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Antioxydant activity, oxidative stability properties of Colza oil, comparison of mechanical agitated and ultrasonic extraction on green tea catechins of *Camellia sinensis* L.

Z. Amrouche[1,2,4*], C. Blecker[2], H. Laribi-Habchi[4], M.L Fauconnier[3], D. El-Hadi[4]

[1] Biological Department, Faculty of Science of the nature and the life, University of Djilali Bounaam, Khemis Miliana- Algeria.
[2] Food science and formulation unit Agro-biotech Gembloux, University of Liege-Belgium.
[3] General and organic chemistry laboratory Agro-biotech Gembloux, University of Liege-Belgium.
[4] Functionnal analysis of chemical process laboratory, Industrial Chemistry Departement, University of Blida 1-Algeria.

*Corresponding author: zoheir.amrouche@yahoo.fr Tel.: + 213 554 95 75 02;

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ABSTRACT/RESUME

Abstract: *Ultrasonic extraction "UE" used to optimize the extraction yield of phenolic compounds "PC" from green tea Camellia sinensis L., and compared with mechanical agitated extraction "MAE".*
UE was applied at different times (15, 10 and 5min) and temperatures (25, 60 and 95°C) and MAE was performed at these experimental conditions (15 min, 95°C, 400 rpm). Results demonstrate that the maximum yield of epigallocatechin 3-gallate "EGCG" extracted by UE was significantly (P < 0.05) higher than that obteined using MAE (136 mg/g vs 100 mg/g, respectively). The optimum conditions for the polyphenol compounds "PC" recovery are obtained using UE during 15 min at 95°C (~134.66 mg/g). Four catechins from extracted PC were identified using high-performance liquid chromatography equipped with a diode array detector and liquid chromatography mass spectrometry "HPLC-DAD & LC-MS": epigallocatechin "EGC", epicatechin "EC", epigallocatechingallate "EGCG", and epicatechin-gallate "ECG". EGCG is the major compound in polyphenol extracts representing 60 %. The antioxidant capacity of the obtained extracts was also studied. Diphényl-2-pycril-hydrazyl "DPPH" scavenging activity is higher for UE than MAE (~ 90 % vs ~85%). Moreover, the PC obtained by UE added to colza oil had a higher oxidative stability, determined by rancimat than those extracted by MAE method (~30.62 h vs ~21.26 h). Results indicate the suitability of UE method for production of PC as potent antioxidant for stabilization of vegetable oils such as colza oil.

I. Introduction

Green tea (*Camellia sinensis* L.) is the second most common beverage in the world next to water. Its habitual consumption has long been associated with health benefits [1]. Green tea is now moving from a traditional beverage to a healthy drink, particularly rich source of polyhydroxy flavan-3-ol derivatives, integrating catechin [2], a source of pharmacologically active molecules, an important member of the antioxidant food group, and a functional food endowed with beneficial health properties [3].
To take profit from these molecules, it is necessary to extract them. The extraction of phenolic compounds from tea is dependent on both time and temperature [4]. The antioxydant activity of polyphenol extracts can be proven by measuring the inhibition of lipid oxidation, for example, the increasing induction period of oil oxidation [5].

Accordingly, due to its strong antioxidant properties, researchers have proposed including green tea as an ingredient in food [6]. Lante and Dario (2013) [7] studied the oxidative stability of the nanoemulsion of the green tea polyphenols comparison in the result; the oxidative stability is made by the colza oil and not by emulsion.

The aim of this work was to optimize the extraction yield of phenolic compounds "PC" from green tea (*Camellia sinensis* L.) by ultrasonic extraction "UE" applied at different time (15, 10 and 5 min) and temperature (25, 60 and 95°C) than compared of mechanical agitated extraction "MAE" time (15min), temperature (95°C) recommended by preceding search. The identification of catechins extracted phenolic compounds using high-performance liquid chromatography equipped with a diode array detector and liquid chromatography mass spectrometry "HPLC-DAD & LC-MS spectrometry". The antioxidant effect and oxidative stability by diphényl-pycril-hydrazyl "DPPH" sacavenging activity and rancimat test respectively.

II. Materials and methods

II.1. Polyphenols extract green tea infusions

II.1.1. Mechanical agitated extraction (MAE)

The commercial green tea leaves were mixed with distilled water (1:10) for 15 min at 95 °C. After each infusion time period, the tea was immediately cooled to room temperature and filtered through filter paper. The filtrate was centrifuged for 15 min on 5.000g at 4°C.The supernatant was then frozen to - 45°C and lyophilized by CHRIST Gamma 2-16 LSCplus, Germany. The lyophilized powder was stored in darkness at 4 °C until used.

