myricetin-3-rhamnoside); NA (naringenin); Q (quercetin); Q-R (rutin); T (Trolox).

**Table 1.** Antioxidant values of phenolic compounds measured by ABTS, DPPH and ORAC methods [2]

It appears that the values obtained of antioxidant activities are not of the same order of magnitude; the ORAC method gives the highest values followed by the ABTS and the DPPH method. Moreover, phenolic compounds having a high antioxidant activity with a given method may have low antioxidant activity with another method. For example, kaempferol 3-glucoside and quercetin have respectively an antioxidant value of 6.6 and 4.2  $\mu\text{mol Trolox}$  with the ORAC method and only 0.2 and 1.8 with the ABTS method. The variability observed is due only to the method used. So, it seems that it is difficult to compare the numerical values of antioxidant activity provided by different methods of determination.

To analyse the effects of the extraction step and the variety of food, the antioxidant activities of extracts resulting from different raw materials such as onions and apples were investigated.

### Case 2: Antioxidant activity of extracts from the food matrix

To highlight on the effect of the origin and the variety of food raw materials, [3] evaluated the total antioxidant capacity (TAC) by three methods (ABTS, FRAP and DPPH) using four varieties of onions with a similar procedure of extraction (methanol:water (70:30 v/v)). The activities obtained are summarized in table 2.

	ABTS ( $\mu\text{mol Trolox/g FW}$ )	FRAP ( $\mu\text{mol Trolox/g FW}$ )	DPPH ( $\mu\text{mol Trolox/g FW}$ )
White onion	11.82 $\pm$ 2.16	4.38 $\pm$ 0.40	3.04 $\pm$ 0.18
Yellow onion	15.22 $\pm$ 2.36	5.32 $\pm$ 0.59	4.56 $\pm$ 0.40
Red onion	28.18 $\pm$ 4.59	5.76 $\pm$ 0.47	5.20 $\pm$ 0.28
Sweet onion	10.56 $\pm$ 1.15	2.48 $\pm$ 0.19	1.42 $\pm$ 0.13

**Table 2.** Antioxidant activity of different onion varieties (*Allium cepa*).obtained with ABTS, FRAP and DPPH methods

These results indicate significant variations of the antioxidant activity depending on the variety tested. Antioxidant activity of the red onion is higher than that of white one. The magnitude of the variation is different according to the method used. For example, there is a factor of 2.4 between the red and white onion with ABTS, whereas this factor is only of 1.3 for FRAP and 1.7 for DPPH. For a given variety, the ABTS values are two times higher than FRAP or DPPH values, while values obtained by FRAP and DPPH are closer and sometimes do not indicate any difference between varieties. This behavior could be attributed to the fact that the active compounds in the origin of the antioxidant activity are not similar for the four varieties tested. This fact will have to be taken into account to compare the antioxidant activity obtained by different methods of determination.

To analyze the effect of the step of extraction, we gathered several results on the antioxidant activity of the golden delicious apple using different methods of determination (ABTS, FRAP and ORAC). The results obtained are given in table 3.

	ABTS	FRAP	ORAC
Ultrasound extraction with methanol	6.7	3.5	7.8
Extraction in plasma	8.3	4.4	9.15
Solvent extraction with methanol	40	–	–
Solvent extraction with methanol/ water (80%v/v)	4.62	–	–
Solvent extraction with acetone (70%), water (28%), and acetic acid (2%)	–	–	26.47

**Table 3.** Total antioxidant capacity of golden delicious apples (in  $\mu\text{mol TE/g FW}$ ) according to [4-7].

These results indicates that for a given method, the extraction procedure has a great impact on antioxidant activity values. For example with the ABTS method, using an extraction with methanol as solvent and assisted by ultrasound, this leads to  $6.7 \mu\text{mol TE/g FW}$ ; while the extraction with a mixture of methanol and water (80%v/v) or with acetone furnishes only  $0.94 \mu\text{mol TE/g FW}$ . The measurement of bioavailability directly in plasma gives a value of  $8.3 \mu\text{mol TE/g FW}$ . Different values are also obtained depending on the method of the extraction used with FRAP or ORAC protocols.

