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Departamento de Química

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica do Doutor Filipe Manuel Coreta Gomes, Investigador Doutorado do Departamento de Química da Universidade de Aveiro e do Doutor Ramiro Daniel Carvalho de Almeida, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

palavras-chave Café, melanoidinas, ácidos clorogénicos, doenças neurodegenerativas, doença de Alzheimer, amiloide-beta

resumo As melanoidinas são compostos de alto peso molecular resultantes de reações de Maillard, características de alimentos termicamente processados, como o pão, a cerveja e o café. A formação de melanoidinas envolve açúcares livres, polissacarídeos, proteínas e compostos fenólicos, que são incorporados na sua estrutura. Os compostos fenólicos podem estar quimicamente ligados ou adsorvidos aos compostos de elevado peso molecular, podendo apresentar potencial antioxidante e neuroprotetor.

Neste trabalho, frações de alto peso molecular (melanoidinas) e baixo peso molecular (compostos fenólicos adsorvidos às melanoidinas) provenientes do café, foram extraídas, fracionadas, caracterizadas quimicamente e avaliadas relativamente ao seu efeito antioxidante e neuroprotetor. Esta análise revelou que as frações de alto peso molecular (melanoidinas) e baixo peso molecular (compostos fenólicos) apresentaram atividade antioxidante. Os compostos de baixo peso molecular mostraram diminuir a agregação de amilóide β, compatível com atividade neuroprotetora. Contudo, esta atividade não se verificou na prevenção da morte de neurónios após estímulo com oligómeros de amilóide β. Futuramente, será pertinente explorar outras matrizes alimentares ricas em melanoidinas, como pão e cerveja, ricas em compostos fenólicos, que fazem parte dos hábitos alimentares diários, avaliando o seu potencial neuroprotetor, assim como os seus mecanismos de ação.

keywords Coffee, melanoidins, chlorogenic acids, neurodegenerative diseases, alzheimer's disease, amyloid-beta

abstract Melanoidins are high molecular weight compounds resulting from Maillard reactions, characteristic of thermally processed foods such as bread, beer and coffee. The formation of melanoidins involves free sugars, polysaccharides, proteins and phenolic compounds, which are incorporated into their structure. Phenolic compounds may be chemically bound or adsorbed to high molecular weight compounds, which may have antioxidant and neuroprotective potential.

In this work, high molecular weight (melanoidins) and low molecular weight (phenolic compounds adsorbed to melanoidins) fractions from coffee were extracted, fractionated, chemically characterized and evaluated for their antioxidant and neuroprotective effects. This analysis revealed that the melanoidins and phenolic compounds fractions have good antioxidant activity. The low molecular weight compounds showed to decrease the aggregation of β-Amyloid, compatible with neuroprotective activity. However, this activity was not observed in the prevention of neurons death after stimulation with β-Amyloid oligomers. In the future, it will be pertinent to explore other melanoidin-rich food matrices, such as bread and beer, rich in phenolic compounds, which are part of daily eating habits, evaluating their neuroprotective potential as well as their mechanisms of action.

o júri

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Abbreviations

3,4-diCQA - 3,4-dicaffeoylquinic acid

3,5-diCQA - 3,5-dicaffeoylquinic acid

3-CQA - 3-caffeoylquinic acid

4,5-diCQA - 4,5-dicaffeoylquinic acid

4-CQA - 4-caffeoylquinic acid

5-CQA - 5-caffeoylquinic acid

AD - Alzheimer's disease

AGEs - Advanced glycation end products

AGPs - Arabinogalactan-proteins

APP - Amyloid precursor protein

ATR - Attenuated total reflectance accessory

BBB - Blood Brain Barrier

CGAs - Chlorogenic acids

CQAs - Caffeoylquinic acids

DAD - Photodiode array detector

diCQAs - diCaffeoylquinic acids

DMSO - Dimethyl sulfoxide

DPPH - 2,2-diphenyl-1-picrylhydrazyl

e.g. – *Exempli gratia* / For example

FID - Free induction decay

FQAs - Feruloylquinic acids

FTIR - Fourier-transform infrared spectroscopy

HMWM - High molecular weight material

HMWMppt - High molecular weight material precipitate

HMWMSn - High molecular weight material supernatant

HPLC - High performance liquid chromatography

LMWM - Low molecular weight material

MR - Maillard Reaction

NFTs - Neurofibrillary tangles

NMDA - N-methyl D-aspartate

NMR - Nuclear magnetic resonance

pCoQAs - p-Coumaroylquinic acids

PD - Parkinson’s disease

PDL - Poly-D-lysine

ROS - Reactive oxygen species

SPE - Solid phase extraction

ThT - Thioflavin T

TSP - Trimethylsilylpropanoic acid

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1. Introduction

Coffee is consumed every day by millions of people making it one of the most well-known beverages globally,[1] and it is estimated that per day, two billion cups are consumed.[2] Coffee and its constituents have shown to be beneficial to human health.[3] Due to their potential biological activity, other coffee compounds besides caffeine, such as polysaccharides, chlorogenic acids, trigonelline, melanoidins, among others have been intensively studied.[3] More than a thousand compounds with different properties and health effects are present in a coffee brew, turning this beverage into one of the most complex food matrices. The content of these compounds can be modulated by coffee species, geographic origin, roasting process and brewing methodologies.[2,4] This beverage can have an impact in several conditions such as type 2 diabetes, liver, cardiovascular and neurodegenerative diseases.[3] In this work, it will be given particular focus to the neuroprotective properties of coffee compounds, as well as their underlying antioxidant activity. Neurodegenerative diseases are not only a medical but also a social problem since the risk of developing this type of conditions increases with age. In fact, it is estimated that in 10 years about 75 million people will live with this type of disorders, with a tendency to continue increasing.[2] Thus it is important to actively study them and develop neuroprotective strategies. In this type of diseases, reactive oxygen species (ROS) is one of the main causes that leads to neuronal damage and neurodegeneration. Antioxidants regenerate oxidized biomolecules and scavenge oxidizing radicals.[5] Since coffee is known to contain antioxidant compounds, such as chlorogenic acids and melanoidins, by measuring their antioxidant potential it may be possible to find a solution to attenuate neurodegenerative diseases.

1.1. Coffee

The origins of coffee go back to Ethiopia, from where it spread to Yemen and then to the rest of the world. In Europe the first coffeehouses only opened in the 17th century.[1,3] Coffee is a plant that belongs to *Coffea* genus and comprises about 125 species. *Coffea arabica* species was classified in 1737 by Linnaeus.[1,3] This specie and *Coffea canephora* (robusta coffee) are both of commercial interest, showing distinct chemical composition of green beans.[1,6,7] The plant reaches maturity after three years of growth giving its fruit, the cherry. The fruit consists of two parts: pericarp and seed. Pericarp comprises the exocarp (skin),

mesocarp (pulp) and mucilage (pectin layer), while the seed includes the endocarp (parchment), the integument (silverskin) and endosperm (bean).[1,4] The coffee fruit structure is shown in Figure 1. Factors such as the amount of light, water availability, climate, soil type and topography affect coffee development.[1,6] Brazil, Vietnam, Colombia and Indonesia are its main producers, accounting for more than half of global production.[1,8]

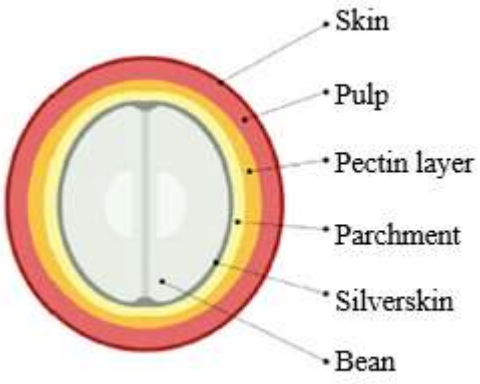


Figure 1: Representation of coffee fruit structure. Adapted from Giovanna *et al.*[4]

The cherry is converted to green bean and then to brown bean by coffee processing. This involves several stages, including harvesting, washing, drying and roasting, before coffee beans are ready to be commercially available.[4] After reaching its full maturity (five to seven years of growth) and, therefore, its full productivity, coffee fruit can be harvested.[1] Then two different approaches can be used, dry or wet processing, with the latter being more resource and time consuming.[1,7,9] The next phase is drying of beans.[9]

During roasting, beans undergo different phases of heating and are cooled to stop the chemical reactions that are occurring when the intended roasting degree is attained.[4,9] Numerous chemical reactions such as degradation and/or formation of coffee compounds, occur within this stage, contributing to coffee aroma, colour and flavor[6,10] Upon roasting, a decrease in carbohydrates, proteins and chlorogenic acids (CGAs) is observed when compared to green beans, whereas lipids, minerals, caffeine, aliphatic acids, trigonelline and free amino acids content is equivalent.[10] In fact, total phenolic content and CGAs levels diminished while roast degree increased. The differences were specifically significant for 5-caffeoylquinic acid, higher in robusta coffee in light and medium roasting and lower in dark roasting.[6] The characteristic brown colour of roasted coffee beans is due to melanoidins content, a compound formed through Maillard Reaction (MR).[9,10] This non-enzymatic

reaction happens in foods and beverages.[11] Additionally, during roasting, there is an expansion of the beans meaning they will increase in volume but decrease in mass, since the cell-wall matrix opens,[12] polymers are destructed[9] and water and volatile substances are lost.[13] Due to these changes, compounds become more extractable, affecting the composition in the beverage. Coffee extraction is an essential process, which plays a determinant role on compounds selectivity, hindering or facilitating the passage to the brew, and thus affecting its final composition. The compounds extracted can be divided into non-volatiles (*e.g.* carbohydrates, melanoidins, lipids, proteins, among others) and volatiles, being the last ones originated from transformation reactions of the non-volatile ones during coffee processing.[14,15]

1.1.1. Chemical composition of coffee

From an economical point of view, *C. arabica* and *C. canephora* are the most explored species of coffee, as mentioned before.[16,17] Even though they belong to the same genus, these two species present distinct characteristics. Brews from arabica beans have better quality than robusta ones, because they contain a higher amount of sucrose that makes the drink more sweet and smooth.[6] Due to their aroma qualities, coffees made from arabica beans represent 75 % of global production. However *C. canephora* has a higher resistance to plagues and a superior antioxidant capacity, given their distinct chemical composition.[16,17] In green beans, the content of caffeine and CGAs is higher on robusta. This tendency is also verified in lipid and trigonelline content. After roasting, and considering the same degree of roast for both coffees, a decrease in CGAs, polysaccharides and sucrose content can be observed, remaining slightly higher in *C. arabica*.[6,17,18] Nevertheless, there are other compounds that take part of coffee beans, such as minerals, like magnesium, calcium and sodium, amino acids, like lysine, glutamic acid and arginine and vitamins.[17] Coffee composition is dependent on coffee species, geographical origin and processing (roasting, grinding and brew preparation).[7,14] Table 1 summarizes the chemical composition of *C. arabica* and *C. canephora* beans and green and roasted beans.

Table 1: Composition (%) of green and roasted beans of *C.arabica* and *C. canhephora*. Adapted from Lopes.[14]

	Green beans		Roasted beans	
Compound	Arabica	Robusta	Arabica	Robusta
Polysaccharides	43.0 – 55.0	37.0 – 48.3	24.0 – 39.0	41.5
Lipids	12.0 – 18.0	8.0 – 13.0	14.5 – 20.0	11.0 – 16.0
Proteins	8.5 – 13.0	8.5 – 13.0	7.5 – 15.0	7.5 – 15.0
Chlorogenic acids	6.5 – 9.2	7.1 – 12.1	2.7	3.1
Sucrose	6.3 – 8.5	0.9 – 4.9	traces	traces
Minerals	3.0 – 4.2	3.6 – 4.8	3.5 – 4.5	4.6 – 5.6
Aliphatic acids	1.7 – 2.9	1.3 – 2.2	2.4	2.5
Caffeine	0.8 – 1.4	1.6 – 4.0	1.3	2.4
Trigonelline	0.6 – 1.2	0.3 – 0.9	0.5 – 1.0	0.3 – 0.7
Free Amino acids	0.2 – 0.8	0.2 – 0.8	-	-
Melanoidins	-	-	16.0 – 23.0	16.0 – 23.0

It is to note that of all the components present in coffee only caffeine is resistant to roasting, not being destroyed during this process. Proteins, CGAs, sugars, trigonelline and lipids undergo transformations, being preserved or potentially degraded.[17] In fact, merely 50 % of original green beans material remains chemically unaltered.[14] The roasting degree is evaluated through the colour acquired from beans and mainly classified into three types: light, medium or dark, depending on the temperature and time of roast.[6,16]

Even though coffee is constituted by a great variety of compounds, in this work the focus will be on melanoidins and compounds that are part of their structure, and their potential neuroprotective activity. Therefore, and due to their relevance, these will be the compounds described in greater detail in the following sections.