II.1.2. Ultrasonics extraction (UE)

Tube which contained 50 mL distilled water, ultrasonic (CPX750, 750w, 20kHz, Julabo Cole Parmer ultrasonic processor, Germany) at low temperature 25°C, 60 °C and 95°C, transmitted a 10000 watts energy (10s/10s) for 15 min, 40 min and 90 min. Infusions were centrifuged at 5000g for 15 min at 4°C and filtered using 0.45µm filter membranes (Millipore, MA, USA). The upper solution was taken and lyophilized by CHRIST Gamma 2-16 LSCplus. The lyophilized powder was stored in darkness at 4 °C until used.

II.2. Phenolic compound analysis

II.2.1. Total phenolics content

Total phenolics of green tea extracts were measured by spectrophotometric analysis (Thermo Electron Corporation) using Folin–Ciocalteu's reagent (Merck, Darmstadt, Germany), described by Kedare and Singh (2011) [8]. This method is based on the reduction of Folin-Ciocalteu reagent by the electrons from the polyphenols [9], reduction of phosphotungstic acid ($H_3PW_{12}O_{40}$) in alkaline solution to phosphotungstic blue (based on $H_3PMo_{12}O_{40}$). The absorbance of formed phosphotungstic blue is proportional to the number of aromatic phenolic groups and is used for their determination, expressed with gallic acid as the calibrant [10]. Total phenolics were expressed as mg gallic acid equivalents (mg GAE)/g of green tea extract.

II.2.2. Total flavonoid content

The quantification of the flavonoids contents are estimated by the $AlCl_3$ (aluminum trichloride solution) method, according to the protocol modified by Kumaran and Karunakaran (2006) [11]. A mix of green tea extract and $AlCl_3$ at a concentration of 20 mg/mL (1:1; v/v), and a drop of acetic acid is added. The all were Incubated at room temperature during 40 min in the darkness. The absorbance is measured in 415 nm. In the same conditions, the standard solution of quercetin is prepared with a concentration equal to 0.1mg/mL. The rate of flavonoids contained in the polyphenols extract is calculated according to the following equation: $X = (A.m_0)/(A_0.m)$, where A is the absorbance extract of green tea, A_0 absorbance of the solution quercetin, m mass of the extract of tea (mg), m_0 mass of the quercetin in the solution. Total flavonoids (X) were expressed as mg quercetin equivalents (mg QE)/g of green tea extract.

II.3. HPLC- DAD & LC-MS analysis of phenolics

High-performance liquid chromatography "HPLC" analysis of freeze-dried polyphenols ultrasonic extraction "UE" and mechanical agitated extraction "MAE" were identified and quantified on an Agilent 1100 series HPLC, equipped with a diode array detector "DAD" (Agilent Technologies, Santa Clara, CA, USA). Separations were performed on a Halo C18 column (4.6 x 75 mm, 2.7µm) (advanced materials technology, USA). The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The column temperature was 30 °C, and the detector was set to a wavelength range of 190–400 nm. The analysis time course was 50 min. The composition of peaks was identified from the times of retention obtained with known standards.

Liquid chromatography mass spectrometry "LC-MS", using a Agilent 1100 series HPLC, Mass detection was performed on ESI esquire HCT (ion trap BRUKER daltonics Germany) coupled to the chromatographic system. Column EC/3 NUCLEODUR 100-3 C18 ec (MACHEREY-

NAGEL Germany). The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The separation was performed on a linear gradient of acetonitrile in water (5–95% B) during 40 min. Injection volumes for all samples were 20 µL. The mass spectra were obtained at a mass-to-charge ratio (m/z) scan range from 100-600. The following MS parameters were used for the analysis: capillary voltage, 4500V; nebulizer pressure, 50 psig; drying gas flow rate, 10 L/min; drying temp 365°C. Samples were analyzed in positive mode. Components of green tea extracts were identified according to their m/z values, UV/Vis absorption spectra and retention times as compared to those found in the literature [2]. The identification of peaks is confirmed by spectre of mass. Quantities of phenolics were calculated from peak areas on chromatograms.

