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Boulmokh Yamina

Intitulée

Secondary metabolites of *Camellia sinensis* L., RP-HPLC analysis and Quantitative Structure Antioxidant Activity Relationships

Soutenue le : 29 Juillet 2021 Devant le Jury composé de :

Mr Affoune A/ Mohamed	Prof.	Université 08 Mai 1945 Guelma	Président
Mme Amira-Guebailia Habiba	Prof.	Université 08 Mai 1945 Guelma	Rapporteur
Mr Gherraf Nouredine	Prof.	Université de Oum el Bouaghi	Examinateur
Mr Benahmed Merzoug	Prof.	Université de Tebessa	Examinateur

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Abstract

In this study, quantitative analyses by HPLC-DAD of *Camellia sinensis* (green tea) samples were performed in order to determine the concentrations of four catechins: (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-Epicatechingallate (ECg), (-)-Epigallocatechin gallate (EGCg), three methylxantines: Caffeine, Theophylline and Theobromine as well as Gallic acid. Two different extraction methods were performed : an infusion in hot distilled water for 5 min and a multistep extraction process using water/MeOH mixtures. The examination of the obtained chromatograms shows good separation of metabolites, especially for tea infusions. Indeed, all peaks have shown good resolution, symmetry,normal Gaussian shapes and linear baselines. Substantial amounts of polyphenols were found in most tea samples. The catechins contents followed the sequence: EGCg > ECg > EC > EGC in both infusions and water/MeOH extracts. Caffeine is the major methylxantine in all tea samples. Methylxantines level followed the sequence: Caffeine > Theobromine > Theophylline. Levels of methylxantines were higher in infusions than the methanol extracts, whereas catechins levels were found to be higher in methanol extracts than in infusions. In the second part of work, we report, a comparative study of the antioxidant potential of EGCg and EC as compared to resveratrol (RSV). The most favorable mechanism by which each molecule exerts the antioxidant activity is determined. Ascorbic acid (AA) was used as a reference. DPPH and FRAP assays were used for experimental evaluation of antioxidant activity and for theoretical calculations, density functional theory (DFT) method was chosen. Three mechanisms were investigated : Hydrogen Atom Transfer (HAT), Single Electron Transfer Proton Transfer (SET-PT) and Sequential Proton Loss Electron Transfer (SPLET). Calculated thermodynamic parameters correlate well with percentage inhibition (I%) and half maximal inhibitory concentration (IC50) values given by the DPPH test. Both experimental and theoretical approaches showed that EGCg is more potent antioxidant than EC and RSV. The most preferential sites are gallate moiety and 4'-OH in EGCg and OH sites of the B ring in EC. The pKa values confirm this finding. All proposed mechanisms are favored for EGCg, SET-PT is preferred antioxidant mechanism for EC and it is the most suitable in the first step for RSV. Flavanols are more potent antioxidants than the stilbene; RSV.

Keywords : Catechins, Methylxantines, Green tea, HPLC-DAD, Quantification, Resveratrol; Antioxidant activity; HAT; SET-PT; SPLET, DPPH, FRAP.

ملخص

في هذه الدراسة ، تم إجراء تحليلات HPLC-DAD الكمية لعينات *Camellia sinensis*(الشاي الاخضر) لتحديد تركيزات أربعة أنواع من الكاتيكين: Epicatechin (EC)، Epigallocatechin (EGC) ، Epicatechin gallate (ECg)، epigallocatechin Gallate (EGCg) ، ثلاثة ميثيل زانتين: الكافيين ، الثيوفيلين ، الثيوبرومين وكذلك حمض الغاليك. تم إجراء طريقتين مختلفتين للاستخلاص: الاستخلاص في الماء المقطر الساخن لمدة 5 دقائق وعملية الاستخراج متعددة الخطوات باستخدام ماء/ ميثانول. يُظهر الكروماتوجرام الذي تم الحصول عليه فصلًا جيدًا عن المستقلبات ، لا سيما بالنسبة لطريقة الاستخراج في الماء الساخن للشاي. في الواقع ، أظهرت جميع القمم لكروماتوجرامية دقة وضوح جيدة وتناظرًا جيدًا وأشكالًا غاوسية عادية وخطوط أساس خطية. تم العثور على كميات كبيرة من مادة البوليفينول في معظم عينات الشاي. يتبع تركيز الكاتيكين التسلسل: EGC < EC < ECg< EGCg في ا الاستخلاص في الماء المقطر الساخن ومستخلصات الميثانول. الكافيين هو الميثيلكسانتين الرئيسي في جميع عينات الشاي. يتبع مستوى الميثيل زانتينات التسلسل: الكافيين > الثيوبرومين > الثيوفيلين. كانت مستويات الميثيلكسانتين أعلى في الاستخلاص في الماء المقطر الساخن منها في المستخلصات الميثانولية ، بينما كانت مستويات الكاتيكين عكس ذلك. في الجزء الثاني من العمل ، تم دراسة مقارنة للقدرة المضادة للأكسدة لـ EGCg و EC مقارنة بريسفيراترول (RSV). تم تحديد الآلية الأكثر ملاءمة التي يمارس بها كل جزيء نشاطًا مضادًا للأكسدة. تم استعمال حمض الأسكوربيك (AA) كمرجع. تم استخدام اختبارات DPPH و FRAP للتقييم التجريبي لنشاط مضادات الأكسدة وللحسابات النظرية ، تم اختيار طريقة النظرية الوظيفية للكثافة (DFT). تمت دراسة ثلاث آليات: نقل ذرة الهيدروجين (HAT) نقل إلكترون واحد متبوعًا بنقل البروتون (SET-PT) وفقدان البروتون المتسلسل متبوعًا بنقل الإلكترون (SPLET). توافقت الخصائص الديناميكية الحرارية المحسوبة جيدًا مع النسبة المئوية للتثبيط (I٪) والتركيزات المثبطة المتوسطة (IC50) التي قدمها اختبار DPPH. أظهرت الأساليب التجريبية والنظرية أن EGCg هو أحد مضادات الأكسدة الأكثر فعالية من EC و RSV. المواقع الأكثر تفضيلاً لنشاط مضادات الأكسدة هي شق (Gallate) لـ EGCg و B-ring 4'-OH في EC. تؤكد قيم pKa هذه النتيجة. جميع الآليات المقترحة مفضلة لـ EGCg، SET-PT هي آلية مضادات الأكسدة المفضلة لـ EC وهي الأكثر ملاءمة في الخطوة الأولى لـ RSV. تعتبر مركبات الفلافانول من مضادات الأكسدة أقوى من مادة الستيلبين. RSV.

الكلمات الرئيسية: الكاتيكين ، الميثيلكسانتين ، الشاي الأخضر ، HPLC-DAD ، القياس الكمي ، ريسفيراترول النشاط المضاد للأكسدة؛ HAT؛ SET-PT ؛ SPLET ، DPPH، FRAP.

Résumé

Dans cette étude, des analyses quantitatives par HPLC-DAD d'échantillons de *Camellia sinensis* (thé vert) ont été réalisées afin de déterminer les concentrations de quatre catéchines : (-) - Epicatechine (EC), (-) - Epigallocatéchine (EGC), (-) - Epicatéchine gallate (ECg), (-) - Epigallocatéchine gallate (EGCg), trois méthylxantines: Caféine, Théophylline et Théobromine ainsi que l'acide gallique. Deux différentes méthodes d'extraction ont été effectuées : une infusion pendant 5 min dans de l'eau chaude distillée et un processus d'extraction en plusieurs étapes utilisant des mélanges eau/MeOH. Les chromatogrammes obtenus montrent une bonne séparation des métabolites, en particulier pour les infusions de thé. En effet, tous les pics ont montré une bonne résolution, une bonne symétrie, des formes gaussiennes normales et des lignes de base linéaires. Des quantités substantielles de polyphénols ont été trouvées dans la plupart des échantillons de thé. Les concentrations des catéchines sont comme suit : EGCg > ECg > EC > EGC dans les infusions et les extraits de eau/MeOH. La caféine est la principale méthylxantine dans tous les échantillons de thé. Les concentrations de méthylxantines suivent la séquence : caféine > théobromine > théophylline. Les taux de méthylxantines étaient plus élevés dans les infusions que dans les extraits méthanoliques, alors que pour les taux de catéchines c'est le contraire. Dans la deuxième partie du travail, nous rapportons une étude comparative le pouvoir antioxydant de l'EGCg et de l'EC par rapport au resvératrol (RSV). Le mécanisme le plus favorable par lequel chaque molécule exerce l'activité antioxydante est déterminé. L'acide ascorbique (AA) a été utilisé comme référence. Les tests DPPH et FRAP ont été utilisés pour l'évaluation expérimentale de l'activité antioxydante et pour les calculs théoriques, la méthode de la théorie fonctionnelle de la densité (DFT) a été choisie. Trois mécanismes ont été étudiés : le transfert d'atome d'hydrogène (HAT), le transfert d'un électron unique suivi par un transfert de protons (SET-PT) et la perte séquentielle de protons suivi d'un transfert d'électrons (SPLET). Les paramètres thermodynamiques calculés sont en bon concordance avec le pourcentage d'inhibition (I%) et les concentrations inhibitrices médianes (IC50) données par le test DPPH. Les approches expérimentales et théoriques ont montré que l'EGCg est un antioxydant plus puissant que l'EC et le RSV. Les sites les plus préférentiels pour l'activité antioxydante sont le groupement gallate pour l'EGCg et le 4'-OH du cycle B dans l'EC. Les valeurs de pKa confirment ce résultat. Tous les mécanismes proposés sont favorisés pour l'EGCg, SET-PT est le mécanisme antioxydant préféré pour l'EC et c'est le plus approprié dans la première étape pour le RSV. Les flavanols sont des antioxydants plus puissants que le stilbène; RSV.

Mots clés : Catéchines, Méthylxantines, Thé vert, HPLC-DAD, Quantification, Resvératrol ; Activité antioxydante; HAT; SET-PT; SPLET, DPPH, FRAP.

I dedicate this thesis to my husband and my parents

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
Abs	Absorbance
AIP	Adiabatic Ioization Potential
B3LYP	Becke, 3-parameters, Lee–Yang–Parr
BDE	Bond Dissociation Enthalpy
Caff	Caffeine
DAD	Diode array detector
DFT	Density funtional theory
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2 Diphenyl 1 picrylhydrazyl
EC	Epicatechin
EGC	Epigallocatechin
ECg	Epicatechin gallate
EGCg	Epigallocatechin gallate
ESP	Electrostatic Potential
ETE	Electron Transfert Enthalpy
FRAP	Ferric reducing antioxidant power
ΔG	Gibbs free energy change
G	Gaussian
GA	Gallic acid
HAT	Hydrogen Atom Transfert
HF	Hartree-Fock
HOMO	Highest Occupied Molecular Orbital
HPLC	High performance liquid chromatography
I	Inhibition
IC50	Half maximal inhibitory concentration
LOD	Limit of detection
LOQ	Limit of qantification
LUMO	Lowest Unoccupied Molecular Orbital
min	Minute
nd	Not detected
O	Ortho
PA	Proton Affinity
PDE	Proton Dissociation Enthalpy
R^2	Regression factor
Rec	Recovery
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Resveratrol
RT	Retention time
S	Sample
SCF	Sel-Consistent Field
SET-PT	Single Electron Transfert- Proton Transfert

LIST OF ABBREVIATIONS

Sol	Solvation
SPLET	Sequential Proton Loss Electron Transfert
TFA	Trifluoroacetic acid
Theobr	Theobromine
Theoph	Theophylline
UV	Ultraviolet

INTRODUCTION

Introduction

According to the source " Market Research World ", around 25,000 cups of tea are drunk in the world every second, i.e. a global consumption of 3.9 million tonnes of tea each year. It is the second most consumed drink in the world after water and its consumption has increased over the last twenty years due to the correlation between tea intake and health benefits.

Among the polyphenolic compounds present in *Camellia sinensis* L. var sinensis (plant of green tea) are catechins. They are powerful antioxidants and their ability to show effective protective effects against oxidative stress is essential for their potential use in therapeutic applications. Green tea catechins include: epicatechin (EC) epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg).

Structurally, catechins consist basically of two phenolic rings and an oxygenated heterocycle. It was reported that antioxidant activity of polyphenols is mainly related to the presence of hydroxyl groups.

In addition to catechins, green tea contains also caffeine, to a lesser degree the two methylxantines: theophylline and theobromine, gallic acid and many other compounds.

Many research papers on tea were focused on the health side. Several other studies have been published on HPLC analysis of tea consumed in different countries.

In the Algerian market, different brands of green tea are available at different prices, with leaves of different shapes and colors. However the color and taste of their infusions are indistinguishable, which prompted us to know the intrinsic difference in the amount of some polyphenols and methylxantines. According to the results obtained from the quantifications of these compounds, we will have an idea on the quality of tea and specifically green tea consumed by the Algerian population.

To the best of our knowledge, no work relating to the investigation and/or the quantification of the components of teas sold on the Algerian market has been reported.

From this observation, it seemed obvious as the first objective of this thesis to quantify by HPLC-DAD, the most important constituents of eight brands of green tea on the Algerian market.

Samples were prepared by both extraction of tea leaves in hydromethanol and infusion in hot water at 85°C for 5 min, the later extraction method aims to determine the concentration of the tea drink. The comparison of the results of both methods of extraction was achieved.

The structure of polyphenolic compounds is linked to their capacity of scavenging the radicals and chelating the metals. It was reported that antioxidant activity of polyphenols is mainly related to the presence of hydroxyl groups. It has also been shown that the more the compound contains OH groups, the higher the antioxidant activity. But it was also reported that even if molecules have the same number and position of OH groups, antioxidant activity is different and this is due to other structural features which influence the antioxidant activity.

Experimental methods can show that a compound exhibits or not antioxidant activity and enable us to compare the antioxidative potential of a set of molecules, but are unable to show which part or site in molecules are responsible for the antioxidant action. Therefore, it is necessary to perform theoretical studies linking the experimental antioxidant capacity to the structure and the physicochemical properties of compounds having antioxidant activity. The most important feature of computational methods such as DFT, as applied to antioxidant activity studies is to show which of the sites of molecules is more responsible for antioxidant activity and the reaction mechanisms by which antioxidants can exert their activity.

DFT is a quantum mechanical technique which can be applied for deep understanding of the mechanisms of antioxidant activity of molecules. This method allows the calculation of five thermodynamic parameters; Bond dissociation enthalpy (BDE), Adiabatic Ionization Potential (AIP), Proton Dissociation Enthalpy (PDE), Proton Affinity (PA) and Electron Transfer Enthalpy (ETE).

Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) energies can be associated with the antioxidant activity of molecules. The spin density is an important parameter characterizing the stability of free radicals, because the energy of a free radical can be efficiently decreased if the unpaired electron is highly delocalized through the conjugated system. The enthalpy of acidity and pKa are parameters strengthening the study of the antioxidant activity of the studied compounds. Molecular electrostatic potential (MEP) mapping through a computer-aided method is a very useful approach to explore the reactivity of compounds.

The second aim of this thesis is to study the antioxidant activity of two green tea catechins; EGCg and EC. The study aims at comparing the antioxidant potential of these molecules and showing, by exploiting the three well-known antioxidant mechanisms, which of the sites in each molecule, is more responsible for antioxidant activity. Resveratrol (RSV) and ascorbic acid (AA) are used for comparison as they present strong antioxidant activity.

INTRODUCTION

This latest study was achieved by a theoretical DFT method using Gaussian 09. Calculated parameters in the gas phase are, BDE, AIP, PDE, PA, ETE, $\Delta H_{acidity}$, pKa and HOMO and LUMO energies. On the other hand, calculation of the first five parameters was performed in presence of water to show how it would influence as solvent, the individual reaction enthalpies. Besides, spin density contours and electrostatic potential maps were plotted. An experimental procedure was also achieved to compare antioxidant activity of EGCg and EC to RSV, AA as a renowned potent antioxidant, is used here as a reference. Experimental antioxidant activity was evaluated on the basis of inhibition of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and ferric reducing power (FRAP) which consists in reducing the colorless Fe^{3+} ion of tripyridyltriazine Fe $(TPTZ)^{3+}$complex into intense blue Fe^{2+} ion, upon reaction with antioxidants. Percentage inhibition of DPPH (I%), IC50 and the reducing power of the studied molecules were evaluated. An attempt was made to correlate experimental results to computational ones. Experimental comparison of antioxidant activity of a sample of green tea (S4) with that of ascorbic acid and resveratrol has been done.

This thesis is structured around four chapters:

- In the first chapter we will present generalities on *Camellia sinensis*, the main constituents of green tea, their extraction methods and their therapeutic effects on health. statistics on published work on tea, catechins and their antioxidant activity will also be a part of this chapter.
- In the second chapter we will focus on theoretical computational methods (Hartree-Fock (HF) and Density Functional Theory (DFT)).
- In the third chapter, the experimental and theoretical methods used in this work will be presented.
- The interpretation of the various results obtained will be detailed in the fourth chapter.
- Finally, a general conclusion will end this work and will give a careful and succinct reading of the results obtained and a presentation of the prospects envisaged.

CHAPTER I

LITERATURE REVIEW

Chapter I

Literature review

Introduction

The aim of this chapter is firstly to give a general overview on *Camellia sinensis* L. (tea plant) with regard to manufacturing, health benefits, chemical composition, catechins and methylxantines extraction methods, free radicals and antioxidant activity mechanism of catechins and secondly to establish statistics of research work carried out on *Camellia sinensis* L., catechins and their therapeutic applications and antioxidant activity.

I.1. Green tea (*Camellia sinensis*)

I.1.1. Origins

The tea plant is known as *Camellia sinensis* (L.) O. Kuntze **(Figure I.1)** of which there are two varieties: var. sinensis and var.assamica **(Hara 2001)** and its birthplace is assumed to be the southwestern China, centered in the Yunnan district **(Yamaguchi and Tanaka 1995)**. The tea plant C. sinensis var. sinensis is usually cultivated for green tea production and C. sinensis var. assamica cultivated for black tea. Tea plants prefer a warm and humid climate with plenty of rainfall and also like diffused light and weak acidic and well-drained soil **(Wan et al. 2009)**.

Figure I.1: Camellia sinensis plant (https://plants.ces.ncsu.edu/plants/camellia-sinensis/) (4/4/2021)

I.1.2. Manufacturing

The quality of fresh tea leaves plays a key role in the characteristics of green tea. During the harvest season, the tender tea shoots are picked by hand or using a mechanical tea picker and delivered immediately to the tea factories. The **Figure I.2** illustrates the process by which green tea is obtained :

- Withering : Freshly plucked leaves are laid out on the floor inside a cool breezy room. The purpose of withering is to reduce the moisture content in the leaves and to allow the flavor compounds to develop.
- Fixation and enzymatic deactivation : It is performed to stop the tea leaf oxidation at desired level by moderately heating it. Indeed, to produce a tea rich in catechins, it is necessary to ban all fermentation and the action of polyphenol oxidase.
- Rolling : Fixed leaves are perfectly and gently rolled, and depending on the style, they are shaped to look wiry, kneaded, or as tightly rolled pellets. During rolling action, essential oils and sap oozes out, intensifying the taste further. More tightly rolled the leaves, the longer they will retain their freshness.
- Drying : It includes sunning, air drying, or baking. Drying enhances a tea's flavor and ensures its long shelf-life. The green tea is then ready for sale.

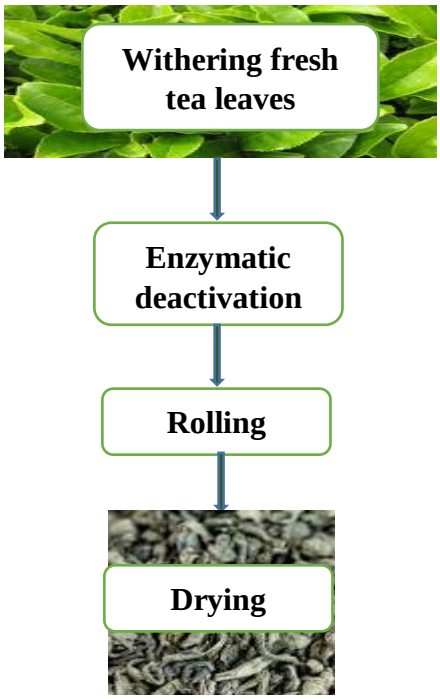


Figure I.2: Process of green tea production (Krieps 2009).

I.1.3. Effects on human health

Over the past thirty years or more, scientists have studied tea plant in respect to potential health benefits.

Analysis of studies performed using human oral consumption of green tea to assess cancer risk showed that these studies gave the most consistent results and were positive to reduce cancer risk in breast, colorectal, esophageal, gastric, lung, ovarian, pancreatic, and prostate **(Ju et al. 2007 and Boehm et al. 2009)**.

Almajano et al. (2008) have studied antioxidant and antimicrobial activities of tea infusions and revealed that the highest radical-scavenging activity was observed for green and white teas. Similar inhibition rate of several microorganisms by these tea varieties was also reported.

Cardiovascular disease (CVD) is a complex disorder involving multiple factors. A study including nearly 50,000 people, reported a decreased mortality rate due to CVD based on consumption of various numbers of cups of tea per day **(Reygaert 2017)**.

In their review article, **Cooper et al. (2005)** reported that green tea consumption had significant effects on food intake suppression, body weight loss, and fat tissue accumulation. In addition, levels of cholesterol and triglycerides were lower for people who consume frequently green tea **(Xu et al. (2020))**.

I.1.4. Composition

I.1.4.1. Catechins

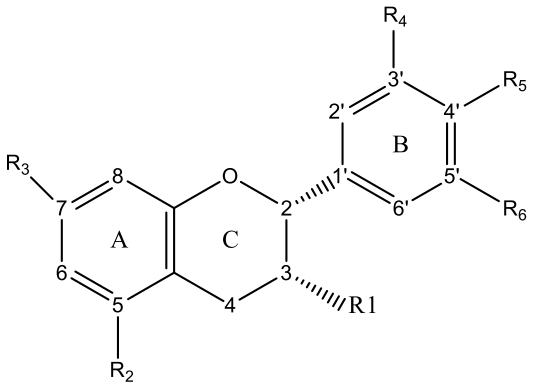
Polyphenols, represent the most interesting group of tea leaf components. In green tea, the polyphenolic fraction is mostly composed of catechins, they are the major components of fresh tea leaves **(Hara 2001)** and they are responsible for the astringent taste and strength of green tea infusion.

The four major polyphenols in green tea are: epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg). Epigallocatechin gallate is considered the most significant active component **(Sinija and Mishra 2008)**.

I.1.4.1.1. Description and structure

Catechins have two asymmetric carbon atoms in positions 2 and 3, on the other hand, some of these molecules are esters of gallic acid in position 3. There is no electron delocalization

between the A and B rings due to the saturation of the heterocyclic ring A. In -(-) Epicatechins **(Figure I.3)**, the substituents at C (2) and C (3) are cis positioned **(Spek et al. 1984)**.



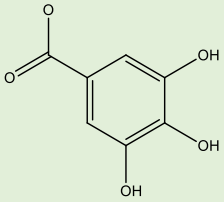
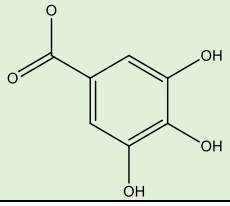
Epicatechins	R1	R2	R3	R4	R5	R6
EC	OH	OH	OH	H	OH	OH
EGC	OH	OH	OH	OH	OH	OH
ECg		OH	OH	H	OH	OH
EGCg		OH	OH	OH	OH	OH

Figure I.3: Molecular structures of (-)- Epicatechin (EC), (-)- Epigallocatechin (EGC), (-)- Epicatechin gallate (ECg) and (-)- Epigallocatechin gallate (EGCg).

I.1.4.1.2. Methods of extraction

I.1.4.1.2.1. Extraction with hot water

Considering that tea is usually consumed as a decoction in hot water, and aiming to evaluate the level of catechins actually consumed by tea drinkers, extraction of tea leaves has been performed using deionized hot water. The presence of heavy metals may decrease the stability of catechins because heavy metals, such as iron, interact strongly with phenolic hydroxyl groups by chelation, rendering them much more sensitive to oxidation **(Tanaka et al. 2013)**. The **Figure I.4** shows different steps for the extraction of green tea catechins in hot water.

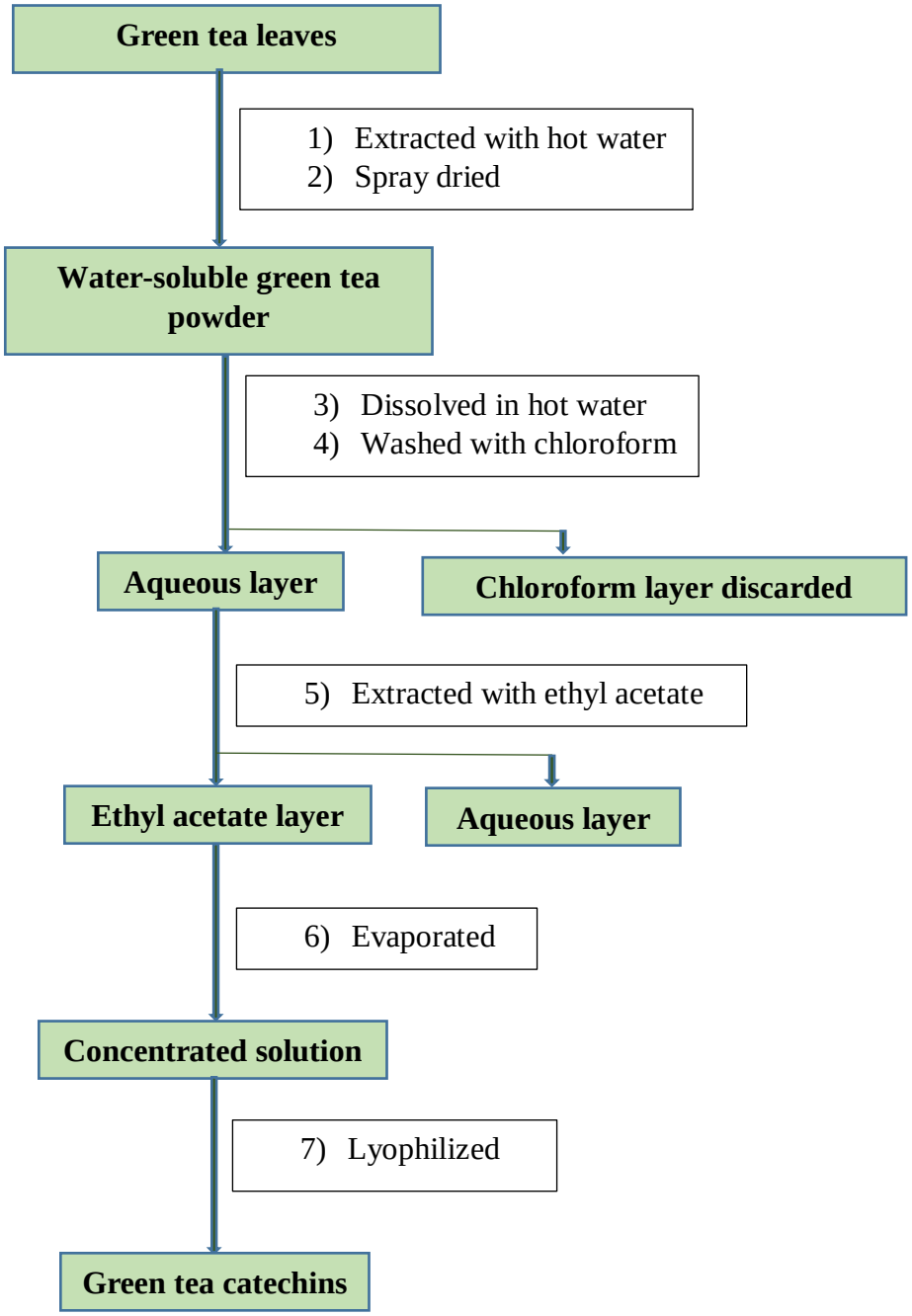


Figure I.4: Extraction of green tea catechins* by hot water *(Hara 2001).

I.1.4.1.2.2. Extraction with organic solvents

To prevent the interactions of catechins with macromolecules, mixtures of water and organic solvents, typically 60% aqueous acetone or 60% aqueous ethanol, were used for extraction in different studies **(Nishizawa et al. 1984)**. A typical scheme for the separation of tea catechins from green tea with aqueous acetone is shown in **Figure I.5**.

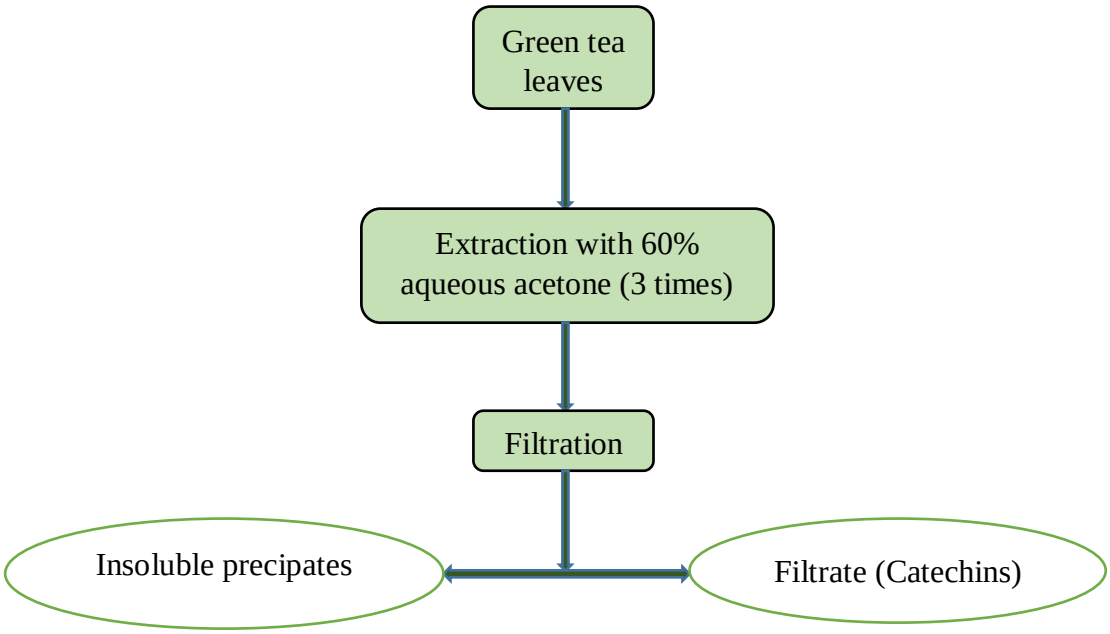


Figure I.5: Extraction of green tea catechins by aqueous acetone (Tanaka et al. 2013).

Simple maceration, ultrasound extraction and accelerated solvent extraction using various solvent systems **(Koch et al. 2020)** can be used to extract polyphenols, mainly catechins from tea. In **Figure I.6**, three extraction methods using different solvents and mixtures are illustrated.

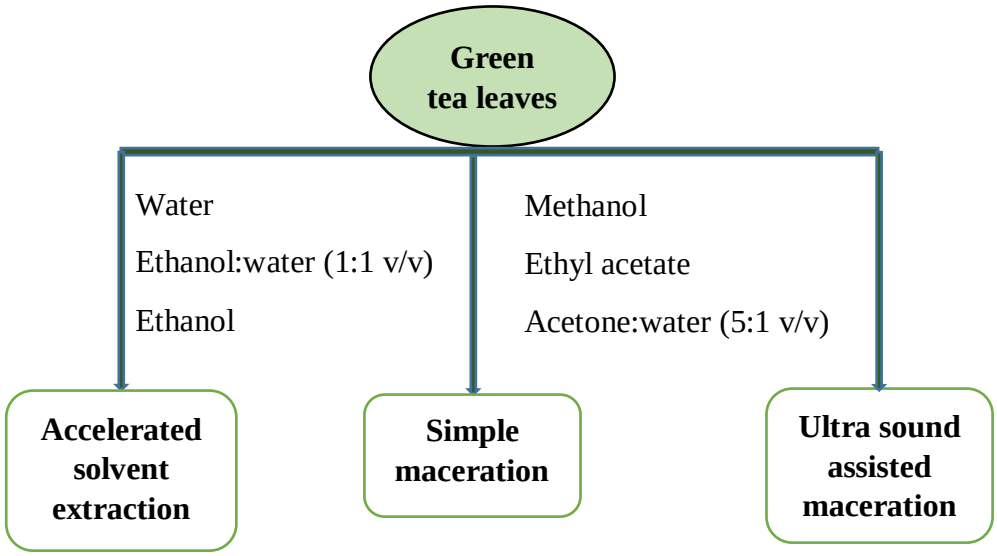


Figure I.6: Extraction of green tea catechins by different solvent mixtures (Koch et al. 2020).

I.1.4.1.3. Beneficial health effects

Catechins are polyphenolic phytochemicals with many important physiological activities in the human body, especially in the prevention of cardiovascular disease **(Chen et al. 2016)**.

Epigallocatechin gallate, the major bioactive catechin in green tea has been studied for almost the past thirty years, initially as a cancer chemoprevention agent and then for its cancer chemotherapeutic ability **(Rady et al. 2018)**.

Chakrawarti et al. (2016) have shown that epigallocatechin gallate has significant antioxidant, anti-carcinogenic, anti-microbial, and neuroprotective properties and has therapeutic potential against various human diseases.

In a review publication, **Fan et al. (2017)** reported the therapeutic benefits of EGCg and especially for the inflammatory bowel disease.

I.1.4.2. Methylxantines

The popularity of tea is in part due to the presence of moderate amounts of caffeine (2.5 - 4.5%). Other methylxanthines such as theobromine and theophylline, are also present but in very small quantities (0.1 and 0.02%, respectively) **(Graham 1992)**. Some authors reported the absence of theophylline in various tea extractions **(Hicks et al. 1996)**.

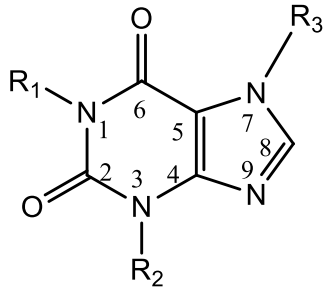
I.1.4.2.1. Description and structure

The tea plant provides alkaloids which are nitrogenous substances, found in plants, and developing biological effects, referred to collectively as methylxanthines, with very large chemical diversity **(Costentin 2010)**.

Methylxanthines are heterocyclic organic compounds built from coupled pyrimidinedione and imidazole rings **(Talik et al. 2012)**.

The most relevant methylxanthines are caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine). They differ in the number (caffeine has three, the others two) and position of N-CH_3 groups **(Figure I.7) (Monteiro et al. 2016)**. It has been proposed that plants started biosynthesizing methylxanthines to protect themselves against pathogens and predators, namely insect **(Monteiro et al. 2019)**.

The range of caffeine levels in the tea plant is affected by all the parameters that bring about variation in plant composition such as soil, raining, weather… etc. Application of nitrogen containing fertilizers can increase caffeine level by as much as 40% **(Balentine et al. 1998)**.



Methylxantine	R1	R2	R3
Caffeine	CH_3	CH_3	CH_3
Theophylline	CH_3	CH_3	H
Theobromine	H	CH_3	CH_3

Figure I.7 : Methylxantines structures (Monteiro et al. 2016).

I.1.4.2.2. Methods of extraction

The isolation of methylxanthines from plant material is mainly based on their solubility in solvents. There are two possible approaches to do this task ; one involving aqueous extraction and the other involving an organic solvent extraction **(Tarka et al. 1998)**.

I.1.4.2.2.1. Extraction with hot water

Methylxanthines are sparingly soluble in water. Theophylline and theobromine are amphoteric compounds whose acidic properties are due to the hydrogen atoms of the imide groups, while the basic properties are due to the nitrogen atom in position 9.

Theobromine is soluble in alkalis (NaOH), while theophylline is soluble not only in alkalis but also in aqueous ammonia. Unlike theohylline and theobromine, caffeine is not acidic and does not form insoluble salts with silver, cobalt, copper or iron ions **(Andreeva et al. 2012)**. The **Figure I.8** illustrates different steps for methylxantines extraction.