1.1.1.1. Melanoidins

Melanoidins are non-volatile, nitrogen containing, high molecular weight material (HMWM), conferring brown colour and aroma to thermally processed foods such as bread,

malt, beer and coffee.[3,6,19–21] The coffee beverage is a great source of melanoidins, where they take up to 25 % of roasted coffee dry matter.[3,20,22,23] These polymeric compounds are the final products of MR.[3,10,18] MR are composed of a network of reactions (Figure 2). Firstly, a carbonyl group from a carbohydrate/reducing sugar reacts with an amino group, from a protein, thus forming an unstable Schiff base.[6,10,11,24] Next, the Schiff base spontaneously rearranges forming the Amadori product.[6,11,24] Afterwards, and depending on the pH, the Amadori product is degraded, resulting in compounds like reductones and furfurals. Since sugars are fragmented, carbonyl and dicarbonyl compounds and amino acids will react to form Strecker aldehydes and aminoketones.[6,11,24] Aldehydes, reductones and furfurals will go through aldol condensation, without intervention of amino acids, followed by reaction with amino compounds and melanoidins formation. This represents the last step of MR.[6,11,24]

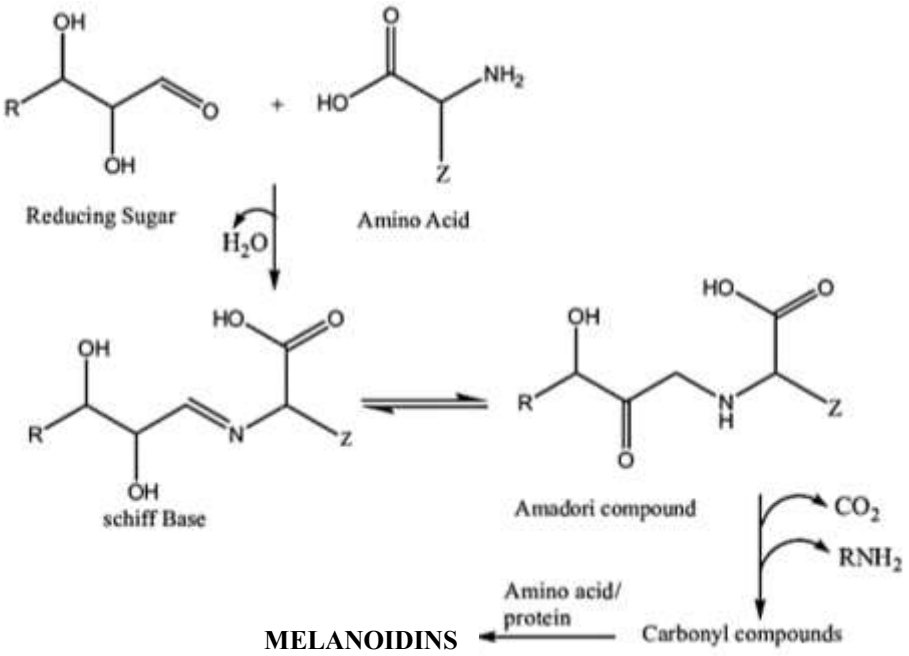


Figure 2: Main steps of Maillard reaction. Based on Augustine and Bent. [25]

Melanoidins are an important part of the human diet, being ingested up to 10 g daily.[26,27] In the case of coffee melanoidins, their amount is dependent not only on how coffee brew is prepared but also by the degree of roasting since the higher it is the more melanoidins will be present.[27] However their bioavailability and absorbability is very limited.[3,27] Since melanoidins are HMWM (>10 kDa) they will resist digestive enzymes and not be absorbed into the gastrointestinal tract, remaining intact and proceeding to the colon,

where they will become substrates for gut microbia.[3,26,27] In fact, an *in vitro* gastrointestinal digestion of coffee melanoidins demonstrated that 86 % were not digested.[3] Therefore melanoidins will not act as biologically active compounds in the bloodstream or organs but can act as carriers of other molecules, such as CGAs, that due to their lower molecular weight (354 Da) can be absorbed in the gastrointestinal tract.[27] When reaching the colon, microbes will ferment melanoidins, releasing some phenolic compounds previously linked to melanoidins backbone and thus increasing phenolics adsorption.[26]

Coffee melanoidins chemical structure is complex, diverse and dynamic since it changes with roasting degree.[10,19,28] Despite all efforts, its origin and structure are not fully known or understood.[10,18,21] Thus, three theories emerge for its formation. Firstly, it has been proposed that melanoidins are a result of polymerization of low molecular weight molecules (LMWM) from the MR, as pyrroles and furans.[10,18] Secondly, it has been suggested that melanoidins are formed through cross-linking of low molecular weight MR products with proteins reactive side chains of amino acids. Lastly, it is theorized that a polymerization through aldol-type condensation led to an establishment of sugar degradation products in the melanoidin structure.[10,18] Polysaccharides, proteins and phenolic compounds can be involved in the formation of melanoidins, being incorporated into their structure.[19,28,29] Coffee melanoidins structure and formation is shown in Figure 3. Due to their characteristics and the structural uncertainty, melanoidins are difficult to isolate and can only be analysed indirectly.[10] Melanoidins properties include antimicrobial, anti-inflammatory, antioxidant, prebiotic, antihypertensive and neuroprotective.[10,19]

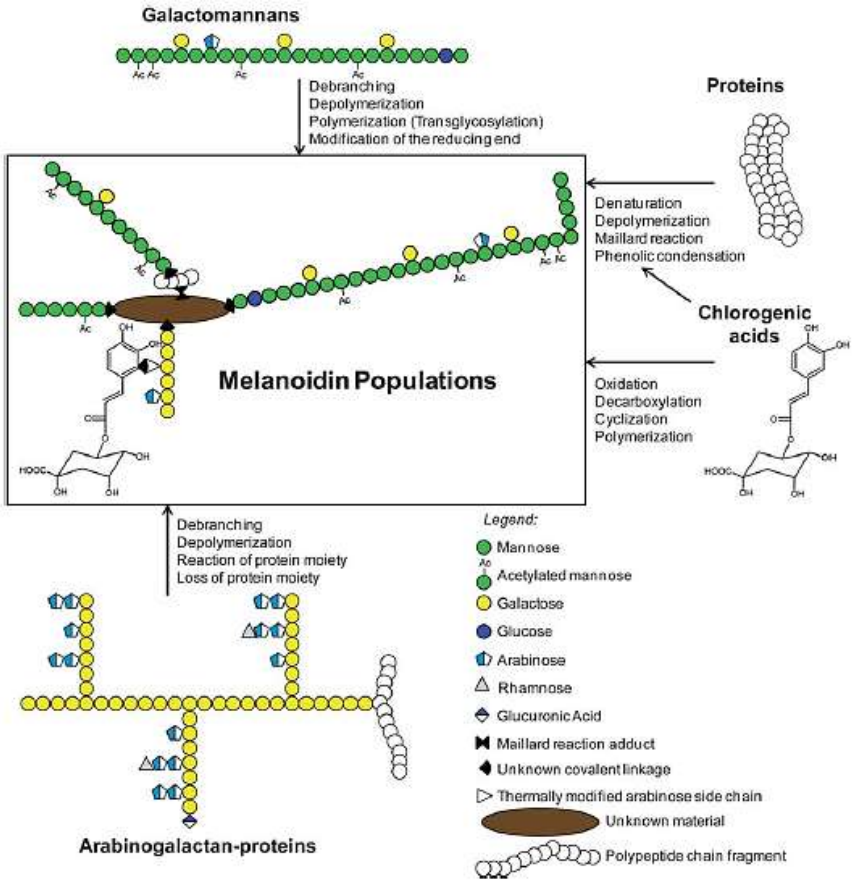


Figure 3: Coffee melanoidin structure and formation. Based on Moreira *et al.*[10]

1.1.1.2. Polysaccharides

Polysaccharides are polymers consisting of long chains of monosaccharide units linked by glycosidic bonds. Mannose, galactose, arabinose and glucose are some examples.[6] Representing about half of green beans weight, polysaccharides are the main carbohydrates found in coffee[10], being their content highly dependent on the roasting process and brewing method.[7] Coffee polysaccharide composition is of great importance for coffee taste, aroma and viscosity.[6,7,13] The two predominant polysaccharides present in coffee are galactomannans and type II arabinogalactans.[7,10,18] Other polymers such as cellulose are also present.[15] Galactomannans consist of a backbone of β-(1 → 4)-linked D-mannose residues, branching at *O*-6 with residues of D-galactose or L-arabinose (Figure 4).[10,12] As for type II arabinogalactans, they are constituted by a backbone of β-(1 → 3)-linked D-galactose residues, some of them substituted at *O*-6 with chains of β-(1 → 6)-linked D-galactose residues (Figure 4). These galactose residues are in turn substituted with terminally (α1 →

5)-linked arabinose, with rhamnose and glucuronic acid. [10,12] Moreover, arabinogalactans can also be covalently linked to proteins, so called arabinogalactan-proteins (AGPs).[10,19]
In green coffee beans arabinogalactans are the predominant polysaccharides extracted with hot water, 62 % against 24 % of galactomannans.[14,30] However, during roasting, these polysaccharides undergo several modifications on their degree of polymerization and branching, which will in turn affect their extractability and solubility in water.[8,9,17] Indeed, these polymers extractability increases from 7 % to 30 % when using roasted beans, with galactomannans predominating over arabinogalactans, due to their higher thermal stability.[14,30] Furthermore, upon roasting, transglycosylation reactions between galactomannans and side chains of arabinogalactans may also occur, originating polymerized products, which can be incorporated into melanoidins structure, and new types of glycosidic linkages.[18]

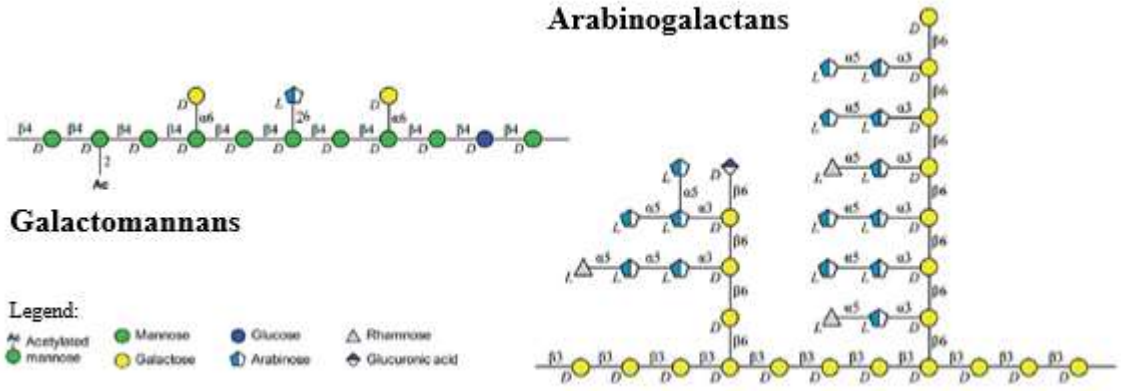


Figure 4: Representation of the structure of galactomannans and arabinogalactans. Based on *Moreira et al.*[9]

1.1.1.3. Proteins

In green coffee, proteins represent approximately 10 % of beans dry weight[10], being 50 % soluble in water and 50 % insoluble.[10,31] Around two thirds of the proteins are free in the cytoplasm, whereas the remaining are associated to arabinogalactans.[15] Accounting for 45 % of total protein content, 11 S-type storage protein is the leading protein of coffee beans.[10] In addition to proteins, peptides and free amino acids also contribute to the characteristic coffee flavor being precursors for volatile compounds formation (furans, aldehydes, pyridines, pyrazines and melanoidins) in the MR.[22,32] More particularly, in espresso coffee, proteins play a role in the formation of foam, enhancing its volume.[31] The main coffee proteins have molecular weights of 58 kDa and 38 kDa. After roasting, a

notorious reduction in total amino acid content can be observed due to protein denaturation and degradation (≤14 kDa), caused by the temperatures of which beans are submitted during this process.[10] However, since coffee does not present crucial amino acids it is not considered a good nutritional source of protein.[22]