II.4. Antioxydant activity DPPH

The radical scavenging activity of the phenolic compounds of the green tea extract was estimated by measuring their capacities to trap the free radical DPPH (1, 1-diphényl-2-pycril-hydrazyl). Its violet color is transformed into yellow as its reduction [12]. A concentration of DPPH is 0.037 mg/mL, prepared from 9.25 mg solubilized in 250mL of absolute methanol. An aliquot of 100 µL of green tea extract with concentration of 5 mg/mL (chosen after preliminary tries) was added to 3 mL of DPPH solution in methanol. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark, and then the absorbance was measured at 517 nm by Thermo Electron Corporation. For each dilution of the extract, the DPPH scavenging activity was calculated as $100.(A_0 - A_1)/A_0$, where A0 is the absorbance of the control without sample at 30 min, and A1 is the absorbance of the sample at 30 min.

II.5. Oxidative stability

The inhibiting effect of polyphenols on lipid oxidation was studied using an accelerated oxidation test [5] by measuring the induction period of Colza oil oxidation with a Rancimat 743 Methrom suiss made. The test substance, 1000 ppm, was added to the reaction vial containing 3g of Colza oil. Pure Colza oil was used as the control. The oil oxidation was induced at 100 °C and an air flow rate of 20 L/h. Volatile compounds released during the oxidation process passed together with the air into a measurement cell filled with demineralized water. The antioxidant activity index was calculated from the measured induction times "IT".

II.6. Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ± standard error. Statistical analyses were performed on Minitab 1.7. A p-value less than 0.05 was considered as statistically significant. Experimental plan with MODE 6.0.

III. Results and discussion

III.1. Yield of the total polyphenols

The extraction of total polyphenols from green tea has been done according to different methods mechanical agitated extraction "MAE" and ultrasonic extraction "UE". The yield of extraction is expressed by the ratio between the weight of the dried residue of extract and the weight of tea (test sample). Results should a significant difference ($P < 0.05$) between the obtained yields from MAE and UE by all ultrasound models, reaching 98 ± 1.15 mg/g (~31.8 %) and 134 ± 2.30 mg/g (~34.7 %), respectively.

According to Sasazuki *et al*. (2008) [13], green tea contains between 30 and 40% of polyphenols: epigallocatechingallate "EGCG", epicatechingallate "ECG", epigallocatechin "EGC", and epicatechin "EC". The solubility of the phenolic compounds seems to depend on their polymerization degree using the instruction with other compounds or the nature of solvent used. This difference can be assigned to different affinities of the solvent extraction for the components of the green tea leaves as well as the different conditions of extraction: the polarity of solvent and the temperature [14].

The objective of the extraction is to release the phenolic compounds existing in the vacuolar structures by the breaking of the vegetal material and by diffusion. The presence of one or several benzoic hydroxylated cycle in all phenolic compounds is responsible for some features used to extract from vegetal material. The yield of the extraction is only relative and it depends on the method and the condition in which the extractions were affected. It must well set down that the methods of the extraction also affect the whole content in polyphenol and the antioxidant capacities. Moreover, ultrasound extraction can significantly increase extraction efficiency [15].

III.2. Analysis of phenolic compounds

III.2.1. Total phenolic content

Levels of polyphenols were measured with the Folin-Ciocalteu method [8]. The extraction of polyphenols was generally evaluated by extraction ratio of gallic acid/dry weight (mg/g) [9]. The content of the phenolic compounds from different methods of extraction have been derived from the calibration curve and expressed in milligrams gallic acid equivalent by gram of green tea (mg GAE/g). The results are illustrated in Figure 1.

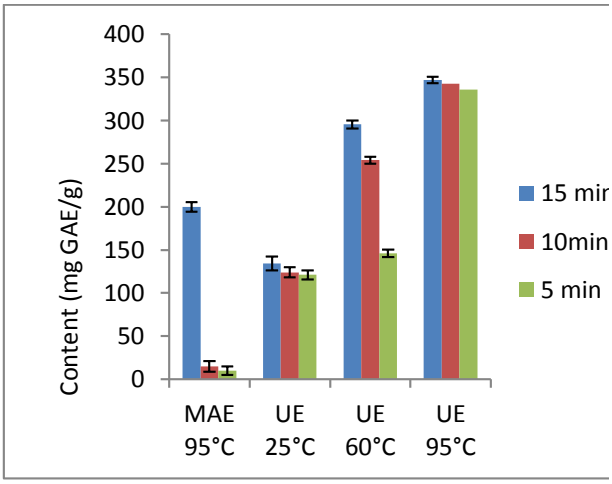

Figure 1. *Content of total polyphenols of MAE 95°C (15, 10 and 5min) and UE time (15, 10 and 5min) temperature (25, 60 and 95°C). MAE: Mechanical agitated extraction; UE: Ultrasonic extraction. Results are expressed as mg GAE/g ±SD*

Results showed that the content in total polyphenols differs according to the process of extraction. The extraction by ultrasound offers the best process with polyphenol content of 347±4.34mg GAE/g, then by MAE in which the content is of 200 ± 5.56 mg GAE/g.