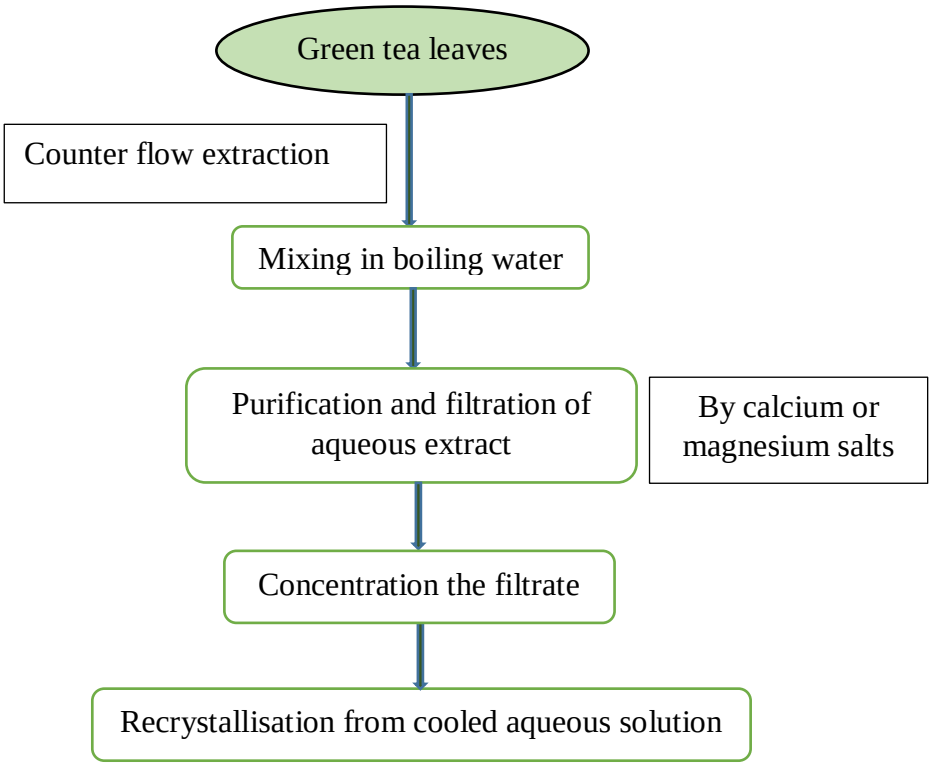


Figure I.8: Extraction of methylxantines in hot water (Andreeva et al. 2012).

I.1.4.2.2.2. Extraction with organic solvents

Liquid extraction using solvents such as methylene chloride, chloroform, methanol and n-hexane, has been used for methylxanthine extraction from natural plants **(Monteiro 2016)**. The **Figure I.9** summarizes the steps of methylxantines isolation from plants by organic solvents.

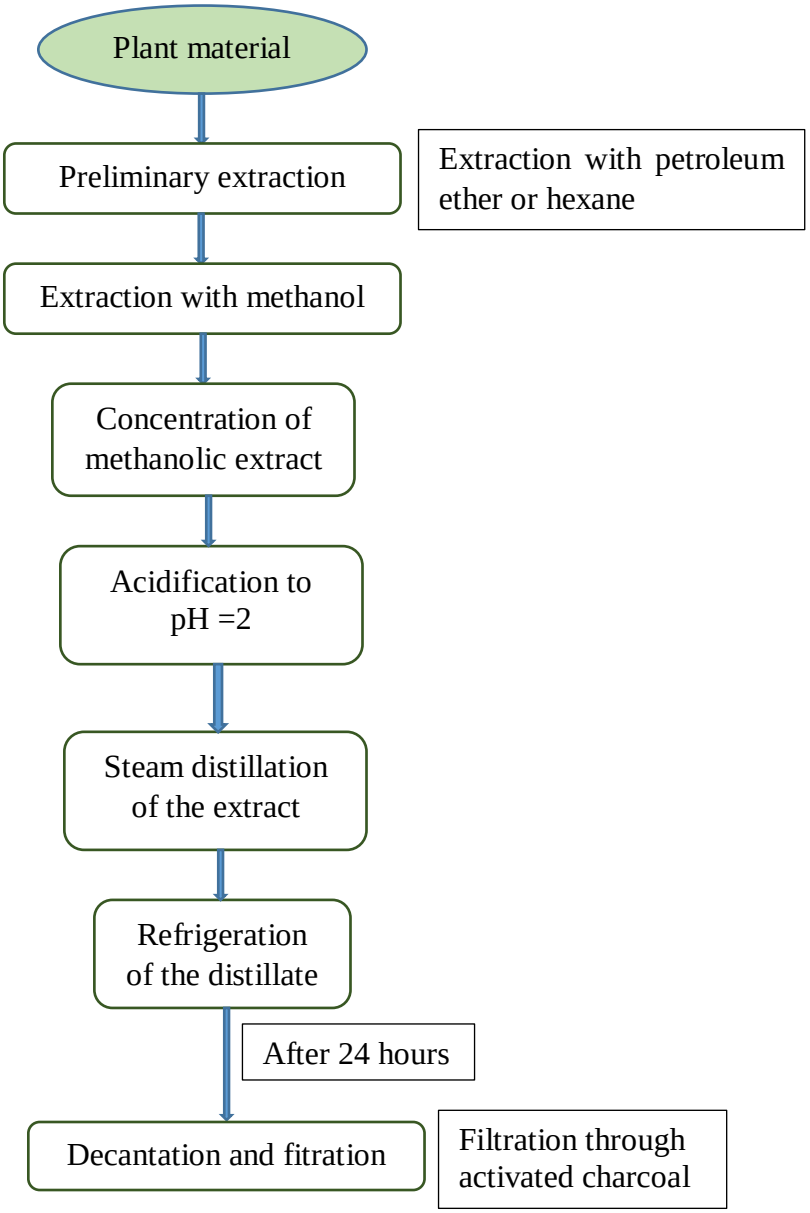


Figure I.9: Method of methylxantines extraction by organic solvents (Tarka and Hurst 1998).

I.1.4.2.3. Effects on human health

It is known that caffeine stimulates the central nervous system (CNS). Both caffeine and theophylline affect cerebral circulation, most likely through their effect as adenosine antagonists and increase the conduction velocity in the heart **(Fredholm 2011)**.

Methylxanthines correct, in part, neurological expressions of Parkinson's disease, thus developing a symptomatic effect **(El Yacoubi et al. 2001)**. Methylxanthines have similar effects to those of antidepressant agents in relation to the blockade of adenosine receptors **(El Yacoubi et al. 2003)**.

I.1.4.3. Gallic acid

Gallic acid **(Figure I.10)** is present in tea leaf, it is a hydroxylated derivative of benzoic acid and is a known reactant during the complex enzymatic and organochemical reactions that occur when tea components are oxidized **(Balentine et al. 1998)**. Gallic acid and quinic acid play key roles in forming esters with various polyphenols.

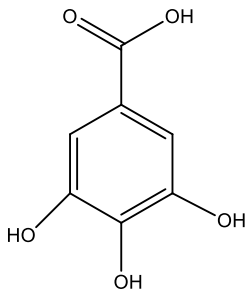


Figure I.10: Structure of gallic acid.

I.1.4.4. Other minor components

The other polyphenols in tea include flavonols (quercetin, kaempferol, myricetin), and their glycosides. Anthocyanidins are also found in tea leaves and the amino acid theanine is unique to tea.

In addition to phenolic compounds, the tea leaf contains vitamins and several minerals. Vitamin C is lost during the processing of the fresh leaf, but carotenoids and vitamin K are present in brewed tea. Tea also contains aluminium, potassium, fluoride and manganese **(Graham et al. 1992)**.

I.1.5. Free radicals

The free radicals, both the reactive oxygen species (ROS) and reactive nitrogen species (RNS), are derived from both endogenous sources (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells etc.) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs and radiations) **(Phaniendra et al. 2015)**.

I.1.6. Reactive oxygen species and oxidative stress

Oxidation by free radicals is the origin for the damage of biological complexes, such as DNA, lipids and proteins, which induces various degenerative diseases, like inflammatory, ischaemic, neuro disorder and cancer **(Anitha et al. 2020)**.

Free radicals are produced due to oxidative stress and are termed as reactive oxygen species which are formed from molecular oxygen in the mitochondrial respiratory chain. Oxidative stress reflects an imbalance between the production of ROS and the action of the antioxidant defense system in charge of their neutralization.

During the successive four steps of one electron reduction **(Reaction I.1)**, three primary species include, the superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) **(Collin 2019)**.

$$O_2 \xrightarrow{+e^-} O_2^{\cdot -} \xrightarrow{+e^-(+2H^+)} H_2O_2 \xrightarrow{+e^-} HO^{\cdot}(+HO^-) \xrightarrow{+e^-(+2H^+)} 2H_2O \qquad \text{(I.1)}$$

ROS can be deleterious for biomolecules and leads to oxidative damages. However, they play an important role in homeostasis, cell signalization, regulation of metabolism, or memory formation via DNA methylation **(Rhee 1999, Zhou et al. 2016)**. In mitochondria, ROS are produced during ATP biosynthesis which is accompanied by electron and proton transfers with molecular oxygen as the final target **(Collin (2019))**.

I.1.7. Antioxidant activity mechanism of green tea catechins

I.1.7.1. Mechanism of epicatechin oxidation

The epicatechin has two different pharmacophores, the catechol group in B ring and the resorcinol group in A ring and it has also the hydroxyl group at position 3 in C ring. The A and B rings in this molecule are not conjugated and ionization of the OH groups of one ring system should not appreciably affect ionization of the OH groups of the other ring **(Janeiro and Brett 2004)**. The **Figure I.11** shows the transformation of epicatechin by oxidation in B ring to quinone form.

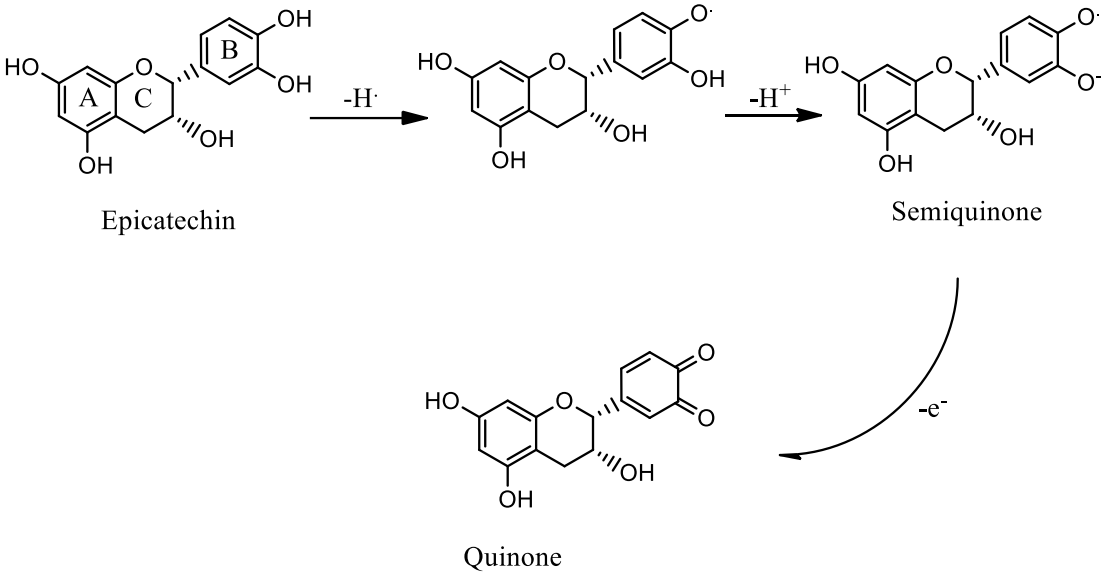


Figure I.11: Mechanism of catechins oxidation (Janeiro and Brett 2004).

It was revealed by spectroscopic methods that the influence of the A ring on the spectral properties of the radicals from the B ring is negligible in flavonoids where the C ring is completely saturated **(Jovanovic et al. 1994)**.

I.1.7.2. Scavenging reactive oxygen species by epigallocatechin gallate

Tea polyphenols have been shown to scavenge reactive oxygen, which may play important roles in carcinogenisis **(Wan et al. 2009)**.

Catechins (AH) can trap peroxyl radicals and thus suppress radical chain reactions and terminate lipid peroxidation **(Terao et al. 1994)**. The progress of the reaction was thought to proceed via the following stages:

1) Radical generation :

$$2R^{\cdot} + 2O_2 \longrightarrow 2ROO^{\cdot} \quad \text{(I.2)}$$

2) Radical trapping :

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad \text{(I.3)}$$

3) Radical termination

$$A^{\cdot} + X^{\cdot} \longrightarrow AX \text{ (Nonradical material)} \quad \text{(I.4)}$$

Among tea catechins, epigallocatecin gallate (EGCg) is most effective in reacting with most reaction oxygen species **(Yang et al. 2002 and He et al. 2018)**. The **Figure I.12** illustrates the scavenging mechanism of EGCg to peroxyl radical.

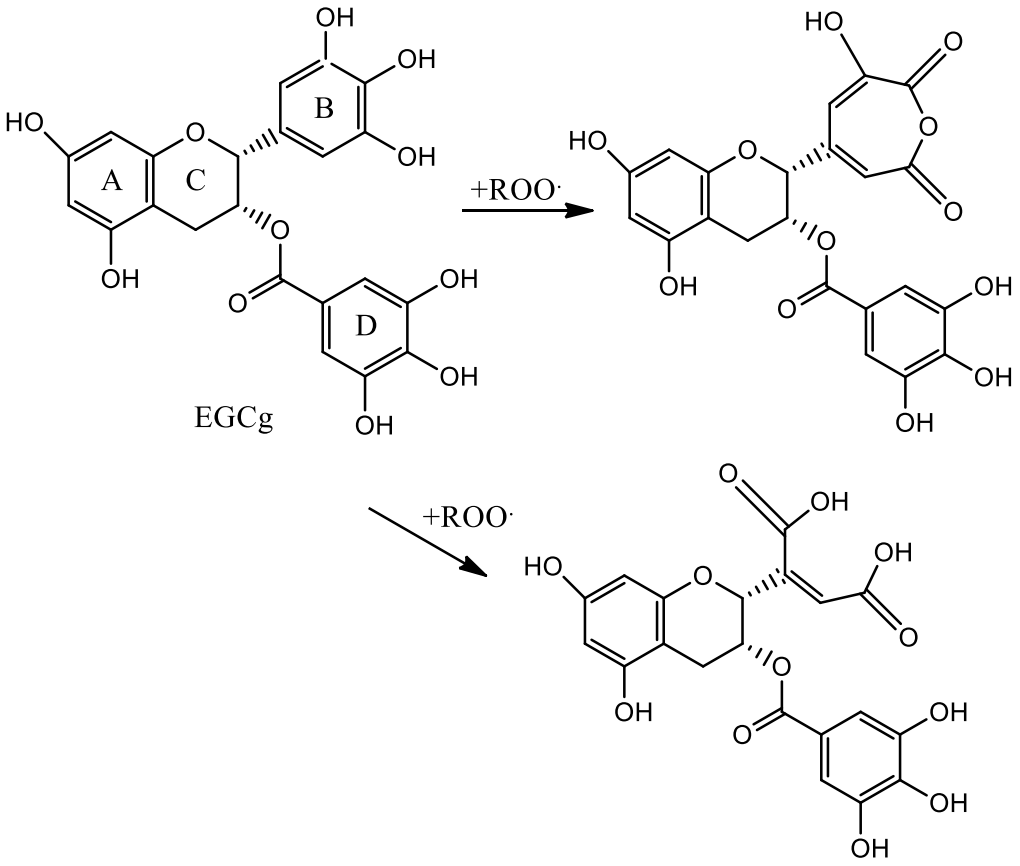


Figure I.12: Scavenging mechanism of EGCg to peroxyl radical (Wan et al. 2009).

I.2. Statistics of publications on tea and catechins

This section briefly traces the works on tea. Several groups of researchers around the world have studied the composition of tea, their applications, methods and solvents for their extraction. In what follows, the interpretation is focused on the few works carried out in 2020 and 2021.

I.2.1. Publications on tea (*Camellia sinensis*)

On **march 2021, Bai et al.** have published a work about biochemical characterization of specific Alanine Decarboxylase (AlaDC) and its ancestral enzyme Serine Decarboxylase (SDC) in tea plants (Camellia sinensis).

The study of **Swathi et al. (2021)** from India, has been focalized on extraction of active constituents from different brands of green tea. Ethanol and water were used as solvents.

Jia et al. (2021) from China developed an optical colorimetric sensor array for the discrimination of chinese teas.

Akbarialiabad et al. (2021), from Iran, analyzed the effect of tea on human health. Their review aimed to compile all the crucial data reporting the investigation on the conspicuous intervention of green tea and related lead compounds for their neurological activities, mechanisms of action, and clinical properties.

In a study published in Toxicon journal by an egyptian team **(El-Sayed Mostafa et al. 2021)**, the histopathological changes, inflammatory cytokines, and oxidative stress markers were found to significantly decrease during concomitant administration of green tea extract.

In **2021**, a review was published by **Kochman et al.** regarding health benefits and chemical composition of Matcha green tea.

A study about phytochemical properties of black tea (Camellia sinensis) and rooibos tea (Aspalathus linearis); and their modulatory effects on key hyperglycaemic processes and oxidative stress black tea has been published by **Xiao et al. (2020)** in the journal of Food Science and Technology.

The objective of **Chaikul et al. (2020)** from Thailand, was to investigate the activity of green tea against skin aging in melanoma cells and human skin fibroblasts.

The histogram below **(Figure I.13)** represents the number of publications from 2012 to 2021 on the general theme *Camellia sinensis* or tea.

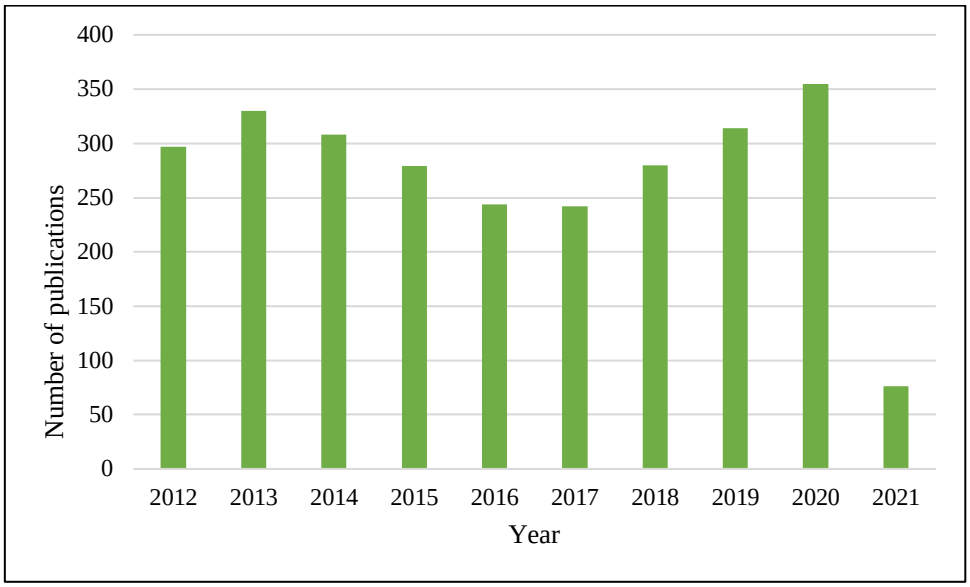


Figure I.13: Evolution of the number of publications on the general theme "Camellia sinensis or tea". Search carried out on 6 April 2021 in the Scopus platform.

I.2.2. Publications on extraction methods of tea

Some authors have published in Food chemistry journal studies about the extraction of polyciclic hydrocarbons from tea. **Deng et al. (2021)** could develop a novel density-tunable liquid-phase microextraction system to directly extract polycyclic aromatic hydrocarbons from tea and other foods. **Zhang et al. (2021)**, as far as they are concerned, their publication is titled ‘’Air-assisted liquid-liquid microextraction based on the solidification of floating deep eutectic solvents for the simultaneous determination of bisphenols and polycyclic aromatic hydrocarbons in tea infusions via HPLC’’.

To reduce the environmental pollution caused by organic solvents and improve the extraction efficiency, a range of natural deep eutectic solvents was explored for the extraction of green tea polyphenols, that was the aim of the study of Cui and his co-authors **(Cui et al. 2021)**.

Rajapaksha and Shimizu (2020), from Japan, evaluated the integrated processes of subcritical solvent extraction of polyphenols from spent black tea which was followed by microencapsulation to improve the stability of obtained extract.

For determination of pyrethroids in tea beverages, **Qian et al. (2020)** investigated the hydrophobic deep eutectic solvents based membrane emulsification-assisted liquid-phase microextraction method.

In the same area**, Bajkacz et al. (2020)** from Poland published in Molecules journal, a study titled ‘’Application of deep eutectic solvents and ionic liquids in the extraction of catechins from tea’’.

The graph in **Figure I.14** below represents the number of publications appeared between 2012 and 2021 whose themes are focused on methods of tea extraction.

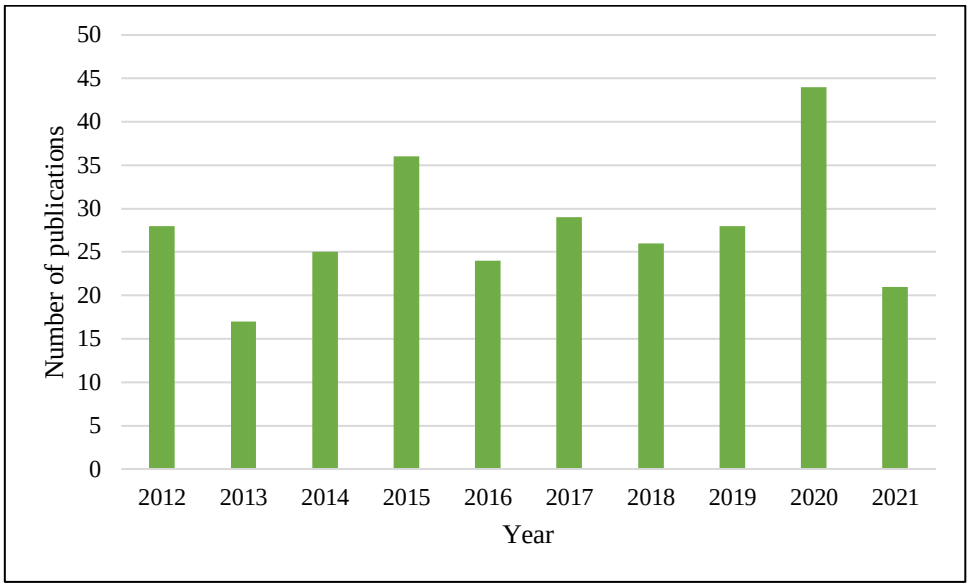


Figure I.14: Evolution of the number of publications on the extraction method of tea theme. Search carried out on 7 April 2021 in the Scopus platform.

I.2.3. Publications on therapeutic effect of epigallocatechin gallate

Constituting the major polyphenol in green tea, epigallocatechin gallate (EGCg) has been the subject of many studies mainly with regard to its therapeutic effects.

Jang et al. (2021), From Yonsei university of South Korea, published in Biochemical and Biophysical Research Communications journal an article about a current topic; COVID-19 pandemic titled ‘’EGCg, a green tea polyphenol, inhibits human coronavirus replication *in vitro*’’.

The study of **Xu et al. (2021)** from China, aimed to fabricate nanovesicles in-situ gel based on EGCg phospholipid complex in order to increase its stability and efficacy. The formation of EGCg phospholipid complex was characterized by Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC).

Zhang et al. (2021), from China, published in International Immunopharmacology journal, an article titled ‘’Epigallocatechin-3-gallate prevents inflammation and diabetes -Induced glucose tolerance through inhibition of NLRP3 inflammasome activation’’.

In Peking university school and hospital of stomatology of China, the study of Li and his coauthors **(Li et al. 2021)** investigated the effect of epigallocatechin gallate (EGCg) on the proliferation, mineralization, inflammation and hypoxia responses of human dental pulp stem cells (hDPSCs) in vitro and its effect on inflammatory pulp tissue in rats *in vivo*.

The **Figure I.15** recapitulates publications focusing on the biological applications of EGCg.

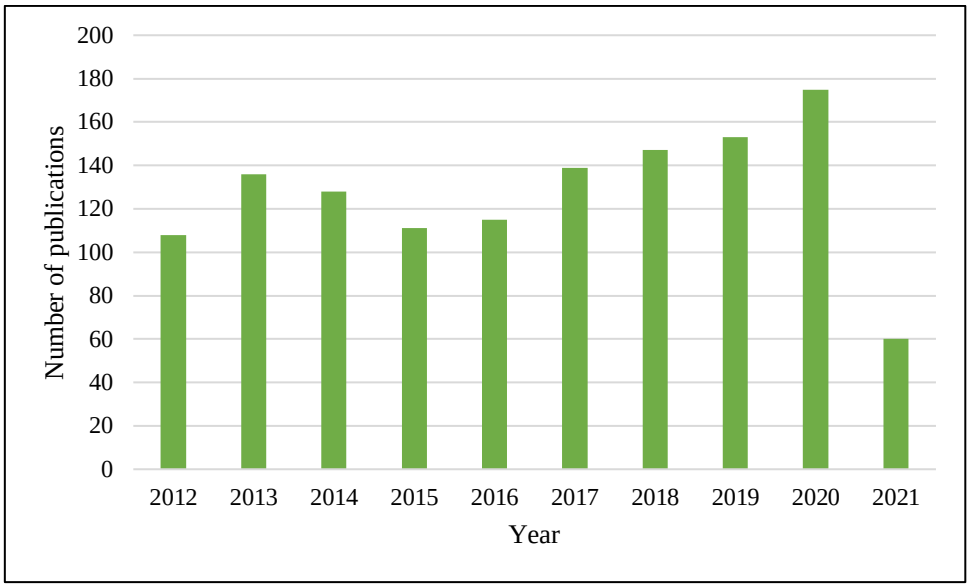


Figure I.15: Evolution of the number of publications on the therapeutic effect of EGCg. Search carried out on 6 April 2021 in the Scopus platform.

I.2.4. Publications on antioxidant activity of catechins

Hu et al. (2021) published a recent study titled ‘’Highly efficient synthesis and characterization of starch aldehyde-catechin conjugate with potent antioxidant activity’’.

The effects of epigallocatechin gallate (EGCg), epigallocatechin (EGC) and epicatechin gallate (ECg) on the chemical and cell-based antioxidant activity, sensory properties, and cytotoxicity of a catechin-free model beverage were modeled using response surface methodology by **Xu et al. (2021)**

In Food chemistry journal, **Ambigaipalan et al. (2020)** published a study which title is ‘’Epigallocatechin (EGC) esters as potential sources of antioxidants’’.

The **Figure I.16** recapitulates publications focusing on the antioxidant activity of tea catechins and polyphenols.

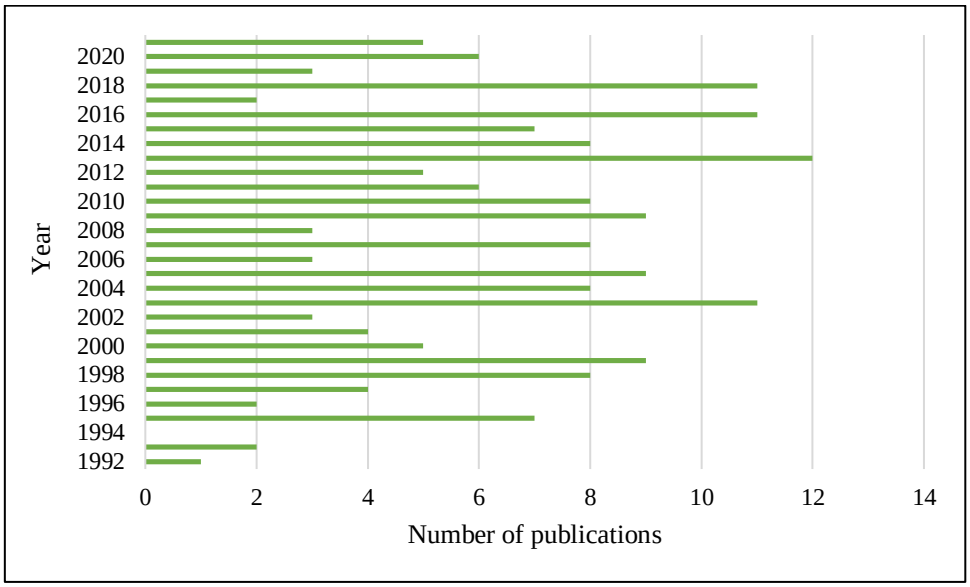


Figure I.16 : Evolution of the number of publications on the antioxidant activity of catechins. Search carried out on 28 April 2021 provided by Google Scholar.

Compared to published work and to the best of our knowledge and according to the availability of currently existing stastics, we can estimate that few publications on the theoretical study of the antioxidant activity of catechins have been carried out. We note that during five years between 2016 and 2020, only four articles were published on this subject **(Jongrungruangchok 2016, Wang et al. 2017, Labidi et al. 2018 and Anitha et al. 2020)**.

The publication of **(Boulmokh et al. 2021)** is considered as we know a considerable contribution on theoretical study of antioxidant activity of epicatechin and epigallocatechin gallate as two constituents of green tea polyphenols.

Conclusion

In this chapter, Essential points have been treated for instance structure, extraction methods and therapeutic effects of catechins and methylxantines which have the major contribution in green tea composition. The mechanisms of antioxidant activity of epicatechin and epigallocatechin gallate have also been viewed. Statistical insight of publications which focused on the tea, its

catechins and theirs applications led us to conclude that our study could be a net contribution to the knowledge of antioxidant activity of tea catechins, basically in the theoretical approach.

CHAPTER II

THEORETICAL CALCULATION METHODS

Chapter II

Theoretical calculation methods

Introduction

This chapter provides a brief view of the density functional theory (DFT) and Hartree Fock (HF) as the two methods used in the theoretical study of antioxidant activity of epigallocatechin gallate and epicatechin as compared to that of resveratrol and ascorbic acid.

Whereas the Hatree Fock methods lead to express the energy of the system as a functional of its wave function (ψ), in the DFT methods, the energy is a functional of the electron density of the system (ρ).

II.1. Schrödinger equation

The Schrödinger **equation (II.1)**, devised by Austrian Erwin Schrödinger in 1925-1926, is a fundamental equation in quantum mechanics based on the ideas put forward in 1924 by L. De Bgroglie **(Bransden and Joachain 1983)**. It was generalized by Paul Dirac a few years later. Initially, it took up the ideas of the mathematicians Hamilton and Felix Klein to extend De Broglie's theory of waves of matter.

The Schrödinger equation shows that the quantification of the energy of atomic systems results from a formulation in terms of eigenvalue problem for a linear differential operator H.

$$\hat{H}\psi = E\psi \qquad \text{II.1}$$

$\hat{H}$: Hamiltonian operator.

ψ : System wave function.

E : System energy.

$$\hat{H} = \hat{T}_e + \hat{T}_n + \hat{V}_{nn} + \hat{V}_{ne} + \hat{V}_{ee} \qquad \text{II.2}$$

Where $\hat{T}$ and $\hat{V}$ represent the operators of kinetic energy and potential energy, respectively **(Sulzer 2012)**.

$$\hat{\mathbf{T}}_{\mathbf{e}} = -\frac{\hbar^2}{2}\sum_{i=1}^{n}\frac{\nabla_i^2}{m_e} \qquad \text{II.3}$$

$$\hat{\mathbf{T}}_{\mathbf{n}} = -\frac{\hbar^2}{2}\sum_{I=1}^{N}\frac{\nabla_I^2}{m_I} \qquad \text{II.4}$$

$$\hat{\mathbf{V}}_{\mathbf{ee}} = \sum_{i=1}^{n}\sum_{j\neq i}\frac{e^2}{4\pi\varepsilon_0 r_{ij}} \qquad \text{II.5}$$

$$\hat{\mathbf{V}}_{\mathbf{nn}} = \sum_{I=1}^{N}\sum_{J\neq I}\frac{Z_I Z_J e^2}{4\pi\varepsilon_0 R_{IJ}} \qquad \text{II.6}$$

$$\hat{\mathbf{V}}_{\mathbf{ne}} = \sum_{I=1}^{N}\sum_{i=1}^{n}\frac{Z_I e^2}{4\pi\varepsilon_0 r_{Ii}} \qquad \text{II.7}$$

$$\left(-\frac{\hbar^2}{2}\sum_{i=1}^{n}\frac{\nabla_i^2}{m_e} - \frac{\hbar^2}{2}\sum_{I=1}^{N}\frac{\nabla_I^2}{m_I} + \sum_{i=1}^{n}\sum_{j\neq i}\frac{e^2}{4\pi\varepsilon_0 r_{ij}} + \sum_{I=1}^{N}\sum_{J\neq I}\frac{Z_I Z_J e^2}{4\pi\varepsilon_0 R_{IJ}} + \sum_{I=1}^{N}\sum_{i=1}^{n}\frac{Z_I e^2}{4\pi\varepsilon_0 r_{Ii}}\right)$$

$$\boldsymbol{\Psi} = \mathbf{E}\,\boldsymbol{\Psi} \qquad \text{II.8}$$

We can convert some terms in atomic units (au) as follows : **(Staemmler 2006)**

$\hbar = 1$; $e = 1$; $m = 1$, $4\pi\varepsilon_0 = 1$. The Schrödinger equation becomes:

$$\left(-\frac{1}{2}\sum_{i=1}^{n}\nabla_i^2 - \frac{1}{2}\sum_{I=1}^{N}\nabla_I^2 + \sum_{i=1}^{n}\sum_{j\neq i}\frac{1}{r_{ij}} + \sum_{I=1}^{N}\sum_{J\neq I}\frac{Z_I Z_J}{R_{IJ}} + \sum_{I=1}^{N}\sum_{i=1}^{n}\frac{Z_I}{r_{Ii}}\right)\boldsymbol{\Psi} = \mathbf{E}\,\boldsymbol{\Psi} \qquad \text{II.9}$$

According to Born-Oppenheimer approximation :

$-\frac{1}{2}\sum_{I=1}^{N}\nabla_I^2 = 0$ and $\sum_{I=1}^{N}\sum_{J\neq I}\frac{Z_I Z_J}{R_{IJ}}$ = Constant, the Hamiltonian can be written as:

$$\hat{H} = \hat{H}_e = \hat{T}_e + \hat{V}_{ne} + \hat{V}_{ee} \qquad \text{II.10}$$

The expression of the electronic Hamiltonian of a polyelectronic system is:

$$\hat{\mathrm{H}}_e = -\frac{\hbar^2}{2}\sum_{i=1}^{n}\frac{\nabla_i^2}{m_e} + \sum_{i=1}^{n}\sum_{j\neq i}\frac{e^2}{4\pi\varepsilon_0 r_{ij}} + \sum_{I=1}^{N}\sum_{i=1}^{n}\frac{Z_I e^2}{4\pi\varepsilon_0 r_{Ii}} \qquad \text{II.11}$$

$$\hat{H}_e = -\frac{1}{2}\sum_i^n \nabla_i^2 + \sum_{i\neq j}^n \frac{1}{r_{ij}} + \sum_i^n v(r_i) \quad \text{II.12}$$

$v(r_i)$: Electron potential i.

ħ : Planck's universal constant.

m_e : Electron mass.

m_I : Mass of the nucleus I.

r_{Ii} : The distance between nucleus I and electron i.

R_{IJ} : The distance between nucleus I and nucleus J.

r_{ij} : The distance between electron i and electron j.

Z_I, Z_J : Nuclear charges of nucleus I and nucleus J respectively.

∇_I^2 (Δ_I) : The Laplacian of nucleus I.

∇_i^2 (Δ_i): The Laplacian of electron i.

II.2. Hartree-Fock method

Because inter-electronic repulsion makes the Schrödinger equation impossible to solve accurately/analytically, the best possible one-electron wave functions was published by Hartree in 1928 and improved two years later by Fock.

The Hartree-Fock method seeks to approximately solve Schrödinger equation, and it assumes that the wave function can be approximated by a single Slater determinant made up of one spin orbital per electron. The Hartree-Fock method (HF) is a particular case of the variational method, in which the trial function for the N-electron atom is a Slater determinant.

The HF method has two variants: the Restricted Hartree-Fock approach or RHF which concerns the so-called "closed" layer systems and the Hartree-Fock unrestricted or UHF approach concerns the so-called "open" layer systems **(Orio 2007)**.

For a system containing non interacting electrons, the Hamiltonian has the form :

$$\boldsymbol{H} = \sum_{i=1}^N \boldsymbol{h}(\boldsymbol{i}) \quad \text{II.13}$$

$$\boldsymbol{h}(\boldsymbol{i})\boldsymbol{\chi_j}(\mathbf{X}_i) = \boldsymbol{\varepsilon_j}\boldsymbol{\chi_j}(\mathbf{X}_i) \quad \text{II.14}$$

h (i) is the operator describing the kinetic energy and the potential energy of electron i.

χ_j is the spin orbital.

ε_j is the spin orbiatl energy.

X_i is space and spin coordinates of electron i.