The analysis of the results of antioxidant activity of pure components and extracts from the food matrix indicates a broad variability in antioxidant values whatever the method used. This variability is also observed for a given method with the variety or the degree of maturation of the food raw material. This variability of the antioxidant activity determination can be attributed to three sources (i) factors related to food products such as the variety, and the growth method. (ii) Factors related to the extraction method such as pH, temperature, solvent, presence of an accelerator and (iii) factors related to the method used for the antioxidant activity determination.

### 3. Extraction techniques

To measure antioxidant activity of food raw materials, the active molecules must be extracted from the food matrix. The processes of extraction of the phenolic compounds are affected by several factors such as the pH, the temperature, the solvent used. Thus, the optimization of this step requires a judicious choice of the set points of these factors. However, in the bibliography few studies have been devoted to the optimization of these factors.

Moreover, these factors need to be adjusted according to the matrix of the raw material and the quantity of antioxidant molecules. To help in the choice of the most suitable method of the extraction, the main processes described in the literature are summarized in table 4. The advantages and the drawbacks of each process are also reported.

Technique	Principle	Tool	Advantages	Drawbacks	Example of use
Solid-liquid extraction (SLE) [8]	Extraction with a solvent. Basically methanol based on maceration then filtration and evaporation of the solvent.	Appropriate solvent.	Simple. Popular.	Toxic solvent (methanol). High extraction time and low yield. Extracts not suitable to be added to food. Not safe for the environment.	Extraction of antioxidants from PistaciaLentiscus.
Ultra-Sound assisted extraction (UAE) [9,10]	Sonication of a sample-solvent mixture.	Ultrasounds with determined frequency and duration. Appropriate solvent.	More effective Reduction of the dependence on the extraction solvent Yields greatly enhanced with ethanol.	Less effective than microwave for example.	Extraction of antioxidants from rosemary leaves.
Microwave assisted extraction (MAE) [11-13]	Extraction of a sample-solvent using microwave energy.	Microwaves with determined power and duration. Appropriate solvent.	Reduced extraction time Minimized solvent volume Saved energy High extraction yields Increased solubility of phenolic compounds in solvents.	-	Tea polyphenols.
Pressurized Liquid Extraction (PLE) [14]	High temperature or pressure.	Appropriate solvent.	Faster extraction procedure Good recoveries of phenolic compounds from solid residues. Reduce waste generation, and improve sample throughput. Reduce extraction time.	-	Extraction of phenolic compounds from olive cakes.
Supercritical fluid extraction (SFE) [15]	Combination of low temperature and high	Supercritical fluid.	Low temperature used Reduced energy consumption	-	Extraction of peach almond oil.

Technique	Principle	Tool	Advantages	Drawbacks	Example of use
	pressure with a supercritical fluid.		Efficiency in solvent use Recycling possibility Prevention of oxidation reactions High product quality. Absence of solvent in solute phase. Flexible process.		
			High extraction capacity. Mild conditions. Low cost.		
Aqueous two-phase extraction (ATPE) [16]	Extraction in an aqueous two-phase system.	Short chain alcohol/hydrophilic organic solvents. Inorganic salts.	Short process time without back extraction. The potential to achieve the desired purification and concentration of the product in a single step.	No reports on the use of the ATPE to extract and purify anthocyanins.	Extraction of mulberry anthocyanins.

**Table 4.** Advantages and drawbacks of the main extraction methods used

Different processes of extraction of active compounds are available. However, the effectiveness of these processes is affected by several factors such as the nature of the solvent, the temperature or the extraction time. The presence of an accelerator of extraction such as microwaves or ultrasounds also plays significant role. The availability of the active molecule will be also taken into account. The analysis of the efficiency of the different processes described above indicates that the use of accelerators provides higher yields than the solid-liquid extraction (SLE) while allowing a low temperature to be maintained. The least advantageous method is the solid-liquid extraction due to the toxicity of solvent and the long time extraction in the majority of cases. The use of microwaves (MAE) as accelerator is highly acclaimed as an alternative method. The use of ultrasounds (UAE) also allows an enhancing of the extraction of active compounds at low temperatures but it leads to lower yields than with microwaves. Other accelerators can be used (ASE, PLE) but they need to increase the pressure and/ or the temperature, which can damage target molecules or alter their properties. Supercritical fluid extraction (SFE) does not use drastic conditions but the molecules extracted must be soluble in liquid CO<sub>2</sub>. The use of a co-solvent may be necessary if antioxidants are poorly soluble in CO<sub>2</sub>. So, the difference in the efficiency of the different extraction methods used for antioxidant activity determination could be at the origin of the variability observed in the bibliography.