The results of polyphenol contents are higher than thouse found by Gervaise (2004) [16], between 219 and 233 mg/g of dried material. The composition of green tea leaves varied according to the climate, variety and especially to the leaves'age [17]. According to Brewer (2011) [18], who has studied the natural antioxidants, in science of human food and nutrition department, Illinois University (USA) has affirmed that the antioxidant activity of green tea is linearly related to the content in polyphenols, which is about 450 mg/g.

III.2.2. Total flavonoid content

The reason for what the dosage of polyphenols has been followed by a dosage of flavonoid is expressed by the big importance of this class, with more than 5000 compounds already described [19]. The dosage of flavonoids has been realized according to the method of $AlCl_3$ by using the Quercetin as a standard. The Figure 2 represents the different values gotten from each mode of extraction, expressed in mg quercetin equivalent by gramme of the dried material of green tea (mg QE/g).

Flavonoid content in the studied organs ranges from 85 to 300 mg QE/ g (Figure 2). It is lower in ultrasonic extraction "UE" 25°C than in "UE" 60°C and mechanical agitated extraction "MAE". Yet, flavonoids represent a larger part of total polyphenol content in UE 95°C, UE 60°C 270 ± 2.08 mg QE/g (80%) than in the MAE and UE 25°C, 145 ± 2 mg QE/g (72%) and 105 ± 4.35 mg QE/g (70%), respectively. According to the data of the Figure 2, the green tea is rich in flavonoids, one of the major compounds of polyphenols. The extraction by dried green tea contains a dose of 290.5 mg QE/g by using ultrasounds (UE 95°C, 15 min).

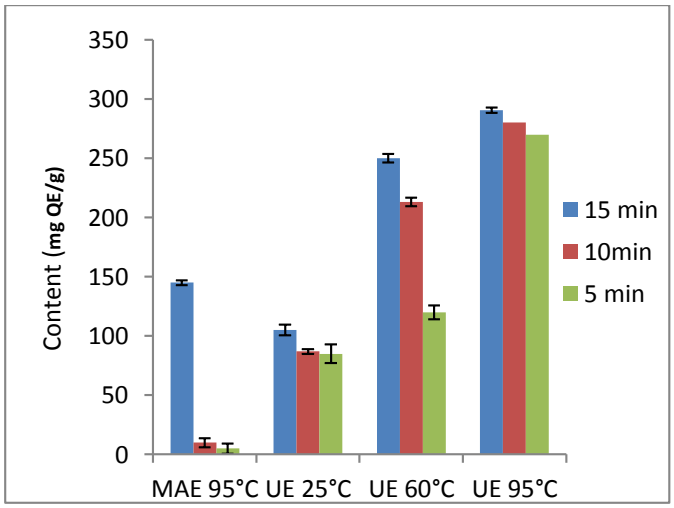

Figure 2. *Content of total flavonoids of MAE 95°C (15, 10 and 5min) and UE time (15, 10 and 5min) temperature (25, 60 and 95°C). MAE: Mechanical agitated extraction; UE Ultrasonic extraction. Results are expressed as mg QE /g ±SD*

III.3. Phenolic composition of thegreen tea extract

Identification by high-performance liquid chromatography equipped with a diode array detector "HPLC-DAD", and liquid chromatography mass spectrometry "LC-MS" of the biochemical composition of total polyphenol extract by ultrasound "UE" and by mechanical agitation "MAE" of green tea were characterized by four main peaks (Figure. 3). These peaks for UE (95°C, 15 min) correspond to epigallocatechines gallates "EGCG" which represent the major part with content of 60.1 %. The rest is composed by epicatechingallates "ECG" 13 %, epicatechin (5%) and epigallocatechin "EGC" 12 %. Our results are

similar to those found by Sajilata et al. (2008) [17], where identification of green tea polyphenols represents a high rate in EGCG compared to other types of tea. These results are consistent with work of Chandrakant et al. (2011) [20] whose EGCG content is of 58.1%, ECG 18.1% and EGC 12%. Therefore, EGCG represents the bulk of green tea polyphenols, whose content is above 50%, as in the case of the current study.