Another term is introduced, the Hartree product wave function (Ψ^{HP}) :

$$\boldsymbol{\Psi^{HP}(X_1, X_2, \ldots\ldots, X_N) = \chi_i(X_1)\,\chi_j(X_2)\ldots\ldots\ldots\chi_k(X_N)} \qquad \text{II.15}$$

$$\mathbf{H\Psi^{HP} = E\Psi^{HP}} \qquad \text{II.16}$$

The eigenvalue E represents the sum of the spin orbital energies :

$$\mathbf{E = \varepsilon_i + \varepsilon_j + \ldots\ldots\ldots\varepsilon_k} \qquad \text{II.17}$$

II.2.1. Slater determinant

In the Hartree-Fock approach, it is assumed, in accordance with the independent particle approximation and the Pauli exclusion principle, that the N-electron wave function is a Slater determinant, or in other words an antisymmetric product of individual electron spin orbitals **(Szabo 1982)**. For an atom with N electrons :

$$\mathbf{\Psi(X_1, X_2, X_3,\ldots\ldots\ldots,X_N)} = \frac{1}{\sqrt{N!}} = \begin{vmatrix} \chi_i(X_1) & \chi_j(X_1) & \ldots & \chi_k(X_1) \\ \chi_i(X_2) & \chi_j(X_2) & \ldots & \chi_k(X_2) \\ \vdots & \vdots & & \vdots \\ \chi_i(X_N) & \chi_j(X_N) & \ldots & \chi_k(X_N) \end{vmatrix} \qquad \text{II.18}$$

$\frac{1}{\sqrt{N!}}$: Normalization factor.

The Slater determinant has N electrons occupying N spin orbitals (χ_i, χ_j, ….., χ_k) without specifying which electron is in which orbital **(Szabo 1982)**.

II.2.2. The Hartree-Fock approximation

The Hartree-Fock approximation is important not only for its own sake but as a starting point for more accurate approximations which include the effects of electron correlation. It is convenient to define a coulomb operator as **(Sherrill 2000)**:

$$\mathcal{J}_j(\boldsymbol{X_1}) = \int \boldsymbol{dX_2} \left|\chi_j(\boldsymbol{X_2})\right|^2 \boldsymbol{r_{12}^{-1}} \qquad \text{II.19}$$

We define an exchange operator in terms of its action on an arbitrary spin orbital χ_i :

$$\text{K}_j(\boldsymbol{X_1})(\chi_i)(\boldsymbol{X_1}) = \left[\int \boldsymbol{dX_2}\, \chi_j^*(\boldsymbol{X_2}) r_{12}^{-1} \chi_i(\boldsymbol{X_2})\right]\chi_j(\boldsymbol{X_1}) \qquad \text{II.20}$$

The Hartree-Fock equation becomes :

$$\left[h(\boldsymbol{X}_1) + \sum_{j\neq i} \mathcal{J}_j(\boldsymbol{X}_1) - \sum_{j\neq i} \text{K}_j\,(\boldsymbol{X}_1)\right]\chi_i(\boldsymbol{X}_1) = \varepsilon_i \chi_i(\boldsymbol{X}_1) \qquad \text{II.21}$$

If we put the Fock operator in the following form :

$$f(\boldsymbol{X}_1) = h(\boldsymbol{X}_1) + \sum_j \mathcal{J}_j\,(\boldsymbol{X}_1) - \text{K}_j(\boldsymbol{X}_1) \qquad \text{II.22}$$

The Hartree-Fock equation is an eigenvalue equation of the form :

$$\boldsymbol{f(i)\,\chi(X_i) = \varepsilon\chi(X_i)} \qquad \text{II.23}$$

Where $\boldsymbol{f(i)}$ is an effective one-electron operator called the Fock operator.

$$\mathbf{f}\,(\mathbf{i}) = -\frac{\mathbf{1}}{\mathbf{2}}\boldsymbol{\nabla_i^2} - \sum_{I=1}^{N} \frac{Z_I}{r_{iI}} + \boldsymbol{\nu^{HF}(i)} \qquad \text{II.24}$$

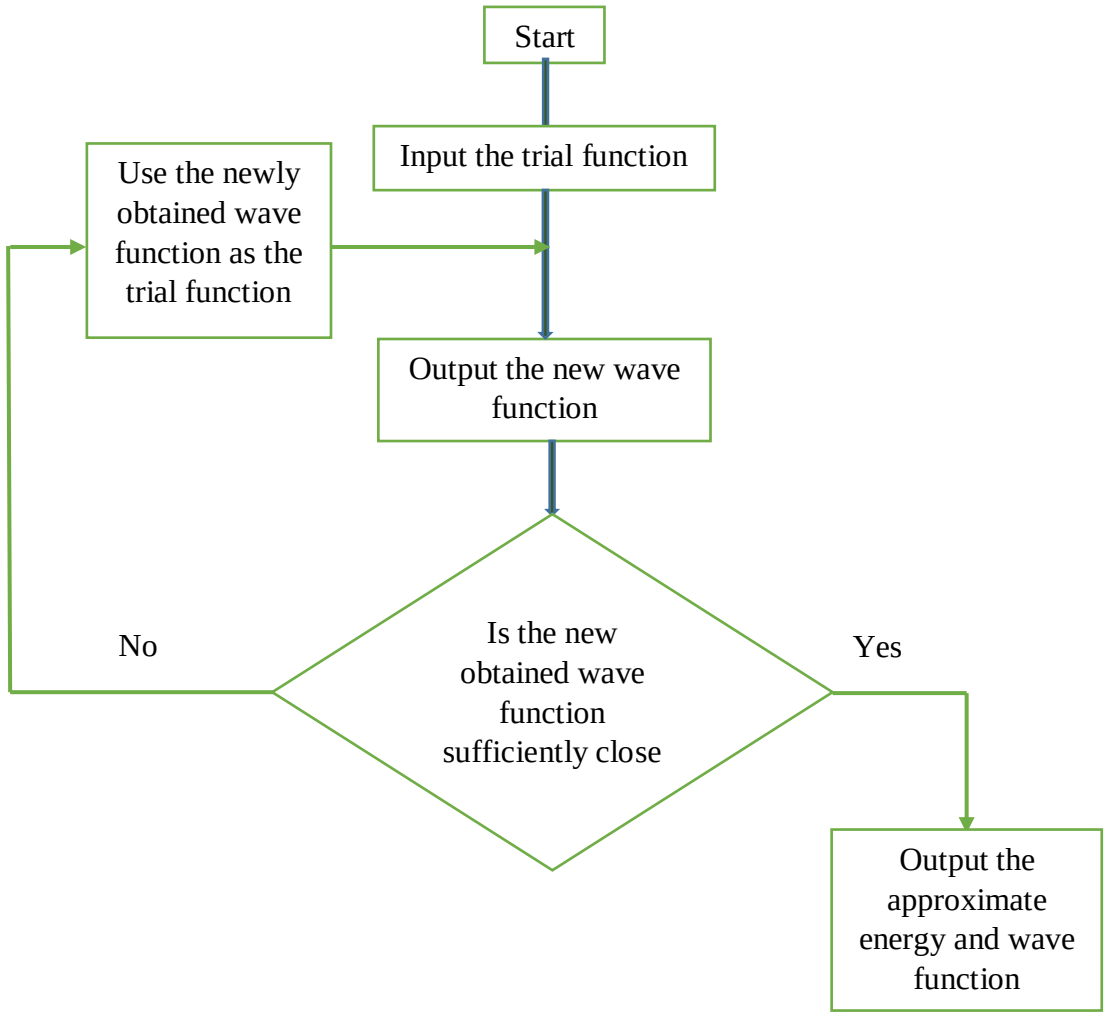


Figure II.1 : Principle of Hartree-Fock method.

II.3. Density functional theory

II.3.1. Definition and basics

Density functional theory (DFT) is an approximate solution to the Shrodinger equation of a many-body system. It is primarily a theory of electronic ground state structure, couched in terms of the electronic density distribution n(r) **(Kohn et al. 1996)**.

As its name suggests, DFT theory uses electronic density instead of wave function and the goal of DFT methods is to determine the functionalities which allow to link electronic density to energy. This theory has become a theoretical tool which has taken a very important place among the methods used for the description and analysis of physicochemical properties for complex systems containing a large number of electrons **(Dreizler and Gross 1990)**.

The DFT solves the Schrödinger equation with N particles by using only the observable ρ ($\vec{r}$), defined in physical space R^3 which replaces a configuration space with 3N variables in which the wave function is defined.

The electronic density ρ (r) is a positive function depending only on the 3 coordinates (x,y,z) of space satisfying the following conditions :

$$\begin{cases} \boldsymbol{\rho}\,(\boldsymbol{r} \to \infty) = \mathbf{0} \\ \int \boldsymbol{\rho}(\boldsymbol{r})\boldsymbol{dr} = \boldsymbol{N} \end{cases} \qquad \text{II.25}$$

II.3.2. The energy functional

In density functional theory, the total energy is written as **(Gill et al. 1992)**:

$$\boldsymbol{E}[\boldsymbol{\rho}(\boldsymbol{r})] = \boldsymbol{E_T}[\boldsymbol{\rho}] + \boldsymbol{E_V}[\boldsymbol{\rho}] + \boldsymbol{E_J}[\boldsymbol{\rho}] + \boldsymbol{E_{XC}} \qquad \text{II.26}$$

where E_T is the kinetic energy of independent electrons having the density p, E_v is the potential energy involving nuclei (nuclear-electron + nuclear-nuclear), E_J is the overall coulomb repulsive energy and E_{XC} is the exchange-correlation energy, representing the energy lowering due to the fact that the complete electron-electron interaction is less than E_J.

II.3.3. Hohenberg and Kohn theorem

The first theorem of **(Hohenberg and Kohn 1964)** consists in giving a theoretical justification to the idea that at a given density corresponds a unique external potential (ν_{ext}(r)) which is determined by the electron density ρ(r). Since ρ fixes the number of electrons, it follows that the electron density ρ(r) also uniquely determines the wave function and all electronic properties of the system. The terms independent of the system are then grouped within a Hohenberg-Kohn functional (E_{HK}). This new functional contains E_T[ρ], electronic kinetic energy and $V_{e\text{-}e}$[ρ], the potential energy due to the interaction between electrons.

$$\boldsymbol{E_{HK}}[\boldsymbol{\rho}(\boldsymbol{r})] = \boldsymbol{E_T}[\boldsymbol{\rho}(\boldsymbol{r})] + \boldsymbol{E_J}[\boldsymbol{\rho}(\boldsymbol{r})] \qquad \text{II.27}$$

$$\boldsymbol{E}[\boldsymbol{\rho}(\boldsymbol{r})] = \boldsymbol{E_{HK}}[\boldsymbol{\rho}(\boldsymbol{r})] + \int \boldsymbol{\rho}(\boldsymbol{r})\boldsymbol{\nu_{ext}}(\boldsymbol{r})\boldsymbol{dr} \qquad \text{II.28}$$

The explicit expressions of these two functionals are not known. On the other hand, we can extract from $E_{e\text{-}e}$ the classical part, Hartree energy $\boldsymbol{E_{e-e}^{cla}[\rho]}$.

$$\boldsymbol{E_{e-e}^{cla} = \frac{1}{2}\int \frac{\rho(r)\rho(r')}{|r-r'|}} \qquad \text{II.29}$$

The **Hohenberg and Kohn (1964)** second theorem which we can state in the following way : Energy $E[\rho_{test}]$, associated with any test density, satisfying the necessary boundary conditions : $\rho_{test}(r) \geq 0$, $\int \rho_{test}(r)dr = N$ and associated with an external potential ν_{ext}, is greater than or equal to the energy associated with the electron density of the fondamental state $E[\rho_{fond}]$.
According to the first theorem, a test density defines its own Hamiltonian and similarly its own test wave function.
From there, we can have a correspondence between the variational principle in its wave function version and in its electron density version such that :

$$\boldsymbol{\langle \psi_{test}|H|\psi_{test}\rangle = E[\rho_{test}] \geq E_{fond} = \langle \psi_{fond}|H|\psi_{fond}\rangle} \qquad \text{II.30}$$

The two theorems of Hohenberg and Kohn therefore offer a theoretical framework for considering the resolution of the Schrödinger equation via electronic density as the main variable.

II.3.4. Equations of Kohn-Sham

During the different treatments of the total energy of the system, we therefore introduced a new functional, called universal because it does not depend on the electronic system, F_{HK}, the functional of Hohenberg and Kohn. As we have seen previously, this functional group includes two terms (T_e and $V_{e\text{-}e}$); which themselves are density functionals. However, their analytical expression for the interacting N electron system is unknown.

Kohn and Sham (1965) thought it was essential to have an expression as precise as possible for the kinetic energy term. To do this, they introduced the idea of fictitious electron system without interaction of the same density ρ(r) that the interacting electron system. Based on this reference system, it is then possible to give an exact expression to the kinetic energy of a system of N electrons not interacting as a density functional ρ(r).

Kohn and Sham introduced an orbital method. In order to evaluate the kinetic energy of N non interacting particles given only their density distribution n(r), they simply found the corresponding potential (ν_{eff}(r) and used the Schrodinger equation :

$$\left(-\frac{\hbar^2}{2m}\nabla^2 + \nu_{eff}(r)\right)\Psi_i(r) = \epsilon_i\Psi_i(r) \qquad \text{II.31}$$

$$\nu_{eff}(r) = \nu(r) - e\varphi(r) + \nu_{xc}(r) \qquad \text{II.32}$$

The electrostatic potential is given by :

$$\varphi(r) = -e\int dr' \frac{n(r')}{|r-r'|} \qquad \text{II.33}$$

$$\nu_{xc}(r) = \frac{\delta E_{xc}}{\delta n(r)} \qquad \text{II.34}$$

Given a practical approximation for E_{xc}(n), one obtains ν_{xc}(r) and can thus find ν_{eff}(r) **(Argaman and Makov 2000)**.

II.3.5. Simplest exchange and the correlation functionals

The density functional theory applied in the context of the orbital approach of Kohn and Sham remains exact in its formalism. Gradually, the unknown part in the functional E[ρ] has been reduced to a universal functional F_{HK}[ρ] and finally to an exchange energy and correlation Exc [ρ]. At this stage, it is necessary to approach the expression of this functional exchange and correlation, so that it offers a description as precise as possible of the system.

II.3.5.1. Local approximation

The local density approximation (LDA) is the simplest and most widely used exchange-correlation functional **(Filippi et al. 1996)**, it can be defined as follows :

$$E_{xc}^{LDA}[\rho] = \int \rho(r)\varepsilon_{xc}\big(\rho(r)\big)dr \qquad \text{II.35}$$

It is the functional for which an exact form is almost known. The approximation of E_{xc} [ρ] is based on the uniform electron gas model where the term ε_{xc} (ρ(r)) is the particle-specific exchange-

correlation energy of the uniform electron gas with density ρ(r). Furthermore, ε_{xc} (ρ(r)) can be considered as the sum of an exchange and correlation contribution :

$$\boldsymbol{\varepsilon_{xc}(\rho(r)) = \varepsilon_x(\rho(r)) + \varepsilon_c(\rho(r))} \quad \text{II.36}$$

II.3.5.2. Generalized gradient approximation

In the generalized gradient approximation (GGA) a functional form is adopted which ensures the normalization condition and that the exchange hole is negative definite.

The typical form for a generalised gradient approximation (GGA) is **(Fillipi et al. 1996)**:

$$\boldsymbol{E_{xc}^{GGA}[\rho] = \int \rho(r)\epsilon_{xc}^{GGA}(\rho(r), |\nabla\rho(r)|, \nabla^2\rho(r))dr} \quad \text{II.37}$$

II.3.6. Functional HF-DFT hybrids

The basic idea behind the hybrid functionals is to mix exchange energies calculated in an exact (Hartree-Fock-like) manner with those obtained from DFT methods in order to improve performance.

Becke has chosen to use the exact exchange (HF) differently by including only a part of it in the exchange-correlation energy **(Becke 1993)**.

$$\boldsymbol{E_{xc} = E_{xc}^{LDA} + a_0(E_x^{HF} - E_x^{LDA}) + a_x\Delta E_x^{B88} + a_c\Delta E_c^{PW91}} \quad \text{II.38}$$

Where the coefficients a_0, a_x and a_c are determined semi-empirically by fitting on the experimental data. E_X^{HF} represents the exact exchange energy obtained from of a Hartree-Fock calculation.
A variant of this hybrid approach, using the approximation of Lee, Yang and Parr (LYP) rather than that of Perdew and Wang (PW).

$$\boldsymbol{E_x^{B3LYP} = a_0E_x^{LDA} + (1 - a_0)E_x^{HF} + a_1\Delta E_x^{B88} + E_c^{LDA} + a_2(E_c^{LYP} + E_c^{LDA})} \quad \text{II.39}$$

Where $a_0 = 0.80$, $a_1 = 0.72$ and $a_2 = 0.81$.

II.4. GAUSSIAN software

GAUSSIAN is a computational chemistry software program initially released in 1970 by John Pople and for more than 30 years “Gaussian” has been the standard program in computational

quantum chemistry, and its development continues vigorously in the hands of Pople's former students **(Pople 2004)**.

Among many other standard capabilities and according to the most recent Gaussian manual, the package can do :

- SCF methods : Restricted, unrestricted, and restricted Open-shell Hartree-Fock.
- Built-in DFT methods : B3LYP and other hybrid functionals, exchange functionals (PBE, MPW, PW91, Slater,…) and correlation functionals (PW91, LYP, PL, P86, B95,…).

The major release history is as follows : Gaussian70, 76, 77, 78, 80, 82, 83, 85, 86, 88, 90, 92, 93, 94, 95, 96, 98, 03, 09 and 16.

In the gas phase or in solutions, and in their ground state or in an excited state, Gaussian is capable of predicting energies and structures of transition sates, bond and reaction energies, molecular orbitals, multipole moments, atomic charges and electrostatic potential, vibrational frequencies,….etc.

Conclusion

Both methods : Density functional theory (DFT) and Hartree-Fock (HF) have approximations to make them applicable in different systems. Gaussian as software allows the calculation of electronic or thermodynamic parameters via both methods.

CHAPTER III

MATERIAL AND METHODS

Chapter III
Material and methods

Introduction

In this chapter, we will present the methods, material, reagents and samples used in the experimental and the theoretical parts of this study.

In the experimental part, we will first present the procedures for the quantification by HPLC of polyphenolic compounds (epigallocatechin gallate, epicatechin gallate, epigallocatechin, epicatechin and gallic acid) and methylxathines (caffeine, theophylline and theobromine) in eight brands of green tea.

Secondly, we will present the procedures applied for the determination of the antioxidant activity of epigallocatechin gallate, epicatechin and a sample of green tea by the inhibition of DPPH radical and by ferric reducing antioxydant power (FRAP) methods.

The theoretical part which is essentially based on the DFT method, is focused on the study of the antioxidant activity of EGCg and EC based on the calculation of the: Bond Dissociation Enthalpy (BDE), Adiabatic Ionization Potential (AIP), Proton Dissociation Enthalpy (PDE), Proton Affinity (PA) and Electron Transfert Enthalpy (ETE), Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) energies, spin density, Electrostatic Potential (ESP), the enthalpy of acidity ($\Delta H_{acidity}$) and pKa of the OHs of the above mentioned molecules.

We should notice that the study of the antioxidant activity by both experimental and theoretical approaches was carried out in comparison with that of resveratrol and ascorbic acid.

III.1. Experimental part

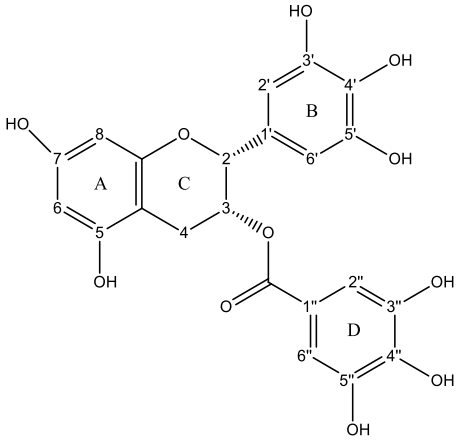
III.1.1. Quantification of green tea metabolites

III.1.1.1. Standards and green tea samples

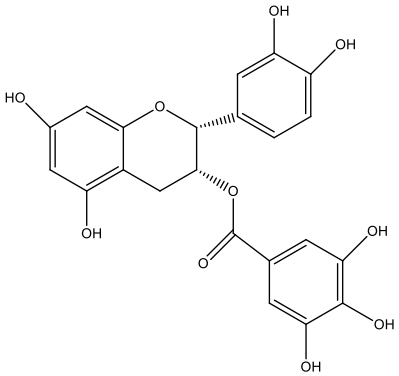
In this study, the standards ((-)-epicatechin (EC), (-)-epigallocatechin, (-)-epicatechingallate (ECg), (-)-epigallocatechin gallate (EGCg), gallic acid (GA), caffeine (Caff), theophylline (Theoph) and theobromine (Theobr)) were of purity greater than 97%. All these molecules were purchased from Sigma-Aldrich (Germany).

Eight commercial green teas (S1- S8), originated in China, at different prices were purchased at Guelma markets.

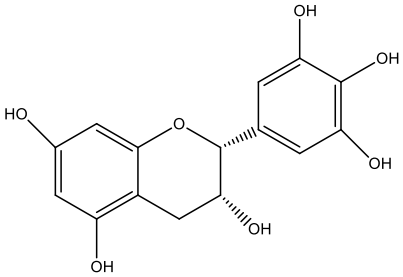
The structures of the standard compounds, their main properties and their UV spectra are presented in **Figure III.1**, **Table III.1** and **Table III.2** respectively.



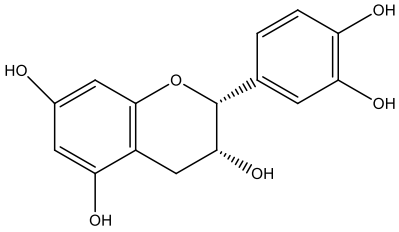
Epigallocatechin gallate



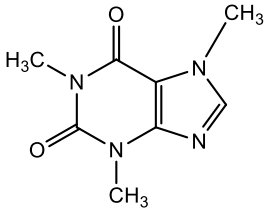
Epicatechin gallate



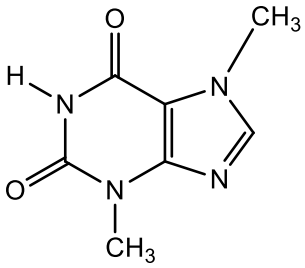
Epigallocatechin



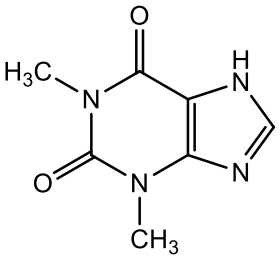
Epicatechin



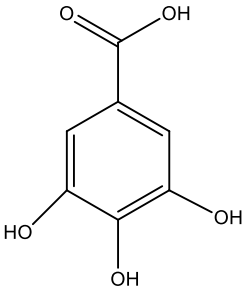
Caffeine



Theobromine



Theophylline



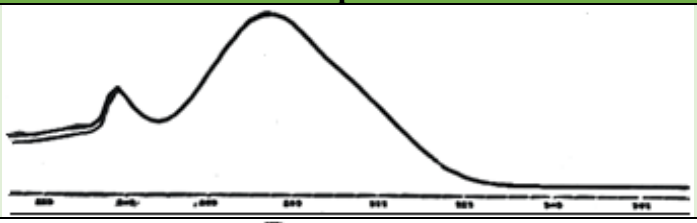
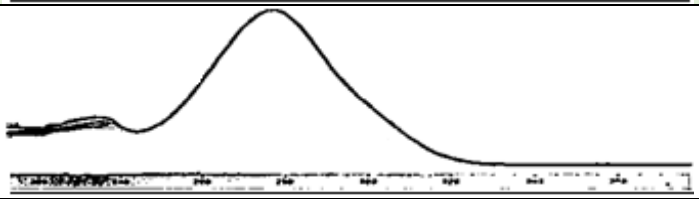
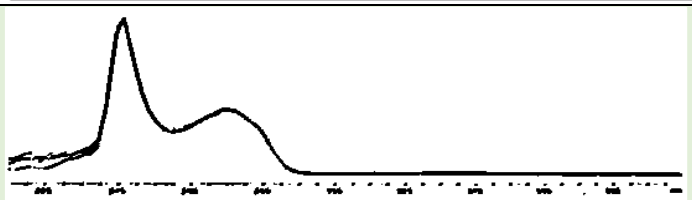
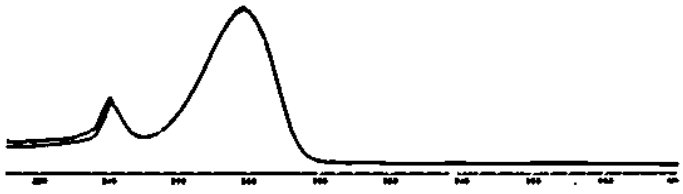
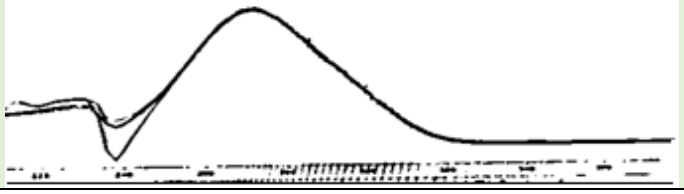
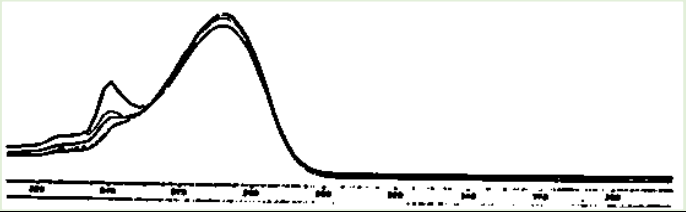
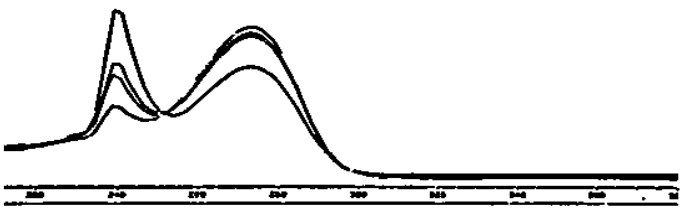
Gallic acid

Figure III.1: Structures of the standard molecules.

Table III.1: Some characteristics of molecules studied (LKT Laboratories 2021, ChemicalBook 2017).

Molecule	Formula	Molecular mass (g/mol)	Appearance	Solubility
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	458.37	White crystalline powder	Soluble in methanol, ethanol (92 mg/mL) or DMSO (92 mg/mL). Slightly soluble in water
Epicatechin gallate	$C_{22}H_{18}O_{10}$	442.37	White crystalline powder	Soluble in water or alcohol.
Epigallocatechin	$C_{15}H_{14}O_7$	306.27	White powder	Soluble in water or alcohol.
Epicatechin	$C_{15}H_{14}O_6$	290.27	White crystalline powder	Soluble in water or alcohol.
Caffeine	$C_8H_{10}N_4O_2$	194.19	White crystalline powder	Soluble in ethyl acetate or pyrrole. Slightly soluble in water (20 mg/mL), solubility is increased by adding dilute acid. Slightly soluble in ethanol (13 mg/mL).
Theophylline	$C_7H_8N_4O_2$	180.16	White crystalline powder	Slightly soluble in water (4 mg/mL). Soluble in ammonium hydroxide (50 mg/mL), alcohol (10 mg/mL), DMSO (15 mg/mL)
Theobromine	$C_7H_8N_4O_2$	180.16	white crystalline powder	Slightly soluble in water <0.1 g/100 mL at 18 ºC
Gallic acid	$C_7H_6O_5$	170.12	White to off white powder	Soluble in ethanol, acetone, DMSO, methanol. Slightly soluble in water (11.5 mg/mL)

Table III.2: UV spectra of pure compounds.

Molecule	UV spectra	λ_{max} (nm)
Epigallocatechin gallate		280
Epicatechin gallate		280
Epigallocatechin		280
Epicatechin		280
Gallic acid		270
Caffeine		273
Theophylline		273
Theobromine		273

The eight brands of green tea studied and their organoleptic properties are presented In **Figure III.2**, and **Table III.3,** respectively.

Figure III.2: The eight brands of green tea used.

Table III.3: Organoleptic properties of studied green tea leaves.

Green tea sample	Color	Shape (form)
S1	Deep green	Needle and curved
S2	Deep green	Needle and curved
S3	Deep green	Needle
S4	Deep green	Needle
S5	Deep green	Needle
S6	Green	Needle and curved
S7	Green	Needle and curved
S8	Green	Needle

III.1.1.2. Solvents and reagents

For extraction and HPLC analyses, HPLC grade methanol and distilled and filtered water were used. In the mobile phase, formic acid of purity > 99 % was added. The two reagents (methanol and formic acid) were purchased from Sigma-Aldrich (Germany). In **Table III.4**, some physical properties of methanol and formic acid are summarized.

III.1.1.3. HPLC apparatus

HPLC analysis was conducted using an Agilent 1260 infinity series apparatus (DE, Germany) **(Figure III.3)**, operating with a quaternary pump system, a flow range of 0.05 - 5.0 ml/min, a pressure operating range (0 - 600 bar) and a recommended pH (1- 12.5). The different modules composing this apparatus are:

(i) A degasser, model G4225A, which comprises four separative vacuum chambers with semi-permeable tubings, a vacuum pump and a control assembly.

(ii) A thermostated column compartment (TCC) which controls the temperature between 10°C below ambient and up to 100°C at 2.5 ml/min, and 80°C at up to 5 ml/min, respectively.

(iii) A diode array detector (DAD) G4212B with a deuterium lamp as the light source operating at a wavelength range from 190 to 640 nm. The entrance to the spectrograph is through a fixed optical slit of 4 nm. Signals can be output at 80 Hz (80 data points/second) for accurate recording of the fastest (narrowed) chromatographic peaks. At the same time, the module can also output full-range spectra to the data system at the same rate of 80 Hz.

The apparatus is equipped with a Zorbax Eclipse Plus C 18 column (analytical 4.6x250mm; 5-Micron) (Agilent, USA). The whole system was monitored by the Chem Station software. The peaks were identified based on their retention times. The peak area of compounds were taken from the chromatograms recorded at 280 nm for the four catechins, 270 for gallic acid and at 273 nm for the three methylxantines studied.

Figure III.3: HPLC (Agilent 1260 infinity) used in analysis (Laboratoire de Chimie Appliquée, Université 8 Mai 1945, Guelma).

III.1.1.4. Preparation of tea extracts

III.1.1.4.1. Preparation of green tea extracts by hydromethanol

The tea extracts were prepared as follows **(Figure III.4)**: in the first step 0.5 g of each tea sample were extracted with 15 ml of 50% methanol/water, under stirring, using an ultrasound bath for 20 min at 30°C, then the sample was centrifuged for 10 min using a universal centrifuge with a maximum speed of 4000 rpm. The supernatant was collected and the solid residue was subjected to the second extraction step with 15 ml of 75% methanol/water and finally with 15 ml of 100% methanol. The extracts were combined, filtered and water was added to obtain a final volume of 100 ml **(Belguidoum et al. 2014)**. For the analyses, we proceeded to dilutions, when necessary.

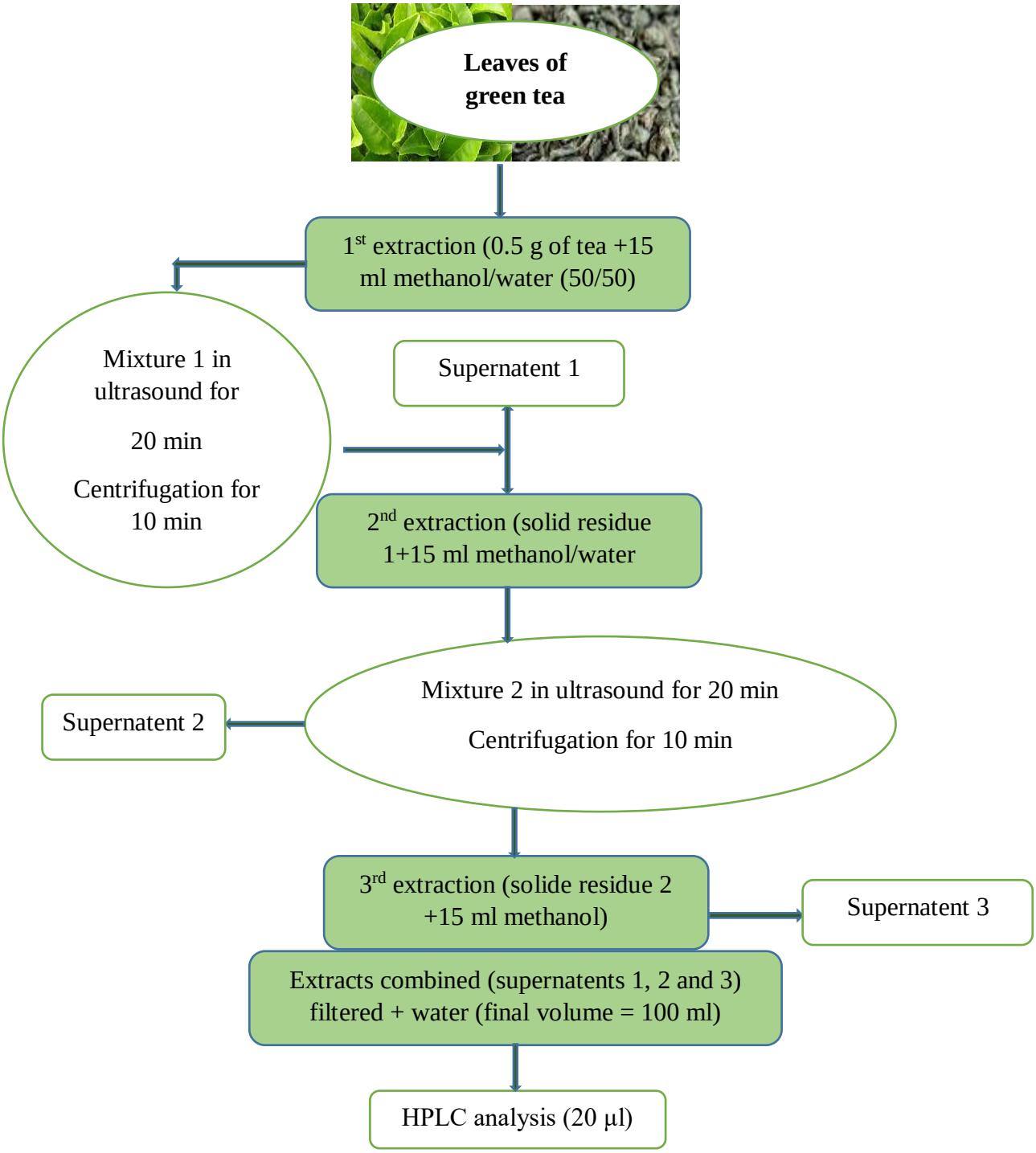


Figure III.4: Steps of preparation of tea extracts by hydromethanol.

III.1.1.4.2. Preparation of green tea leaves infusions

2 (Two) g of green tea leaves were allowed to soak in 100 ml of hot distilled water (85° C), for 5 minutes. This extraction method aims to determine the concentration of the tea drink. Dilutions were carried out when needed.

The **Figure III.5** illustrates the steps for preparation of tea extracts infusion.

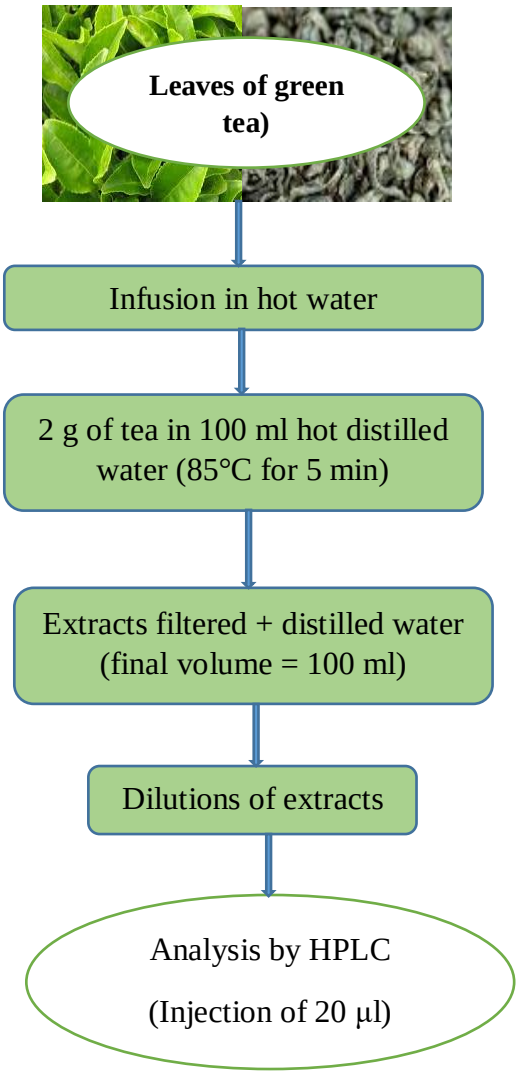


Figure III.5: Steps for preparation of tea extracts by infusion in hot water.