1.1.1.4. Chlorogenic acids

Coffee represents a great source of CGAs.[6,22] These polyphenols result from the esterification of *trans*-cinnamic acids, such as caffeic, ferulic, and *p*-coumaric acids, with a quinic acid.[10,22,33] The esterification can occur in the carbons 3-, 4- and 5- of the quinic acid, with one or more cinnamic substituents, contributing to a great variety of compounds. Esters may also be formed with hydroxyl in position 1- but it is not usual.[22] With the possibility of several different combinations, depending on the nature and number of substituents, four subclasses of compounds can be obtained: caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), p-coumaroylquinic acids (pCo-QAs), and dicaffeoylquinic acids (diCQAs).[22,33,34] Caffeoylquinic acids are the most abundant class, with 5-caffeoylquinic acid (5-CQA) being the major CGA found in coffee.[3,6] Besides this CGA, 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA) are the ones found in greater quantity.[33] Some of the CGAs present in coffee are shown in Figure 5. Since these phenolic compounds are thermolabile, upon roasting they will suffer several transformations, including isomerization, epimerization, lactonization, degradation and incorporation into melanoidins through covalent or noncovalent bonds.[3,22,33] The CGAs adsorbed to HMWM are 3-CQA, 3-CoQA, 5-CQA, 3,4-diCQA and 3,5-diCQA.[35] If roasting is too harsh, up to 90 % of the chlorogenic acids initially present in coffee beans can be lost.[16] *C. canephora* has higher levels of chlorogenic acids when compared to *C. arabica*, contributing to the defences of the plant against insects, microorganisms and UV radiation.[6,22] These polyphenols provide bitterness, astringency and acidity to the coffee beverage.[22] Part of these compounds is absorbed in the small intestine, while the rest is metabolized in the large intestine.[3,4] Their consumption through coffee is associated with neuroprotective, antimicrobial and antioxidant properties.[3,4,6]

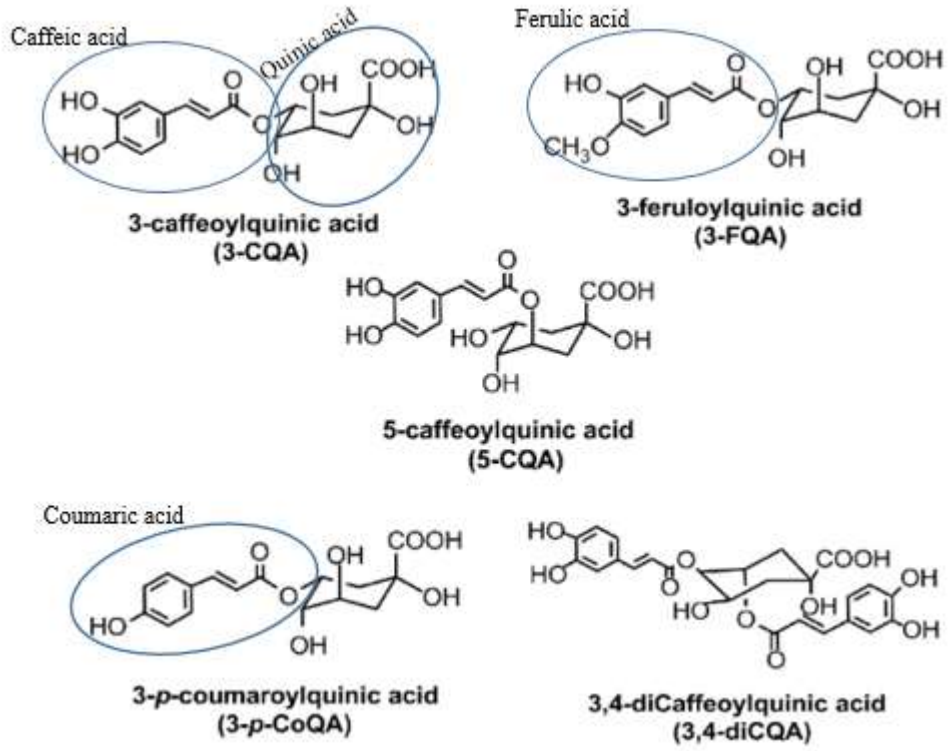


Figure 5: Structure of some chlorogenic acids present in coffee. The remaining ones, not shown, are formed by the esterification of carbons 4- or 5- of quinic acid with caffeic, ferulic or coumaric acid. Adapted from Liang and Kitts. [33]

1.1.2. Coffee and neurodegenerative diseases

1.1.2.1. Positive impact

Coffee has been linked to several positive impacts in human health, mainly due to the variety of bioactive compounds present in its composition. Anti-inflammatory, antioxidant, antidiabetic, cardiovascular and neuroprotective properties are among the several health properties associated to coffee consumption.[3] Some studies have shown the beneficial effects of moderate coffee intake in decreasing the risk of developing neurodegenerative diseases, as is the case of Wu *et al.*[36] meta-analysis of nine cohort studies, which in addition revealed that this relationship is dose dependent. The lower risk was associated with daily consumption of 1-2 cups of coffee.[36] After examination of brain autopsies, Gelber *et al.*[37] observed that the highest consumptions had the lower risk of developing neuropathological lesions. In the same study, midlife consumption and late life risk of neuropathology and cognitive impairment did not show significant associations.[37] Haller *et al.*[38] reported correlations between chronic coffee consumption and a beneficial cognitive performance

and between moderate to heavy intake of coffee and a higher cerebral blood-flow.[38] Finally, a study conducted by Ding *et al.*[39] showed that coffee consumption decreases the risk of dying from neurodegenerative diseases.[39] More specifically, coffee intake seemed to lower Parkinson's non motor symptoms,[40] lower levels of Aβ accumulation in Alzheimer's [41] and decrease the risk of amyotrophic lateral sclerosis.[42] These studies suggest that coffee is a preventive factor in neurodegenerative diseases but further research is still needed.[2]

The coffee compound that is most extensively studied towards neuroprotection is caffeine. Its neuroprotective effects are mainly related to its antagonistic activity at adenosine receptors present in the brain.[2] However, and when comparing neuroprotective effects between caffeinated and decaffeinated coffee, no differences were detected which suggests that other compounds besides caffeine, such as chlorogenic acids, may have a significant contribute on the positive effects of this beverage against neurodegenerative diseases.[43]

1.1.2.2. **Negative impact**

Despite the various benefits displayed in the previous topic, coffee consumption can be associated with not so positive properties to human health. A class of compounds that has been studied for its role in neurodegenerative diseases are advanced glycation end products (AGEs). Even tough not directly related with coffee, AGEs originate from Maillard reaction as a result of rearrangements of end-products.[44] AGEs can be classified according to their origin, either endogenous, being produced within the body in physiological or pathological circumstances, or exogenous, which includes AGEs from foods. Even though the biological function of both types of AGEs is analogous, there is a higher content of AGEs in exogenous sources comparatively to endogenous ones.[44,45] The chemical structures of some AGEs are represented in Figure 6.

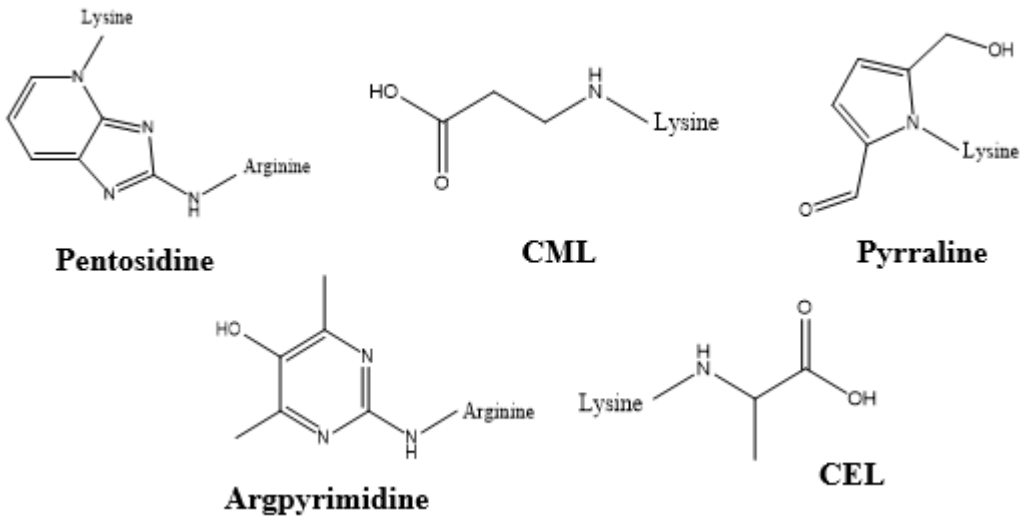


Figure 6: Chemical structures of AGEs. Abbreviations: CML: N-carboxymethyllysine and CEL: N-carboxyethil-lysine.

AGEs can be associated with neurodegeneration, since they promote inflammation and oxidative stress and are capable of producing ROS which leads to modifications in proteins function and structure, dysfunction at a cellular level and even apoptosis.[45,46] During aging these compounds progressively accumulate in the brain due to high oxidative stress but oxidative stress can also be a consequence of AGE formation.[45] In Alzheimer's disease (AD) the presence of AGEs contribute to the processing of the amyloid precursor protein (APP), increasing its formation and, therefore of amyloid β, and can also lead to the hyperphosphorylation of tau protein. Additionally, it can accelerate the cross-liking between Aβ and AGEs, which lessens the ability of microglia to clear the senile plaques.[44,45] In Parkinson's disease (PD), AGEs increase the aggregation of α-synuclein oligomers and the formation of Lewy bodies, the two hallmarks of this disease, and lead to dopamine neurons death.[44,45]

1.2. Neurodegenerative diseases

Neurodegenerative diseases, such as AD and PD, are characterized by deterioration of neurons over time, leading to loss of structure and function and, ultimately, cell death.[47,48] The deterioration of brain cells is related to the deposition of proteins that will change the physicochemical properties of the brain and peripheral organs.[49] Fused-in sarcoma protein, Aβ, α-synuclein, prion protein, TAR-DNA-binding protein 43 kDa and tau protein are the

most common proteins involved in the pathogenesis of such diseases.[49] AD and PD are disabling, slowly advancing, prolonged and the most frequent disorders in a group that also includes diseases such as dementia with Lewy bodies, amyotrophic lateral sclerosis, Huntington's disease and others.[2,48] The risk of these diseases is due to the combination of several factors.[47,48] Age is considered the biggest risk factor in neurodegenerative diseases due to the natural degradation of neurons over time. Other factors such as lifestyle, environmental conditions and genetics also influence the incidence of these disorders.[48] An accumulation of oxidative stress may lead to an acceleration of aging process and development of neurodegenerative diseases.[50] As these diseases affect approximately 17 million people in the world, it is vital to deeply explore their causes and develop new treatments and forms of prevention, as there is no cure available and treatments only alleviate the symptoms.[2,47,51]

1.2.1. Case of study: Alzheimer

1.2.1.1. Disease mechanism and current treatments

Alzheimer's disease is a very complex and multifactorial neurodegenerative disease,[48,52] characterized by the deposition of senile plaques of Aβ peptides extracellularly and by accumulation of neurofibrillary tangles (NFTs) of hyperphosphorylated microtubule-binding tau proteins intracellularly.[2,48,52,53] Age, environmental factors and/ or mutations are the factors leading to an incorrect processing of these proteins, becoming misfolded and acquiring changed conformations.[53] Since their conformation is altered, neurons will present anomalous functions and reduce in number causing a decrease in communication between brain cells and eventually their death.[2,53] Some of the lesions caused by Alzheimer's pathology are synaptic and neuronal loss and cerebral amyloid angiopathy.[48] AD can be classified into two types, as inherited, and therefore with a genetic origin, and sporadic, with an unknown basis but representing the majority of cases.[2] Patients with this disease experience progressive loss of cognitive and motor functions associated with language, memory and rational thought in consequence of neurodegeneration of the brain.[2,54] However it takes years of neuronal changes until symptoms become perceptible.[48,54] At a molecular level, changes start with altered cleavage of amyloid precursor protein, APP, located in the cell membrane.[53,54] APP, containing three domains, a N-terminal domain, Aβ domain and a C-terminal domain, can be processed by either a non-amyloidogenic or an amyloidogenic

pathway.[53,55] When cleaved by β-secretase and γ-secretase (amyloidogenic processing pathway), this protein, generates Aβ peptides that will form oligomers and diffuse to synaptic gaps affecting synaptic signalling (Figure 7).[53,54] However in non-amyloidogenic pathway APP is cleaved by α-secretase, that cuts inside the Aβ domain and, after the cleavage from γ-secretase, a peptide, p3, that is non-toxic gets released instead of Aβ, representing the key differences between both processing pathways.[53,55]