The main phenolic compounds in green tea are catechins, including (−) epigallocatechin-3- gallate "EGCG", (−) epigallocatechin "EGC", (−) epicatechin-3-gallate "ECG", and (−) epicatechin "EC", with EGCG being the highest accounting for 60% to 65% of the entire catechin content [15, 21].

The green tea MAE contains a relatively complex mixture containing about 72% catechins. The main components are epigallocatechin-3-gallate "EGCG" (40.25%), epicatechingallate (20.57%) and epigallocatechin (23.69%). Minor catechin components are epicatechingallate (16.44%) and epicatechin/catechin (9.39%). Green tea extract containing 60% EGCG was approved by the US Food and Drug Administration as the first botanical drug [22]. Phenolic composition of green tea compound "GTC" is presented in Table 1. The results obtained after HPLC-DAD and LC-MS of the commercial extract by ultrasound, green tea compound "GTC" confirm that EGCG and catechin derivatives constitute more than75% of the green tea phenolic mixture and it was almost depleted of phenolic acids (3.16%), compared to the loose green tea leaves extract 24.98% [23]. EGCG is the major catechin in green tea and accounts for 50% to 80% [1]. Furthermore, chemical modification of an EGCG pharmacophore may modify relative therapeutic activities so that combinatorial supplementation may synergistically enhance beneficial health effects.

Table 1. *Proportion of major catechins in MAE and UE (15min, 95°C)*

Compound	MAE (%)	UE(%)
−Epigallocatechin « «EGC »	23.69 ± 0.28	12 ± 0.25
–Epigallocatechin-3-gallate « EGCG »	40.25 ± 0.32	60.1 ± 0.17
−Epicatechin « EC »	09.39 ± 0.2	05 ± 0.2
−Epicatechin-3-gallate « ECG »	16.44 ± 0.52	13 ± 0.45
Autres	10.23 ± 0.64	09.89 ± 0.26

The results are expressed as averages of three independent measurements ± SD. MAE: Mechanical agitated extraction; UE: Ultrasonic extraction.

We may conclude that sonication enhances extraction of EGCG by about 30% compared to MAE, in agreement with the report of Wijngaard *et al*. (2012) [24], which found only 20% of more catechin that could be extracted from apple pomaces using ultrasound. Physical and chemical forces included in conventional solvent extraction are different from those included in ultrasound extraction. This case modifies the temperature and pressure within the bubble, stimulating the system to produce the necessary energy for chemical reactions. Mechanical forces which result cavitation participates in the demolition of plant cell walls, as a result reinforcing extraction of secondary metabolites as catechins. It is obvious that the competence of the operation is influenced by many factors, like acoustic intensity, extraction time, solvent type, and temperature inside the extraction container. Although, some studies report that testing for the presence of phenolic compounds in tea under different infusion conditions [4, 19, 25] have been undertaken earlier, no study details the changes in relation with the extension of infusion time. The various extraction conditions influence efficiency, taking into account that ultrasonic extraction can improve EGCG recovery. The chemical composition of tea leaves varies with species, season, age of the leaf, climate, and horticultural practices [19, 26].