III.1.1.5. Solutions of standards

The aim of this step is to build the calibration curve of the pure compounds (standards) to be quantified later in the tea samples.

Standard solutions were prepared by diluting the pure compounds in a methanol/water mixture (50:50, V/V) at four different concentrations (1; 0.75 ; 0.5 and 0.25 mg/ml).

III.1.1.6. HPLC analysis

III.1.1.6.1. HPLC gradient

The mobile phase has been chosen after trying several mixtures already published **(Table III.4)**. A binary gradient elution **(Table III.5)** was used. The mobile phase was composed of 0.5% formic acid in water (V/V) (solvent A) and 0.5 % formic acid in methanol (V/V) (solvent B). The flow rate was set at 1 ml/min.

Table III.4: Mobile phases used in current and previous studies.

Solvant A	Solvent B	Reference
0.5% Formic acid in water	0.5 % Formic acid in methanol	**Current study**
Water-acetic acid	Methanol	**Zuo et al. 2002**
Acetonitrile	Acetonitrile and phosphoric acid	**Yang et al. 2007**
Water/methanol/formic acid	Acetonitrile/formic acid	**Bonoli et al. 2003**
5%(v/v) Acetonitrile and 0.035 % (v/v) TFA	50% (v/v) acetonitrile and 0.025 % (v/v) TFA	**El Shahawi et al. 2012**
Mixtures of acetonitrile and 20 mM KH_2PO_4 in isocratic mode		**Friedman et al. 2006**

Table III.5: Analytical HPLC gradient used.

Time (min)	0	10	20	25	27	29
%A	75	55	50	0	0	75
%B	25	45	50	100	100	25

III.1.1.6.2. Injection of standards and tea samples

20 µl of each standard and tea solution were injected in triplicate in the HPLC apparatus which is equipped with an automated sample injector allowing for the injection of up to 100 samples per sequence.

III.1.1.6. 3. Validation of analytical method

III.1.1.6.3.1. Calibration curves

Calibration curves were plotted using linear regressions of data composed of peak area of the standard (Y-axis) and concentration of the standard (X-axis). Microsoft Excel (2013) was used for this purpose.

III.1.1.6.3.2. Limit of detection and limit of quantification

The lower limits of the analytical range also called the limits of detection and quantification (LOD and LOQ) are two fundamental aspects of method validation **(Vial et al. 2003)**. They are used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure **(Shrivastava and Gupta 2011)**.

The limit of detection (LOD) and the limit of quantification (LOQ) were expressed as the two equations below **III.1** and **III.2** respectyvely **(Bartolomeo and Maisano 2006**, **ICH 1995):**

$$\mathbf{LOD = 3.3 \times \sigma / S} \qquad \text{III.1}$$

$$\mathbf{LOQ = 10 \times \sigma / S} \qquad \text{III.2}$$

Where σ is the standard deviation of the responses obtained from the triplicate analyses of each standard, and S is the slope of the calibration curve. The slope S was estimated from the calibration curve, employing the mean value of the peak area of each standard.

III.1.1.6.3.3. Recovery capacities

The recovery is the percentage of the rescue of the analyte in a sample. Using a chromatographic method, the experimental design and the calculation methodology depend on the aim to determine the effectiveness of the sample preparation. In any case, the recovery must be always determined using real samples and should be ideally 100% **(Peris-Vicente et al. 2015)**. The recovery should be measured at several concentration levels **(Peris-Vicente et al. 2013)**, it is calculated as follows:

$$\% \, \mathbf{Rec} = \frac{(C_S - C)}{C_A} . \mathbf{100} \qquad \text{III.3}$$

C_S: The concentration of an analyte detected in the spiked sample (mg/ml).

C: The concentration of an analyte detected in the sample before the spiking (mg/ml).

C_A: The true added concentration of an analyte (mg/ml).

The recovery capacities of the used method of the present study were determined in quadruplicate by spiking tea infusions of S4 sample with three concentrations (0.2, 0.1 and 0.05 mg/ml) of each standard.

III.1.2. Antioxidant activity

III.1.2.1. Reagents and apparatus

All reagents ((-)-epicatechin, (-)-epigallocatechin gallate, trans-resveratrol, ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), trichloroacetic acid, ferric chloride, potassium phosphate) were purchased from Sigma-Aldrich (Germany). A UV-visible spectrophotometer (DR6000 HACH, France) was used for reading the absorbance.

III.1.2.2. DPPH radical scavenging with catechins

The DPPH system offers a stable radical generating procedure. It is sensitive enough to detect active principles at low concentrations. The antioxidant process of catechins is thought to be divided into the following two stages:

$$DPPH^{\cdot} + AH \longleftrightarrow DPPH\text{-}H + A^{\cdot} \qquad \text{III.4}$$

$$H^{\cdot} + X^{\cdot} \longrightarrow \text{Non radical materials} \qquad \text{III.5}$$

AH is the antioxidant, $A^{\cdot}$ is the antioxidant radical and $X^{\cdot}$ is another radical species or the same species as $A^{\cdot}$. Although the first step is a reversible process, the second step is irreversible and produces stable radical termination compunds **(Wan et al. 2009)**.

As shown in **Figure III.6** below, an initial one-electron oxidation of epicatechin on the B ring by a DPPH radical generates epicatechin phenoxyl radical. This phenoxyl radical can be tautomerized to the corresponding *O*-quinone, which is then subjected to nucleophilic attack by C-6 carbon of another epicatechin to form a new compound. This mechanism reveal that both the B-ring and A-ring are the principal sites of antioxidant activity of epicatechin in the DPPH oxidant system **(Wan et al. 2009)**.

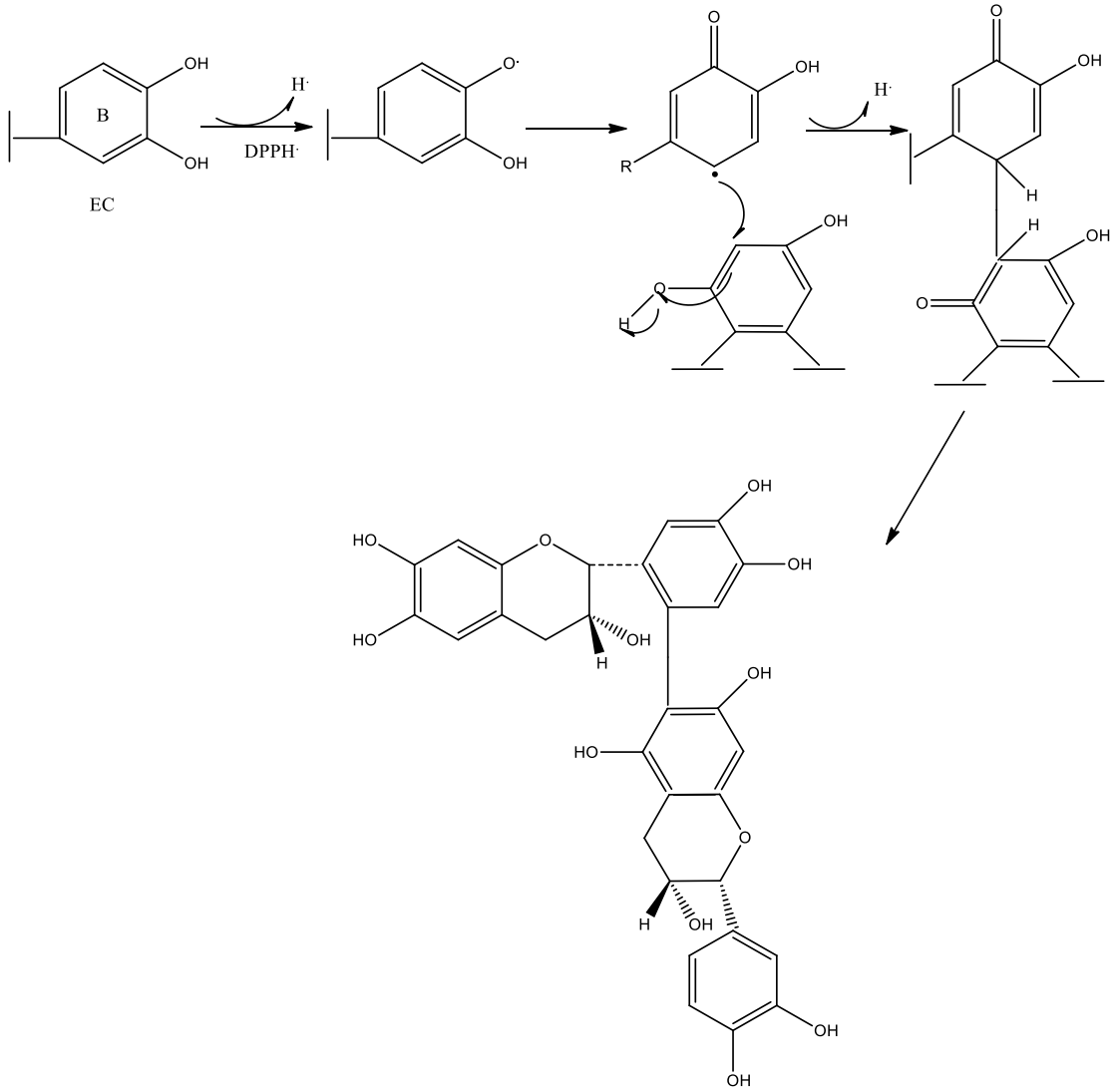


Figure III.6 : Scavenging mechanism of epicatechin to DPPH (Wan et al. 2009).

As shown in **Figure III.7** below of antioxidant mechanism, attacked with DPPH epigallocatechin gallate, phenoxyl radical was generated and tautomerized to *O*-quinone. This quinone attacks the C-2' carbon of another epigallocatechin gallate to form theasinensin **(Wan et al. 2009)**.

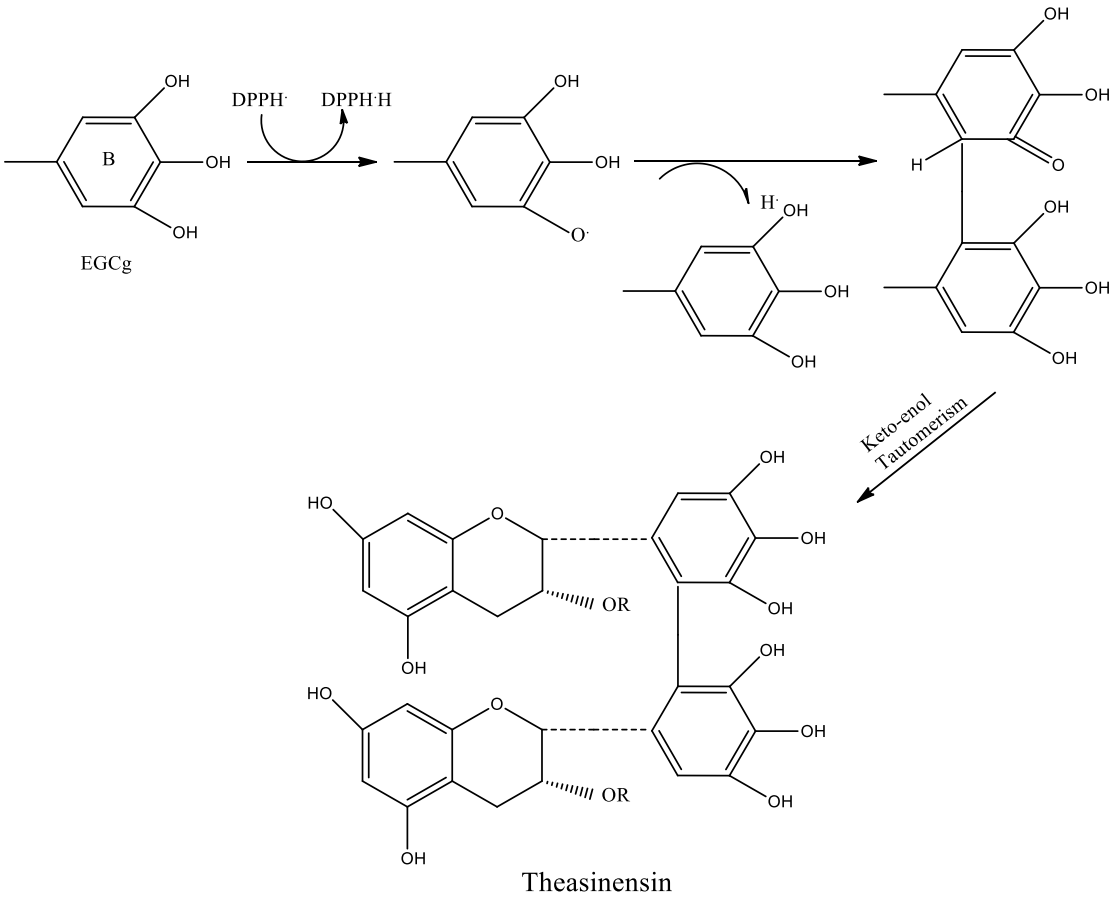


Figure III.7: Scavenging mechanism of epigallocatechin gallate to DPPH (Wan et al. 2009).

III.1.2.3. DPPH radical scavenging method

The experimental protocol **(Figure III.8)** is based on conditions previously established by **Bougandoura and Bendimerad (2013)** with small modifications. The DPPH solution is prepared by solubilizing 2 mg of this product in 100 ml of methanol. A volume of 1.95 ml of this solution is added to 50 µl of each of the methanolic solutions of EGCg, EC, RSV, AA and the sample S4 (green tea) at various concentrations (0.05 to 1 mg/ml).

In parallel, a negative control is prepared by mixing 50 µl of methanol with 1.95 ml of methanolic solution of DPPH. After 30 min of incubation in the dark and at room temperature, the absorbance is read at 517 nm. For each concentration of the four molecules' solutions, the assay is carried out in triplicate. The results were expressed as percent inhibition (I%).

$$\mathbf{I\% = [(Abs_{control} - Abs_{test}) / Abs_{control}] \times 100} \quad \text{III.6}$$

Abs_{test}: Absorbance of the samples.

$Abs_{control}$: Absorbance of the negative control.

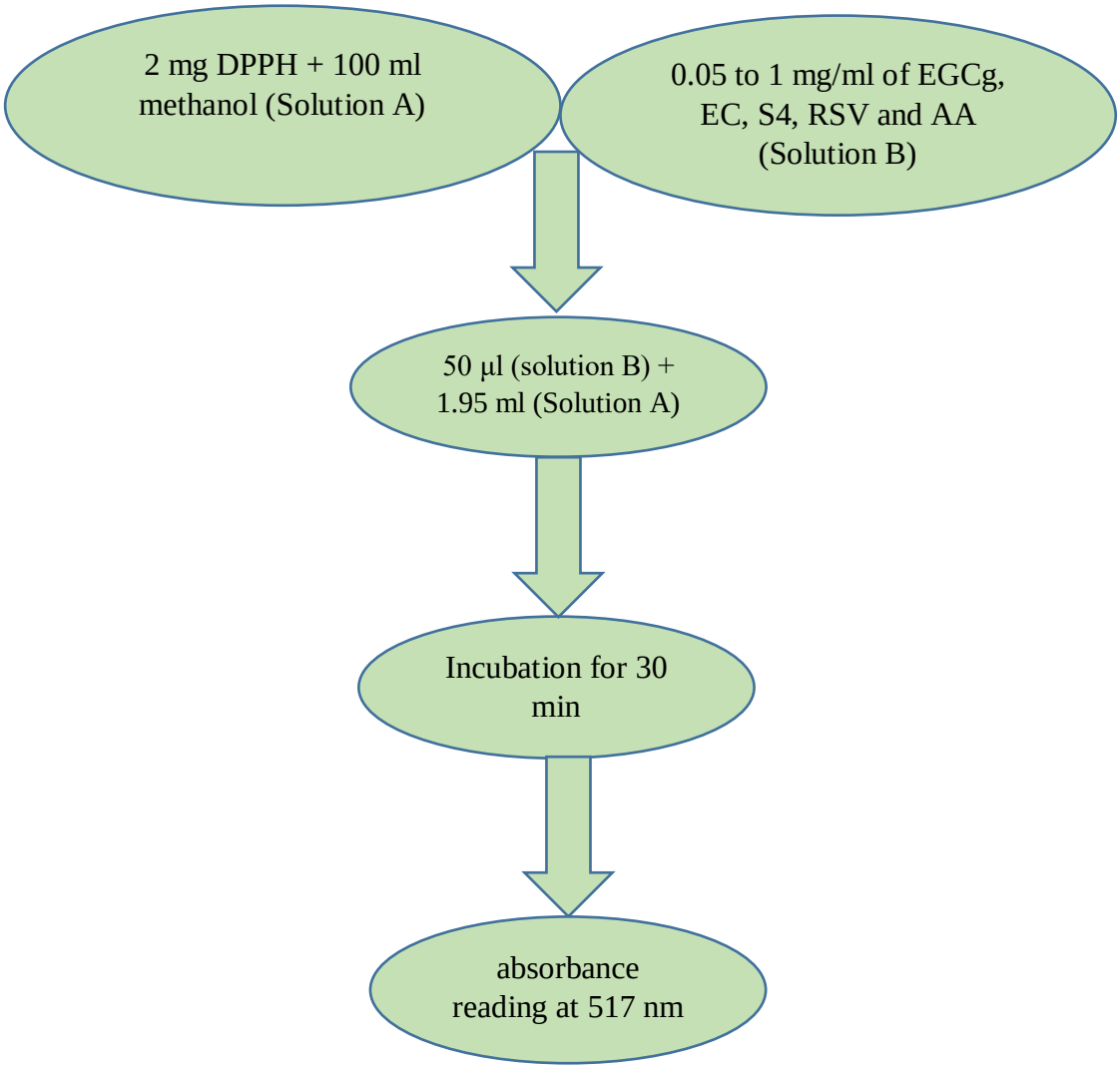


Figure III.8: Steps of antioxidant activity test by DPPH method.

III.1.2.4. Chelating iron ions

Guo et al. (1996) found that the chelated ratios of EGC, EGCg, ECg and EC with iron (III) 3:2, 2:1, 2:1 and 3:1 respectively. The **Figure III.9** shows the principle of the FRAP method using EGCg. The coordinate bonds in EGCg iron complexes are formed by the gallate (D) and B rings, both have the same effect.

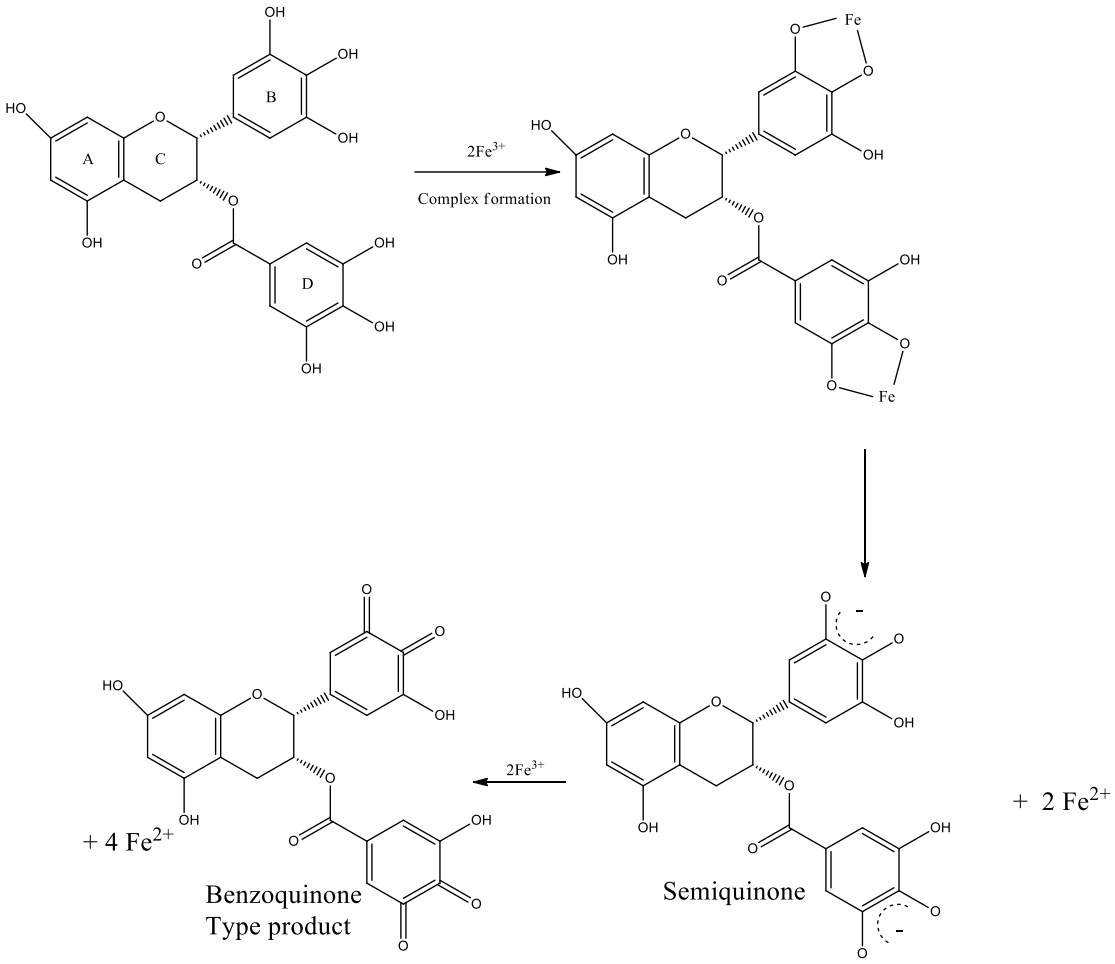


Figure III.9: principle of the FRAP method with EGCg (Ryan and Hynes 2007).

III.1.2.5. FRAP method

The experimental protocol **(Figure III.10)** used is based on conditions previously optimized by **Ferreira et al. (2009)** with small modifications. One milliliter of each methanolic solution of EGCg, EC, RSV and AA at different concentrations (from 0.005 to 1 mg/ml) is mixed with 2.5 ml of a 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide $K_3Fe(CN)_6$.

The mixture is incubated at 50°C for 20 minutes. After that, 2.5 ml of 10% trichloroacetic acid are added to stop the reaction and the mixture was centrifuged at 4000 rpm for 10 min. The upper layer (2.5 ml) is combined with 2.5 ml of distilled water and 0.5 ml of solution of $FeCl_3$ 0.1% (ferric chloride) freshly prepared. The absorbance of the reaction medium was read at 700 nm. An increase in absorbance corresponds to an increase in the reducing power of tested compounds.

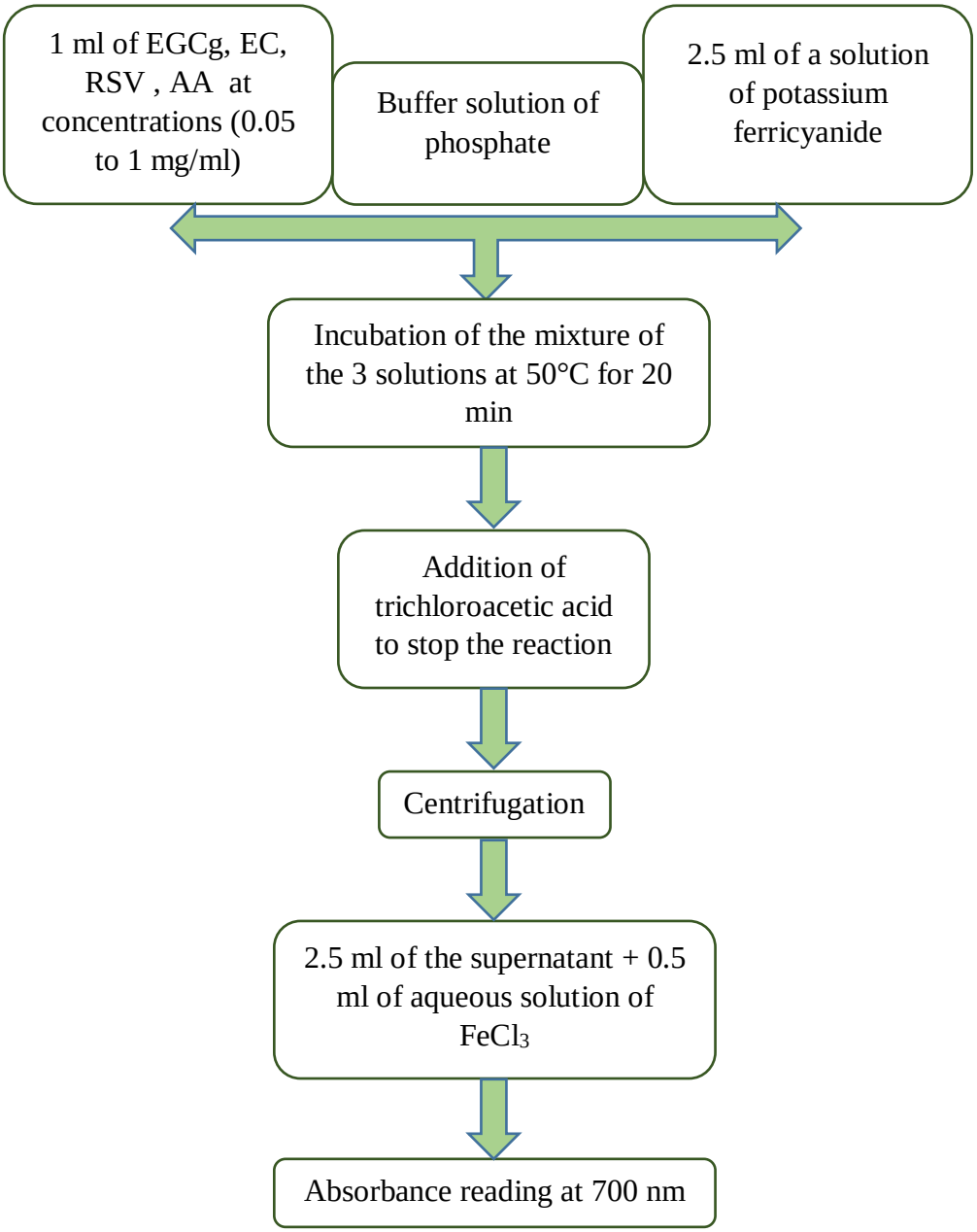
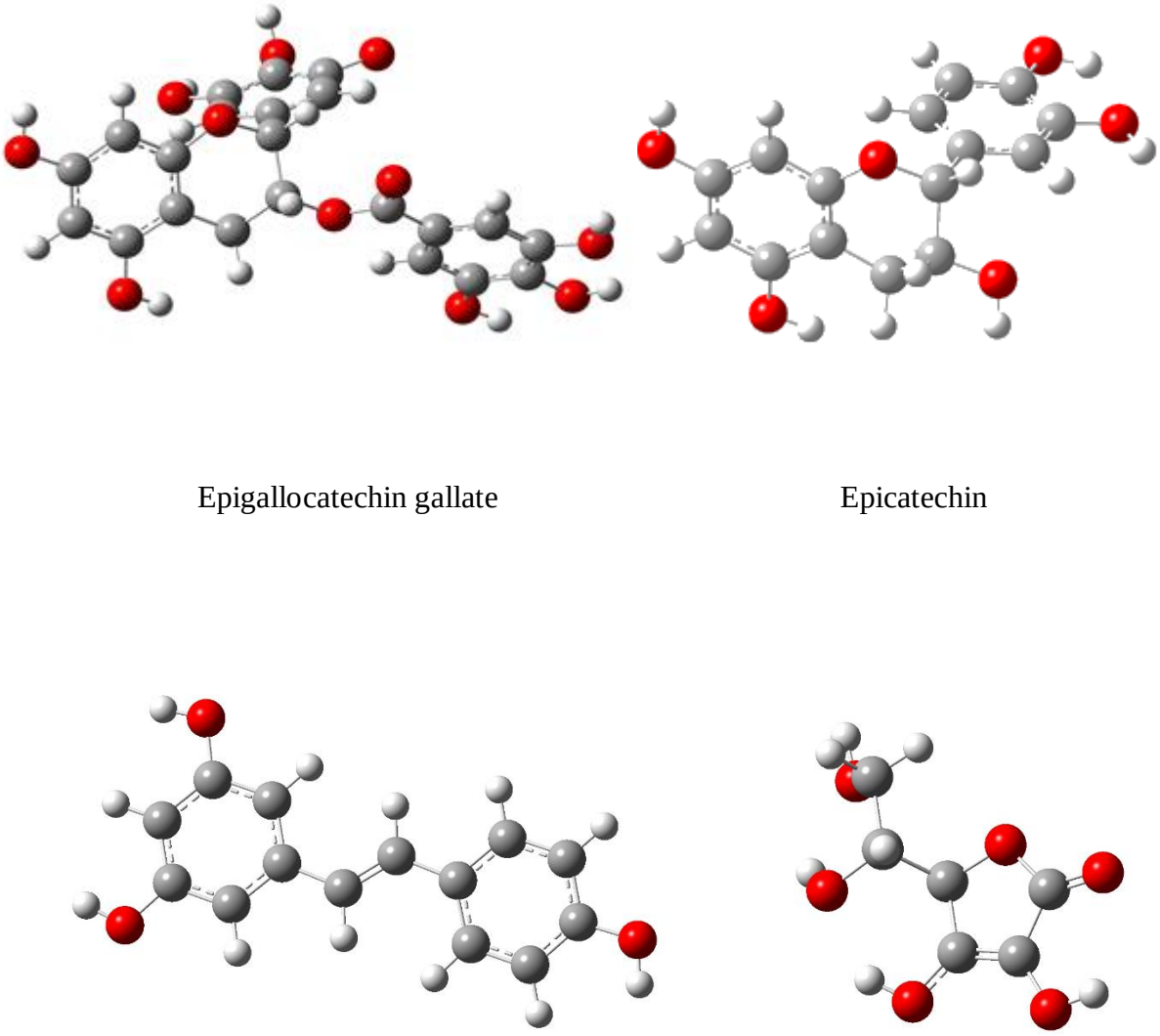


Figure III.10: Steps of antioxidant activity test by FRAP method.

III.2. Theoretical part

In this study, optimization energies of different neutral molecules **(Figure III.11)**, radicals, cations and anions were carried out by DFT method with 6-311++G(d,p) basis set and B3LYP functional, with the aim of calculating BDE, AIP, PDE, PA, ETE, $\Delta H_{acidity}$, pKa as thermodynamic parameters.



Epigallocatechin gallate

Epicatechin

Resveratrol

Ascorbic acid

Figure III.11: B3LYP/6-311++G(d,p) fully optimized geometries of studied molecules.

The B3LYP is known to provide accurate results of structure and thermodynamic properties of phenolic compounds **(Anitha et al. 2020)**.

The feasibility of different mechanisms is studied by the calculation of five parameters using the following equations:

For HAT:

$$\mathbf{BDE = H(ArO^{\cdot}) + H(H^{\cdot}) - H(ArOH)} \quad \text{III.7}$$

For SET-PT:

$$\mathbf{AIP = H(ArOH^{+\cdot}) + H(e^{-}) - H(ArOH)} \quad \text{III.8}$$

$$\mathbf{PDE = H(ArO^{\cdot}) + H(H^{+}) - H(ArOH^{+\cdot})} \quad \text{III.9}$$

For SPLET:

$$\mathbf{PA = H(ArO^{-}) + H(H^{+}) - H(ArOH)} \quad \text{III.10}$$

$$\mathbf{ETE = H(ArO^{\cdot}) + H(e^{-}) - H(ArO^{-})} \quad \text{III.11}$$

HOMO and LUMO were calculated using two basis sets: 6-311G(d,p) and 6-311G++(d,p).

Spin density was determined using both Hartree Fock (HF) and DFT methods. The calculation was carried out using the basis 6-311G (d,p) with and without diffuse function.

To determine polyphenols' acidity, two methods were investigated, the first one is the calculation of pKa and the second one is the determination of the enthalpy difference between the anion (A^-) and its neutral species (HA).

pKa was determined by application of the following thermodynamic cycle **(Pliego Jr 2003)** :

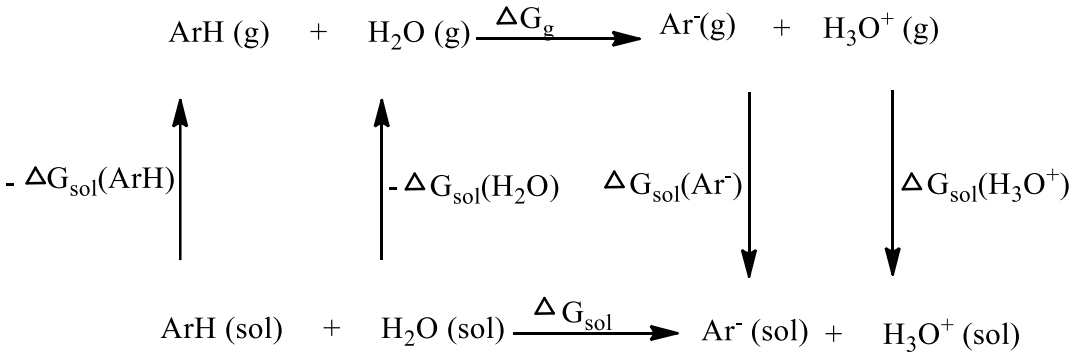


Figure III.12 : Thermodynamic cycle of polyphenolic compound hydrolysis (Pliego Jr 2003).

ΔG_{sol} values for H_3O^+ and H_2O were determined experimentally and reported as -110.2 kcal/mol **(Pliego Jr 2003)** and -6.31 kcal/mol **(Kelly et al. 2005)** respectively.

$$\Delta G_{sol} = \Delta G_g + \Delta G_{sol}(Ar^-) + \Delta G_{sol}(H_3O^+) - \Delta G_{sol}(ArH) - \Delta G_{sol}(H_2O) \quad \text{III.12}$$

$$\Delta G_{sol} = \Delta G_g + \Delta G_{sol}(Ar^-) + \Delta G_{sol}(H_3O^+) - \Delta G_{sol}(ArH) - \Delta G_{sol}(H_2O) \quad \text{III.13}$$

$$\mathbf{pk_a} = \frac{\Delta G_{sol}}{1.364} - 1.744 \quad \text{III.14}$$

$$\mathbf{pk_a\ (corrected) = pk_a\ (calculated) - 4.54} \quad \text{III.15}$$

The gas-phase acidity is computed at 298 K as the enthalpy difference between the anion (A^-) and its neutral species (HA):

$$\mathbf{\Delta H_{acidity} = H(A^-) - H(HA)} \quad \text{III.16}$$

Calculations and structures were performed by Gaussian 09 **(Frisch et al. 2009)** and Gauss View 5.0.

Conclusion

After detailing the equipment and methods used in this chapter for the experimental part and the theoretical part, we can see that the analysis method chosen for the quantification of polyphenols and methylxantins in green tea which has been suitably validated is simple and effective. The experimental protocols used for quantification or the antioxidant activity are clear and precise. The calculation of different parameters to evaluate the antioxidant activity of two green tea catechins was perfectly carried out using both DFT and HF.

CHAPTER IV

RESULTS AND DISCUSSION

Chapter IV
Results and discussion

Introduction

In this chapter, we will present in details the results obtained for this study it comprises three main axes:

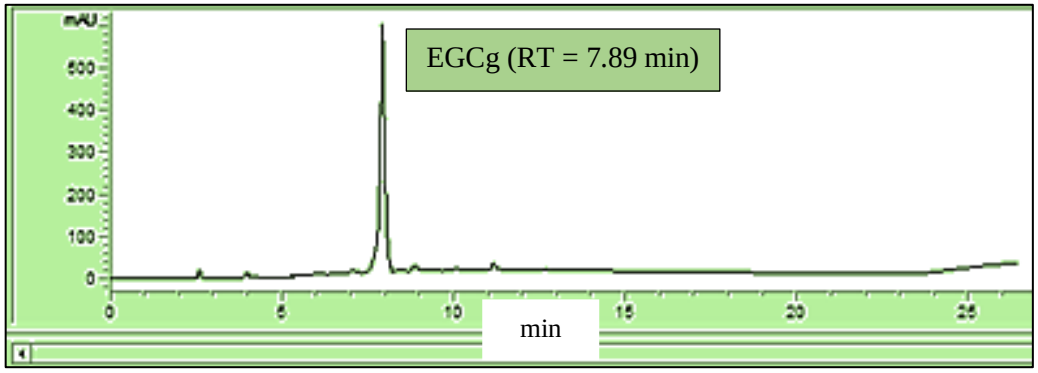
- Quantification of catechins, methylxantins and gallic acid in eight brands of green tea.
- Experimental comparison of antioxidant activity of epicatechin, epigallocatechin gallate and a sample of green tea with that of ascorbic acid and resveratrol.
- Computational study of the antioxidant activity of the two catechins cited above and the comparison with that of resveratrol and ascorbic acid.