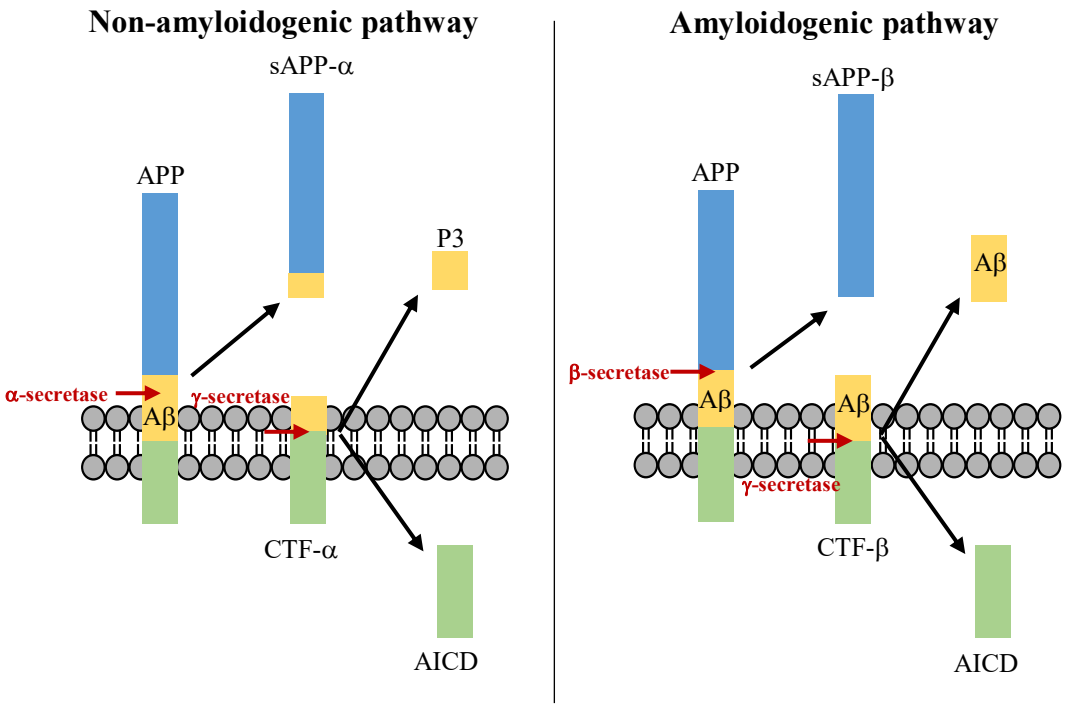


Figure 7: APP processing pathways.

The processed Aβ peptides will accumulate and polymerize into Aβ fibrils later aggregating to form Aβ plaques (Figure 8), a characteristic hallmark of AD.[53,54] The 40 amino acids (Aβ 40) and 42 amino acids (Aβ 42) peptides are the two most abundant species.[55] The latter aggregates more quickly and is therefore considered the largest component of senile plaques, in addition to being potentially more toxic.[54] It is usually used as *in vitro* model to evaluate the effect of potential neuroprotective compounds.

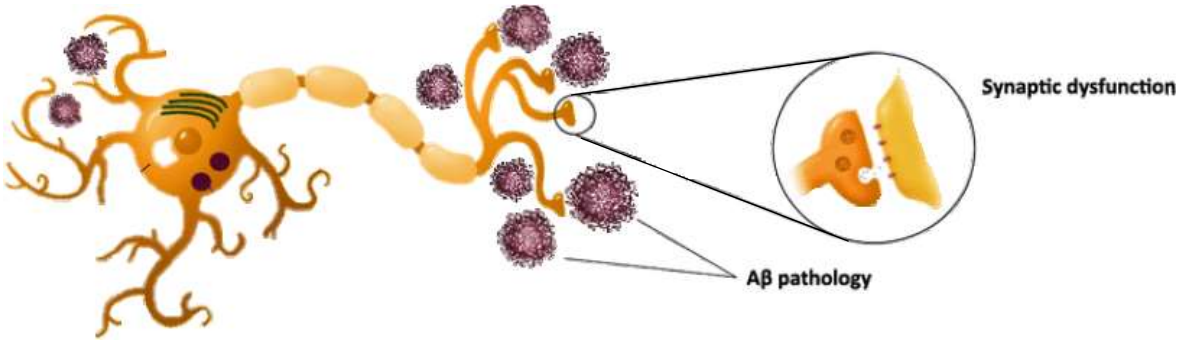


Figure 8: Schematization of the characteristic hallmarks of AD. Adapted from Milà-Alomà *et al.* [56]

Neurons are constituted by microtubules and, usually, tau protein is responsible for their stabilization, and organization. However, when tau becomes hyperphosphorylated, it loses its function, detaching from the tubulin assemblies. This will cause tau proteins to aggregate and polymerize into NFTs (Figure 9).[53,54] The formation of senile plaques and NFTs occurs in different parts of the brain, later spreading to the remaining regions.[54] Their presence promotes the appearance and activation of microglia that fails to eliminate Aβ plaques, ultimately contributing to neurotoxicity.[53] Oxidative stress can induce a cycle of pathogenesis since it can intensify the production and aggregation of Aβ and stimulate tau phosphorylation.[50]

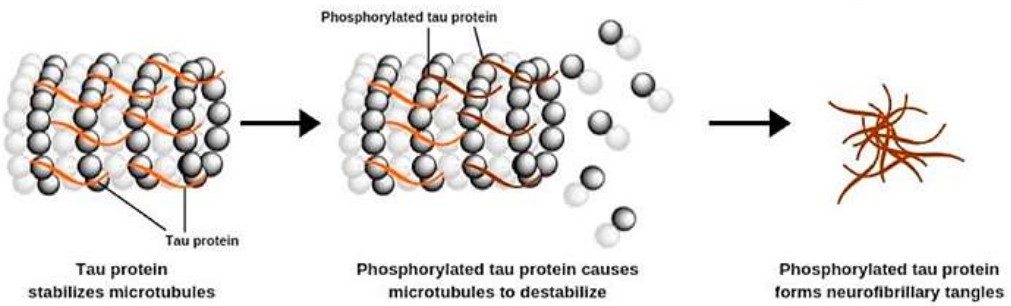


Figure 9: Formation of neurofibrillary tangles. Based on Grodzicki and Dziendzikowska.[57]

The treatments available for AD only lessen symptoms, namely the change in behaviour and loss of logical thinking and memory, not preventing the progress of the disease.[48,58,59] This phenomenon is due to the presence of a semipermeable blood brain barrier (BBB). Since BBB selectively transports molecules into the brain, an effective delivery of neurotherapeutic agents becomes difficult and larger doses of drugs will be needed to achieve a concentration positively affecting the brain.[53] Current treatments include cholinesterase inhibitors and N-methyl D-aspartate (NMDA) receptor antagonists.[58,59] Cholinesterase inhibitors (Rivastigmine, Donepezil and Galantamine) bind to

acetylcholinesterase preventing this enzyme to break acetylcholine. Thus, there will be more acetylcholine available in the brain, improving the transmission of signals between neurons and reducing memory loss.[59] However, with the development of the disease, less acetylcholine is produced by the brain and, therefore, the drug can lose its effect.[58] These inhibitors are used in mild to moderate symptoms.[58,59] NMDA receptor antagonists (*e.g.*, Memantine) help with cognitive and behavioural symptoms, reducing abnormal brain cell activity trough obstruction of electrical current over NMDA receptors, located on the surface of neurons.[59] Since the two categories of treatments have different mechanisms of action they can be prescribed in combination.[58] Neuroprotective therapies depend on the pathology of the illness and can be from three different sources, natural, synthetic or existing drugs.[48] Aducanumab is the only disease-modifying drug that has been approved for AD, being an Aβ directed antibody for the reduction of senile plaques.[58] Neuroprotective strategies should target the main causes of the disease by slowing or inhibiting the pathological cascade that leads to neuronal dysfunction and regenerate neurons functions and structure.[48]

Some of the major approaches being studied are disaggregation and inhibition of protein aggregates, stimulation of autophagy, immunomodulation and recombinant proteins. The later presents some disadvantages such as reduced membrane permeability and bioavailability and unpredicted side effects, blocking its pharmacological application.[48] The other therapies still remain in an experimental phase. Some issues may become evident when translating the studied dosages for human use, such as the need to use higher doses, inability to pass through biological membranes and complications in delivering therapeutic agents.[48] Continuous studies show that compounds like alkaloids, terpenoids and polyphenols, present in food sources, may have neuroprotective effects by slowing the pathogenetic cascade.[48]

1.2.1.2. Bioactive compounds with an effect on Alzheimer's

Besides factors like environment or age, a good nutrition rich in bioactive compounds is important to prevent the appearance of neurodegenerative diseases like AD.[57] Bioactive compounds are able to control metabolic processes and therefore improve health. Components like phenols, vitamins and carotenoids may decrease scavenging free radicals and inflammation and regulate cell signalling pathways.[60] In the class of phenolic compounds, genistein, resveratrol, oleuropein and curcumin are some of the molecules reported to have an effect on AD. Genistein (Figure 10) was found to inhibit synthesis of

ROS thus reducing oxidative stress in the brain. Moreover, it can diminish senile plaque formation and Aβ synthesis by acting as an α-secretase promotor and a β-secretase inhibitor.[57] Resveratrol (Figure 10) was shown to stimulate α-secretase activity, giving cells protection against Aβ-induced cell apoptosis and lowering accumulation of Aβ peptides.[60] Oleuropein (Figure 10) affected tau metabolism suggesting that it might inhibit formation of tau aggregates, toxic for cells. This mechanism is most likely due to existence of aldehyde groups in the tautomeric forms of its aglycone metabolite. Oleuropein was also able to reduce Aβ levels by decreasing the expression of an enzyme involved in Aβ synthesis.[57] Curcumin (Figure 10) is an antioxidant and therefore capable of reducing oxidative stress. Adding to this it was demonstrated to decrease microglia activity and inflammation and prevent Aβ-induced tau hyperphosphorylation.[57,60] Curcumin can inhibit Aβ aggregation *in vitro* by disrupting the β-sheet structure of aggregates and dissociate fibrillary Aβ40. Chemically this anti-aggregating activity is possible due to the two phenyl groups with polar substituents separated by 8-16 Å.[54] Since its chemical structure is similar to a substance used for senile plaques staining, the phenol Congo red can bind to Aβ and inhibit oligomers formation.[57] Reinke and Gestwicki [61] studied which characteristics of curcumin are responsible for this activity. It was demonstrated that a second aromatic group, the hydrogen-bonding properties and linker length were indispensable features for activity and that alternating or inhibiting only one of these caused a major activity loss.[61] Furthermore, the study found that simple aromatics do not exhibit any activity, losing the hydroxyl group on the aromatic rings eliminates activity and that there are a common prevalence of polar functional groups in other reported ligands.[61] Additionally, Malafaia *et al.*[54]demonstrated that, for example, changing the diketone moiety of curcumin to an isoxazole isostere, in curcumin-derived isoxazoles, improved the activity on Aβ secretion. Anti-aggregating activities of tau and Aβ were also intensified by replacing the aromatic ring with other cyclic moieties. In the vitamins class, vitamin D and E were shown to have some effects on AD. The first helped clear Aβ in the hippocampus trough phagocytosis.[57] Adding to this vitamin D presents anti-inflammatory activity and is able to regulate neurotransmitters.[57,60] The second is an antioxidant and so protects DNA and cellular membranes from free radicals.[57,60] Lastly, of carotenoids, lycopene, quercetin, astaxanthin and hesperidin showed antioxidant, anti-cytotoxic and anti-apoptotic properties.[57,60] Quercetin also reduced Aβ aggregation.[60]