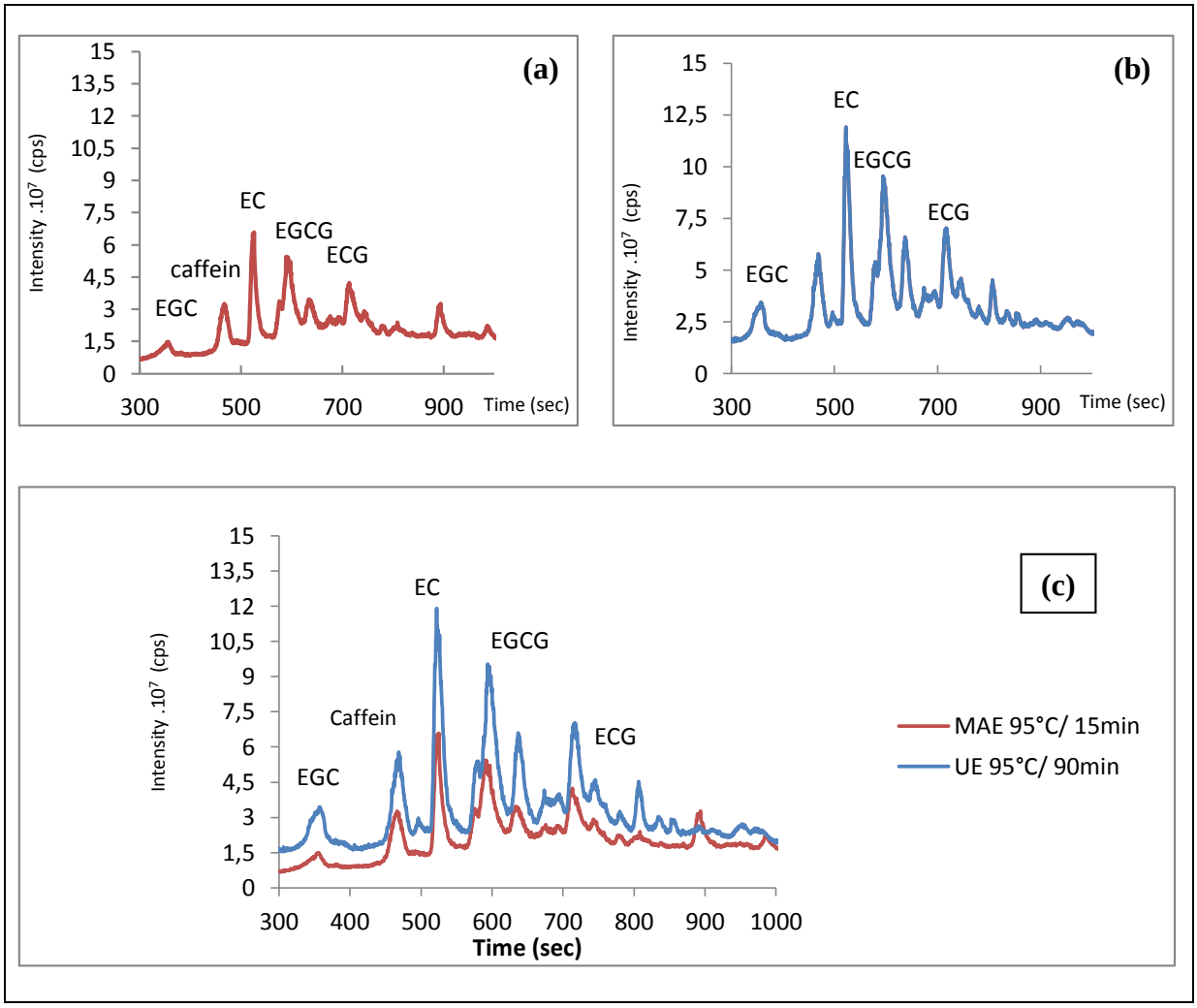

Figure 3. *Chromatograms LC-MS of MAE and UE. (a) MAE (95°C, 15 min). (b) UE (95°C, 15 min). (c) illustrated superposed chromatograms of (a) and (b).* --- *(a);* --- *(b)*

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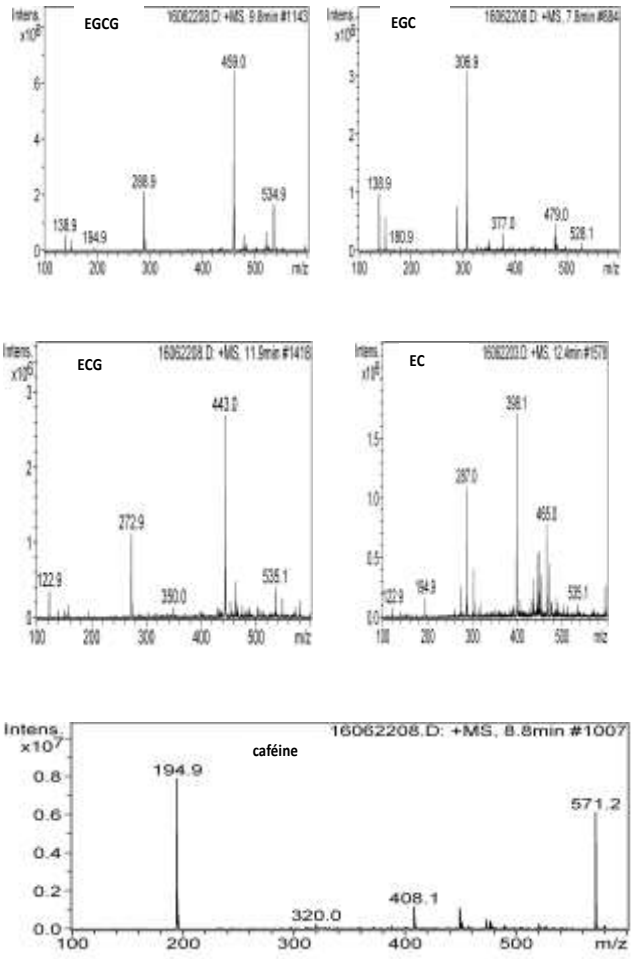