IV.1. Experimental part

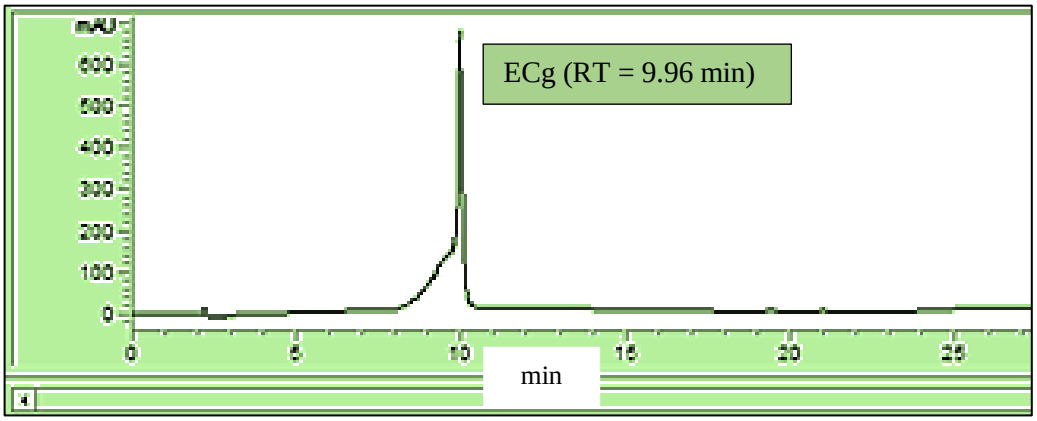
IV.1.1. HPLC analysis

IV.1.1.1. Chromatograms of pure compounds

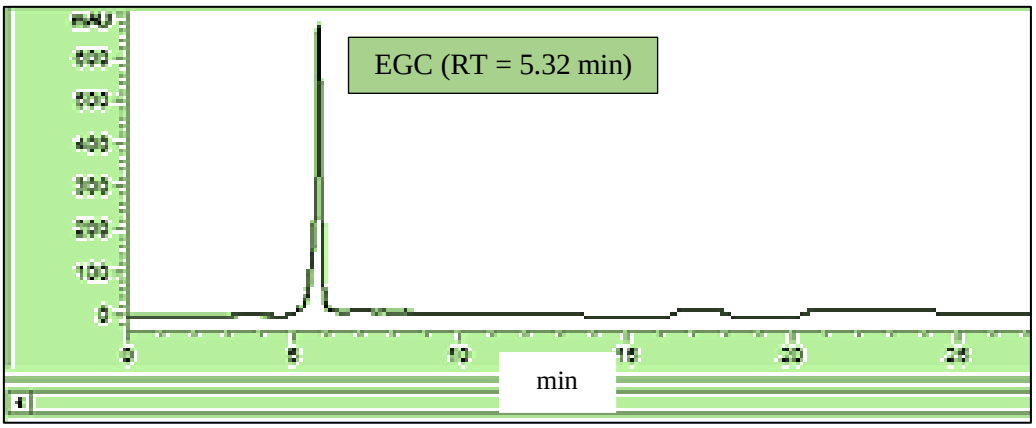
The **Figure IV.1** represents the HPLC chromatograms of standards which are pure compounds used in this study and are: epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC), epicatechin (EC), gallic acid (GA), caffeine (Caff), theophylline (Theoph) and theobromine (Theobr).



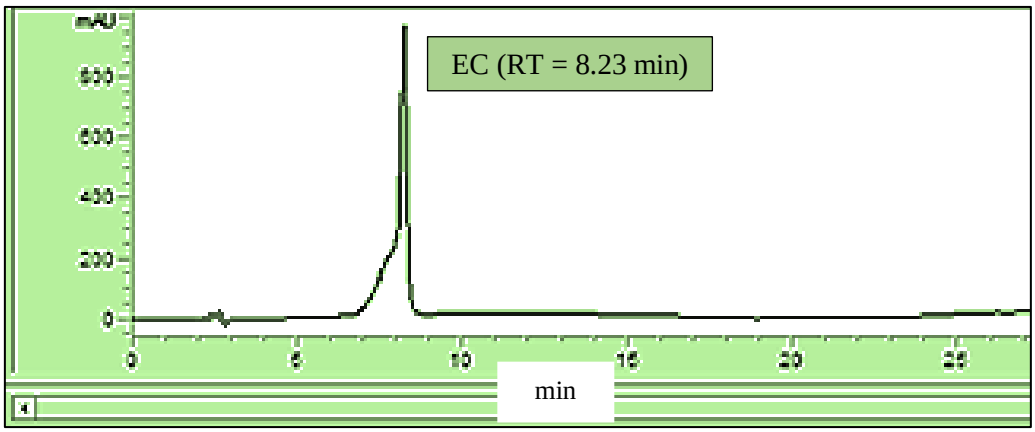
(a)


mAU
600
500
400
300
200
100
0
ECg (RT = 9.96 min)
0
5
10
15
20
25
min

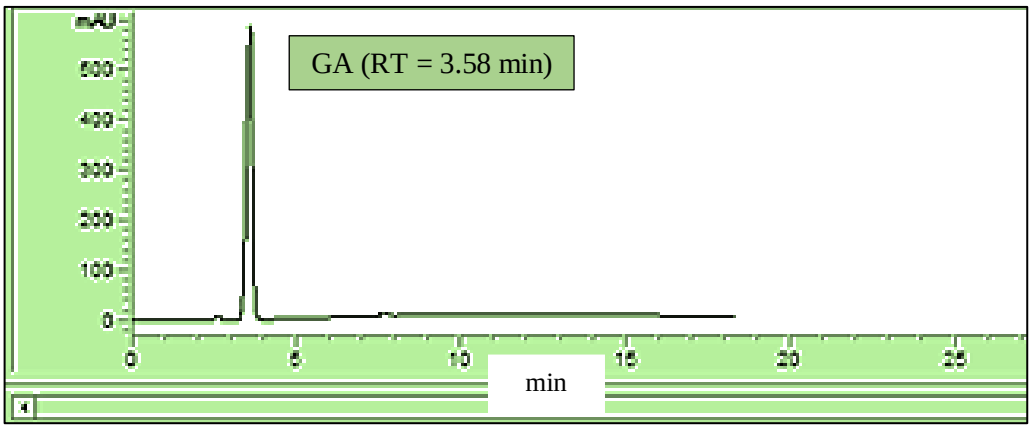
(b)


mAU
600
500
400
300
200
100
0
EGC (RT = 5.32 min)
0
5
10
15
20
25
min

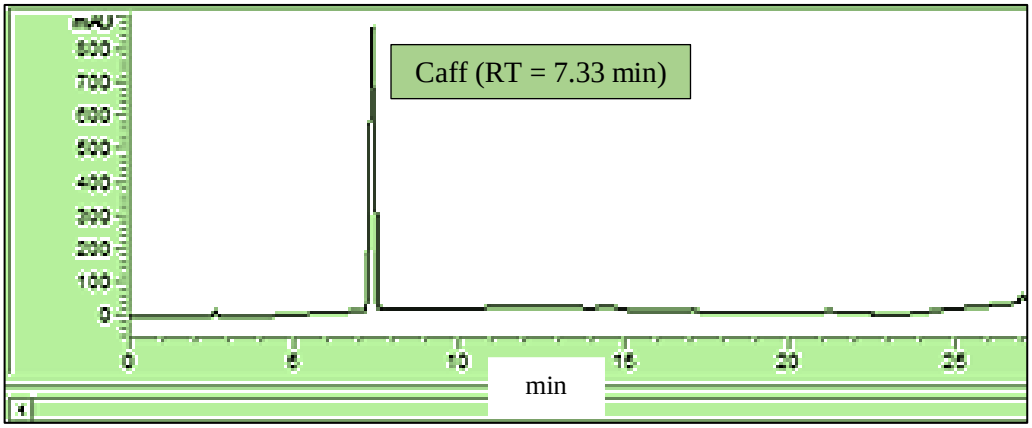
(c)


mAU
800
600
400
200
0
EC (RT = 8.23 min)
0
5
10
15
20
25
min

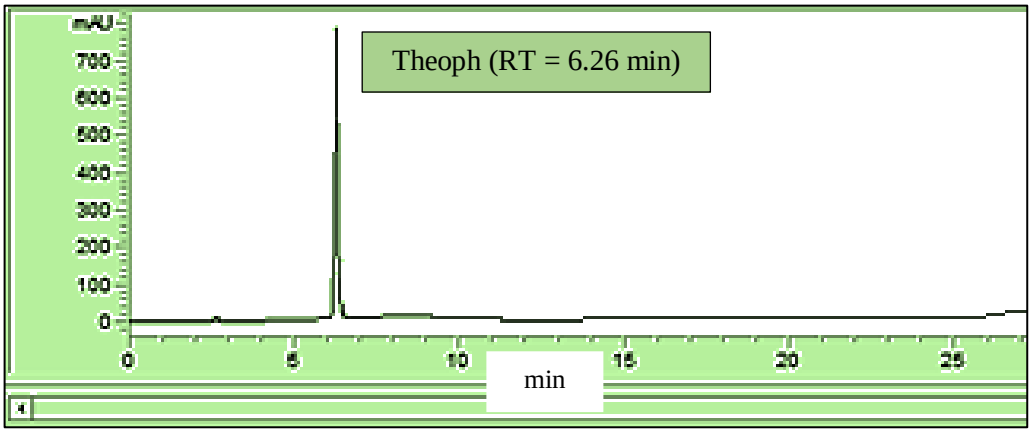
(d)


GA (RT = 3.58 min)
mAU
500
400
300
200
100
0
0
5
10
15
20
25
min

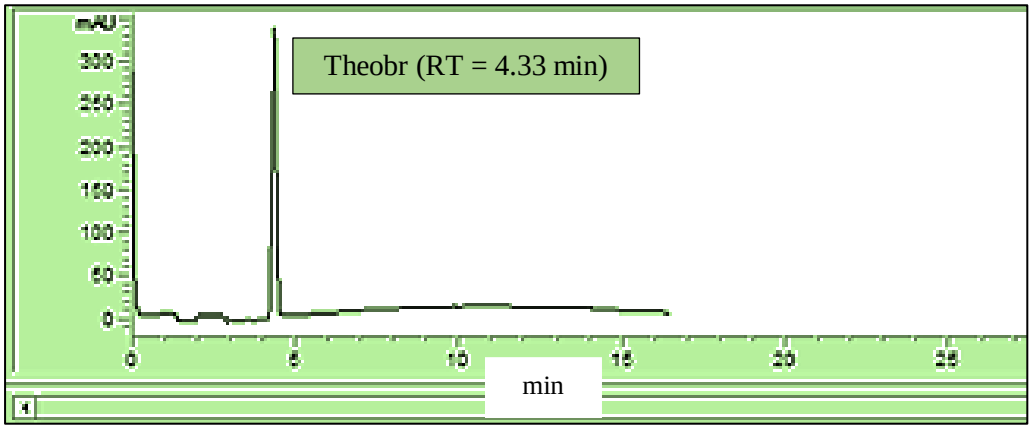
(e)


Caff (RT = 7.33 min)
mAU
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
min

(f)


Theoph (RT = 6.26 min)
mAU
700
600
500
400
300
200
100
0
0
5
10
15
20
25
min

(g)



(h)

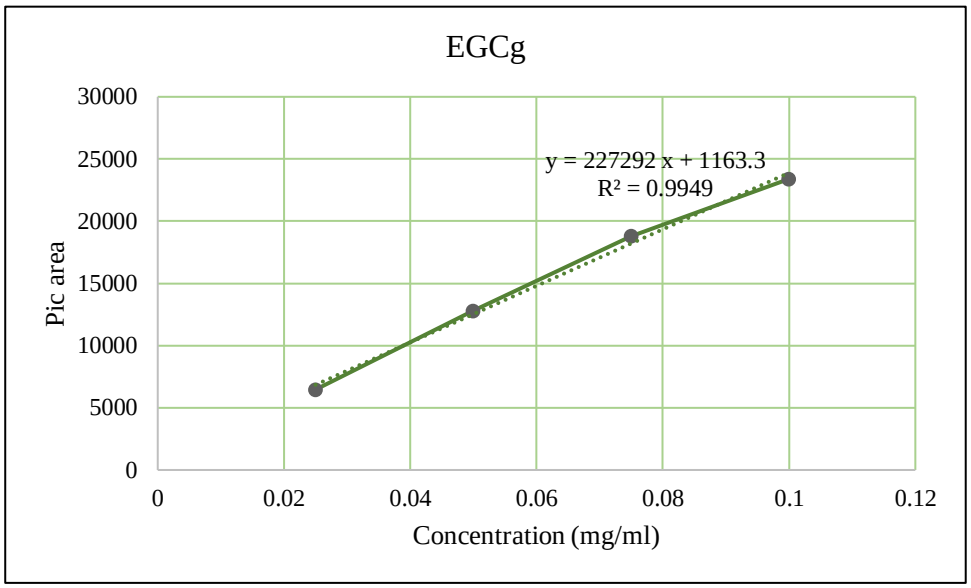
Figure IV.1: Chromatograms of pure compounds (a) EGCg, (b) ECg, (c) EGC, (d) EC, (e) GA, (f) Caff, (g) Theoph and (h) Theobr.

IV.1.1.2. Method validation

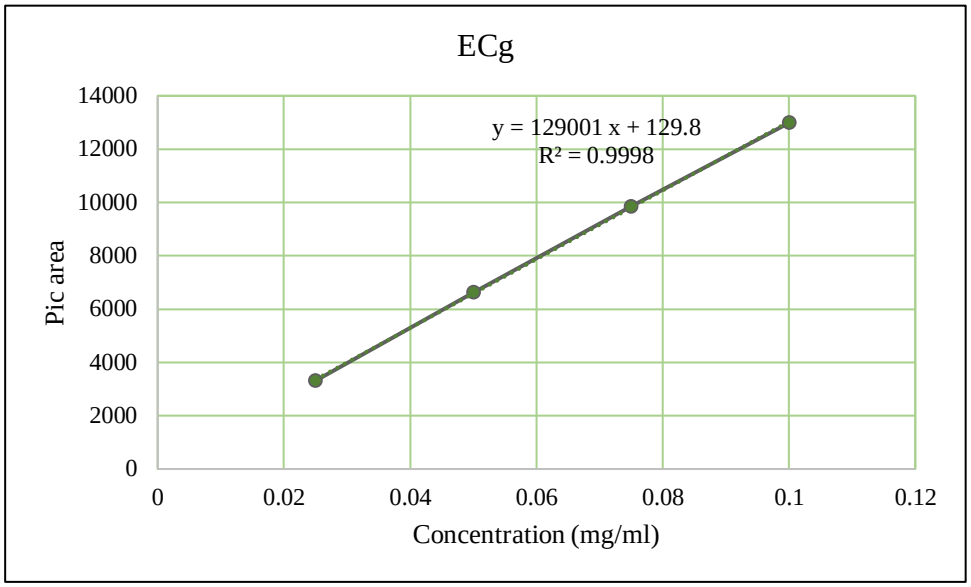
The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Typical validation characteristics considered in this study are : linearity, limit of detection, limit of quantification and the recovery rate.

IV.1.1.2.1. Linearity

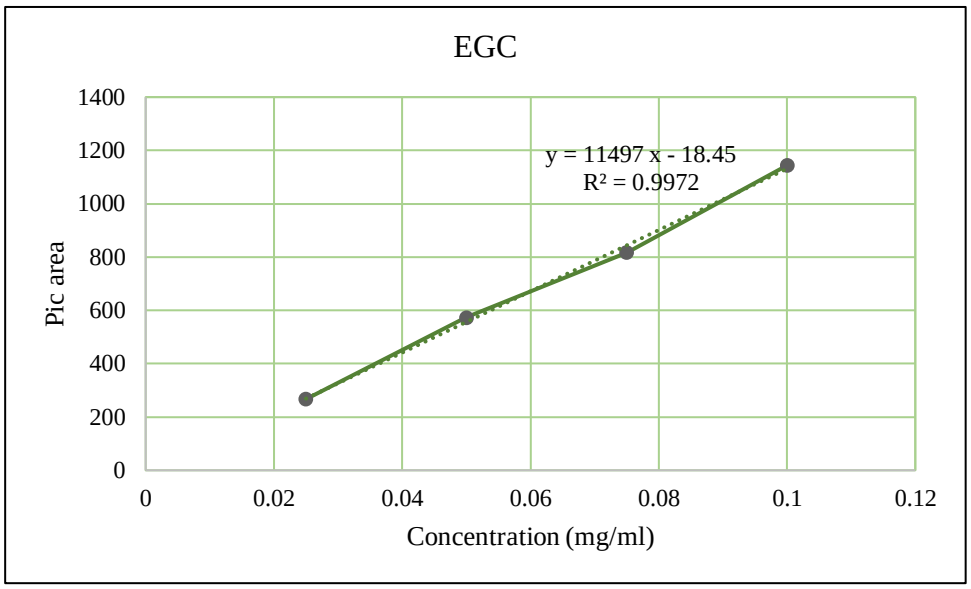
Figure IV. 2 shows the calibration curves of the studied pure compounds.



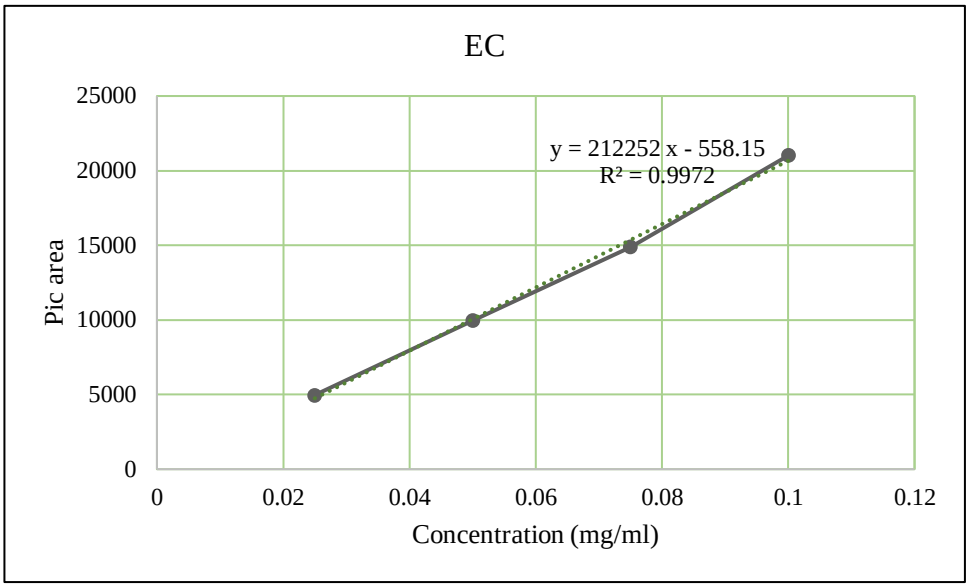
(a)


ECg
y = 129001 x + 129.8
R² = 0.9998
Pic area
Concentration (mg/ml)
0
2000
4000
6000
8000
10000
12000
14000
0
0.02
0.04
0.06
0.08
0.1
0.12

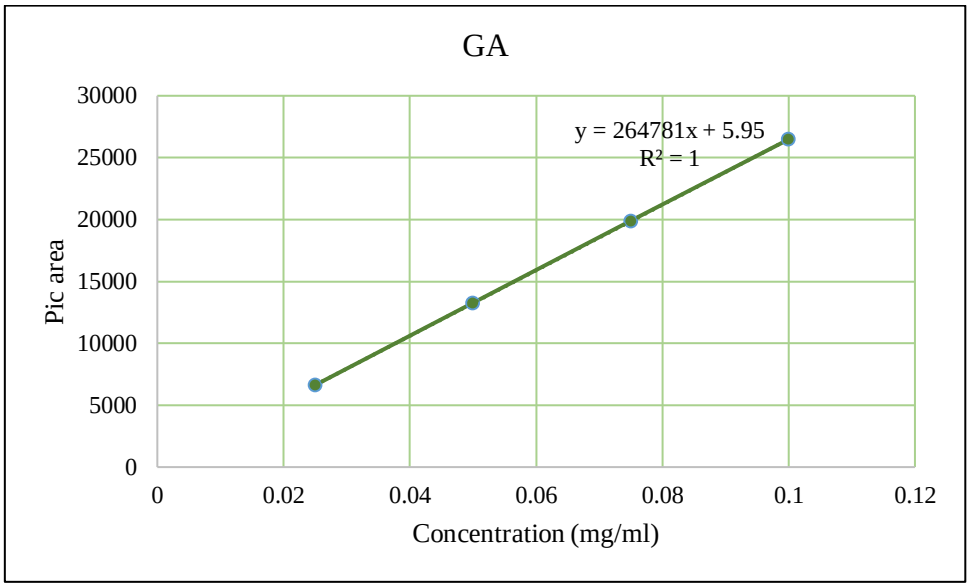
(b)


EGC
y = 11497 x - 18.45
R² = 0.9972
Pic area
Concentration (mg/ml)
0
200
400
600
800
1000
1200
1400
0
0.02
0.04
0.06
0.08
0.1
0.12

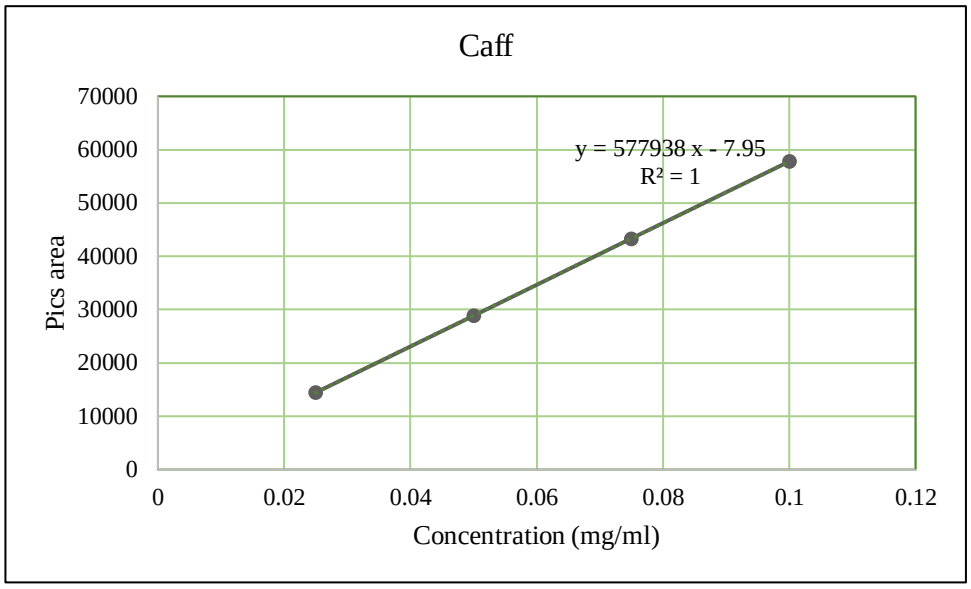
(c)


EC
y = 212252 x - 558.15
R² = 0.9972
Pic area
25000
20000
15000
10000
5000
0
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration (mg/ml)

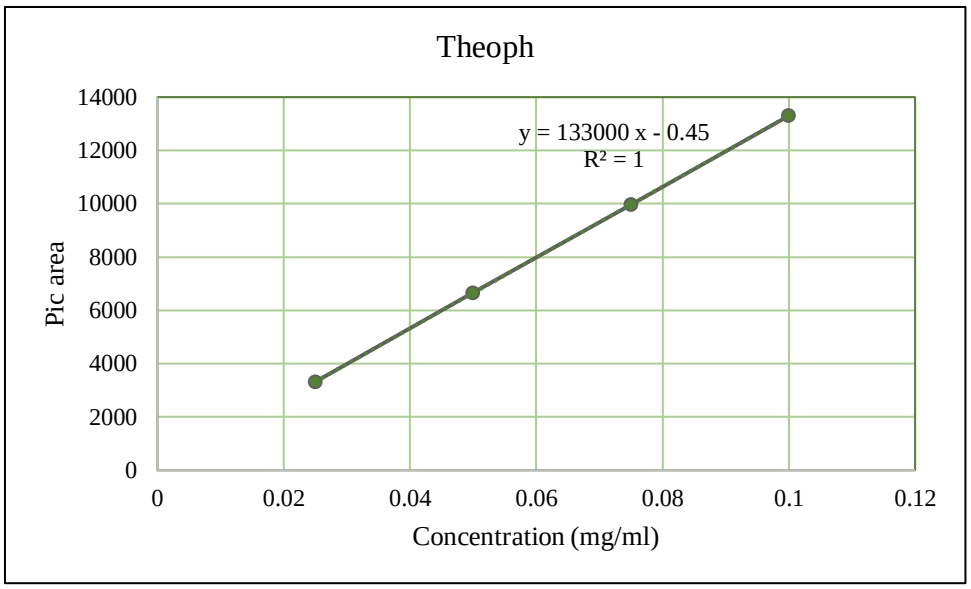
(d)


GA
y = 264781x + 5.95
R² = 1
Pic area
30000
25000
20000
15000
10000
5000
0
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration (mg/ml)

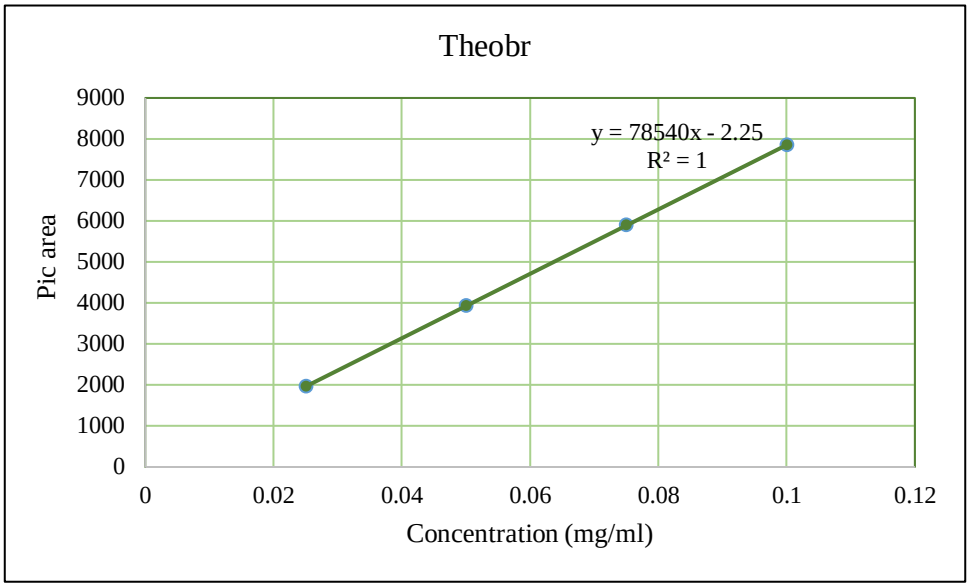
(e)


Caff
y = 577938 x - 7.95
R² = 1
Pics area
70000
60000
50000
40000
30000
20000
10000
0
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration (mg/ml)

(f)


Theoph
y = 133000 x - 0.45
R² = 1
Pic area
14000
12000
10000
8000
6000
4000
2000
0
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration (mg/ml)

(g)



(h)

Figure IV.2 : Calibration curves of pure compounds (a) EGCg, (b) ECg, (c) EGC, (d) EC, (e) GA, (f) Caff, (g) Theoph and (h) Theobr.

As can be seen from **Figure IV.2**, the calibration curves show complete linearity for methylxantines (R^2 =1) and gallic acid and regression factors R^2> 0.99 were obtained for all studied catechins.

IV.1.1.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

We calculated the detectable and quantifiable concentrations in the compounds of tea studied. This is what we call "limit of detection (LOD) and limit of quantification (LOQ)". The estimated values are shown in **Table IV.1**. Limit of detection values estimated for the method used, ranged from 0.05 to 1.52 µg/ml and those for quantification from 0.15 to 4.61 µg /ml.

Table IV.1: Limit of detection LOD and limit of quantification LOQ.

Compound	EGCg	ECg	EGC	EC	GA	Caff	Theoph	Theobr
LOD (µg/ml)	0.09	0.32	1.52	0.08	0.07	0.13	0.05	0.36
LOQ (µg/ml)	0.27	0.99	4.61	0.25	0.23	0.40	0.15	1.09

IV.1.1.2.3. Recovery rates of studied molecules

The recovery rates of the method used were evaluated by spiking the tea extracts with three different concentrations of pure compounds (0.005, 0.1 and 0.2 mg /ml). The results are collected in **Table IV.2**.

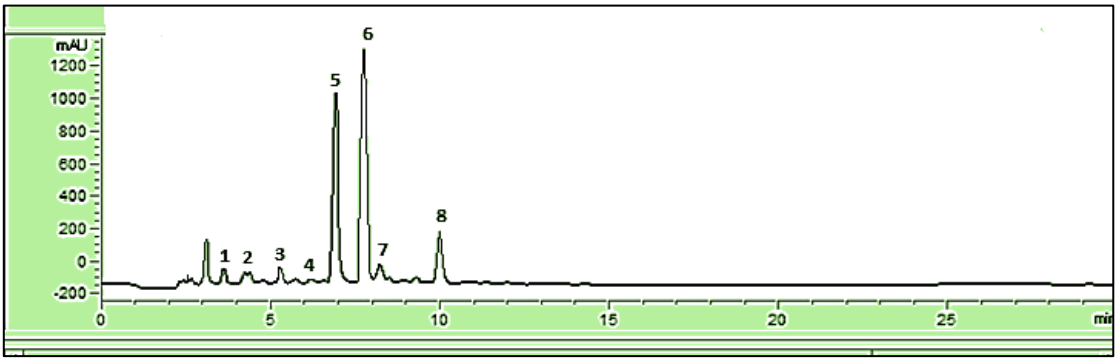
The results of the standards spiking carried out on the green tea infusion of the sample S4 show very good recovery. The minimum recovery is greater than 97% for all molecules. Indeed, the recovery rates of standards added to the sample tested at different concentrations are reproducible, with satisfactory values.

Table IV.2: Recovery values of the eight studied molecules.

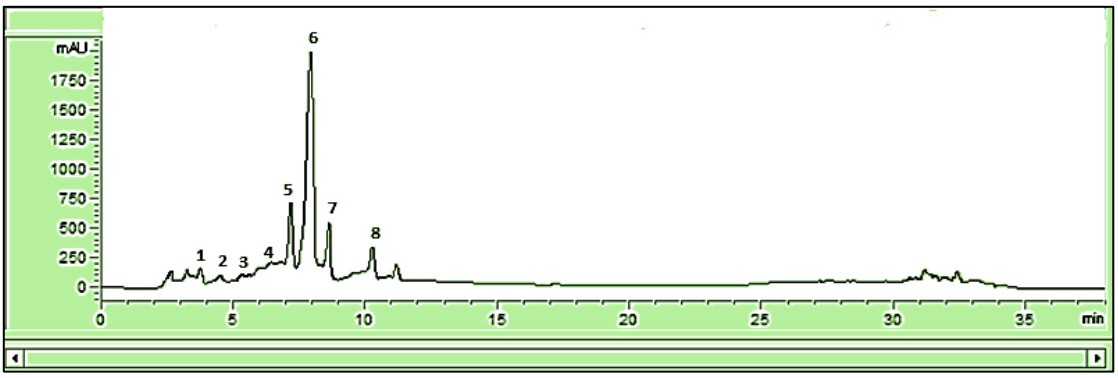
Compound	Spike level (mg/ml)	n	Recovery (%)
Epicatechin	0.02	4	97.4 ± 1.4
	0.01	4	97.5 ± 2.0
	0.005	4	100.3 ± 2.1
Epigallocatechin	0.02	4	97.8 ± 1.2
	0.01	4	99.7 ± 3.1
	0.005	4	98.5 ± 3.2
Epicatechingallate	0.02	4	100.3 ± 2.2
	0.01	4	105.5 ± 2.5
	0.005	4	98.7 ± 2.1
Epigallocatechin gallate	0.02	4	100.7 ± 1.9
	0.01	4	99.3 ± 1.7
	0.005	4	98.7 ± 1.8
Caffeine	0.02	4	104.2 ± 1.7
	0.01	4	102.6 ± 2.3
	0.005	4	100.4 ± 2.0
Theophylline	0.02	4	99.3 ± 1.8
	0.01	4	98.2 ± 1.3
	0.005	4	100.3 ± 1.4
Theobromine	0.02	4	102.6 ± 4.3
	0.01	4	99.1 ± 1.1
	0.005	4	99.7 ± 1.1
Gallic acid	0.02	4	97.2 ± 1.8
	0.01	4	97.9 ± 1.2
	0.005	4	99.3 ± 3.1

IV.1.1.3. Effect of solvent extraction on HPLC separation.

Two different methods of extraction were used: the first one was an infusion in hot water in order to simulate the tea drink preparation and to conclude about the catechins and methylxantines content of a cup of tea. This method has already been used and reported by several authors **(Friedman et al. 2006 and Theppakorn et al. 2014)**. It's worth noting here, that compounds were greatly separated and chromatograms obtained for the infusions showed high resolution and selectivity and good peak shape. In the case of multistep extraction method in which, hydromethanol mixtures were used, the compounds were well separated but the baselines were not as linear as those obtained in the case of infusions **(Figure IV.3)**.



(a)



(b)

Figure IV.3 : HPLC chromatograms of a tea sample : (a) Extraction by infusion in hot water, (b) Extraction by hydromethanol, 1: GA, 2: Theobr, 3: EGC, 4: Theoph, 5: Caff, 6: EGCg, 7: EC, 8: ECg.

The good resolution of the chromatogram in the case of infused green tea compared to the extract by hydromethanol could be due to the temperature difference between the first extraction (in hot water) and the second extraction which was performed at room temperature, and this observation was also reported by **Schuster et al. (2013)**, who claimed that higher column temperatures reduce mobile phase viscosity and increase solvent diffusion rate, therefore increasing column efficiency.

IV.1.1.4. Concentrations of individual and total catechins

IV.1.1.4.1. Extraction in hydromethanol

The concentrations in mg/g of the catechins analyzed are summarized in **Table IV.3**. The analysis of the results shows that the level of EGCg is the highest among all studied metabolites. The maximum and minimum amounts of EGCg were 91.26 mg/g, found in the sample S4 and 43.19 mg/g, found in sample S3, respectively. Our results are in agreement with the literature in terms of EGCg concentrations **(Friedman et al. 2006)**.

Concentrations of ECg (15.81 and 10.55 mg/g) and EC (14.65 and 10.98 mg/g) for samples S6 and S7 respectively, are almost the same. The highest concentration values of ECg (35.17 mg/g) and EC (22.63 mg/g) are found in S4 sample.

Concentrations of catechins in the eight studied brands of tea followed the sequence:

EGCg > ECg > EC > EGC.

Additionally, we calculated the total catechins (TC) values, which is the sum of individual concentrations of the catechins. The sample S4 has the highest TC value (151.89 mg/g). This result shows the high quality of this green tea brand because it contains high level of antioxidant compounds (EGCg, ECg, EGC and EC). It should be noted that the cost of sample S4 is 30 times higher than that of S7 having a TC level of 73.77 mg/g. The lowest TC value (63.32 mg/g) was detected in sample S3. The different methods of manufacture account for the marked difference in the chemical compositions of green teas, and even among green tea products the effects of plant variety, growth conditions, and processing method would be expected to produce quite wide variations in the chemical compositions of the resulting products **(Astill et al. 2001)**.

Table IV.3: Catechins and total catechins content in mg/g in hydromethanol extracts of green tea samples.

Sample	EGCg	ECg	EC	EGC	TC
S1	47.31 ± 1.10	27.66 ± 0.60	19.24± .44	1.76 ± 0.14	95.97 ± 2.28
S2	48.31 ± 0.50	15.49 ± 0.11	13.45± .84	1.51 ± 0.055	78.76± 1.505
S3	43.19 ± 1.13	11.22 ± 0.12	7.43 ± 0.59	1.48 ± 0.06	63.32 ± 1.90
S4	91.26 ± 0.14	35.17 ± 0.07	22.63± .43	2.83 ± 0.047	151.89± .687
S5	88.54 ± 0.21	24.24 ± 0.05	16.37± .32	3.94 ± 0.068	133.09± .648
S6	54.41 ± 0.13	15.81 ± 0.13	14.65± .59	4.38 ± 0.07	89.25 ± 0.92
S7	48.32 ± 0.39	10.55 ± 0.087	10.98± .51	3.92 ± 0.09	73.77± 1.077
S8	46.53 ± 0.88	14.74 ± 0.055	11.30± .38	3.20 ± 0.07	75.77± 1.385

IV.1.1.4.2. Infusion in hot water

The concentrations in mg/g of catechins in eight brand of tea are presented in **Table IV.4**. The major component in tea infusions as in methanol/water extracts is EGCg whose levels varied from 20.01 mg/g to 39.72 mg/g with the highest concentration (39.72 mg/g) detected in sample S4. Catechins' contents follow the sequence:

EGCg > ECg > EC > EGC.