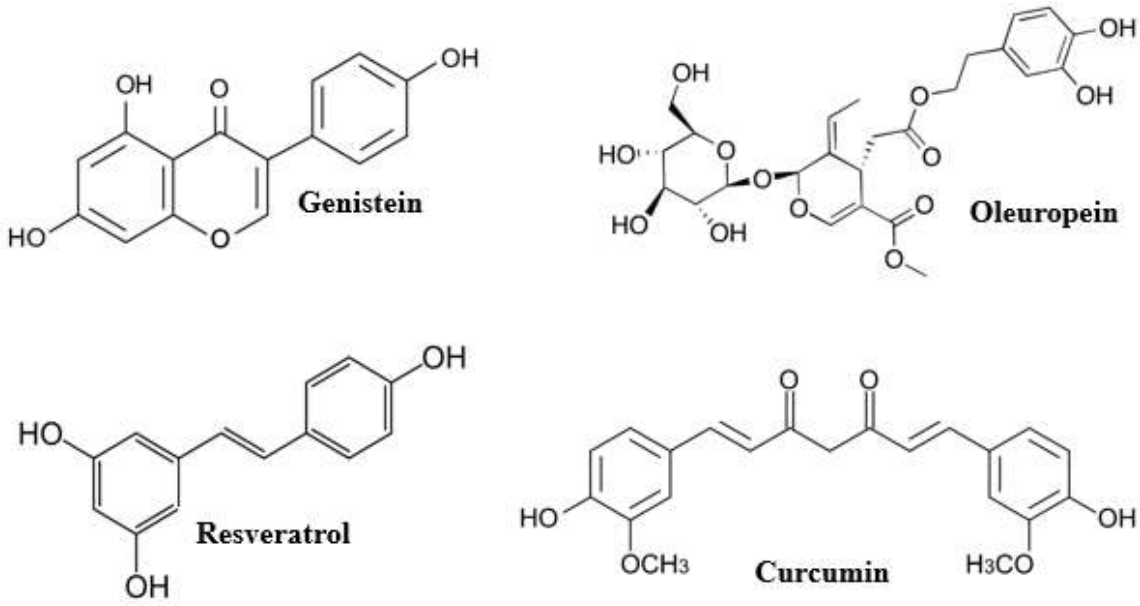


Figure 10: Structure of bioactive compounds with effect on AD. Based on Zhang et al.[62], Trollope et al.[63], Elama et al.[64] and Farazuddin et al.[65]

Comparing the structure of these phenolic compounds with the structure of CGAs presented above (Section 1.1.1.4. Figure 5) it is possible to infer that they may also have an effect on AD due to the presence of aromatic and hydroxyl groups, which have been identified as determinant molecular structure features for activity. As they have two aromatic groups, diCQAs might be the CGAs with the highest neuroprotective activity and, therefore, the most promising for the study on AD.

1.2.1.3. Known effects of melanoidins and chlorogenic acids on Alzheimer's

Coffee consumption seems to be related with a decreased risk in developing AD.[66] Melanoidins and CGAs are two of the compounds present in coffee that exhibit neuroprotective activity and thus are being studied for that purpose. Melanoidins were demonstrated to interact with Aβ 1–42, promoting cell survival in Aβ 1–42-induced toxicity.[67] Moreover melanoidins prevented oxidative stress induced death of neurons and revealed anti-fibrillation activity. Since melanoidins are Aβ oligomer ligands, they directly interact with the target, resulting in their ability to impede neurotoxicity and fibrillation, as mentioned.[67] However, studying melanoidins effect on neuronal cells might not be relevant, at least without gastrointestinal digestion, since as mentioned before, due to their size they

will not be absorbed in gastro-intestinal tract and therefore will not reach the brain and neither the intended target, the neurons. On the other hand, bioactive compounds such as phenolic compounds either adsorbed or covalently linked to melanoidins may have potential to target these tissues. The neuroprotective effects of CGAs against Aβ peptide have also been studied. In PC12 cells this compound have showed neuroprotection to Aβ 25–35 induced neuronal damage and suppression of Aβ 1–42 aggregation.[68–70] Furthermore, in hippocampal neurons and homogenized mouse brain CGAs inhibited acetylcholinesterase activity.[71,72] CGAs have also been reported to reduce neurons loss in the hippocampus and to increase memory in APP/PS2 transgenic mice. Saitou *et al.*[73] studied the consumption of a drink with CGA or a placebo, during 16 week, and its effect on cognitive functions. For this purpose, a sample of 38 healthy people with memory complaints was taken. Results showed an improvement on some functions such as attention, executive function and motor speed indicating that CGA regular ingestion might be beneficial. Another study that investigated the intake of CGA during 6 months also reported better cognitive functions (motor speed, mental flexibility, verbal memory and attention).[74] Lastly, and using a transgenic mouse model of AD it was showed that 5-CQA reduced accumulation of Aβ plaques by degrading them.[75]

1.3. Objective of this work

Melanoidins are high molecular weight compounds with importance on the organoleptic profile of coffee beverage, as well as contributing for soluble fibre. Moreover, they have been related with improvement of key physiological functions promoted by coffee consumption as well as other food sources. Melanoidins also known to have in their composition low molecular weight compounds, such as phenolic compounds, which can be absorbed and/or covalently linked. These compounds are known to have antioxidant and neuroprotective activity. In this work, coffee with different roasting stages and its resulting high and low molecular weight fractions were extracted and chemically characterized, to unveil their possible antioxidant and neuroprotective effects. The most pertinent samples, will be tested regarding their effect on Aβ aggregation peptide behaviour and on the neuron viability after its stimulation with Aβ oligomers.

2. Materials and Methods

2.1. Coffee samples

Coffee beans (Arabica Brasil) used for the experiments were submitted to three different roasting degrees, light (222.8 ºC, 16 min), medium (227.9 ºC, 17.55 min) and dark (233.1 ºC, 18.14 min). To grind the roasted coffee beans, a coffee grinder was used and set for grinding level 1 (fine). The extraction of the expresso coffees (40 mL per coffee) was performed with distilled water, from 6.0 g of grounded coffee, using a tamper of 51 mm to pressure the coffee powder into the coffee portafilter basket, in a coffee machine model (Flama 10) operating at 15 bar.

2.2. Separation/Fractionation of coffee compounds

The following schematics (Figure 11) represents, briefly, the experimental work carried out in order to meet the objectives proposed above.

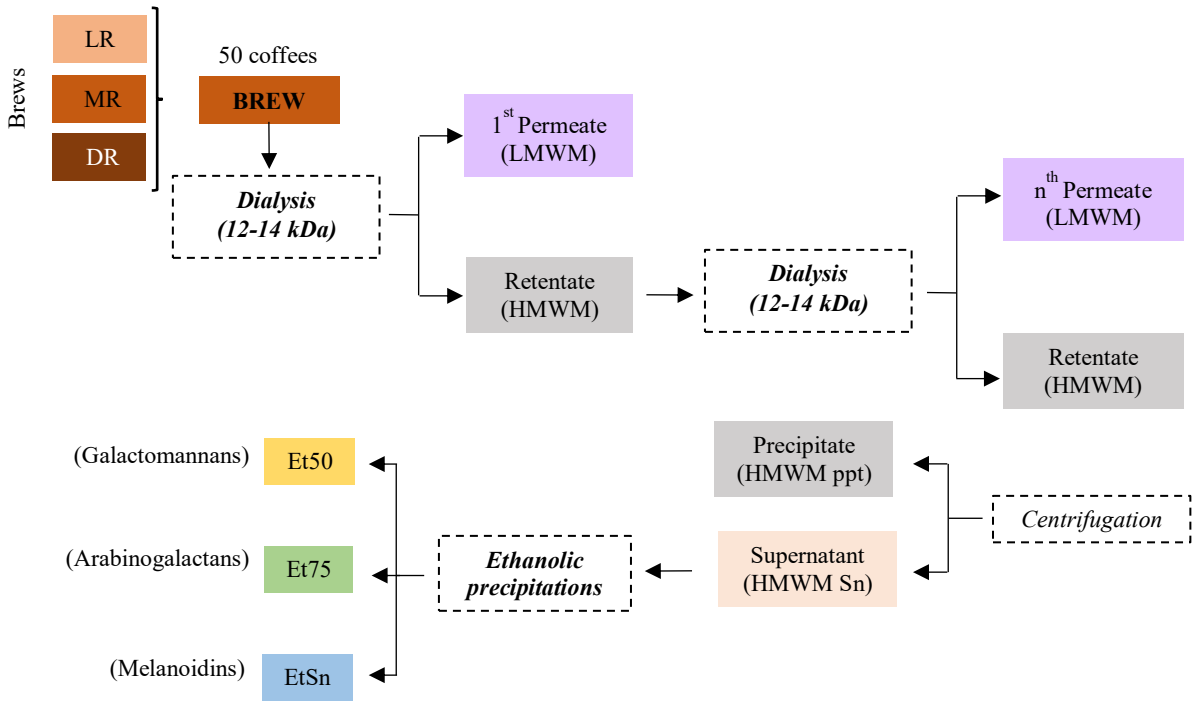


Figure 11: Schematic representation of the separation/fractionation methodology used to obtain coffee HMW and LMW compounds.

2.2.1. Dialysis

The coffee sample, corresponding to 50 coffees, was dialyzed (12-14 kDa cut-off) in distilled water with continuous stirring at 4°C. The first water change took place approximately 6 hours after the start of the dialysis. This permeate, which constitutes the LMWM, was collected, concentrated, frozen and then lyophilized. The same procedure was followed for the first four waters. The rest were discarded. During the dialysis process, approximately 13 water renewals were performed, at the end of which the water was colorless and its conductivity was close to that of distilled water. The content of the dialysis membranes, the retentate, which corresponds to the HMWM, was collected and concentrated to 200 mL. Then this material was centrifuged (1°C, 25 min, 15 000 rpm). The resulting precipitate (HMWMppt) was frozen and lyophilized during several hours and the supernatant (HMWMSn) was further fractionated by ethanolic precipitations.

2.2.2. Ethanolic precipitations

The volume of HMWMSn resulting from the previous procedure was submitted to sequential ethanolic precipitations. First, 50 % ethanol was added to the existing volume. The solution was mixed and then allowed to stand for 1h30 at 4°C for the precipitation to occur. After this period, the solution was centrifuged (4°C, 25 min, 15 000 rpm) and the residue named Et50 was resuspended in water, concentrated by rotary evaporation at 40 ºC to remove ethanol completely, frozen and lyophilized. This fraction is mainly rich in galactomannans. Sequentially, a volume of 75 % ethanol was added to the supernatant and left again for 1h30 at 4°C, then, it was further centrifuged (1°C, 25 min, 15 000 rpm). The same procedure was performed for the resulting precipitate, named Et75. This fraction is mainly rich in arabinogalactans. The supernatant, called EtSn, is the fraction soluble in 75 % of ethanol and corresponds to the melanoidins.

2.3. Characterization of extracts

2.3.1. Neutral sugar analysis

The amount of individual sugars present in the coffee brew, LMWM, and Et50, Et75 and EtSn fractions was evaluated through neutral sugar analysis. This procedure consisted in three stages: hydrolysis of glycosidic linkages, reduction of aldehyde group to alcohol and acetylation of alcohol groups. In the hydrolysis, to the samples previously freeze-dried (1-2

mg) were added 72 % H_2SO_4 and incubated for 3 h at room temperature with stirring, followed by 1h incubation at 120°C with final concentration 2 M H_2SO_4. As internal standard 2-deoxyglucose (1 mg.mL^{-1}) was used and for neutralization a 25 % NH_3 solution was added. The samples were reduced with 15 % (m/v) $NaBH_4$ in 3 M NH_3 during 1 h at 30°C and added acetic acid. Then they were acetylated with 1-methylimidazole and acetic anhydride (30 min, 30°C). The extraction of the alditol acetates was achieved by two washes with distillate water and dichloromethane (centrifugation for 30 s at 3000 rpm) followed by two washes with distillate water (centrifugation for 30 s at 3000 rpm). After evaporation the alditol acetates present in the samples were dissolved in anhydrous acetone and injected in the GC-FID with a DB-225 capillary column.