Figure 4. *Mass spectra of catechins*

III.4. DPPH radical scavenging activity

Diphényl-pycril-hydrazyl "DPPH" is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form, DPPH-H, by the reaction. The total phenolic extract of *C.sinensis* showed a concentration-dependent antiradical activity by reducing the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine derivative. The total polyphenols of green tea extracted by ultrasound (UE 95°C, 15min) is higher than mechanical agitated extraction (MAE 95°C, 15min) where present rates 90.22 ± 0.27 % vs 85 ± 0.81%, respectively (Figure 5). This informs us about the effectiveness of phenolic compounds, which is in some cases, not related to their higher rate. Comparing the results with those of the standard antioxidant of butyl hydroxy toluene "BHT" in Figure 6, the liquor of green tea extract by ultrasound seems to have an antioxidant activity close to that of BHT (92%).

However, scavenging activity of gallic acid, a known antioxidant used as positive control, was relatively high. Epigallocatechingallate "EGCG" antioxidant is the major chemical constituent of *C.sinensis*. Total phenolic extract showed potent DPPH free radical-scavenging activity. Natural polyphenols have chain-breaking antioxidant activities and are believed to prevent many degenerative diseases, including cancer and atherosclerosis [27, 28]. In food systems, antioxidants are useful in retarding lipid peroxidation and secondary lipid peroxidation product formation [29], and thus help to maintain flavor, texture, and, in some cases, the color of the food product during storage.