Total catechins varied from 30.79 mg/g in sample S8 to 62.02 mg/g in sample S4. It should be noted that EC and EGC levels are very close to each other for all samples.

Table IV.4: Catechins and total catechins content in mg/g in infusions of green tea samples.

Sample	EGCg	ECg	EC	EGC	TC
S1	27.69 ± 0.05	13.72 ± 0.02	3.60 ± 0.08	3.13 ± 0.005	48.14± 0.155
S2	20.47 ± 0.032	11.29 ± 0.032	2.65± .096	2.59 ± 0.01	37.00 ± 0.17
S3	23.49 ± 0.035	9.30 ± 0.017	2.84± .081	2.56 ± 0.01	38.19± 0.143
S4	39.72 ± 0.05	12.21 ± 0.025	5.46± .045	4.63 ± 0.008	62.02± 0.128
S5	34.04 ± 0.015	11.81 ± 0.01	4.22± .086	3.19 ± 0.011	53.26± 0.122
S6	29.64 ± 0.035	10.92 ± 0.026	2.80±0.057	2.59 ± 0.006	45.95± 0.124
S7	25.30 ± 0.04	8.42 ± 0.015	3.48 ± 0.07	2.79 ± 0.032	39.99± 0.157
S8	20.01 ± 0.01	6.44 ± 0.015	2.32±0.086	2.02 ± 0.005	30.79± 0.116

IV.1.1.5. Concentrations of individual, total methylxantines and gallic acid

IV.1.1.5.1. Extraction in hydromethanol

The methylxantines studied are caffeine, theophylline and theobromine. The caffeine content of aqueous methanol green tea extracts were found to vary from 12.71 to 20.98 mg/g, this result is in a concordance with those reported by **Friedman et al. (2006)** and **Zhao et al. (2011)**.

Theobromine concentrations, varying from 1.92 mg/g to 5.57 mg/g, are higher than those of theophylline ranging from 0.19 to 0.82 mg/g. In addition, theophylline was detected in three samples only (S3-S5) and not detected in the remaining samples. This result is in agreement with that of **Friedman et al. (2006)**.

Total methylxantines concentration varied from 17.32 mg/g in sample S7 to 26.12 mg/g in sample S4.

We have also determined, the content of a phenolic acid usually detected in green tea samples, it is gallic acid. The concentrations of gallic acid varied from 0.6 to 3.8 mg/g **(Table IV.5)**.

Table IV.5: Concentration of methylxantines, total methylxantines and gallic acid in mg/g in hydromethanol extracts of green tea samples.

Sample	Caffeine	Theophylline	Theobromine	TM	Gallic acid
S1	20.98 ± 0.055	nd	2.95 ± 0.14	23.93 ± 0.195	0.80 ± 0.047
S2	16.32 ± 0.035	nd	1.92 ± 0.18	18.24 ± 0.215	0.60 ± 0.065
S3	15.07 ± 0.03	0.82 ± 0.08	2.56 ± 0.25	18.45 ± 0.36	1.06 ± 0.040
S4	20.25 ± 0.027	0.30 ± 0.14	5.57 ± 0.15	26.12 ± 0.317	3.80 ± 0.045
S5	12.71 ± 0.036	0.19 ± 0.12	5.28 ± 0.17	18.18 ± 0.326	3.18 ± 0.037
S6	17.44 ± 0.043	nd	3.69 ± 0.21	21.13 ± 0.253	1.74 ± 0.022
S7	14.01 ± 0.045	nd	3.31 ± 0.23	17.32 ± 0.275	2.50 ± 0.045
S8	19.76 ± 0.03	nd	2.95 ± 0.15	22.71 ± 0.18	1.69 ± 0.037

IV.1.1.5.2. Infusion in hot water

The concentrations of caffeine, theobromine and theophylline in tea infusions ranged from 14.19 to 29.88 mg/g, 1.78 to 9.10 mg/g and 0.23 to 1.35 mg/g, respectively. The high concentration of theobromine in tea infusions is indicative of higher solubility of this molecule in water at 85°C compared to methanol/water mixtures at room temperature. This result is in agreement with that reported by **Zhong et al. (2017)**. Total methylxantines concentration varied from 17.43 mg/g in sample S5 to 38.58 mg/g in sample S6.

Gallic acid, the only phenolic acid studied here, was found in concentrations ranging from 0.94 to 2.70 mg/g **(Table IV.6)**.

Table IV.6: Concentration (mg/g) of methylxantines, total methylxantines and gallic acid in infusions of green tea samples.

Sample	Caffeine	Theophylline	Theobromine	TM	Gallic acid
S1	29.88 ± 0.01	0.91 ± 0.01	2.93 ± 0.015	33.72 ± 0.035	1.33 ± 0.013
S2	19.68 ± 0.02	0.86 ± 0.012	2.30 ± 0.007	22.84 ± 0.039	1.26 ± 0.002
S3	18.24 ± 0.03	0.73 ± 0.01	2.19 ± 0.02	21.16 ± 0.06	0.94 ± 0.005
S4	26.53 ± 0.055	0.23 ± 0.005	7.54 ± 0.02	34.30 ± 0.08	2.70 ± 0.04
S5	14.19 ± 0.03	0.64 ± 0.01	2.60 ± 0.015	17.43 ± 0.055	1.66 ± 0.019
S6	28.13 ± 0.01	1.35 ± 0.026	9.10 ± 0.02	38.58 ± 0.056	2.31 ± 0.03
S7	20.51 ± 0.015	0.73 ± 0.011	1.78 ± 0.015	23.02 ± 0.041	1.55 ± 0.005
S8	25.18 ± 0.11	1.07 ± 0.03	6.81 ± 0.18	33.06 ± 0.32	1.70 ± 0.005

IV.1.1.6. Comparison of the concentrations of metabolites in both types of extracts

The mean values of concentration for EGCg and ECg found in methanol/water extracts are almost two-fold greater than those found for infusions, whereas EC concentration mean value is four times higher in MeOH/Water mixtures than in infusions. EGC content mean value is almost the same for both types of extracts **(Table IV.7)**.

Total catechins mean value for methanol extracts (95.22 mg/g) is roughly two-fold that of infusions (44.41 mg/g). This is due to the affinity of the studied metabolites with the methanol water mixtures and to the efficiency of the exhaustive extraction method used. This result is in agreement with the literature **(Friedman et al. 2006)**.

Nevertheless, concentrations of caffeine, theobromine and theophylline in infusions are higher than those found for the hydromethanol extracts. The mean values of caffeine, theobromine, and theophylline concentrations (in mg/g) in MeOH/water extracts are 17.06, 3.52 and 0.43, respectively, whereas concentrations (in mg/g) of same metabolites in infusions are 22.79, 4.40 and 0.815, respectively. Caffeine solubility is higher in water at higher temperatures (85°C) than in methanol at room temperature. This observation was also reported by **Shalmashi and Golmohammad (2010)** and **Zhong et al. (2017)**. Total methylxanthines mean value was found to be 28.006 mg/g for tea infusions and 21.01 mg/g for MeOH/water extracts.

Table IV.7: Concentration mean values in mg/g of studied molecules in tea infusions and MeOH /Water extracts.

Molecule	Extraction method	
	Hydromethanol	**Infusion in hot water**
EGCg	58.48 ± 0.56	27.54 ± 0.033
ECg	19.36 ± 0.15	10.51 ± 0.02
EC	14.50 ± 0.51	3.42 ± 0.075
EGC	2.87 ± 0.075	2.93 ± 0.01
Total catechins	95.22 ± 1.30	44.41 ± 0.139
Caffeine	17.06 ± 0.037	22.79 ± 0.035
Theophylline	0.43 ± 0.11	0.815 ± 0.014
Theobromine	3.52 ± 0.185	4.40 ± 0.036
Total methylxanthines	21.01 ± 0.11	28.006 ± 0.028
Gallic acid	1.92 ± 0.042	1.68 ± 0.014

Total catechins and methylamines concentrations in green tea methanol/water extracts and infusions are shown in **Figures IV.4** and **IV.5**, respectively.

We should notice that levels of total catechins found in MeOH /H_2O green tea extracts were higher than those in the infusions for the same green tea sample, while the opposite was true for total methylxantines.

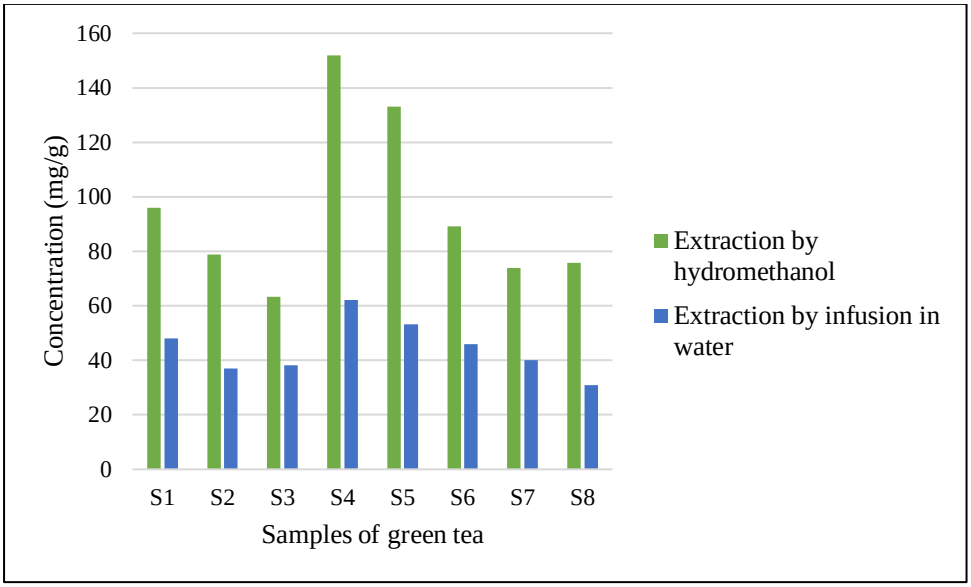


Figure IV.4: Total catechins concentrations in MeOH/water extracts and infusions of green tea.

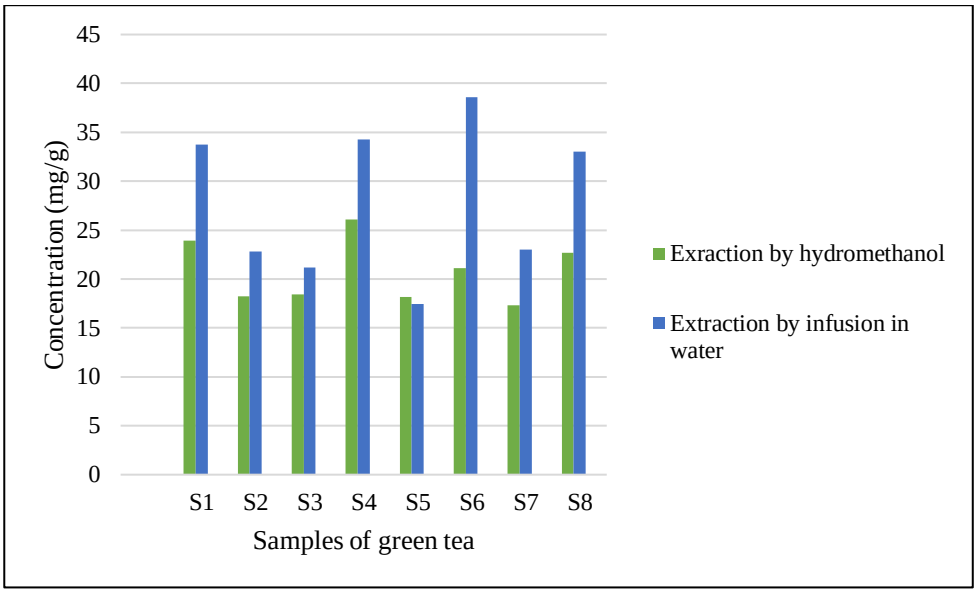
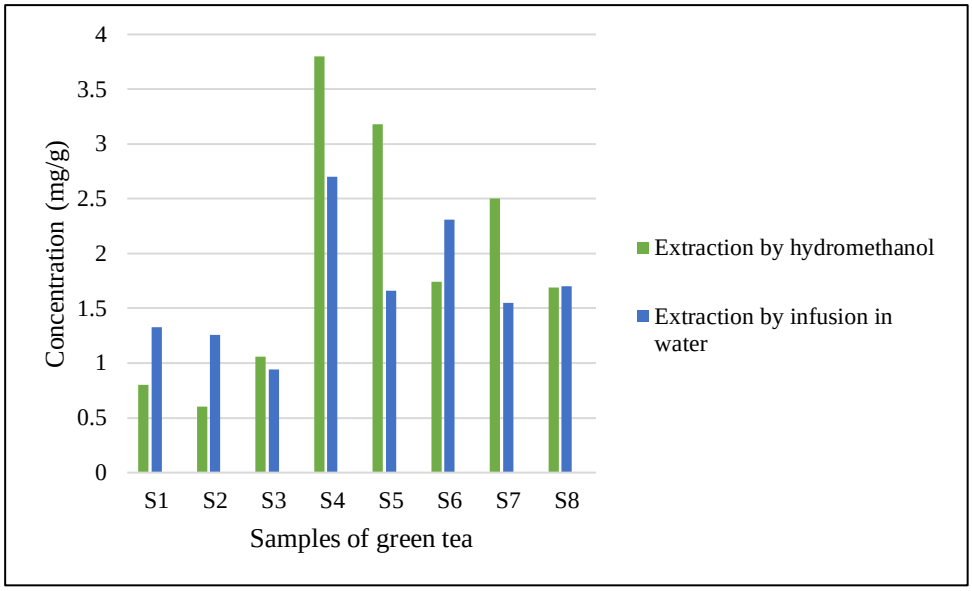


Figure IV.5: Total methylxantines concentrations in MeOH/Water extracts and infusions of green tea.

For gallic acid, concentrations in samples S3, S4, S5 and S7 were found to be higher in hydromethanol extracts than in infusions **(Figure IV.6)**. **Daneshfar et al. (2008)** reported that the solubility of gallic acid in methanol is higher than in water at the same temperature, and the gallic acid solubility in different solvents increases smoothly with temperature, it could then be concluded that the solubility of metabolites is not the lonely factor that governs the extraction yield but other interactions could play an important role too.



IV.6: Gallic acid concentrations in green tea MeOH/Water extracts and infusions.

In the **Figure IV.7**, illustrated a global representation which summarizes the above results.

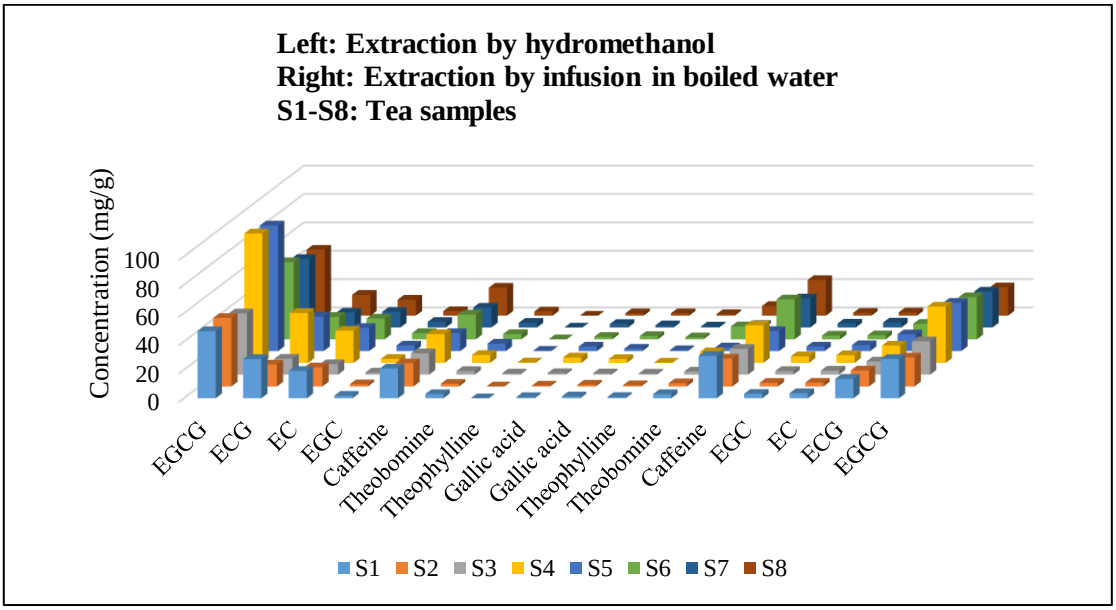


Figure IV.7: Global representation of studied metabolites' concentrations in both MetOH/Water extracts and infusions in hot water.

IV.1.1.7. Comparison of results of the present study to literature

The results of this study were validated by comparison to published ones (details are listed in **Table IV.8**.

Table IV.8: Comparison of green tea extracts content in mg/g of the present study with literature.

Compound	Present study		Previous studies	
	Extraction by infusion in hot water			
	Min	Max	Min	Max
EGCg	20.01	39.72	1.01 [a]	53.6 [b]
ECg	6.44	13.72	0.216 [a]	27.1 [b]
EGC	2.02	4.63	0.1 [b]	23.2 [a]
EC	2.32	5.46	0.1 [b]	10.2 [a]
Caffeine	14.19	29.88	0.3 [b]	29.9 [b]
Theobromine	1.78	9.10	0.04 [b]	1.9 [b]
Theophylline	0.23	1.35	0.1 [b]	0.6 [b]
Gallic acid	0.94	2.70	0.37 [c]	1.66 [c]
Compound	**Extraction by hydromethanol**		**Extraction by hydroalcohol**	
	Min	**Max**	**Min**	**Max**
EGCg	43.19	91.26	7.0 [b]	144.21 [d]
ECg	10.55	35.17	2.4 [b]	40.5 [b]
EGC	1.48	4.38	1.4 [b]	47.08 [d]
EC	7.43	22.63	0.3 [b]	15.40 [d]
Caffeine	12.71	20.98	0.5 [b]	29.84 [d]
Theobromine	1.92	5.57	0.1 [b]	2.6 [b]
Theophylline	0.19	0.82	0.2 [b]	0.6 [b]
Gallic acid	0.60	3.80	0.37 [e]	1.4 [f]

[a] (El-Shahawi et al. 2012), [b] (Friedman et al. 2006), [c] (Wang et al. 2000), [d] (Zhao et al. 2011), [e] (Zuo et al. 2002), [f] (Cabrera et al. 2003).

All concentrations of studied metabolites are found to be in the same range of published data, excluding Theobromine and Theophylline maximum values (9.10 mg/g and 1.35mg/g) both, found for infusion in hot water of sample S6, EC (22.63 mg/g), Theobromine (5.57 mg/g) and Gallic acid (3.80 mg/g) for hydromethanol extract of sample S4 and Theophylline (0.82 mg/g) for sample S3. We should also mention that in most cases, the minimum values of this study are far higher than minimum values found by other authors, indicating the high quality of tea samples studied.

To summarize, if we consider the Level of these metabolites as a criterion for quality control, we can conclude that , in general the tea brands commercialized in the Algerian markets are in the same norm as those consumed elsewhere and can't be subjected to adulteration like coffee, in which we found in a previous study many additives and dangerous metabolites created during roasting.

IV.1.2. Antioxidant activity

IV.1.2.1. Evaluation of antioxidant activity of EGCg and EC

The antioxidant activity of the two catechins (EGCg and EC) was carried out in comparison with that of ascorbic acid (AA) and resveratrol (RSV) as renowned powerful antioxidants.

IV.1.2.1.1. Scavenging and inhibition of the DPPH radical

Results of DPPH free radical percentage inhibition for EGCg, EC, RSV and AA are shown in **Figure IV.8**.

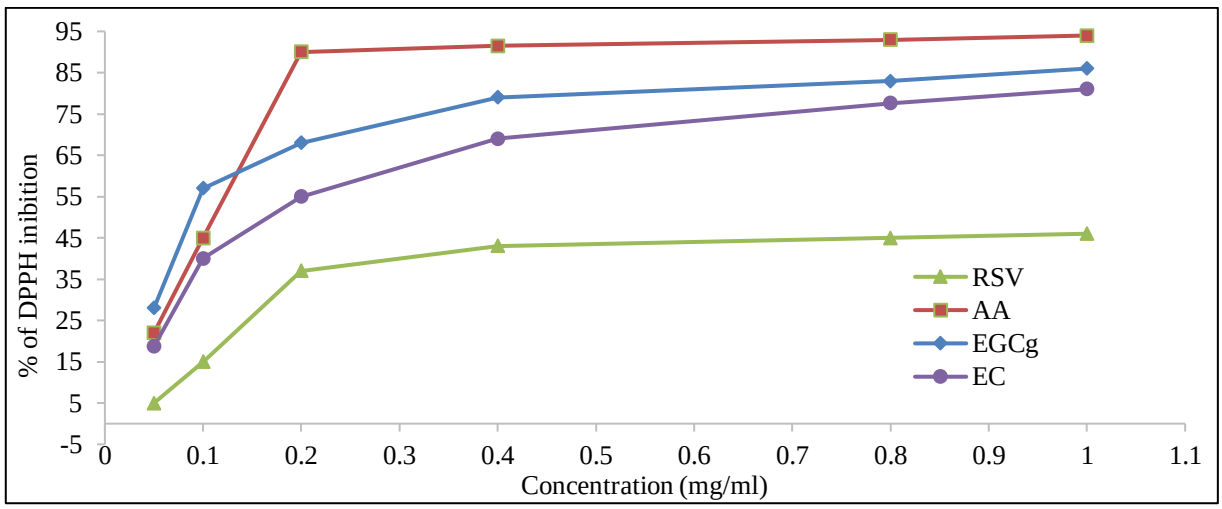


Figure IV.8: Percent inhibition of DPPH vs concentration of EGCg, EC, RSV and AA.

The free radical percentage inhibition increases with increasing concentration. According to **Figure IV.8**, for the concentration range of 0.05 to 0.1 mg/ml, the percentages inhibition (I%) of the DPPH radical by EGCg, ranging from 28 to 57%, were higher than those of RSV (5 to 15%) and AA (from 22 to 45%). For EC, I% (from 18.7 to 40%) were lower than those of AA but higher than those of RSV.

For concentrations from 0.2 to 1.0 mg/ml, I%, ranging for EGCg from 68 to 86% and for EC from 55 to 81%, were higher than those of RSV (37-46 %) but lower than those of AA (90-94%). These results indicate that EGCg is more effective than EC for radical scavenging activity. This result is in agreement with that reported by **He et al. (2018)**. It's worth noting that some authors such as **Guo et al. (1999) and Nanjo et al. (1996)** revealed the importance of the gallate moiety and its three hydroxyl groups in scavenging the DPPH radical.

Our results also show that EGCg as well as AA are more susceptible to protons donation to neutralize the DPPH radical.

IV.1.2.1.2. Determination of IC50

The IC50 is the concentration of the antioxidant required to reduce the original amount of radical by 50% **(Zaiter et al. 2016)**. IC50 value which decreases with increasing antioxidant activity of the samples tested was determined graphically from the corresponding linear regression equation. According to the results illustrated in **Figure IV.9** bellow, and taking into account IC50 values, AA showed the most strong antioxidant activity (IC50 = 0.026 mg/ml) followed by EGCg (IC50 = 0.047 mg/ml), EC (IC50 = 0.289) and lastly RSV (IC50 = 0.939).

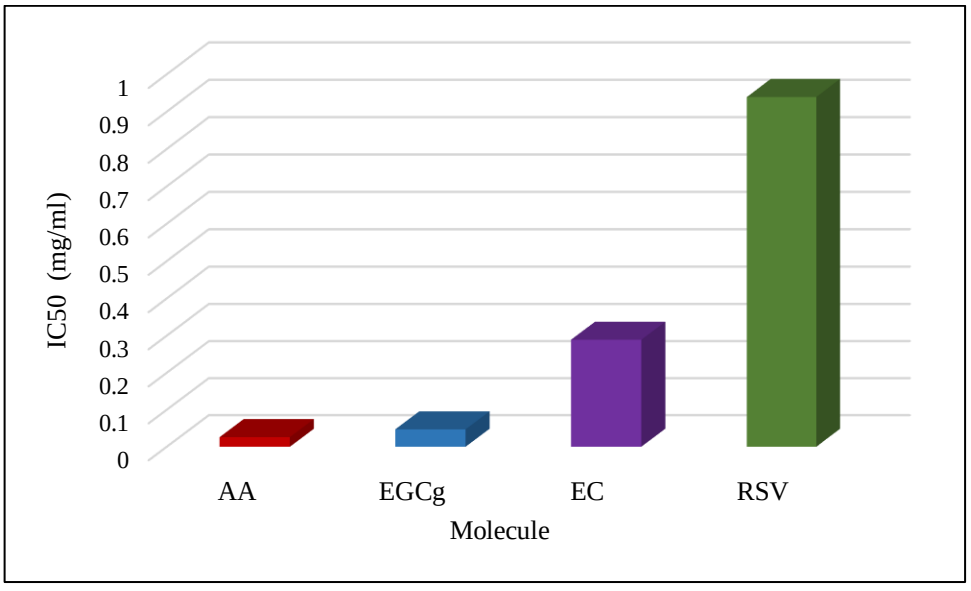


Figure IV.9: IC50 values of EGCg, EC, RSV and AA.

IV.1.2.1.3. Ferric reduction antioxidant power

Catechins were most reactive and showed the highest stoichiometry of Fe^{3+} in the ferric reduction antioxidant power **(**FRAP) assay compared to other flavonoids **(Grzesik et al. 2018)**.

The results obtained allowed us to plot the curves representing the variation of the reducing power expressed as the absorbance vs concentration **(Figure IV.10)**.

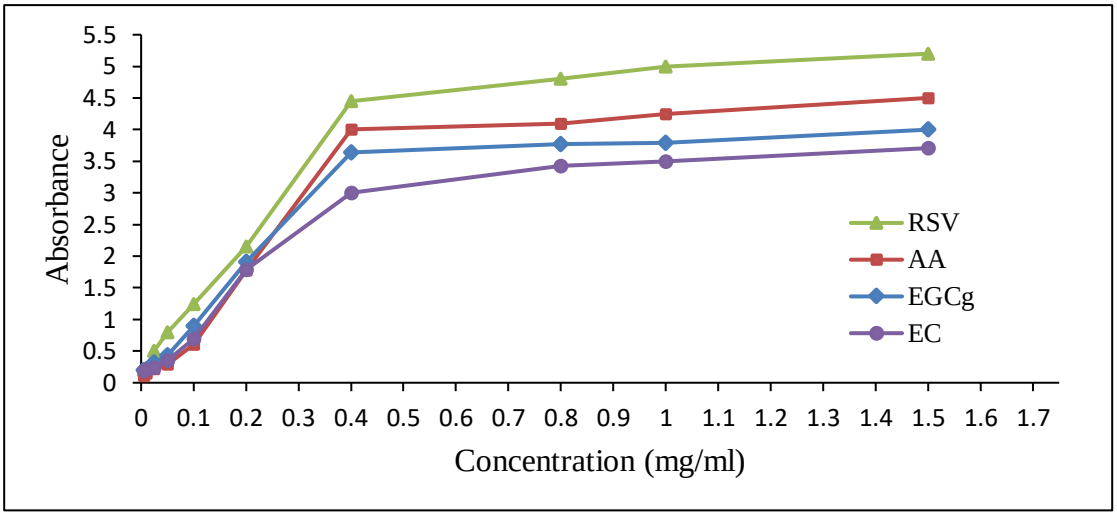


Figure IV.10: Reducing power of EGCg, EC, RSV and AA.

The increase in the absorbance of the reaction medium means an increase in the reduction of iron which increases with increasing concentration of tested molecules.

In view of these results, EGCg has an antioxidant activity greater than that of EC, this is obviously due to the presence of gallate moiety which contains more OH groups susceptible to donate electrons but also to other features as revealed by theoretical calculations presented below. This result is consistent with literature. Indeed, many authors already reported that catechin gallate esters (EGCg) are more effective for FRAP scavenging than EC **(He et al. 2018)**.

According to **Figure IV.10**, for concentrations ranging from 0.005 to 0.2 mg/ml, the reducing power of RSV and the two catechins (Abs = 0.197-1.907 for EGCg and Abs = 0.187-1.786 for EC) is greater than that of AA. Above these concentrations, the reducing capacity of catechins increases but remains lower than that of AA. Among studied compounds, RSV is the most potent for reducing the ferric cation.

IV.1.2.2. Evaluation of antioxidant activity of green tea

IV.1.2.2.1. Comparison of DPPH Inhibition of green tea with EGCg, EC, RSV and AA

The sample S4 was chosen among the eight brands of green tea studied, since it has the highest level of catechins (cf **Tables IV.3 and IV.4** above). The **Table IV.9** shows the mass percentages of the four catechins in this samlpe (S4).

Table IV.9: Mass percentages (%) of the four catechins in green tea (S4).

Molecule	**EGCg**	**ECg**	**EGC**	**EC**	**TC**
Mass percentage (%)	9.12	3.51	0.28	2.26	15.17

The total catechins percentage is found to be 15.17 %, which is in concordance with the literature **(Wei et al. 2018)**.

According to the **Figure IV.11**, we can notice that for all concentrations (from 0.05 to 1 mg/ml), green tea sample S4 percentage inhibition of DPPH radical is comparable to pure EGCg, EC, lower than AA, but higher than RSV.

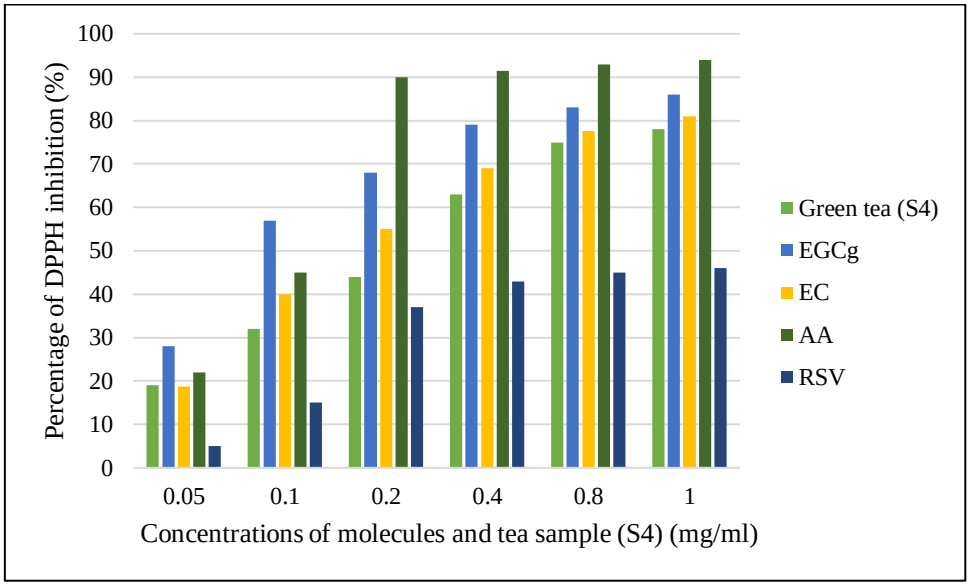


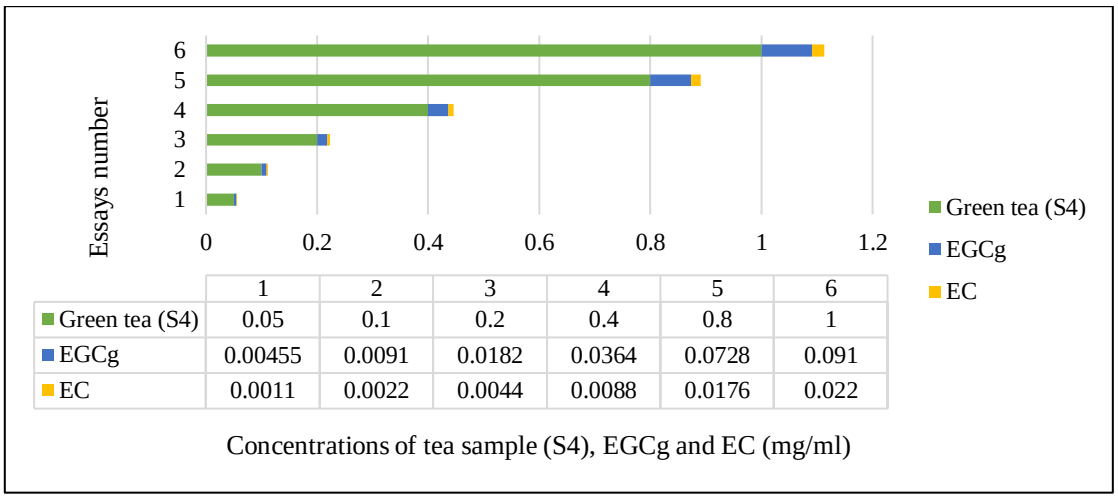
Figure IV.11: Percent inhibition of DPPH radical vs concentration of green tea sample (S4), EGCg, EC, RSV and AA.

This finding confirms that green tea is comparable to pure molecules recognized as strong antioxidants such catechins.

For a concentration of 0.05 mg/ml, S4 and EC have almost the same power of inhibition of DPPH (19 and 18.7 % respectively).

IV.1.2.2.2. Estimation of the antioxidant activity of EGCg compared to that of S4

The **Figure IV.12** shows the concentrations of EGCg and EC when the green tea sample (S4) has the concentrations from 0.05 to 1 mg/ml.



	1	2	3	4	5	6
Green tea (S4)	0.05	0.1	0.2	0.4	0.8	1
EGCg	0.00455	0.0091	0.0182	0.0364	0.0728	0.091
EC	0.0011	0.0022	0.0044	0.0088	0.0176	0.022

Figure IV.12: Sample tea (S4) and corresponding EGCg and EC concentrations.

For the two concentrations (0.8 and 1 mg/ml) of S4, the concentrations of EGCg are 0.0728 and 0.091 mg/ml respectively. If we consider the linearity of the evolution of inhibition of DPPH by EGCg between the two concentrations above (0.0728 and 0.091 mg/ml), we can draw the line with the concentrations from 0.05 to 0.1 mg/ml **(Figure IV.13)** having the equation ($y = 580\ x - 1$) which allow us to calculate the percentage of inhibition of EGCg at 0.0728 and 0.091 mg/ml (49 and 66 %) respectively.

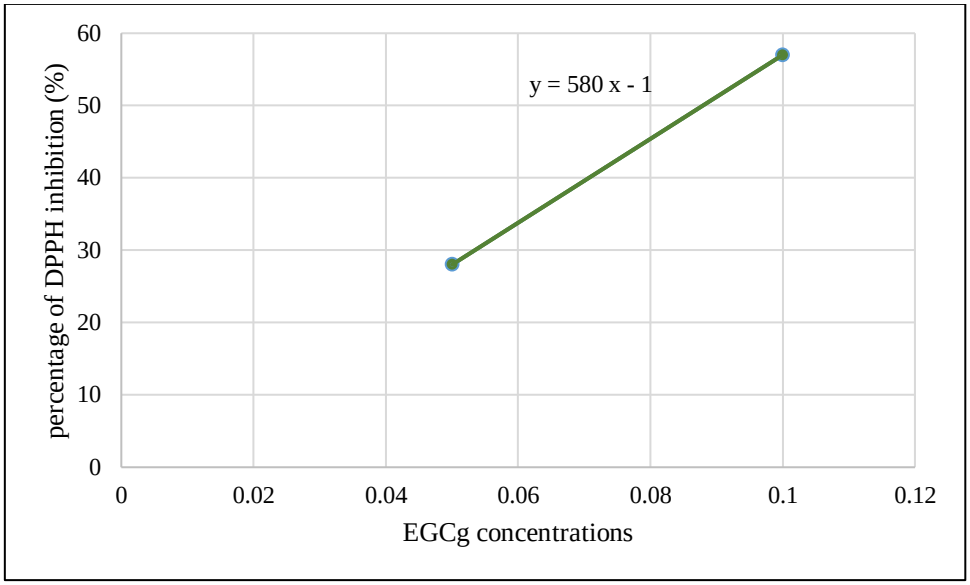


Figure IV.13: Percent inhibition of DPPH vs concentration of EGCg from 0.05 to 0.1 mg/ml.

The **Figure IV.14** illustrates the percentages inhibition of DPPH by EGCg (49 %, 66 %) and and green tea (S4) (75 %, 78 %) for the concentrations of (S4): 0.8 and 1 mg/ml.