2.3.2. Fractionation of LMW compounds

The LMWM corresponding to the second water of dialysis was fractionated by solid phase extraction (SPE) using a C18 cartridge. SPE is a liquid-solid chromatography that uses solid particles as stationary phase to chemically separate different components of a sample, while passing a solvent (mobile phase). Despite following liquid chromatography principles, SPE is a technique used for the preparation of a sample to be submitted for analytical testing.[76] This procedure consisted in four stages: conditioning, loading, washing and eluting. Before usage, the column was conditioned with 50 mL of ethanol, 50 mL of distillate water and 50 mL of acidic water, which contained 2 % of acetic acid (pH = 3). The sample was loaded (200 mg/mL) and washed with 100 mL of acidic water. The rest of the material was obtained through washing with the following solutions, 100 mL of 95/5 water/methanol (fractions SPE 3 and 4), 100 mL of 80/20 water/methanol (fractions SPE 5 and 6), 100 mL of 50/50 water/methanol (fractions SPE 7 and 8) and finally 100 mL of 100 % methanol (fractions SPE 9 and 10). The collected fractions were further evaporated and analyzed by HPLC and NMR.

2.3.3. Chlorogenic acid (5-CQA) analysis

Approximately 2 mg of lyophilized LMWM was dissolved in 1 mL of ultrapure water. The solution was filtered through a 0.22 μm filter and then 0.05 mL of the filtrate was injected into high performance liquid chromatography (HPLC). This technique is based on the separation of components of a sample through the differences in the speed of which they

pass through a separation column, facilitating their quantitative analysis. HPLC is characterized by a mobile phase, the solvent used to separate the compounds of the sample, and a stationary phase corresponding to the column that separates the components. The fastest compounds to be eluted have the stronger affinity to the mobile phase.[77] For this purpose, a HPLC with a photodiode array detector (DAD) equipped with C18 column (Waters Sherisorb S10 ODS2, 4.6 mm x 250 mm, 10 μm) was used. The eluent A was formic acid 5 % and eluent B was methanol. The elution occurred at a flow rate of 0.8 mL min^{-1}. In the first minutes of elution the gradient was composed of 90 % eluent A and 10 % eluent B. The 5-CQA peak was detected at 325 nm. To quantify the samples, a calibration curve was prepared (R^2 = 0.982).

2.3.4. NMR analysis

LMWM fractions obtained by dialysis (1st, 2nd and 3rd waters) and from fractionation by SPE (fractions 2, 4 and 6) were characterized by ^{1}H nuclear magnetic resonance (NMR). This is a highly accurate, reproducible and non-destructive technique that uses the magnetic properties of atomic nuclei of liquid samples to analyse their chemical structure. Based on the principle that nuclei with nuclear spin different from zero generate a magnetic field, this technique can be characterized by three main steps, the alignment of the nuclear spins when applied a constant external magnetic field, its perturbation by irradiation in the radiofrequency region and the detection and analysis of the emitted signal.[78] When the sample to be tested is inserted into the NMR spectrometer, the spins of its atoms will adopt a lower energy configuration and, therefore, will be positioned parallel to the external magnetic field. Then, an external magnetic field is applied in the form of a radiofrequency pulse inducing a transition of the nucleus to a higher level of energy, altering the spin. When returning to its lowest energy level, the nuclei emit a signal named as free induction decay (FID). Finally, FID is converted into a NMR spectrum by a Fourier transform.[78] ^{1}H NMR spectra were obtained using a 500 MHz Bruker spectrometer, operating at room temperature, using a 90° pulse angle sequence, a spectral width of 7500 Hz, acquisition time of 1.0 s, a relaxation delay of 5 s and 128 acquisition scans. Spectra were processed with MestreNova 6.1.1 (Mestrelab Research, Santiago de Compostela, Spain). Assignments were made by comparing chemical shifts, coupling constants and coupling behavior of neighboring atoms, with available literature. Quantification was done using as internal standard

trimethylsilylpropanoic acid (TSP) at a known concentration, typically 0.5 mM, and using deuterated water (D_2O) as solvent for deuterium lock purposes.

2.3.5 FTIR analysis

To further characterize the fractionated samples, light and dark roasted brews, LMW, SPE 4 and SPE 6 fractions were submitted to Fourier-transform infrared spectroscopy (FTIR) analysis. FTIR is a non-destructive technique that uses infrared radiation to identify the molecular structure of analysed compounds and the mathematical principal of the Fourier transform to convert data into spectra.[79] Samples analysis was performed in an infrared spectrometer with the wavenumber 4000 – 600 cm^{-1}. For the measurement of absorbance, the previously freeze-dried samples were placed covering the crystal of the attenuated total reflectance accessory (ATR). After each measurement ATR was cleaned with water followed by ethanol at 70 %. Each sample had five replicates.

2.4. Properties of extracts

2.4.1. Antioxidant activity

The antioxidant activity of the coffee brews, LMWM and Et50, Et75 and EtSn fractions was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. At room temperature, DPPH is a free radical that presents a purple colour in solution. When in the presence of an antioxidant, DPPH is reduced, changing its colour to yellow. That change is measured in a spectrometer at 517 nm.[80] Different concentrations of the samples (1; 0.75; 0.5; 0.4; 0.25; 0.1; 0.05; 0.01) were prepared for this method. In a 96-well microplate were added 50 µL of sample and 250 µL of DPPH solution (8.6 ×10-5 M prepared in ethanol). The microplate was kept in the dark for 30 min followed by the measurement of absorbance at 517 nm. To assess the antioxidant activity the percentage of inhibition was calculated through Equation 1.[14]

Equation 1

$$\%\ inhibiton = \frac{A_0 - (A - A_b)}{A_0} \times 100$$

Where A_0 represents the absorbance of the control (DPPH and distillate water), A the absorbance of the sample (DPPH and sample) and A_b the absorbance of the blank of the sample (sample and ethanol).

2.4.2. Light scattering assay

Aβ 1-42 was dissolved in dimethyl sulfoxide (DMSO) to obtain a 250 μM stock solution. This solution was further dissolved in buffer Tris-HCl 10 mM, pH 7.4, 150 mM NaCl and buffer PB 50 mM, NaOH 10 mM, H_2O (2:1:1) to make a solution of Aβ 2.5 μM in 1 % DMSO. Samples of Aβ dissolved in each buffer and buffer alone were added into a quartz 96-well microplate and a spectral scanning (200-700 nm, step 5 nm, temperature 37ºC) was measured, at different time frames and with and without prolonged heating in a 37ºC water bath.

2.4.3. SEM analysis

For better characterization of the tested samples a scanning electron microscope (SEM) assay was performed, since it produces high resolution images of the surface of the sample by using electrons for imaging.[81] Samples to be tested included buffer phosphate and Tris-HCl, with or without Aβ 1-42, chlorogenic acid in the presence of Aβ 1-42 and coffee with two different degrees of roasting, light and dark, also with Aβ 1-42. The resulting images were obtained at diverse resolutions: 50.0 μm, 30.0 μm, 10.0 μm and 5.00 μm.

2.4.4. Thioflavin assay

Thioflavin T (ThT) is a dye frequently used to track Aβ fibril formation because it binds to β sheet structures in amyloid aggregates, altering the fluorescence intensity.[82] In this fluorescence assay, ThT was excited at 410 nm, producing a fluorescence signal at 485nm. Aβ 1-42 (10 μM, 2% DMSO) affinity to different buffers, such as PB (50 mM), HEPES (50 mM) and Tris (50 mM), was tested by adding 5 μL of Aβ (503 μM), 25 μL of ThT (50 μM; final concentration 7.5 μM) and 220 μL of the buffer to be tested, with or without NaCl (150 mM), in a 96-well microplate The microplate was kept at 37 °C during 20 h, while fluorescence was measured every 15 min. Then, Aβ 1-42 (503 μM, 2 % DMSO) was dissolved in buffer PB (50 mM) (selected from previous test) and different coffee

samples obtained from previous methods, coffee (62.5 µg/mL), LMW fractions (62.5 µg/mL) and fractions SPE 4 and 6 (62.5 µg/mL). Experiences with CGA (62.5 µg/mL) and caffeine (62.5 µg/mL) were also made. Samples were incubated at 37°C in a 96-well microplate during 15 h. Fluorescence intensity was verified every 15 min. The system used to add the samples to the microplate is presented in Table 2. Each sample had two or three replicates.

Table 2: Schematization of the volumes added to each well.

Solvent to be added / Description of samples	Buffer PB (50 mM)	Buffer PB (67 mM)	ThT (125 µM)	Aβ 1-42 (503 µM)	DMSO	Sample
Aβ 1-42	184 µL	–	12 µL	4 µL	–	–
Aβ 1-42 control	184 µL	–	12 µL	–	4 µL	–
Coffee samples (coffee, LMW, SPE 4 and 6, CGA, caffeine)	–	140 µL	12 µL	4 µL	–	48 µL
Coffee samples control (without Aβ)	–	140 µL	12 µL	–	4 µL	48 µL
Coffee samples control (without ThT)	16 µL	140 µL	–	–	4 µL	48 µL
Buffer PB	184 µL	–	12 µL	–	4 µL	–
Buffer PB control	196 µL	–	–	–	4 µL	–
ThT	188 µL	–	12 µL	–	4 µL	–

2.4.5. Neurotoxicity assays

To assess the effect of CGAs and LMW on cells we performed resazurin (for more detail read section 2.4.5.1.) and Hoechst (for more detail read section 2.4.5.2.) assays. Primary rat cortical cultures were prepared has described previously[83] and cells maintained in an incubator (at 37 °C) until DIV-10, then stimulated with the desired compounds and the microplate was returned to the incubator.

2.4.5.1. Resazurin assay

At DIV-13 the Resazurin assay was performed. This compound measures the metabolic activity of cells based on the conversion of resazurin to resofurin. This reduction reaction can be measured by either absorbance or fluorescence.[84] The media in each well of the 96-well microplate was aspirated and replaced with 100 μL of fresh medium containing 5 μg/mL of resazurin. Two additional samples were prepared, the control with just neuronal cells and the blank containing only resazurin. After a 4 h incubation fluorescence was measured (excitation at 560 nm and emission at 590 nm) in a Tecan M200 plate reader.

2.4.5.2. Hoechst assay

Hoechst 33342 is a cell permeable dye that binds to DNA, specifically rich adenine-thymine (A-T) regions of DNA, and is used to label the nucleus of cells.[83] At DIV-13 cells were fixed with 4 % PFA for 10 min. Next the wells were washed three times with PBS (200 μL, 3 min per wash) and incubated with 200 μL fluorescent dye Hoechst 33342 (diluted 1:1000 in PBS). The microplate was kept 10 min protected from light. Afterwards was washed two times with PBS. PBS from last wash remained in the microplate. Cells in the microplate were imaging by microscopy (Olympus IX2-UCB). Cell count was performed using the ImageJ software.

3. Results and Discussion

3.1. Separation of coffee compounds: extraction yields

The extraction of coffee compounds (Table 3) into high molecular weight (HMW) and low molecular weight (LMW) material, in either one of the roasting degrees, resulted in similar yields (around 20 % w/w per cup for HMW and 80 % w/w per cup for LMW). After fractionation of HMWM, the yield of the obtained fractions (HMWMppt and HMWMSn) was different for the three roasting degrees. There was higher recuperation of HMWMppt fraction in medium roasted coffee (15 % w/w per cup), while the highest recovered percentage of HMWMSn fraction was observed in light roasted coffee (17 % w/w per cup). In the dark roasted coffee the extraction yield of HMWMppt and HMWMSn fractions was similar, 11 % w/w per cup. The fractions that resulted from the ethanolic precipitation of HMWMSn, (Et50, Et75, EtSn) also presented similar extraction yields across all roasting degrees.

Table 3: Extraction yields of the separation of coffee compounds.