Thus, the choice of a method of extraction needs to take into account the nature of the food matrix and the structure of the molecule to be extracted. The physico-chemical factors of the extraction must be also adjusted carefully. In conclusion there is a great need to standardize the methods of extraction by establishing different protocols and pay attention to different conditions.

#### 4. In vitro methods for antioxidant activity measurement

An antioxidant is usually defined as a molecule which delays, prevents or removes oxidative damage to a target molecule [17], thus an antioxidant is assessed according to its ability to neutralize free radicals as for example in equation 1 to avoid oxidative degradations.



AO: antioxidant molecule,  $\text{FR}\cdot$ : free radicals



AH : antioxidant molecule,  $\text{R}\cdot$ : free radicals

Free radicals are reactive oxygen species produced either through numerous biological reactions: mitochondrial respiratory chain or any inflammatory conditions, or from numerous environmental factors such as pollutants, U.V., alcohol, smoking, stress, drugs,... Free radicals are useful if they are in low quantity; they allow the elimination of old cells of the living organism by oxidation reactions or participating in the body's defense. However if they are too numerous, they attack other cells inducing a rapid aging of these cells which causes damage to living organisms. To avoid these reactions, antioxidants can neutralize free radicals and protect our cells. When antioxidant quantity is not enough to neutralize free radicals, it leads to the oxidative stress which has a great importance in the development of chronic degenerative diseases including coronary heart disease, cancer and the degenerative processes associated with aging.

Antioxidants can neutralize radicals by two different mechanisms. The final product will be the same but reactions occurring are different. Radicals can be deactivated either by hydrogen donation (Hydrogen Atom Transfer HAT) or by electron transfer (Single Electron Transfer SET). HAT and SET mechanisms may occur in parallel, the predominant mechanism being determined according to antioxidant structure and properties, solubility, partition coefficient, and system solvent [18]. A wide variety of one-dimensional methods have been developed to measure antioxidant activity in vitro. The methodological diversity is due to the use of a broad range of conditions for antioxidant activity measurement. This diversity has led to widely conflicting results that are extremely difficult to interpret.



#### 4.1. Systems based on SET

SET-based methods involve two components in the reaction, i.e. the antioxidant and the oxidant. These methods measure the ability of an antioxidant to reduce any compound (metals, radicals) by electron transfer according to equations 3 and 4.



SET reactions are pH dependent. Indeed, relative reactivity in SET methods is based primarily on deprotonation and the ionization potential of the reactive functional groups. Ionization potential decreases when pH increases, so SET reactions are favored in alkaline environments. SET reactions are usually slow and can require a long time to reach their final state, so antioxidant capacity calculations are based on the decrease in product concentration rather than their kinetic.

- **ABTS (2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay**

ABTS assay is a spectrophotometric method which measures the ability to an antioxidant to scavenge a free radical cation ABTS<sup>•+</sup>. This method was developed by [19] and adapted by [20] to generate directly the radical ABTS<sup>•+</sup> through a reaction between ABTS solution (7mM) with potassium persulfate (2,45 mM) in water. The reaction mixture, which is allowed to stand at room temperature for 12-16 h before use, produces a dark blue solution. Thus, the mixture is diluted with ethanol or phosphate buffered saline (pH 7.4) to a final absorbance of 0.7 at 734 nm (wavelength the most used) and 37 °C. The assay is based on the discoloration of ABTS<sup>•+</sup> during its oxidation by antioxidant compounds, thus reflecting the amount of ABTS radicals that are scavenged within a fixed time period (generally 6 min). The absorbance of the reaction mixture between radicals and antioxidants is compared to that of the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). When Trolox is used as standard, this assay is also called Trolox Equivalent Antioxidant capacity (TEAC) assay.