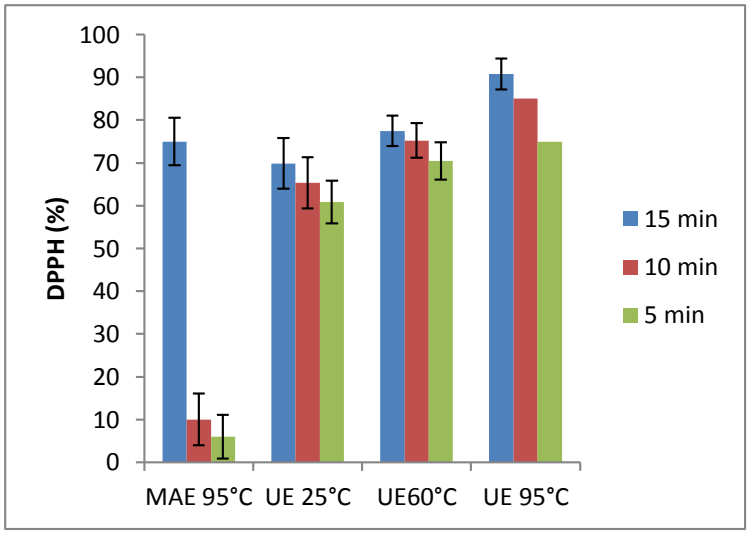

Figure 5. *Antioxidant activity of catechins extracted UE and MAE*

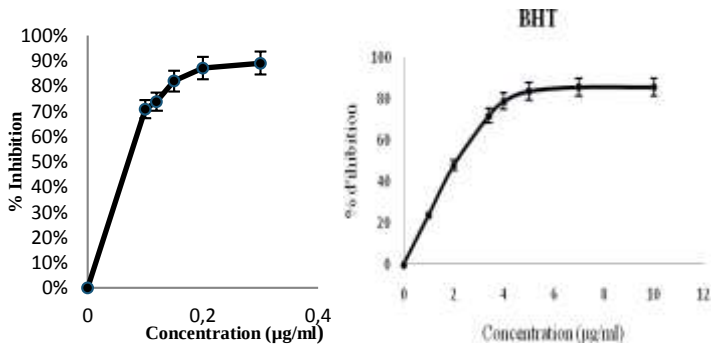

Figure 6. *Comparison antioxidant activity of UE (95°C, 15 min) with BHT.*

III.5. Oxidative stability

Oxidative stability of ultrasonic extraction "UE" and mechanical agitated extraction "MAE" polyphenols in colza oil was determined using the Rancimat test. Oxidative stability of 5 and 10min for "MAE 95°C" was not achieved because the antioxidant activity is too low and not considered. The result of oxidative stability of UE polyphenols in time (15, 10 and 5min) and temperature (25, 60 and 95°C), then, for the MAE polyphenols (15min, 95°C) in colza oil (Figure 7). The induction time of MAE (15min, 95°C) is 21.26±0.7 hours; the highest time of induction is of 30.62±0.69h, for UE (15 min, 95°C). However, UE methods present an upper time of induction than one of the colza oil (17.35± 0.68 h) as shown in Figs 7 and 8. Research clearly indicates that antioxidants play a very important role in increasing the oxidative stability of oil. As a result, the polyphenols green tea extract is important, as hot water allows for better scavenging of oxidative radicals than cold water [25], likely due to increased extraction of polyphenols. More recent evidence suggests that antioxidant efficacy is influenced by the polarity of the compounds [30]. The high antioxidant activity, however, means a high reactivity of substances which could hinder their rapid degradation.

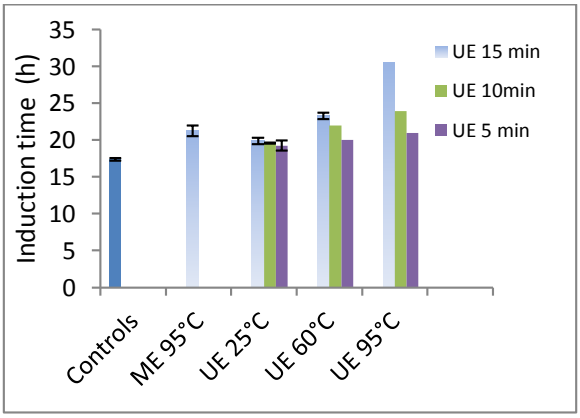

Figure 7. *Oxidative stability of catechins UE and MAE using the rancimat test. UE times (15, 10 and 5min), temperatures (25, 60 and 95°C) and MAE (15min, 95°C) catechins. Results are expressed as h ±SD*

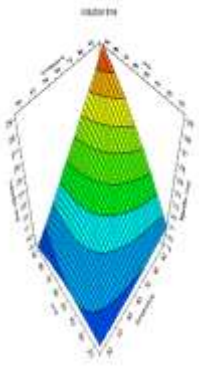

Figure 8. *Induction time of UE temperatures (25, 60 and 95°C), times (15, 10 and 5min)*

The Rancimat assay is based on oil oxidation by air oxygen at higher temperature (100°C in our case), and is certainly critical for thermolabile substances. Thus, the polyphenols resist or could be partially destroyed at the experimental temperature, observing a positive correlation between total polyphenols extract from *C.sinensis* and colza oil oxidative activity. UE plays a key role in the lipid oxidation activity.The antioxidative proprieties depend mostly on the donor-proton capacity of substances; total catechin extract is powerful antioxidants [12].

Studying the influence of infusion temperature and time duration on polyphenol content and antioxidant capacity of polyphenols green tea can contribute to inform us how to extract polyphenols effectively [31]. There is a correlation between the total polyphenols content and the antioxydant capacity [32, 33]. Increasing the EGCG content in total catechin extracts presents practical benefits, and the antioxidant activity of colza oil retards lipid oxidation.

IV. Conclusion

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative process. Green tea *Camellia sinensis* is a richer source of phenolics. Epigallocatechin 3-gallate "EGCG" is the most abundant and potent in green tea catechin, but the extraction efficiency of these compounds strongly depends on the time and temperature extraction. The results of this study confirm that ultrasound extraction UE improves the yield of EGCG antioxidant from green tea. Results showed a simple method for extraction and oil storage (non chemical natural conservative), adding polyphenols green tea to food products for a safe, natural antioxidant that prevent rancidity and promotes good health.

In this case, there is a significant difference in phenolic content and oxidative stability between mechanical agitated extraction "MAE" and ultrasonic extraction "UE" green tea extracts. Liquid chromatography mass spectrometry "LC-

MS" analysis of catechins, which are the dominant phenolics in the extract, confirmed the fact that green tea is a richer source of EGCG phenolics. The major polyphenol in *Camellia sinensis*, epigallocatechingallate "EGCG", was more likely to be responsible for most of the observed antioxidant activity. In food systems, antioxidants are useful in retarding lipid oxidation and protect during storage.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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