		Yield (% w/w per cup)	Yield (% w/w HMWM total)	Yield (% w/w HMWM Sn)
Light roast	HMWM $_{total}$	24.83		
	LMWM	75.17		
	HMWM $_{ppt}$	8.02	32.31	
	HMWM $_{Sn}$	16.81	67.69	
	Et50	0.04		22.60
	Et75	0.03		18.40
	EtSn	0.03		16.36
Medium roast	HMWM $_{total}$	17.47		
	LMWM	82.53		
	HMWM $_{ppt}$	14.83	84.92	
	HMWM $_{Sn}$	8.94	51.16	
	Et50	0.02		20.00
	Et75	0.04		43.25
	EtSn	0.03		32.41
Dark roast	HMWM $_{total}$	21.91		
	LMWM	78.09		
	HMWM $_{ppt}$	11.30	51.59	

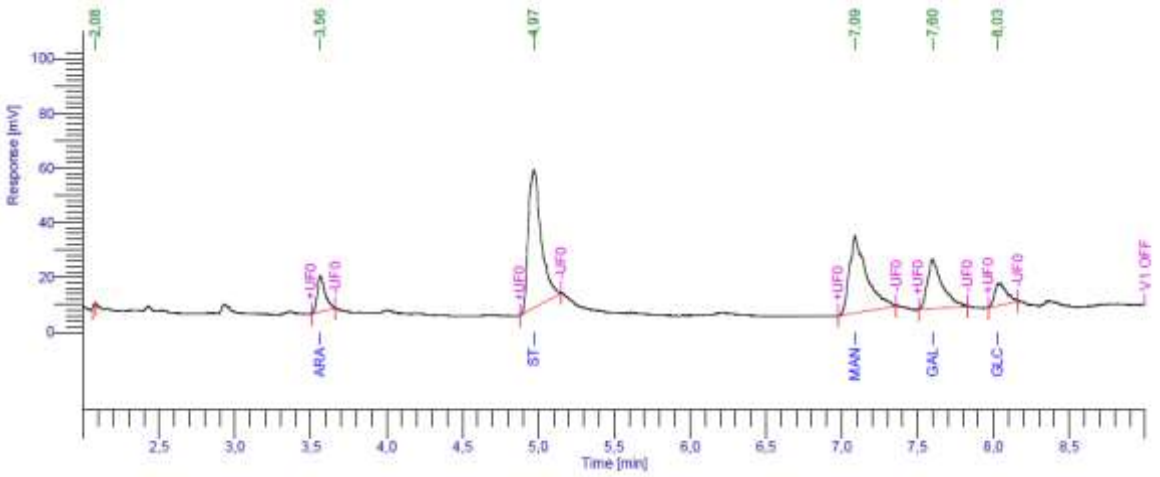
	HMWM $_{Sn}$	11.00	50.21	
	Et50	0.04		33.96
	Et75	0.03		31.06
	EtSn	0.03		25.65

In relation to the total HMWM, the resulting fractions HMWMppt and HMWMSn, presented differences in yields in each roasting degree. HMWMSn was the most recovered fraction in light roast coffee, with a yield of, approximately, 68 % w/w, while in medium roast about 85 % w/w of HMWMppt was obtained. In dark roasted coffee was recovered almost the same amount of HMWMppt and HMWMSn (50 % w/w of HMWM total). Of all recovered HMWMSn, the majority was Et50 fraction in light (around 23 % w/w) and dark (around 34 % w/w) roasted coffees and Et75 in medium roasted ones (43 % w/w).

3.2. Characterization of coffee and its compounds

3.2.1. Sugar composition of coffee, HMW and LMWM

Coffee fractions with different roasting degrees (light, medium and dark) were submitted to sugar analysis to understand the effect of roasting on sugar composition. The studied coffee fractions were coffee brews, HMW (Et50, Et75 and EtSn) and LMW fractions collected from dialysis (first, second, third and fourth dialysis water). The following results were obtained from GC-FID chromatograms (Figure 12).



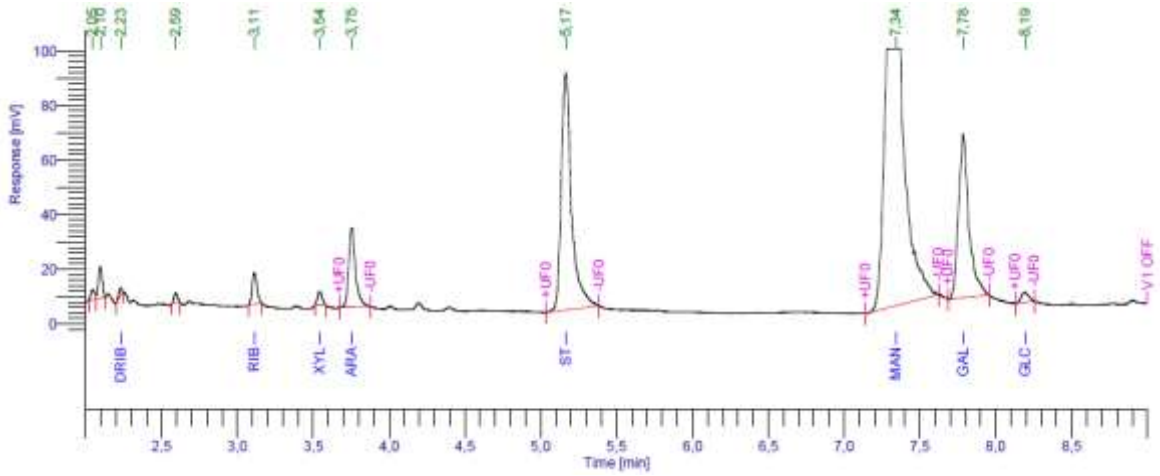
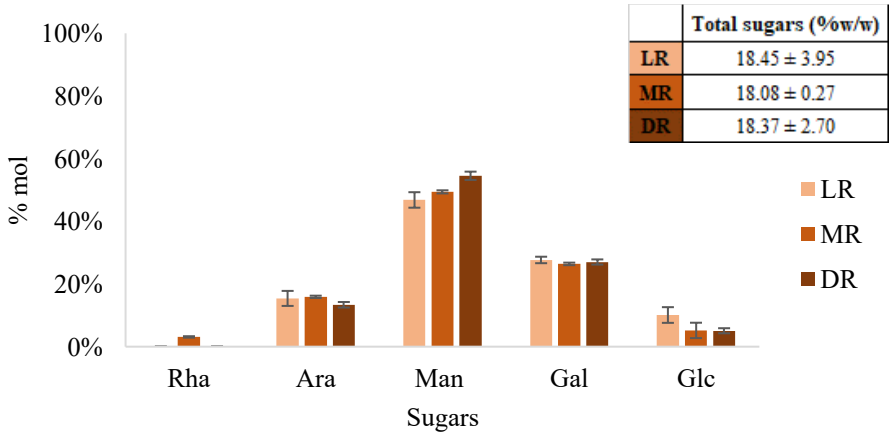


Figure 12: Chromatograms representative of sugar analysis of coffee performed by GC-FID. The first chromatogram corresponds to light roast coffee brew and the second chromatogram corresponds to Et50 fraction of light roasted coffee. (Ara – Arabinose; ST – Internal standard; Man – Mannose; Gal – Galactose; Glc – Glucose)

The sugar analysis of the coffee brews (Figure 13) showed this beverage as mainly constituted by mannose (50 % mol), galactose (27 % mol) and arabinose (15 % mol), which is consistent with reported literature.[7,85] The results did not show significant differences in each sugar content when comparing the three roasting degrees. This tendency was also followed by Et50, Et75 and EtSn fractions where sugar composition was independent of the roasting degree. Furthermore, the total sugar composition was similar in light, medium and dark coffee.



	Total sugars (%w/w)
LR	18.45 ± 3.95
MR	18.08 ± 0.27
DR	18.37 ± 2.70

Figure 13: Sugar (% mol) and total sugars (% w/w) composition in coffee. (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

Coffee polysaccharides constitute a major portion of the brew and are greatly affected by roasting.[7,10] In Et50 fraction the sugar with the highest amount was mannose (70 % mol) followed by galactose (20 % mol) (Figure 14). These results are in line with the ones described in literature,[7,23,31] since this fraction contains galactomannan-like polysaccharides, which are predominantly constituted by those two sugars. There is not a major variation on each sugar composition across the roasting degrees however, and comparing with light and dark, Et50 from medium roast has a lower amount of total sugars of 45 % w/w against 63 % w/w of the remaining roasting degrees.

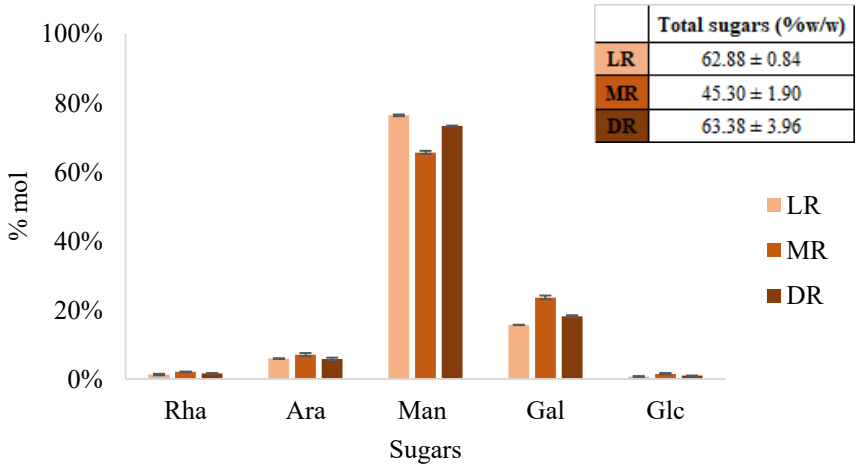


Figure 14: Sugar (% mol) and total sugars (% w/w) composition in Et50 fraction. (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

Galactomannans and type II arabinogalactans are the two main polysaccharides present in coffee.[7] The content of these polysaccharides is determined through the following equations[86].

Equation 2

$$GM = \frac{(Man + 0.05 \times Man)}{100} \times \frac{g_{sugar}}{g_{sample}}$$

Equation 3

$$AG = \frac{(Ara + Gal - 0.05 \times Man)}{100} \times \frac{g_{sugar}}{g_{sample}}$$

Where GM represents arabinogalactans and AG represents arabinogalactans.

When calculating the content of arabinogalactans 5 % of total mannose that is part of galactomannans was subtracted to the total amount of arabinose and galactose. It was also

taken into account that all arabinose is part of arabinogalactans. Table 4 displays the polysaccharides composition.

Table 4: Content of polysaccharides (g polysaccharide/ g sample) present in Et50 fraction. (LR – Light roast; MR – Medium roast; DR – Dark roast)

	Galactomannans content	Arabinogalactans content
LR	50.4 %	11.2 %
MR	31.2 %	12.4 %
DR	48.8 %	12.9 %

Galactomannans are predominant over arabinogalactans in every roasting degree, as expected.[7] Roasting process did not degrade polysaccharides since their content did not variate much between roasting degrees.

In Et75 the predominant sugars were galactose (70 % mol) and arabinose (15 % mol) (Figure 15), showing that this fraction has arabinogalactan-like polysaccharides, as seen in literature.[7,23,31] This fraction also does not present much variability between sugars in any of the studied roasting degrees. Contrarily, total sugar content shows discrepancies, with light roast having the highest value (68 % w/w).

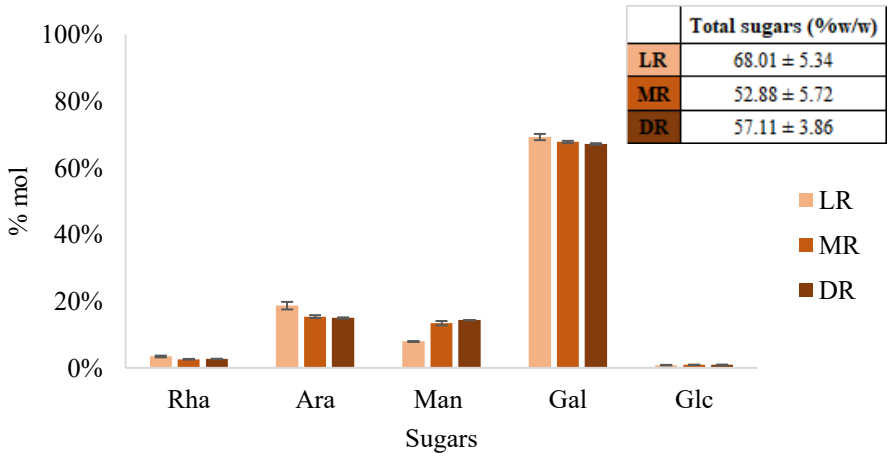


Figure 15: Sugar (% mol) and total sugars (% w/w) composition in Et75 fraction. (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

Content of polysaccharides of Et75 (Table 5) was calculated from Equations 2 and 3. This fraction has a higher content of arabinogalactans in every roasting degree, which is in accordance with the sugar analysis.