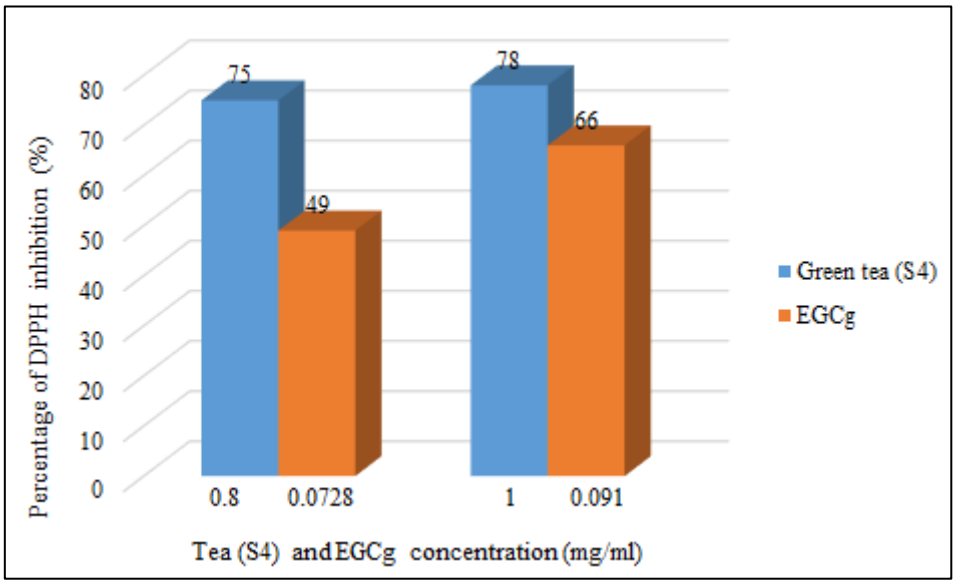


Figure IV.14: Representation of percentage inhibition of DPPH by S4 (0.8 and 1 mg/ml) and EGCg.

These results show that the antioxidant activity of green tea (S4) is mainly due to EGCg with a percentage ranging from 65 to 85 %.

IV.2. Theoretical approach for evaluation of antioxidant activity of EGCg and EC

In this part, theoretical evaluation of the antioxidant activity of EGCg and EC was deeply investigated and compared to that of RSV and AA as renowned potent antioxidants.

IV.2.1. Mechanisms of antioxidant activity of EGCg and EC in gas phase

Three mechanisms have been studied in order to determine the most suitable for evaluating the antioxidant activity of EGCg and EC in comparison with RSV as a potententl antioxidant and AA included as a reference. Calculations have been performed in the gas phase and in water as selected solvent.

a) Hydrogen Atom Transfer (HAT)

To study HAT mechanism, bond dissociation energies (BDEs) were calculated. BDE is a numerical parameter characterizing the stability of the bond in the hydroxyl group. The weaker this bond, the higher the antioxidant activity and hence, the more favorable the reaction with free radicals. Therefore, the molecules with low BDE values are expected to show high antioxidant activity. Calculated BDE values for studied compounds are presented in **Table IV.10**.

Our results reveal that for EGCg, the lowest value of BDE (300.45 kJ/mol) was observed for 4'-OH, followed by 4''-OH (307.78 kJ/mol) and 5'-OH (331.76 kJ/mol). 5''-OH and 3'-OH sites have almost identical BDE values (337.58 and 337.63 kJ/mol respectively), indicating that these two OH groups are similar in their stability and probability of H-atom donating. These results are in concordance with literature **(Wang et al. 2017)**.

For EC, the most interesting sites in the B ring are 4'-OH with a BDE value of 336.01 kJ/mol and 5'-OH with a BDE value of 338.41 kJ/mol. These two BDEs are closer to those of 3'-OH and 5''-OH of EGCg but greater than 4'-OH and 4''-OH in EGCg. This is one of the reasons for which EGCg is better antioxidant than EC.

For RSV, the BDE (351.44 kJ/mol) of 4'-OH is the lowest, followed by the 3-OH (369.93 kJ/mol) and finally 5-OH (371.60 kJ/mol). We can notice that all BDE values of RSV are greater than 325 kJ/mol, this result is in agreement with literature **(Lu et al. 2013, Mikulski and Molski 2010)**.

It should be mentioned that five of the eight OHs of EGCg have BDE values less than those of RSV and our results also show that the lowest BDE value of RSV is higher by 51 kJ/mol than that of EGCg, and that is one of the reasons for which EGCg is more potent antioxidant than RSV. For

EC, 4'-OH and 5'-OH have BDEs less than all OH BDEs of RSV and this is why EC is better antioxidant than RSV according to the HAT mechanism.

Table IV.10: BDE, AIP, PDE, PA and ETE of EGCg, EC, RSV and AA in kJ/mol obtained at B3LYP/6-311++ G(d,p) level of theory, in the gas phase.

Molecule		BDE	AIP	PDE	PA	ETE	Ring
EGCg	5-OH	375.88	708.88	995.01	1444.48	259.41	A
	7-OH	377.41		996.54	1463.07	242.36	A
	3'-OH	337.63		956.76	1409.52	270.74	B
	4'OH	300.45		919.59	1387.30	241.17	B
	5'-OH	331.76		950.89	1410.37	249.41	B
	3''-OH	333.02		952.15	1337.46	323.57	D
	4''-OH	307.78		926.91	1358.64	277.15	D
	5''-OH	337.58		956.71	1322.19	343.41	D
EC	3-OH	439.59	707.28	1060.32	1494.76	272.85	C
	5-OH	364.98		985.71	1445.44	247.56	A
	7-OH	378.23		998.96	1471.66	234.58	A
	5'-OH	338.41		959.14	1433.73	232.70	B
	4'-OH	336.01		956.74	1430.54	233.48	B
RSV	3-OH	369.93 343.13[a] 348.2[b] 345.26[c]	672.24 658.72[a] 683.73[e] 674.23[f] 656.51[g]	1025.70 989.82[c] 1375.12[h] 1024.93[i]	1457.54 1443.85[a] 1423.70[c] 1473.03[i]	240.40 207.32[a] 242.02[c] 210.63[i]	A
	5-OH	371.60 342.13[a] 353.6[b] 345.26[c]		1027.37 989.82[c] 1026.02[i]	1459.98 1441.55[a] 1423.70[c] 1474.99[i]	239.64 210.75[a] 242.02[c] 201.60[i]	A
	4'-OH	351.44 325.49[a] 325.7[b] 325.62[c]		1007.21 970.17[c] 1348.39[h] 1006.71[i]	1433.49 1412.29[a] 1396.95[c] 1443.27[i]	245.97 222.33[a] 249.12[c] 222.16[i]	B
AA	2-OH	362.75 324.12[d]	794.81 995.6[d]	895.96	1474.07	216.69	Inside the pentacycle
	3-OH	357.23 289.46[d]		890.43	1370.95	314.29	Inside the pentacycle
	5-OH	463.27 398.75[d]		996.48	1370.95	420.34	Outside the pentacycle
	6-OH	452.29 417.86[d]		985.50	1355.51	424.80	Outside the pentacycle

[a] (Lu et al. 2013), [b] (Nazarparvar et al. 2012), [c] (Thi and The 2020), [d] (Jabeen et al. 2018), [e] (Albuquerque 2015), [f] (Leopoldini et al. 2011), [g] (Mikulski et al. 2010), [h] (Mikulski et al. 2010), [i] (Benayahom et al. 2014).

BDE values found for OHs in the pentacycle of AA are lower than those outside the pentacycle, this result is in agreement with literature **(Jabeen et al. 2018)**. As can be clearly seen from results in **Table IV.10**, BDE values of EGCg in gallate moiety (ring D), EC and RSV in the B ring are lower than those of AA in pentacycle. On the other hand, BDEs of EGCg, EC and RSV in A and C rings are lower than those of AA outside the pentacycle. AA has been proven experimentally as a potent antioxidant, our results led us to conclude that the HAT mechanism is favored for EGCg, EC and RSV but not for AA.

b) Single Electron Transfer-Proton Transfer mechanism (SET-PT)

The adiabatic ionization potential (AIP) parameter is related to the SET mechanism. It describes the process of electron donation by the antioxidant. Molecules with low AIP values are more susceptible to ionization and have stronger antioxidant properties. The values of AIP for studied molecules are presented in **Table IV.10**. It should be mentioned that a slight difference in AIP values between EGCg and EC (1.6 kJ/mol) is observed, however the difference in AIP values between RSV and EC is rather higher and equals 35 kJ/mol. Hence, AIP values follow the sequence :

$$RSV < EC \approx EGCg < AA.$$

To study single electron transfer followed by proton transfer, PDE values were calculated. PDE is an important physical parameter describing the antioxidative properties of the compound investigated as it shows its ability to donate protons. As a rule, compounds with lower PDE values are more susceptible to proton abstraction and hence are the most potent antioxidants.

The calculated PDE for EGCg, EC, RSV and AA in the gas phase are presented in **Table IV.10**. Our results indicate that lowest PDE values for EGCg are found for OHs in the B ring and in the gallate moiety and 4'-OH is the most acidic. This result reinforces that of HAT transfer and led to conclude that the 4'-OH in the B ring is the most susceptible to H-abstraction. The other values of PDE for EGCg are closer to those of EC and are greater than 950 kJ/mol. These results also corroborate with those of BDE. The PDE values of all OHs of RSV are greater than 1000 kJ/mol and are higher than those of both EGCg and EC OHs, except PDE of 3-OH in EC.

As shown in **Table IV.10**, the 4'-OH of RSV is more acidic than the other two hydroxyl groups and these results are in agreement with those reported by **Fukuhara et al. (2008)** and **Mikulski et al. (2010)**, who claimed that the 4'-OH group of RSV is the most reactive and determines its

biological activity. It can be noticed from our results that OH acidity in the pentacycle of AA is comparable to that of 4'-OH in EGCg. On the other hand, PDE values for OH groups outside the pentacycle in AA are comparable to those of OHs in A ring of EGCg and EC. The sequence for PDE is:

EGCg ≈ AA < EC < RSV.

It should be inferred from these results that SET-PT is the most preferred antioxidant mechanism for catechins and the most suitable in the first step for RSV.

c) Sequential Proton Loss Electron Transfer mechanism (SPLET)

The SPLET mechanism is primarily governed by the ease of deprotonation, which can be described by the proton affinity (PA) values, and secondarily by the ease of electron transfer from the anions, described by the ETE.

Regarding the deprotonating step, PA values in **Table IV.10**, show that EGCg OHs in the gallate moiety are deprotonated considerably easier than the other OH sites and overall, PA values in the gallate lie in a very narrow (1322.19-1358.64 kJ/mol) range.

Additionally, with the exception of the gallate moiety, the data of **Table IV.10** confirm that low proton affinities are related to OH groups in the B ring as it was ordinary in most polyphenols such as flavonoids and stilbenes **(Lu et al. 2013 and Zheng et al. 2018)**. PA values of hydroxyls in the B ring of EGCg are higher than those of the gallate moiety but lower than those of EC and RSV. The most acidic protons in EC are 4'-OH and 5'-OH, followed by protons of the A ring and lastly by the proton of 3-OH in the C ring. The lowest PA value (1433.49 kJ/mol) for RSV was found for 4'-OH. This result was expected since previous results confirmed the high reactivity of this site.

It's worth noting that the calculated PA values of AA are comparable to those of 4'-OH and hydroxyls of the gallate moiety of EGCg, so EGCg deprotonates as much easily as AA and hence, EGCg can be qualified as a potent antioxidant.

After deprotonation, the anions may proceed to form the corresponding radicals by electron transfer measurable by electron transfer enthalpy (ETE). From the thermodynamic cycle for SPLET, it follows that the larger the PA, the lower the ETE and vice versa.

Our results show that all ETE values for the OH groups in gallate moiety of EGCg are higher than those of the B ring in EGCg and all OHs of EC and RSV. In EC, it was observed that the lowest ETEs were found for anions formed from OH groups located in the B ring. The highest value (424.80 kJ/mol) was found for the 6-OH in AA.

The results of PA and ETE confirm that the SPLET mechanism is more favored for EGCg than EC and RSV.

IV.2.2. Effect of water medium on thermodynamic parameters

The results of BDE, AIP, PDE, PA and ETE in water, for EGCg, EC, RSV and AA are presented in **Table IV.11**.

In view of the results obtained from thermodynamic parameters, calculated in the aqueous phase for the studied molecules, we can stipulate that calculated BDEs in the gas phase and in water are close to each other for all compounds. The largest deviation between BDE values in the two phases is only 17.77 kJ/mol, which is in concordance with literature **(Lengryel et al. 2013)**.

It has been stressed that the gas phase and water BDE values follow the same trend except for 3'-OH and 5''-OH sites in EGCg and for 2-OH and 3-OH for AA, however in the two environments; the most preferred sites for antioxidant activity are in gallate moiety for EGCg and in B rings for all polyphenolic compounds.

From results presented in **Table IV.11**, we can notice that PDE and PA values in water medium, ranging from -34.36 to 110.08 kJ/mol and from 87.48 to 221.57 kJ/mol, respectively, are dramatically lower than those found in the gas phase (890.43 – 1060.32 kJ/mol for PDE and 1322 –1494.76 kJ/mol for PA), this is due to the high solvation enthalpies of proton **(Xue et al. 2014)**.

Table IV.11 : BDE, AIP, PDE, PA and ETE in kJ/mol obtained at B3LYP/6-311++ G(d,p) level of theory, in water, for EGCg, EC, RSV and AA.

Molecule		BDE	AIP	PDE	PA	ETE	Ring
EGCg	5-OH	363.27	470.67	17.49	152.53	335.63	A
	7-OH	366.82		21.04	161.85	329.86	A
	3'-OH	338.45		-7.32	131.08	332.26	B
	4'OH	313.89		-31.87	117.00	321.78	B
	5'-OH	334.94		-10.82	131.20	328.64	B
	3''-OH	338.06		-7.71	104.25	358.70	D
	4''-OH	321.22		-24.54	110.92	335.19	D
	5''-OH	342.98		-2.79	87.48	380.38	D
EC	3-OH	447.34	462.15	110.08	221.57	350.66	C
	5-OH	362.69		25.43	157.38	330.19	A
	7-OH	369.19		31.94	169.17	324.91	A
	5'-OH	340.79		3.53	145.83	319.85	B
	4'-OH	338.36		1.10	144.62	318.63	B
RSV	3-OH	368.78 343.97[a]	422.05 519.61[a] 497.16[b]	72.55 82.63[c]	164.55 202.73[d]	329.12 333.56[d]	A
	5-OH	370.68 344.93[a]		73.52 84.39[c]	165.71 202.73[d]	329.86 333.56[d]	A
	4'-OH	344.12 322.36[a]		46.96 59.48[c]	156.23 193.11[d]	312.78 315.17[d]	B
AA	2-OH	344.98	504.24	-34.36	153.18	316.69	Inside the pentacycle
	3-OH	350.51		-28.83	103.52	371.88	Inside the pentacycle
	5-OH	465.17		85.82	103.52	486.54	Outside the pentacycle
	6-OH	455.19		75.84	95.51	484.57	Outside the pentacycle

[a] (Lu et al. 2013), [b] (Mukilski et al. 2010), [c] (Benayahoum et al. 2014), [d] (Thi and The 2020).

Due to their charge, cationic radicals are sensitive to the polarity of water, as expected, the AIP values in water (422.05 - 504.24 kJ/mol) are considerably lower than those of the gas phase (672.24 – 794.81 kJ/mol) for all studied molecules.

According to the SPLET mechanism, electron transfer tendency is studied as the second step by estimating ETEs. It can be seen that the values of this thermodynamic parameter of all compounds

are higher in the water medium (312.78 to 486.54 kJ/mol) than the gas phase (216.69 to 424.80 kJ/mol). This is in line with results obtained by many authors such as **Anitha et al. (2020)**.

PDE, PA and AIP values follow the same sequence for both phases while ETE values in the gas phase do not always follow the same trend as in water medium.

We can conclude from the values obtained by the calculation of BDE, PDE and PA in the aqueous phase that the second step of SET-PT and the first step of SPLET mechanisms are more dominant and preferred than HAT mechanism in all studied molecules.

IV.2.3. Frontier orbitals (HOMO and LUMO)

High occupied molecular orbital (HOMO) and lower unoccupied molecular orbital (LUMO) are among the most important parameters of the molecular electronic structure. According to front orbital theory in DFT **(Sadsivam and Kumarisan 2011)**, the higher the HOMO energy of a molecule, the easier it loses electrons and the faster the reaction of electron donating is. On the other hand, the lower LUMO energy of a molecule indicates its ability to accept electrons.

We present in **Table IV.12**, HOMO and LUMO energies of EGCg, EC, RSV and AA.

As shown in **Table IV.12**, The use of diffuse function caused a decrease in HOMO and LUMO energies for all molecules. Results indicate that EC is slightly more electron-donating than EGCg.

Our results also show that EGCg and RSV have the same LUMO energy (-1.65 eV) in presence of diffuse function. We should mention that our results for HOMO and LUMO values of RSV are in good concordance with literature **(Thi and The 2020, Cao et al. 2003)**.

The lowest values of HOMO energy for both basis set were found for AA. When diffuse function was used, HOMO energy for AA decreased slightly from -6.13 to -6.45 eV which is closer to the value reported in the literature (-6.329 eV) **(Chidiebere 2015)**. On the other hand, the value of LUMO of AA is greater than that of EGCg and RSV but lower than that of EC with or without diffuse function.

Table IV.12 : HOMO and LUMO energies of EGCg, EC, RSV and AA obtained at B3LYP/6-311G(d,p) level of theory with and without diffuse function.

Basis set : 6-311++G(d,p)	**HOMO (eV)**	**LUMO (eV)**	**Gap (eV)**
EGCg	-6.10	-1.65	4.45
EC	-5.99	-0.76	5.23
RSV	-5.62 −5.618[a] -5.58[b]	-1.65 −1.661[a] -0.62[b]	3.97 3.95[a] 4.96[b]
AA	-6.45	-1.07	5.38
Basis set : 6-311G(d,p)	**HOMO (eV)**	**LUMO (eV)**	**Gap (eV)**
EGCg	-5.89	-1.22	4.67
EC	-5.78	-0.22	5.56
RSV	-5.46 -5.21[c] -5.48[d] -5.21[e]	-1.44 -1.15[c] -1.45[d] -1.17[e]	4.02 4.03[c] 3.95[d] 4.04[e]
AA	-6.13	-0.71	5.42

[a] (The and Thi 2020), [b] (Albuquerque et al. 2015, B3LYP/6-31+G(d,p)), [c] (Queiroz et al. 2009, B3LYP/6-31G(d)), [d] (Cao et al. 2003, B3LYP/6-31G(d,p)), [e] (Benayahoum et al. 2013, B3LYP/6-31G(d;p)).

The high reactivity of compounds is characterized by a small energy gap ($\varepsilon_G = E_{HOMO} - E_{LUMO}$) between the HOMO and the LUMO energies and also by a low LUMO energy, this means that these compounds can behave as soft electrophiles **(Lewars 2003, Hatch 2000)**. Reactivity of the studied compounds follows the trend:

RSV > EGCg > AA ≈ EC.

The contours of HOMO and LUMO for the neutral species of studied compounds are displayed in **Figure IV.15**. The π -cloud in the HOMO of EGCg is distributed on the A and C rings, and the π-cloud in the LUMO is distributed on the D ring (gallate moiety). For EC, the π-cloud in the HOMO and LUMO is almost distributed on the three rings. For RSV, it can be noticed that the HOMO and LUMO are distributed on the whole molecule. For AA, The HOMO is concentrated on the pentacycle while the LUMO is distributed on almost the whole molecule.

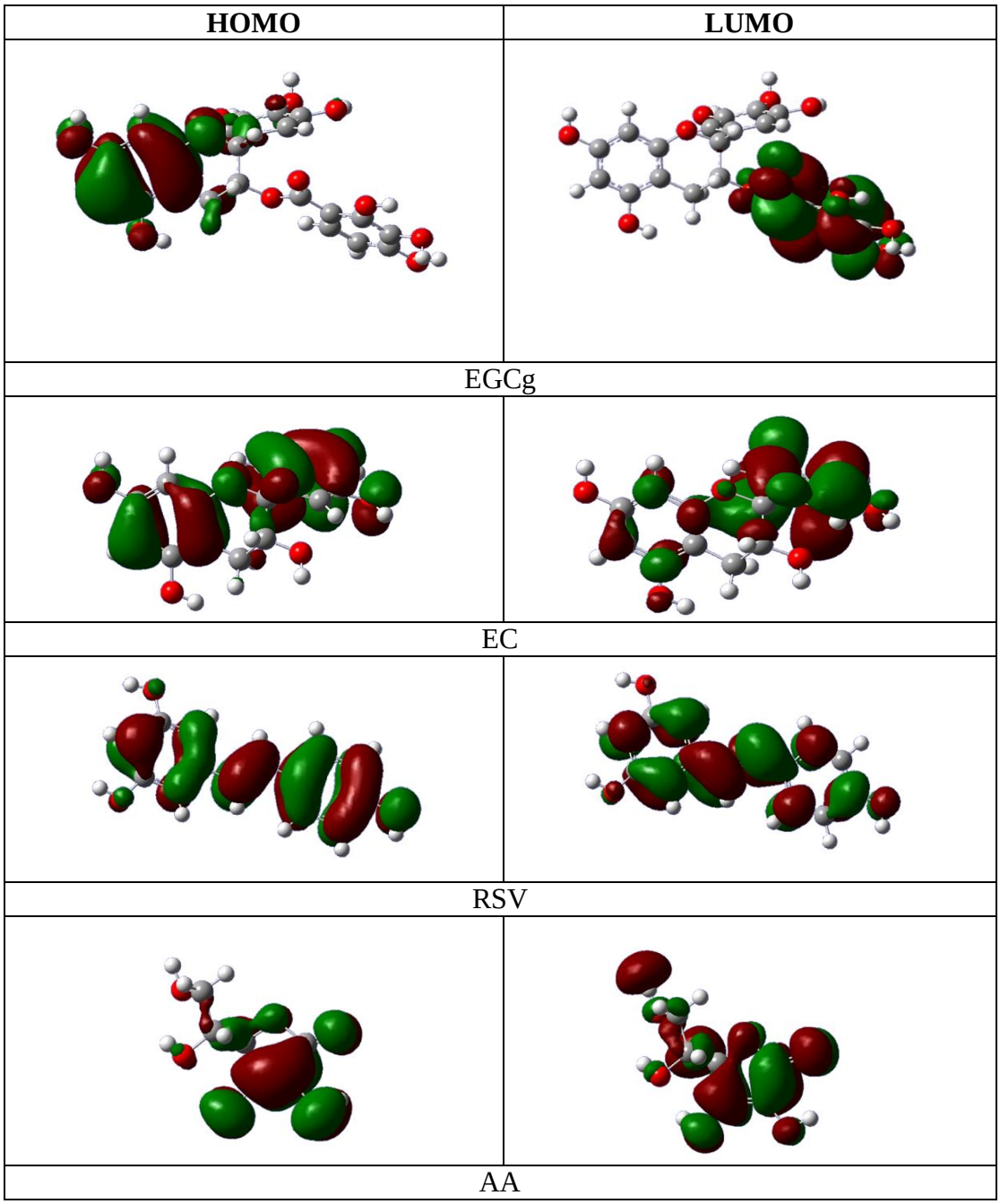


Figure IV.15: HOMO and LUMO frontier orbitals of EGCg, EC, RSV and AA.

IV.2.4. Spin density

The antioxidant activity of the compounds can be determined by an interpretation of the spin density values of the radical species studied. Total spin density characterizing the distribution of electron spin in free radicals is responsible for radicals' stability and it is one of the most important quantum properties of radicals. The more delocalized the spin density in the radical, the easier the

radical is formed and thus the lower the BDE **(Akhtari et al. 2015)**. In our study, spin density was calculated by HF and DFT, because according to some authors **(Filatov and Cremer 2005, Giner and angeli 2016, Macetti et al. 2018)**, DFT overestimates spin density values.

The **Table IV.13** summarizes Mulliken spin densities on oxygen atom in radicals of EGCg, EC, RSV and AA.

Table IV.13: Mulliken spin densities on oxygen atom in radicals of EGCg, EC, RSV and AA obtained by UHF/6-31G, B3LYP/6-311G(d,p) and B3LYP/6-311++G(d,p).

Radical	Method		
	UHF/ 6- 31G	DFT B3LYP/6-311G(d,p)	DFT B3LYP/6-311++G(d,p)
EGCg-5-O	0.21	0.38	0.37
EGCg-7-O	0.20	0.48	0.47
EGCg-3'-O	0.19	0.42	0.41
EGCg-4'-O	0.23	0.39	0.38
EGCg-5'-O	0.19	0.36	0.35
EGCg-3''-O	0.18	0.39	0.39
EGCg-4''-O	0.24	0.41	0.40
EGCg-5''-O	0.19	0.41	0.40
EC-3-O	0.10	0.72	0.70
EC-5-O	0.19	0.38	0.37
EC-7-O	0.17	0.41	0.40
EC-5'-O	0.18	0.35	0.34
EC-4'-O	0.19	0.34	0.34
RSV-3-O	0.18	0.39	0.38
RSV-5-O	0.19	0.40	0.39
RSV-4'-O	0.13	0.31	0.30
AA-2-O	0.23	0.39	0.40
AA-3-O	0.15	0.26	0.26
AA-5-O	0.03	0.70	0.68
AA-6-O	0.01	0.85	0.84

The results of spin density calculated by DFT at B3LYP/6-311G(d,p) including or not diffuse function are almost the same, but they are not in harmony with those calculated by HF method. From results in **Table IV.13**, we can see that the values of spin density for radicals formed after H-abstraction obtained by HF/6-31G method, are almost half of those obtained by DFT B3LYP/6-311G(d,p).

For EGCg , spin densities allow us to conclude that the 3’’-O radical in gallate moiety is the most stable, followed by 3’-O, 5’-O and 5’’-O radicals. The most stable radicals in EC and RSV are 3 -O and 4’-O; respectively according to HF calculations. But according to DFT results, 4’O in EC and 5’-O in EGCg are the most stable. In AA, we recorded the lowest spin density value by HF method but at the same time the highest by DFT attributed to the 6-O radical.

According to results displayed in **Table IV.13** and combining the three mechanisms and spin densities evaluated by DFT, we can conclude that the pharmacophores designated for each molecule are:

- 4'-OH, 5'-OH and 3"-OH for EGCg

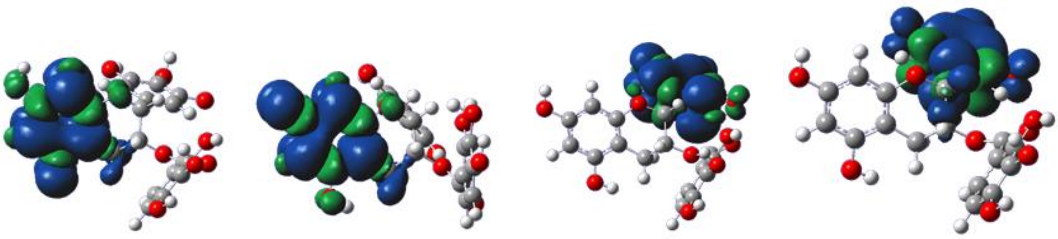
- 5’-OH and 4'-OH for EC

- 4'-OH for RSV

- 3-OH and 2-OH for AA

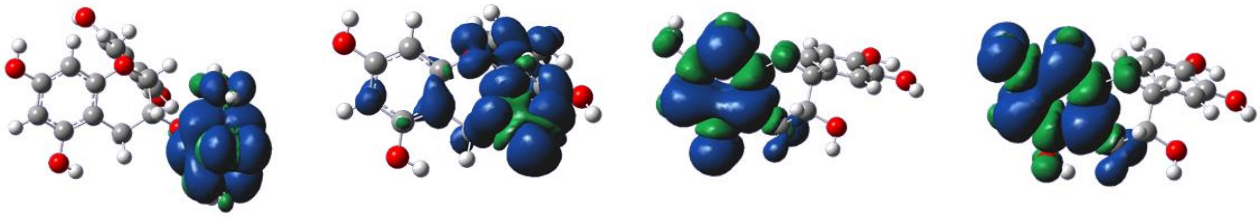
If we take a closer look at spin density images represented in **Figure IV.16**, we find that for EGCg, the spin density on 5-O and 7-O radicals is concentrated on the A ring. For 3’-O, 4’-O and 5’-O radicals, spin density is distributed on the B ring. For 3’’-O, 4’’-O and 5’’-O radicals, the unpaired electron is located on the gallate moiety.

For EC, the unpaired electron of 3-O position is distributed over the B and C rings. For 5-O and 7-O radicals, the spin density is located on both A and C rings. 5’-O and 4’-O spin densities are located on the B ring only.

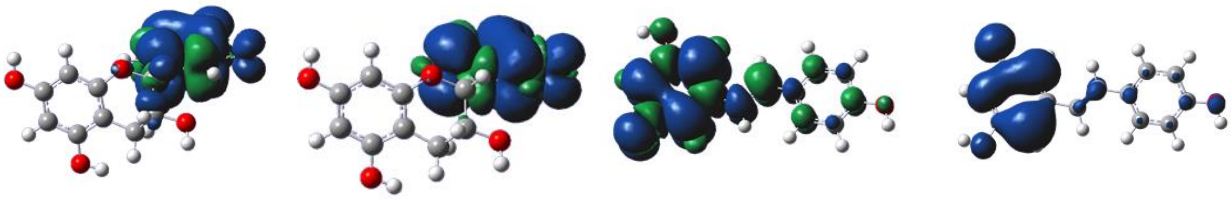


EGCg-5-O EGCg-7-O EGCg-3’-O EGCg-4’-O

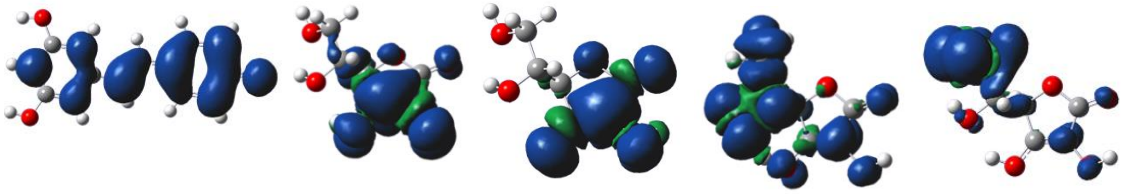
EGCg-5’-O EGCg-3’’-O EGCg-4’’-O



EGCg-5’’-O EC-3-O EC-5-O EC-7-O



EC-4’-O EC-5’-O RSV-3-O RSV-5-O



RSV-4’-O AA-2-O AA-3-O AA-5-O AA-6-O

Figure IV.16: Spin density images of EGCg, EC, RSV and AA.

On the other hand, in 4'-O radical of RSV, the unpaired electron is disposed on the whole molecule as shown in spin density contours **(Figure IV.16)**, while for the 3-O and 5-O radicals, the unpaired electron is mainly located on the A ring and ethylene bridge **(Figure IV.16)**.

For AA, the unpaired electron in 2-O and 3-O radicals is mainly located on the pentacycle, while in the 5-O radical, unpaired electron is almost delocated over the whole radical. However, the spin density in 6-O radical is concentrated outside the pentacycle.

IV.2.5. Electrostatic potential maps

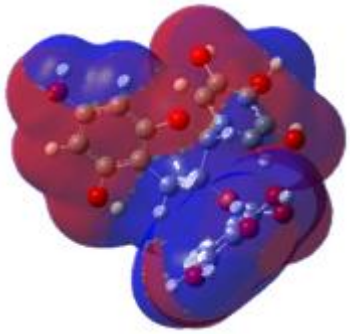
Electrostatic potential (ESP) is another important parameter, which is fundamental for understanding the chemical reactivity and the atomic structure of molecules **(Hubschle and Smaalen 2017)**. In the ESP mapped surface, the negative potential energy (nucleophilic region) is colored in red and positive potential energy (electrophilic region) is colored in blue.

Figure IV.17 illustrates the ESP maps of studied compounds. Regarding EGCg, the gallate part of the molecule is electrophilic, all OH groups are nucleophilic. For EC, the A ring and the 4'-OH site can accept an electrophilic attack.

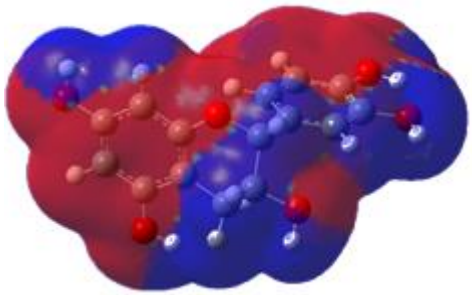
If we consider the ESP map of RSV, we can see the dominance of the nucleophilic region with a small electrophilic character.

The ESP map of AA shows a predominance of the electrophilic region. The OH groups of the pentacycle have a nucleophilic character.

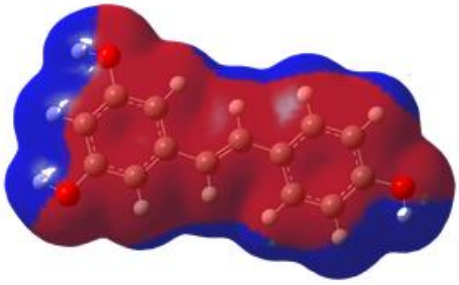
Overall, the results of the ESP show a concordance with the results of frontier orbitals (HOMO and LUMO).



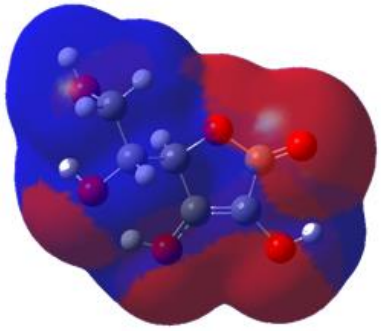
Epigallocatechin gallate



Epicatechin



Resveratrol



Ascorbic acid

Figure IV.17: Molecular electrostatic potential mapped on the isodensity surface of EGCg, EC, RSV and AA at B3LYP/6-311++G(d,p) level of theory in gaseous phase.

IV.2.6. Determination of acidity

To determine the acidity of polyphenols, two methods were investigated: (i) calculation of pKa and (ii) determination of the enthalpy difference between the anion (A^-) and its neutral species (HA).

As shown in **Table IV.14**, for our phenolic compounds (EGCg, EC and RSV), the pKa values of the OH sites belonging to the A ring are between 10.04 and 11.46. Regarding the B ring, the pKas of the OH sites are : 8.45 for 5'-OH of EC and 8.24 for 4'-OH of EC and RSV.

TableIV.14: Enthalpy of acidity in kJ/mol and pKa values for OH groups in EGCg, EC, RSV and AA.

Molecule		$\Delta H_{acidity}$	pka	Ring
EGCg	5-OH	1438.28	10.04	A
	7-OH	1456.87	11.44	A
	3'-OH	1403.32	6.70	B
	4'OH	1381.10	4.21	B
	5'-OH	1404.17	6.64	B
	3''-OH	1331.26	1.81	D
	4''-OH	1352.44	3.07	D
	5''-OH	1315.99	-0.65	D
EC	3-OH	1488.56	21.18	C
	5-OH	1439.24	10.30	A
	7-OH	1465.46	11.46	A
	5'-OH	1427.53	8.45	B
	4'-OH	1424.34	8.24	B
RSV	3-OH	1451.34	11.21	A
	5-OH	1453.78	11.44	A
	4'-OH	1427.29	8.24	B
AA	2-OH	1467.87	9.45	Inside the pentacycle
	3-OH	1364.75	0.26	Insidethe pentacycle
	5-OH	1364.75	0.26	Ouside the pentacycle
	6-OH	1349.31	-0.98	Outside the pentacycle

The pKa values in A and B rings are consistent with the PDE values which were found to be lower in the B ring than those in the A ring, for all three phenolic compounds.

The pKa values of the OH groups in the gallate moiety are the lowest. All pKa values for EGCg in the B ring are lower than 7.00, indicating that all OH sites of EGCg are more acidic than those of EC and RSV in the B ring.

It can be concluded that the high antioxidant activity of EGCg is in part related to the strong acidity of the OH groups, especially in the gallate moiety.

One of the antioxidant mechanisms of polyphenols is chelation of metals. Metals are entrapped in these polyphenols–metals complexes and are hence prevented from being involved in some

reactions, such as the production of free radicals. Chelation of metals often occurs through deprotonated hydroxyls in the polyphenols.

The determination of the acidity of these compounds is an important thermodynamic parameter that must be taken into account. The smaller the energy required to deprotonate the OH groups (acidity), the easier the metals chelation will be.

On the basis of B3LYP/6-311++G(d,p) increasing acidity values, an order can be given by comparing the smallest enthalpy value of each molecule:

EGCg (1315.99 kJ/mol) < AA (1349.31 kJ/mol) < EC (1424.34 kJ/mol) < RSV (1427.29 kJ/mol).

Calculated acidity values are in the same trend of pKa values :

For EGCg : 5''-OH<3''-OH<4''-OH<4'-OH<3'-OH<5'-OH<5-OH< 7-OH.

For EC: 4'-OH<5'-OH<5-OH<7-OH<3-OH.

For RSV: 4'-OH<3-OH<5-OH.