The major advantages of this method are its simplicity to perform and its applicability in lipid and aqueous phases [21]. Thus this method has been widely used in testing antioxidant capacity in food samples. Moreover, the ABTS radical is stable over a wide pH range and can be used to study pH effects on antioxidant mechanisms [22]. This method can be automated and adapted to the use with microplates which allows the carrying out of this measurement with better precision and time.

A major disadvantage of this method is that only the rapid oxidation reactions can be measured because incubation time is often short (6 min). Thus, antioxidants whose constant rates of radical scavenging are low can be undervalued in comparison with their real antioxidant capacity. Moreover, imprecisions on ABTS values can be increased by the fact that variations can occur according to the preparation of ABTS<sup>•+</sup>, and the medium temperature which has to be controlled.

- **DPPH (2,2-diphenyl-1-picrylhydrazyl) assay**

DPPH is one of the oldest and most popular technique used to measure the antioxidant activity of a compound. This method was first described by [23] and subsequently modified by numerous researchers. This method measures the reducing ability of antioxidants toward DPPH $\cdot$ . DPPH $\cdot$  is commercially available and does not have to be generated as for ABTS assay. The antioxidant effect is proportional to the disappearance of DPPH $\cdot$  in a methanolic solution. DPPH solution being purple, the absorbance of the mixture can be followed by spectrophotometry at 515 nm. Assay time may vary from 10±20min up to 6h. Other techniques such as electron spin resonance (EPR) can be used [18].

Like ABTS, this method is simple and can be automated. However, values found by the DPPH method have to be considered as apparent antioxidant activities because (i) DPPH color can be lost via either radical reaction (HAT) or reduction (SET) as well as unrelated reactions, (ii) steric accessibility also influences the reaction, thus small molecules are favored because they have a better access to the radical site and other compounds such as carotenoids can interfere in the measurement of the antioxidant activity [24].

- **Ferric reducing ability of plasma (FRAP)**

The FRAP assay is different from the others as there are no free radicals involved but the reduction of ferric iron (Fe<sup>3+</sup>) to ferrous iron (Fe<sup>2+</sup>) is monitored. FRAP assay was initially described by [25] for measuring reducing power in plasma and subsequently adapted and modified by numerous researchers to measure antioxidant power of botanical extracts [26]. When an Fe<sup>3+</sup>-TPTZ (2,4,6-tripyridyl-s-triazine) complex is reduced to Fe<sup>2+</sup> by an antioxidant under acidic conditions, it forms an intense blue color with absorption maximum at 593nm. Thus the antioxidant effect can be followed by a spectrophotometer.

A major advantage of the FRAP assay is its simplicity, speed and robustness. The validity of this assay was proved in order to quantify samples with hydrophilic and lipophilic antioxidants. As for ABTS assay, only rapid reactions will be taken into account until the incubation time in this method is short (4-6 min). The FRAP assay measures only reactions following the SET mechanism, antioxidant hydrogen donator may go unmeasured by this assay. This method is thus used in parallel with others to determine the action mechanisms of antioxidants. Protein and thiol antioxidants, such as glutathione cannot be measured by the FRAP assay.

- **CUPric Reducing Antioxidant Capacity (CUPRAC) assay**

The CUPRAC assay has many similarities to FRAP, Cu is used instead of Fe. This assay is based on the reduction of Cu (II) to Cu (I) by the antioxidants present in the sample. Cu (I) forms a complex with neocuproine (2,9-dimethyl-1,10-phenanthroline) with a maximum absorbance at 450 nm. A dilution curve generated by uric acid standard is used to convert sample absorbance to uric acid equivalents [18]. Phenanthroline complexes have very limited water solubility and must be dissolved in organic solvents. Cuprac values are comparable to TEAC values, whereas FRAP values are lower. The CUPRAC assay has many advantages [27]. Indeed, the CUPRAC assay is more selective due to its lower redox potential. Sugars and citric acid cannot interfere in the assay because they are not oxidized in CUPRAC. The CUPRAC

reagent is much more stable than other radicals such as DPPH, ABTS. The redox reaction giving rise to a coloured chelate of Cu(I)-Nc is relatively insensitive to a number of parameters such as air, sunlight, humidity, and pH. The CUPRAC reagent can be adsorbed on a membrane to build an optical antioxidant sensor.

A variant of CUPRAC assay is Bioxytech using bathocuproine instead of neocuproine [18].

#### 4.2. Systems based on HAT

The HAT-based methods involve a synthetic radical generator, oxidisable molecular probe and an antioxidant compound. This method measures the ability of an antioxidant to quench free radicals by hydrogen donation as in equation 2. Assays that are based on HAT mechanisms measure competitive kinetics [22].

Antioxidant with hydroxyl component OH donates an H atom to an unstable free radical to give a more stable radical. HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. The presence of reducing agents, including metals, is a complication in HAT assays and can lead to erroneously high apparent reactivity [18].

- **Oxygen radical absorbance capacity (ORAC)**

The ORAC assay has been used widely in measuring the net resultant antioxidant capacity (or peroxy radical absorbance capacity) of botanical and other biological samples.

The ORAC assay was developed by [28] for the determination of reactive oxygen species in biological systems. [29] modified the method using fluorescein (FL) as a more stable and reproducible fluorescent probe. This method exists under several adaptations but the principle always remains the same: using a fluorescent probe and AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) to generate peroxy radicals. A HAT reaction occurs between antioxidant samples (or standard) and the peroxy radicals generated by thermal degradation of AAPH. These reactions lead to a loss of fluorescence measured at 515 nm.

The final results (ORAC values) were calculated using the differences between blank and sample areas under the quenching curves of fluorescein, and were expressed as micromoles of Trolox equivalents (TE).

The ORAC method is superior to similar methods because it combines inhibition time and inhibition degree of free radicals. The ORAC using fluorescein is specific for antioxidants and is sensitive, precise and robust. This assay can model reactions of antioxidants with lipids in both food and physiological systems and it can be adapted to detect both hydrophilic and hydrophobic antioxidants with minor modifications. However, the need of a fluorometer, which may be not routinely available, is considered as a disadvantage of this method. The long analysis time has also been a major criticism even if this assay can be automated.

- **$\beta$ -carotene bleaching test**

This assay was developed by [30] and modified by other researchers. This assay is based on the generation of a stable  $\beta$ -carotene radical from  $\beta$ -carotene peroxy radical; the latter

coming from lipids (linoleic acid for example) in the presence of ROS and O<sub>2</sub>. Thus, the assay measures the ability of an antioxidant to quench β-carotene radical by donating hydrogen atoms. It results in the bleaching of the solution which can be followed with a spectrophotometer at 470 nm.

The main advantage of this assay is its applicability in both lipophilic and hydrophilic environments. Another advantage is that the carotenoid bleaching assay can detect either the antioxidant or pro-oxidant action of a compound under investigation. Lastly, the carotenoid bleaching assay can be automated by the use of microplates. However, a major limitation is that the discoloration of β-carotene at 470 nm can occur through multiple pathways, thereby complicating the interpretation of results. Other carotenoids such as crocin bleach only using the radical oxidation pathway but crocin is not commercially available. The use of molecules commercially available provide repeatable and reliable data between laboratories

- **Total peroxy radical-trapping antioxidant parameter assay (TRAP)**

The total peroxy radical-trapping antioxidant parameter (TRAP) assay was introduced by [31] to measure the total antioxidant status of human plasma. This assay monitors the ability of an antioxidant to interfere with the reaction between peroxy radicals generated by AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) and the target. The oxidation is monitored by oxygen uptake measurement. Results are expressed as time necessary to consume all radicals in comparison with a standard (Trolox). Many modifications were realized on this assay to react with lipids, or to be followed by fluorimetry or to take into account interference from lipids and proteins in plasma [32]. Despite its simplicity, the TRAP assay leads to imprecise results because of difficulties to maintain the endpoint over the period of time. Several modifications were developed by using chemiluminescence methods [33].