The value of acidity for EGCg is the smallest (1315.99 kJ/mol). This finding is in agreement with all calculated parameters.

Conclusion

This study was carried out to control the quality of green tea consumed by Algerian citizens. The first part of the study concerned the evaluation the levels of four catechins (epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin), three methylxantines (caffeine, theobromine and theophylline) and gallic acid in two extracts obtained by different extraction methods (infusion in hot water and extraction in hydromethanol) .

The second part of this study is an experimental overview of the antioxidant activity of green tea extract , epigallocatechin gallate and epicatechin compared to that of resveratrol and ascorbic acid.

The third part is a detailed theoretical study of the antioxidant activity of the two catechins cited above as compared with resveratrol and ascorbic acid.

From the results obtained we can make the following conclusions:

- The levels of total catechins in MeOH / H_2O green tea extracts were higher than those in infusions for the same sample of green tea, which is totally the opposite for total methylxantins.
- Green tea antioxidant activity is comparable to pure EGCg and EC, lower than AA, but higher than resveratrol, indicating the health benefits of tea consumption.
- The results of experimental antioxidant activity assay by FRAP and the theoretical study show that the two flavonoids of tea, epigallocatechin gallate and epicatechin are more potent antioxidants than resveratrol, hence, consumption of green tea can replace stilbenes containing beverages.
- Overall, the quality of tea consumed by the Algerian population is in the same norm worldwide and is not subjected to fraudulent actions such is the case for coffee.

CONCLUSION

Conclusion

The aim of this work was the quality control of green tea samples consumed by the Algerian population, through a quantitative analysis of polyphenols and methylxanthines and determination of antioxidant activity of tea extracts and pure metabolites.

Quantitative analyses of green tea samples from Algerian market were carried out to determine levels of catechins (EGCg, ECg, EC, EGC), methylxantines (Caffeine, Theobromine, Theophylline) and Gallic acid in these samples. Green tea samples were subjected to two methods of extraction; a multistep extraction procedure using methanol/water mixtures, at room temperature and infusion in hot distilled water at 85°C, for 5 min.

Catechins, the main tea antioxidants are found in high concentrations in both green tea extracts in which EGCg is the predominant compound. Catechins level followed the sequence:

EGCg > ECg > EC > EGC.

Methylxantines were found in higher concentrations in infusions than in MeOH/ water extracts, because of their high solubility in hot water compared to methanol, at room temperature. Methylxantines level followed the sequence Caffeine > Theobromine > Theophylline.

It's worth noting here that the most expensive among green tea samples studied, has the highest total catechins level.

All the green tea samples studied are originated from China, nevertheless, differences in the level of catechins and methylxantines studied were found, this is due to several factors among which, the soil quality where the tea plant was grown, the season and type of harvest, the drying process…etc. The use of nitrogen containing pesticides is an important factor that greatly influences the methylxanthines level in tea plant.

Additionally, infusion in hot water is suitable for extraction of green tea methylxantines, whereas methanol/water mixtures are favorable to extraction of polyphenols.

It's worth noting that the results of this study were compared to those published in the specialized literature and we should mention that in most cases, the concentrations of the five polyphenols and the three methylxantines are in concordance with those reported by other authors, either for green tea hydromethanol extracts or for infusions in hot water.

CONCLUSION

It could be concluded for this first part of work, that the consumption of a cup of tea, provides substantial amounts of polyphenolic antioxidants. Nevertheless, tea consumption should be moderated, since in addition to antioxidants, studied samples of tea beverage contains to a lesser extent, methylxantines such as caffeine and theobromine having several adverse health effects.

In the second part of this thesis, the antioxidant activity of tea extracts and flavonoids, epigallocatechin gallate (EGCg) and epicatechin (EC) were determined by experimental and theoretical methods and compared to that of a stilbene, resveratrol (RSV) and ascorbic acid (AA) both of them known for their high antioxidant potential.

Results of the study of antioxidant activity, according to the iron reduction method, show that EGCg has a very interesting power to reduce the Fe^{3+} ion especially at very low concentrations. The DPPH free radical scavenging assay showed that the EGCg has an anti-radical activity greater than RSV but less than AA. Using DFT calculations in the gas phase, enthalpy values (BDE, AIP, PDE, PA, ETE and acidity) and pKas of EGCg revealed that the gallate moiety and 4'-OH in the B ring are the most favored sites and are responsible for the higher antioxidant potential of these molecules compared to RSV.

For EC, the two sites 5'-OH and 4'-OH in the B ring are the most preferential active sites for the three mechanisms (HAT, SET-PT and SPLET) and show an antioxidant capacity lower than EGCg but greater than RSV.

The three mechanisms of antioxidant activity (HAT, SET-PT and SPLET) investigated are favored for EGCg. SET-PT is the most preferred antioxidant mechanism for the two catechins and it is the most suitable in the first step for resveratrol. The SPLET mechanism is favored for EC but to a lesser degree compared to EGCg.

Our results also show that there is a strong relation between BDE and spin density for EC and RSV since these two parameters follow the same trend for all radicals.

In water as solvent, the second step of SET-PT and the first step of SPLET are the most thermodynamically favored mechanisms compared to HAT mechanism for the studied molecules.

Evaluation of acidity of hydroxyl groups by determining both pKa and enthalpy difference between the anion (A^-) and neutral species (HA) showed that higher antioxidant activity of EGCg is in part related to strong acidity of its OH groups, indeed, all OH sites of EGCg, especially in the

gallate moiety are more acidic than those of EC and RSV. Results of pKa of studied molecules reinforce the other computed thermodynamic parameters.

The reactivity of molecules was evaluated by mapping ESP surfaces which showed that resveratrol presents a nucleophilic character, EGCg and EC are rather electrophilic but possess some nucleophilic regions which indicates that studied molecules are good proton donors.

It should be stressed that Mulliken spin density for radicals obtained by DFT method agree better with the other calculated parameters than those obtained by HF method.

Moreover, the use of diffuse function has almost no impact on the spin density values; however it caused a decrease in HOMO and LUMO energies.

Regarding the concordance of experimental and theoretical results, there is a good correlation between DPPH scavenging assay and calculated thermodynamic and electronic parameters.

Finally, we can conclude that the two flavonoids EGCg and EC are more potent antioxidants than the stilbene RSV.

The experimental study by DPPH of a green tea sample reveals that the antioxidant activity of green tea is mainly due to the presence and quantity of epigallocatechin gallate.

This study could be extended to peruse the effect of different attracting and withdrawing substituents on this trend.

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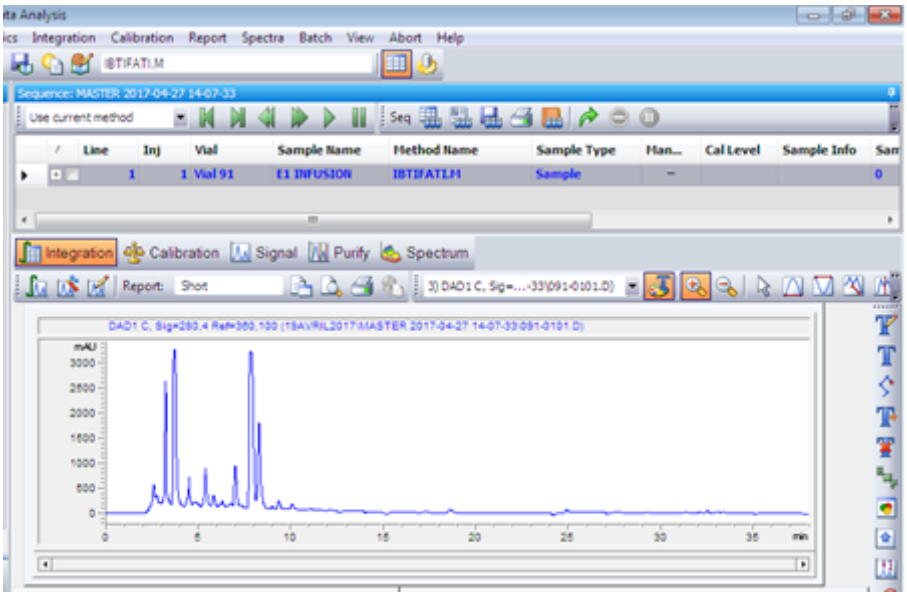
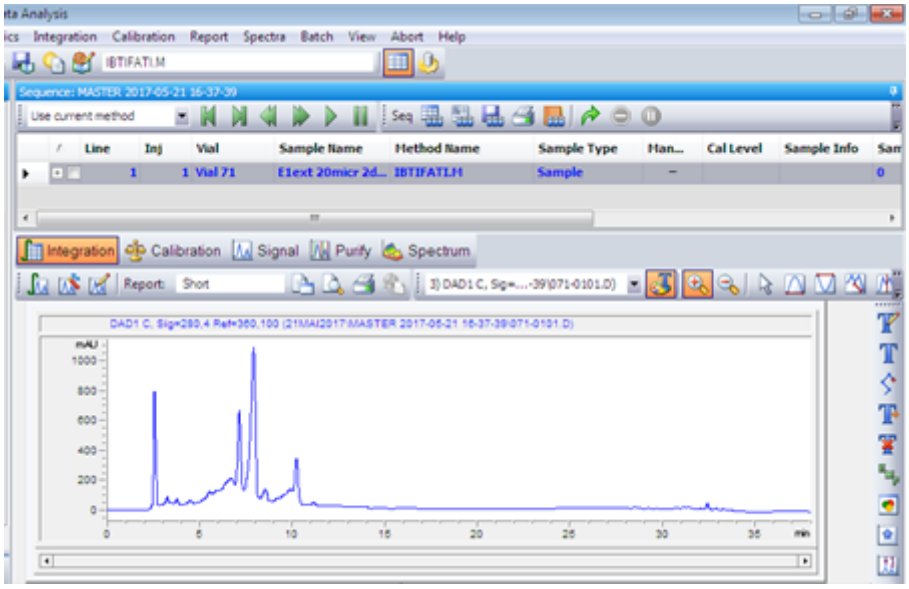
ANNEXES

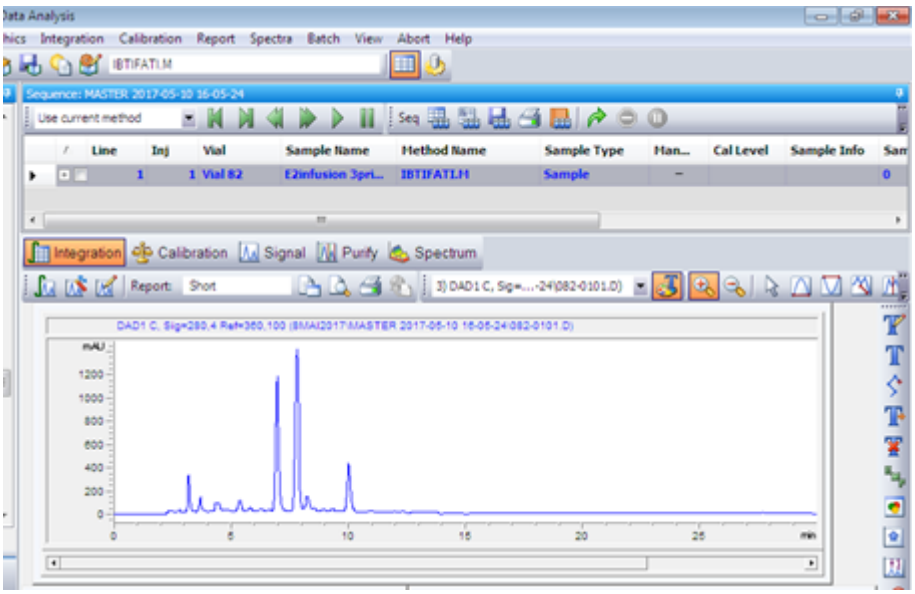
A 1 : Green tea chromatogramms obtained by both extraction in hydromethanol and in hot water (HPLC-Agilent 1260 infinity)



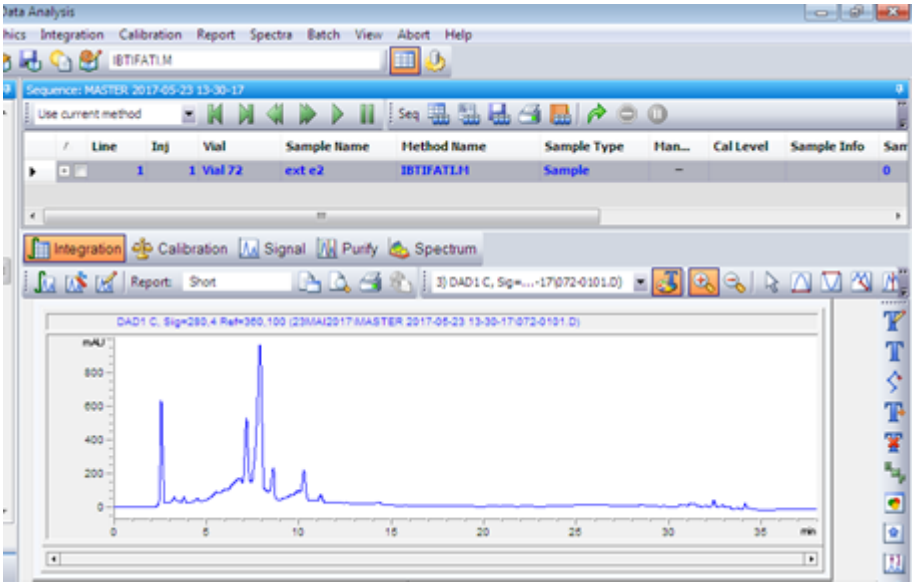
S1 (Water 85°C).



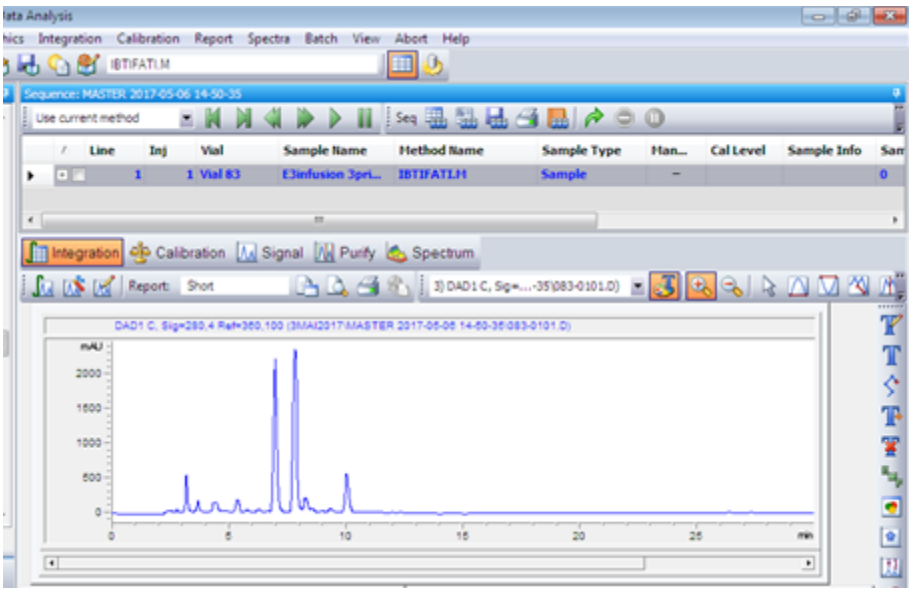
S1 (MeOH/Water).



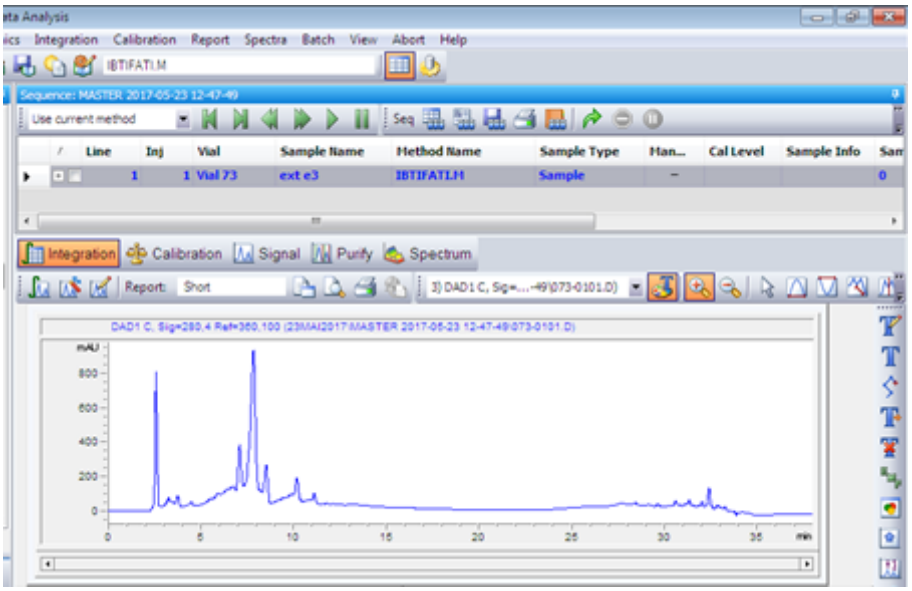
S2 (Water 85°C).



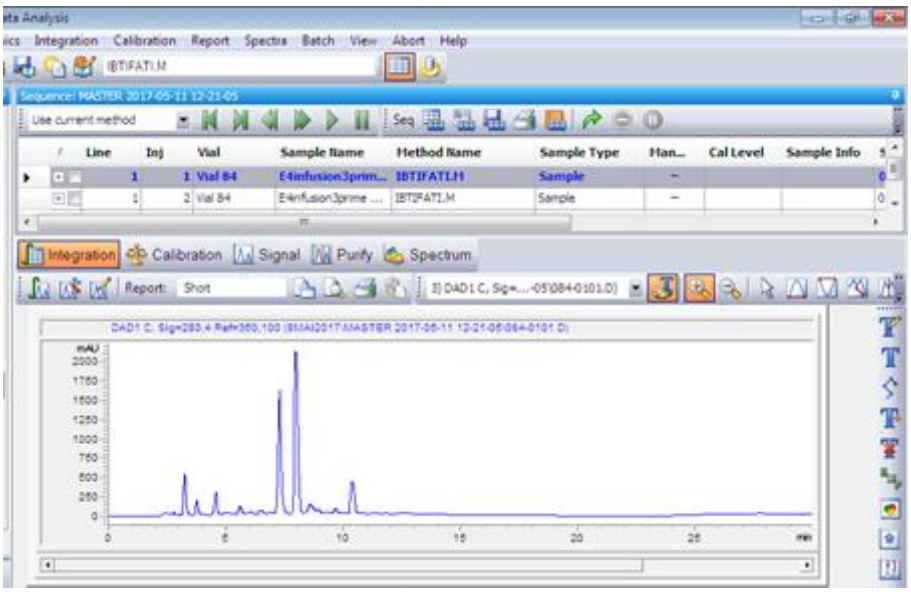
S2 (MeOH/Water).



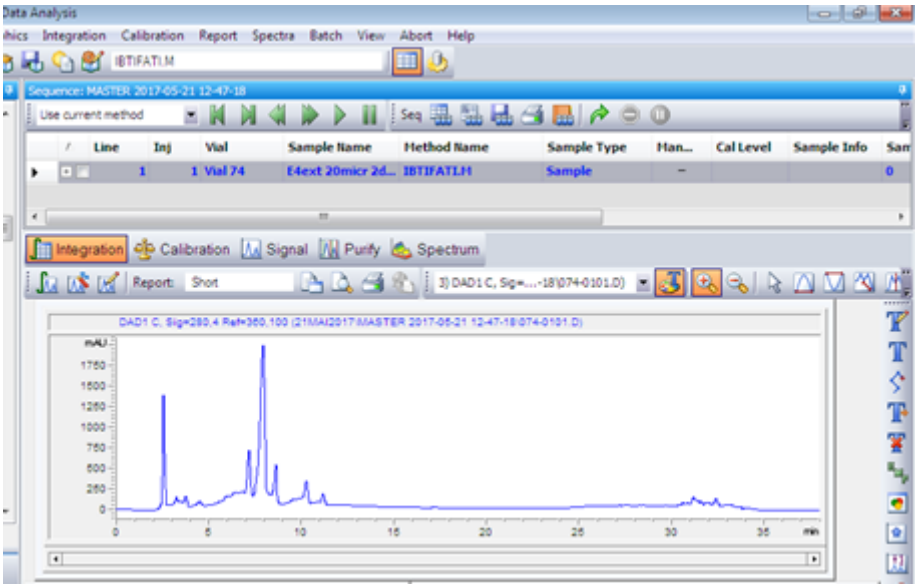
S3 (Water 85°C).



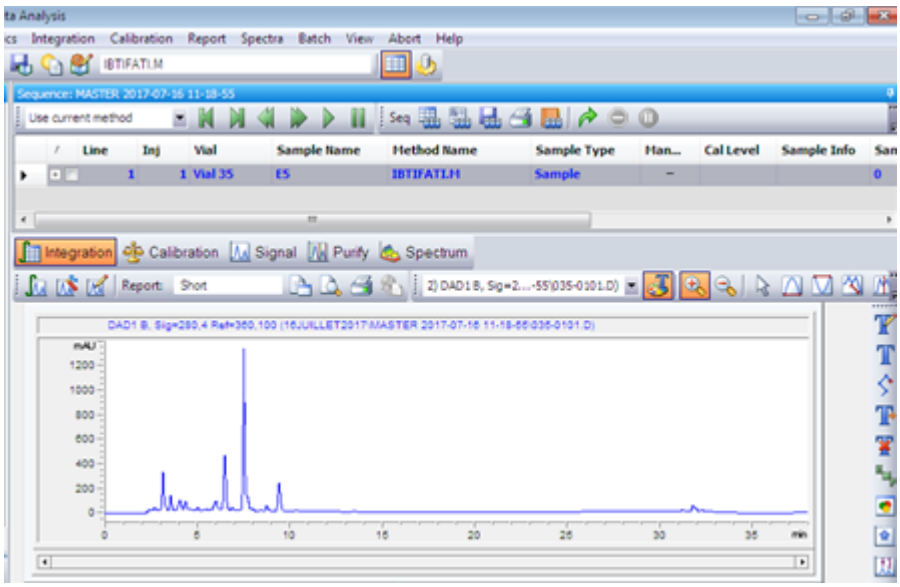
S3 (MeOH/Water).



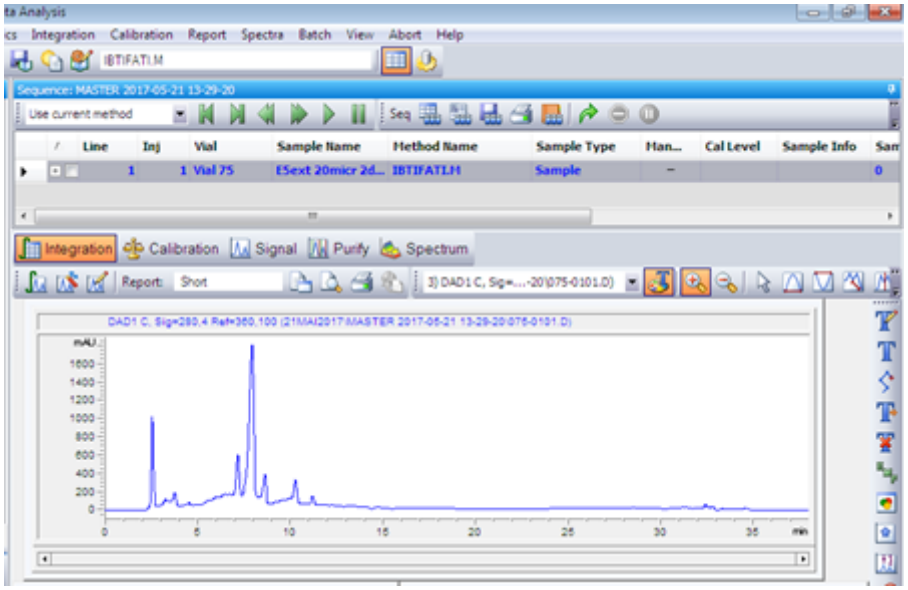
S4 (Water 85°C).



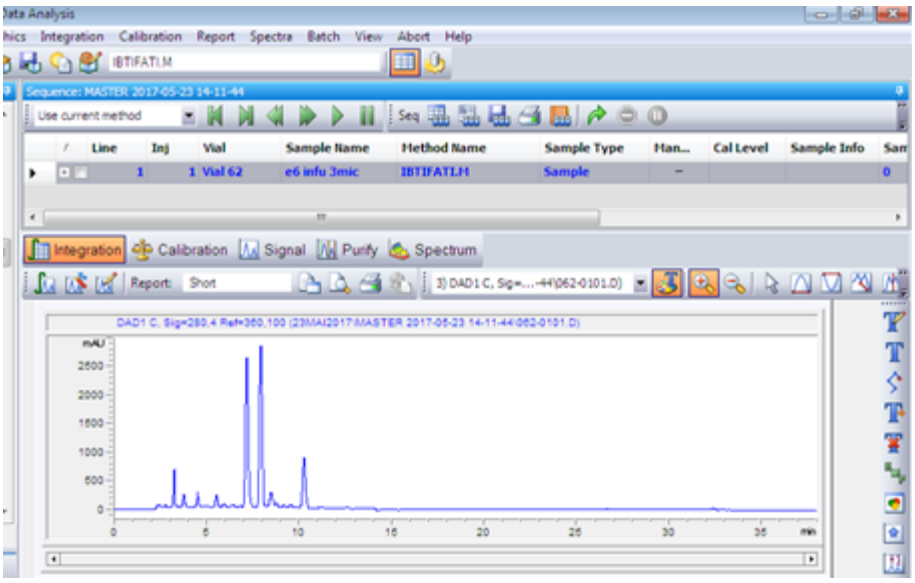
S4 (MeOH/Water).



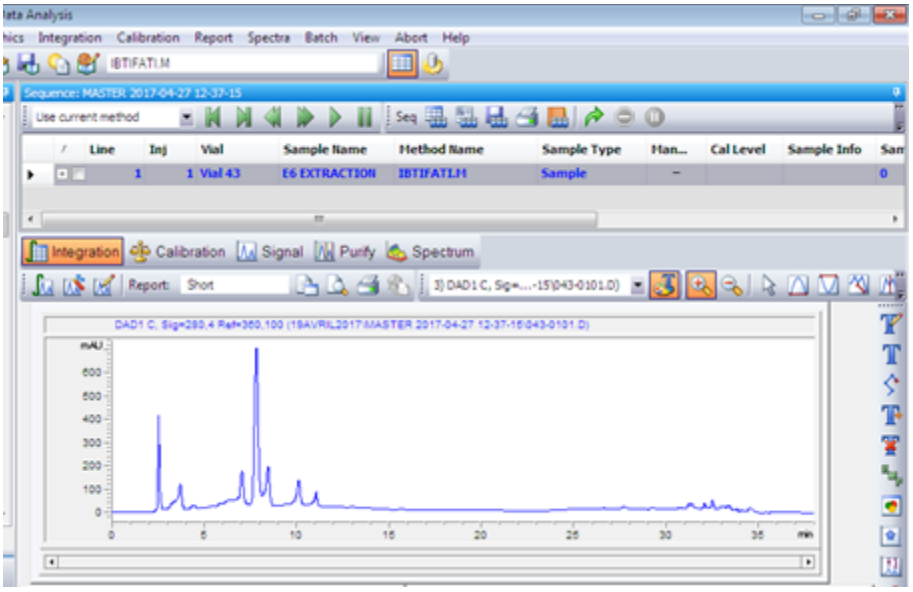
S5 (Water 85°C).



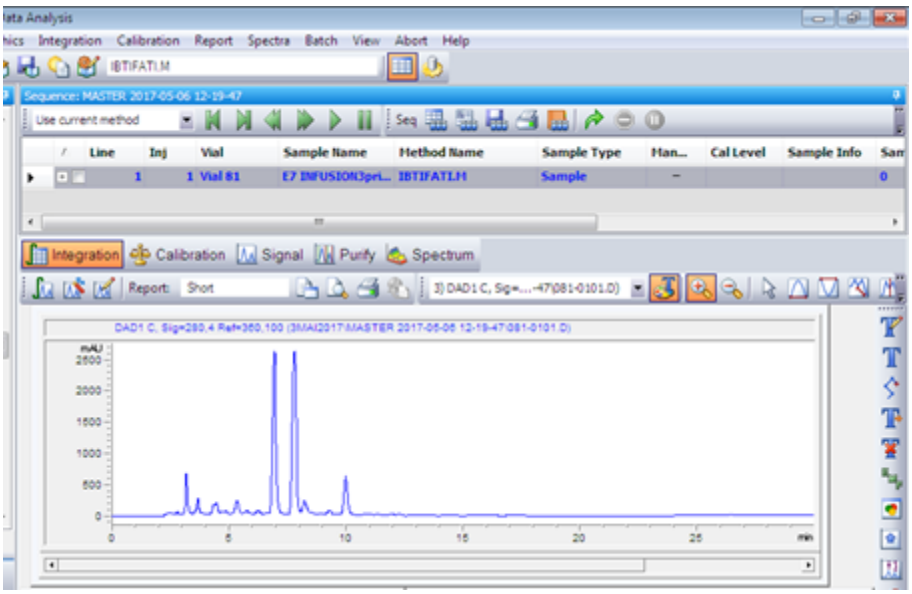
S5 (MeOH/Water).



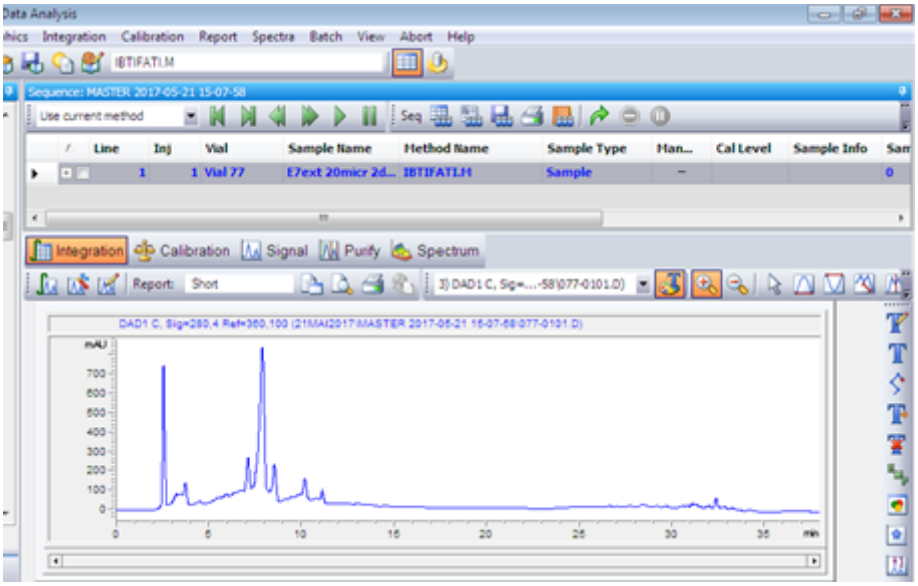
S6 (Water 85°C).



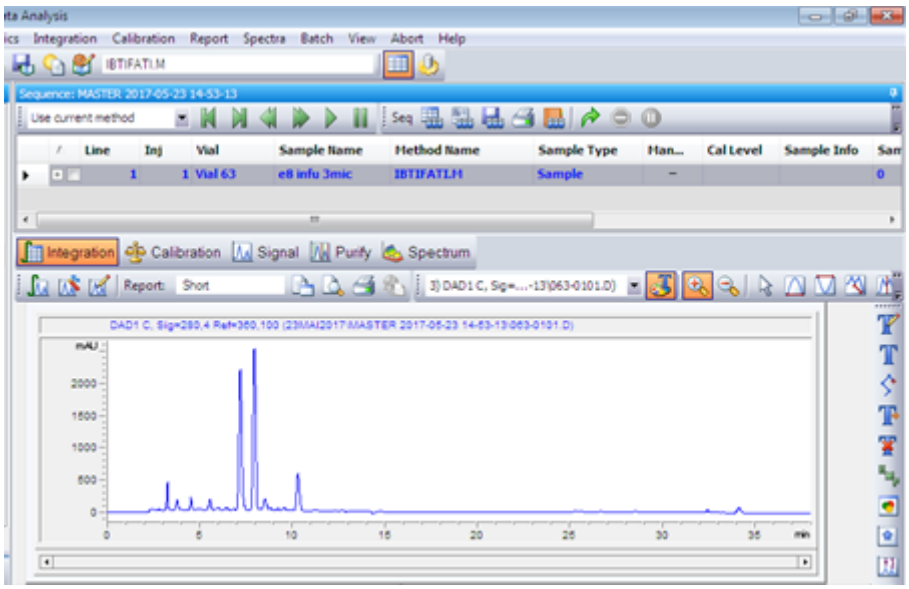
S6 (MeOH/Water).



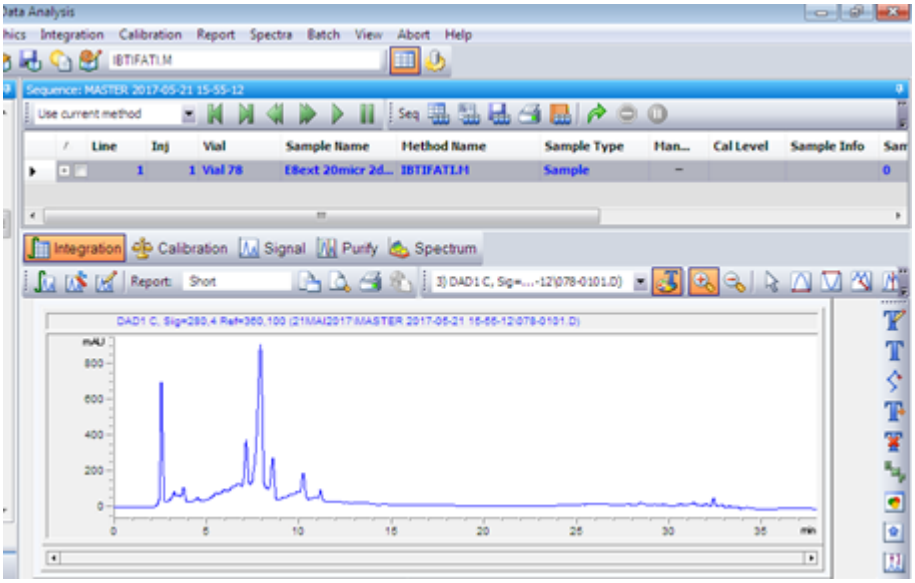
S7 (Water 85°C).



S7 (MeOH/Water).



S8 (Water 85°C).



S8 (MeOH/Water).

A 2 : HPLC data of pure compounds.

Epigallocatechin gallate

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	23390.7	23397.3	23410.2	23399.4	9.91
0.075	18809.3	18815.4	18812.2	18812.3	3.05
0.05	12800.6	12801.1	12807	12802.9	3.55
0.025	6457.2	6455.8	6471.5	6461.5	8.68

Epicatechin gallate

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	12981.6	12983.9	12988	12984.5	3.24
0.075	9855.1	9852.2	9848.4	9851.9	3.36
0.05	6620	6622.2	6623.5	6621.9	1.76
0.025	3311.6	3310.9	3310.8	3311.1	0.43

Epigallocatechin

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	1140.3	1139.1	1151.7	1143.7	6.95
0.075	813.2	818	819.5	816.9	3.29
0.05	570.4	575.1	573.5	573	2.38
0.025	268.2	267.8	264.7	266.9	1.91

Epicatechin

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	21008.8	21011.3	21011.7	21010.6	1.57
0.075	14890.9	14889.4	14895.7	14892	3.29
0.05	9960.7	9962.8	9960.4	9961.3	1.30
0.025	4963.5	4962.4	4973.6	4966.5	6.17

Gallic acid

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	26488.1	26475.9	26476.6	26480.2	6.85
0.075	19876.9	19868.2	19867	19870.7	5.40
0.05	13240.1	13238.6	13254.2	13244.3	8.60
0.025	6627.3	6620.2	6624.2	6623.9	3.55

Caffeine

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	57793.1	57780.4	57791.7	57788.4	6.96
0.075	43333.6	43320.1	43333.3	43329	7.70
0.05	28890.5	28888.4	28916	28898.3	15.36
0.025	14430.2	14424.5	14456.6	14437.1	17.12

Theophylline

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	13274.6	13308.1	13317.9	13300.2	22.70
0.075	9962.3	9965.8	9979.2	9969.1	8.92
0.05	6649.3	6652.1	6674.4	6658.6	13.75
0.025	3313.9	3325.2	3321.8	3320.3	5.79

Theobromine

Concentrations (mg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean value	Standard deviation
0.1	7863.9	7839.2	7840.9	7848	13.79
0.075	5892.8	5889.7	5898.9	5893.8	4.68
0.05	3926.9	3930.2	3918.2	3925.1	6.19
0.025	1959.7	1962	1955.9	1959.2	3.08