## 5. Discussion – Conclusion

Many results on the determination of the antioxidant activity of purified molecules and / or food raw material have been published over the last few years. However, the obtained data present a broad variability even for a given method or a given molecule. To overcome these problems, some authors have proposed other alternatives by developing new methods, or new ways to process data and express the results. [34] proposed the « quencher method », where the antioxidant activity is directly measured from the solid sample without the extraction step. Free radicals are mixed with the food sample and a spectrophotometric method (ABTS, DPPH) was used. [35] developed the global antioxidant response (GAR) method which uses an in vitro approach with enzymatic digestion, designed to mimic digestion through the gastrointestinal tract aimed at releasing antioxidants in foods. [36] suggested a general method of standardization of estimations of total antioxidant activity (TAA) by extrapolating parameters to zero sample concentration based on a pseudo-first-order kinetics model. Accurate results were obtained in comparison with the ABTS method. Moreover, several papers deal with the standardization of the extraction procedures and the results analysis for a given method in order to minimize the observed variability [37]. However, it appears difficult to find a universal

method knowing that many kinds of antioxidants and radicals are present. Four general sources of antioxidant have been identified: (i) enzymes (superoxide dismutase, glutathione peroxidase), (ii) large molecules (albumin, ferritin), (iii) small molecules (phenols, ascorbic acid, carotenoids) and (iv) and some hormones (estrogen, melatonin). Many kinds of free radicals can be found, for example  $O_2^{\cdot-}$ ,  $HO^{\cdot}$ ,  $NO^{\cdot}$ ,  $RO(O)^{\cdot}$ ,  $LO(O)^{\cdot}$ . Moreover, the stability, the selectivity of the radicals and the reaction mechanisms can be also different. Thus, it is possible that no single method may be able to express the antioxidant capacity of different antioxidants taken independently or in a mixture [18]. Previous studies demonstrated that it is not appropriate to use one-dimensional methods to evaluate the antioxidant activity of multifunctional food such as fruits and vegetables, since they contain a large diversity of natural antioxidants.

The determination of antioxidant activity in the food matrix needs a sample preparation to extract the active molecules and then an accurate method of measurement and an expression of the results. (i) During sample preparation, precautions must be taken to avoid the loss of antioxidants due to the drastic conditions of extraction. A determination of all food constituents is necessary because a certain interference with antioxidants can occur. Antioxidant capacity values should only be compared where the method, the solvent and the analytical conditions are similar [38]. Indeed, some authors underlined that there is an effect of the solvent used for the extraction or used to solubilize antioxidants on the result of the antioxidant activity evaluation [39-42]. This is due to interference of the reaction mechanism and the solvent [38]. (ii) The method to measure the antioxidant activity must be chosen according to the nature of the active molecules present in the samples. Some methods described in part 3 are more appropriate for some kinds of antioxidants. For example the DPPH method is more adequate to lipophilic systems. Moreover, several assays must be carried out to determine a value of antioxidant activity. (iii) Results of antioxidant activity measurement can be expressed as EC50 (quantity of antioxidant necessary to assure 50% depletion of free radicals), tEC50 (time to reach 50% depletion of free radicals), tEC50 (time to reach 50% depletion of free radical) or AE (antiradical efficiency defined as the inverse of the product between EC50 and tEC50). Thus, taking these 3 parameters into account can be relevant to have a more comprehensive evaluation of antioxidant activity [38].

The determination of the antioxidant capacity by *in vivo* methods is not always feasible but it appears interesting because it simulates an environment closer to that really happening in biological systems. Methods using HAT reactions will be preferred to SET reactions because peroxy radicals used in HAT assays are the predominant free radicals found in lipid oxidation and biological systems. To elucidate a full profile of antioxidant capacity against various ROS, the development of different methods specific for each ROS/ RNS may be needed. [18] proposed a comparison of different *in vitro* methods; conclusions given that ORAC, TRAP and LDL are considered to be the most biologically relevant assays [18] because the antioxidant capacity measured reflects closer the *in vivo* action of the antioxidants. So, it appears clearly that the antioxidant activity determination needs a standardization of the procedure used and a combination of at least two or three methods. The use of only one method does not reflect the antioxidant activity of food raw material due the variability of the molecules that act as antioxidant.

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