Table 5: Content of polysaccharides (g polysaccharide/ g sample) present in Et75 fraction. (LR – Light roast; MR – Medium roast; DR – Dark roast)

	Galactomannans content	**Arabinogalactans content**
LR	5.6 %	59.5 %
MR	7.9 %	46.1 %
DR	8.6 %	46.5 %

EtSn fraction (Figure 16) is richer in galactose and arabinose. These sugars are present in similar amounts of, approximately, 40 %mol which suggests the presence of arabinogalactans in this fraction. This fraction is constituted of melanoidins, which have incorporated polysaccharides such as arabinogalactans in their structure.[10] Roasting degree did not affect total sugar composition.

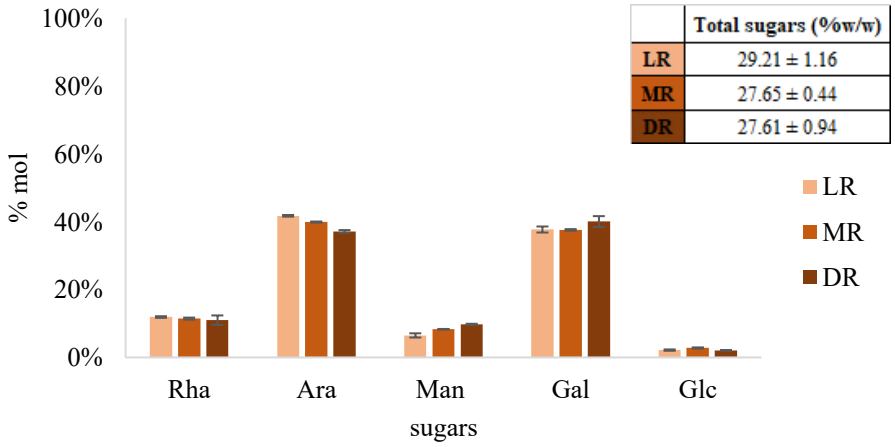


Figure 16: Sugar (% mol) and total sugars (% w/w) composition in EtSn fraction. (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast

Of the polysaccharides present in this EtSn (Table 6) arabinogalactans are predominant, as it was previously suggested by the higher quantity of arabinose and galactose in this fraction.

Table 6: Content of polysaccharides (g polysaccharide/ g sample) present in EtSn fraction. (LR – Light roast; MR – Medium roast; DR – Dark roast)

	Galactomannans content	**Arabinogalactans content**
LR	2.0 %	23.1 %
MR	2.4 %	21.3 %
DR	2.8 %	21.2 %

In the characterized fractions, EtSn exhibited the lowest amount of polysaccharides (28 % w/w) because it also contains proteins and phenolic compounds incorporated into their structure.[19,28,29] Et50 and Et75 presented a similar content of polysaccharides, namely galactomannans and arabinogalactans.

Polysaccharides are HMW compounds being retained by dialysis membrane (12-14 kDa cut-off) during dialysis. Thus, LMW, permeate of dialysis, does not contain polysaccharides but free sugars. The sugar content of the collected LMWM is similar in all roasting degrees (Figures 1, 2 and 3 in Annexes), with exception of the 3rd collected water where there is a slight discrepancy on the sugar level. As seen in Figure 17, medium roasting LMW has a higher content of mannose (39 % mol) and arabinose (29 % mol) than other roasting degrees. Total sugar composition was not affected by roasting.

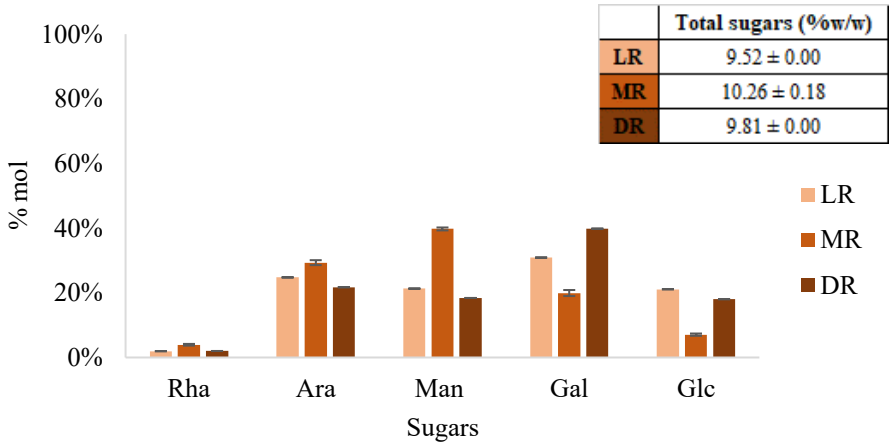


Figure 17: Sugar (% mol) and total sugars (% w/w) composition in LMW material collected from dialysis (third collected water). (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

3.2.2. Chlorogenic acid content in coffee and LMWM

Coffee beans are still regarded as a significant source of CGA in the human diet, despite the fact that a large portion of CGA is destroyed/transformed during roasting.[87] 5-CQA continues to be the most abundant CGA in roasted coffee.[3] CGA concentration is considerably impacted by the brew process, in particular by roasting conditions, resulting in a wide range of CGA levels in diverse coffee brews.[87] Therefore it is important to determine CGAs content to assess which LMW fraction might have better antioxidant and neuroprotective potential. This determination was made by HPLC, where 5-CQA peak was detected at 325 nm (Figure 18).

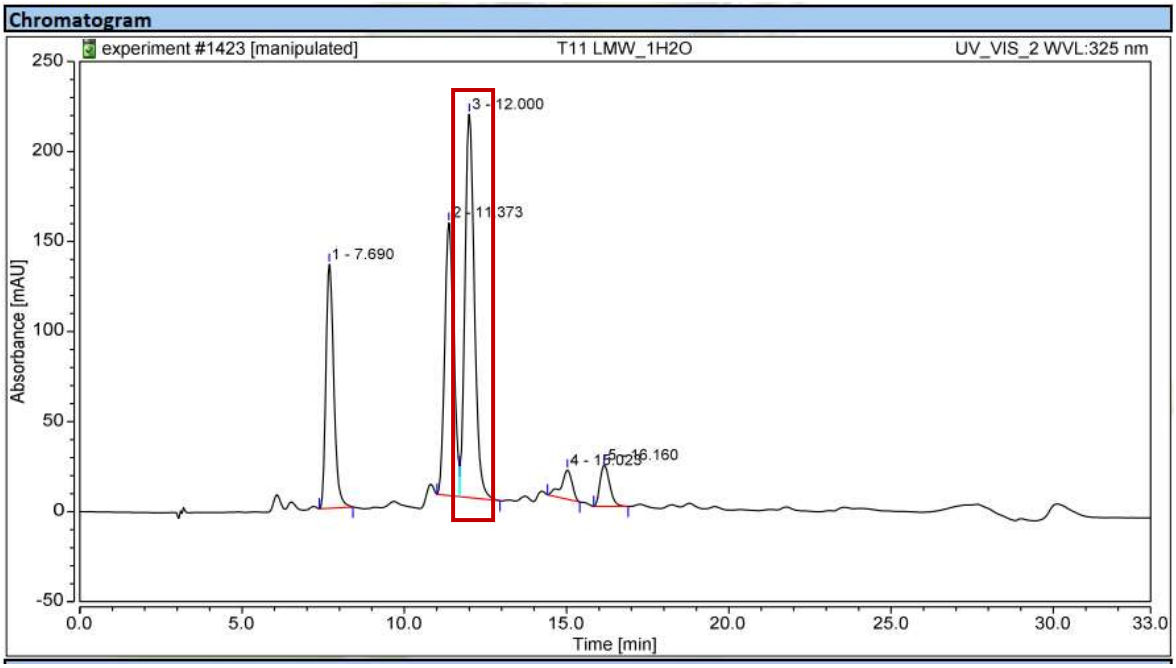


Figure 18: Chromatogram representative of CGA quantification performed by HPLC. Highlighted in red is the 5-CQA peak.

The CGA analysis showed that the chlorogenic content decreases the harsher the roasting (Figure 19). Dark roasted coffee has a lower content of 5-CQA than medium which, in turn, has a lower content than light roasted coffee. CGAs are degraded during roasting giving rise to other compounds, increasing the content of phenolic compounds.[6] There is an increase on chlorogenic acid content from 1st to 2nd H_2O. 2nd and 3rd waters present a similar 5-CQA content reaching its maximum on either of these fractions. From 3rd to 4th collected waters there is an accentuated decrease of CGA in the sample.

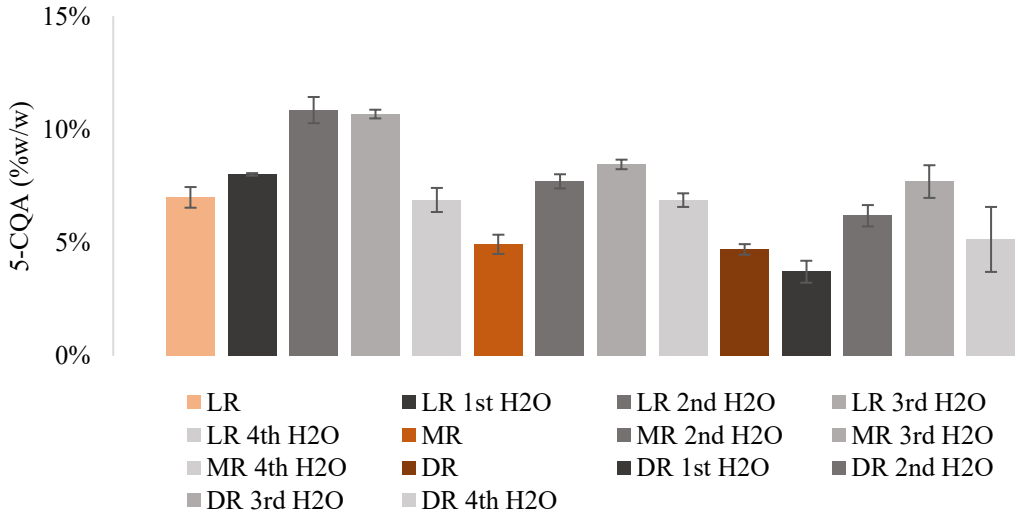


Figure 19: Chlorogenic content (% w/w) in coffee and LWW fractions. (LR – Light roast; MR – Medium roast; DR – Dark roast)

3.2.3. Fractionation and chemical characterization of LMWM

Since LMW 2nd collected waters presented some of the highest contents in CGAs, as proved by HPLC, this dialyzed fraction was chosen to proceed, being fractionated by SPE to obtain a purer fraction enriched in CGAs. The SPE yields are presented in Table 7. In both roasting degrees the most obtained fraction was number 1, eluted with acidic water, followed by fraction 5 in LR and fraction 6 in DR, both eluted with 80/ 20 water/ methanol.

Table 7: Yields obtained from the fractionation of LMW compounds trough SPE. (LR – Light roast; DR – Dark roast)

	LR LMWM 2nd H_2O	**DR LMWM 2nd H_2O**
Fraction number	$m_{obtained}$ (mg)	$m_{obtained}$ (mg)
1	136.9 ± 18.5	140.5 ± 6.2
2	12.7 ± 0.7	9.1 ± 2.0
3	15.9 ± 4.8	6.3 + 0.1
4	10.0 ± 3.5	5.2 ± 2.5
5	40.7 ± 4.5	27.0 ± 2.5
6	15.0 ± 5.2	26.2 ± 12.8
7	17.4 ± 6.4	30.3 ± 3.4
8	3.7 ± 0.9	4.4 ± 2.0

9	4.1 ± 0.8	6.2 ± 1.6
10	1.0 ± 0.5	0.7 ± 0.1
Total	**257.3**	**255.9**

To verify that SPE was effective in purifying LMW, an NMR analysis was conducted. LMW was analyzed by NMR (Figure 4 in Annexes). In the spectra it is possible to identify different classes of compounds expected to be present in the sample. As the CGAs are predicted to be eluted in the first fractions[88], SPE 2, 4 and 6 were analyzed by NMR. The resulting spectra will be presented below.

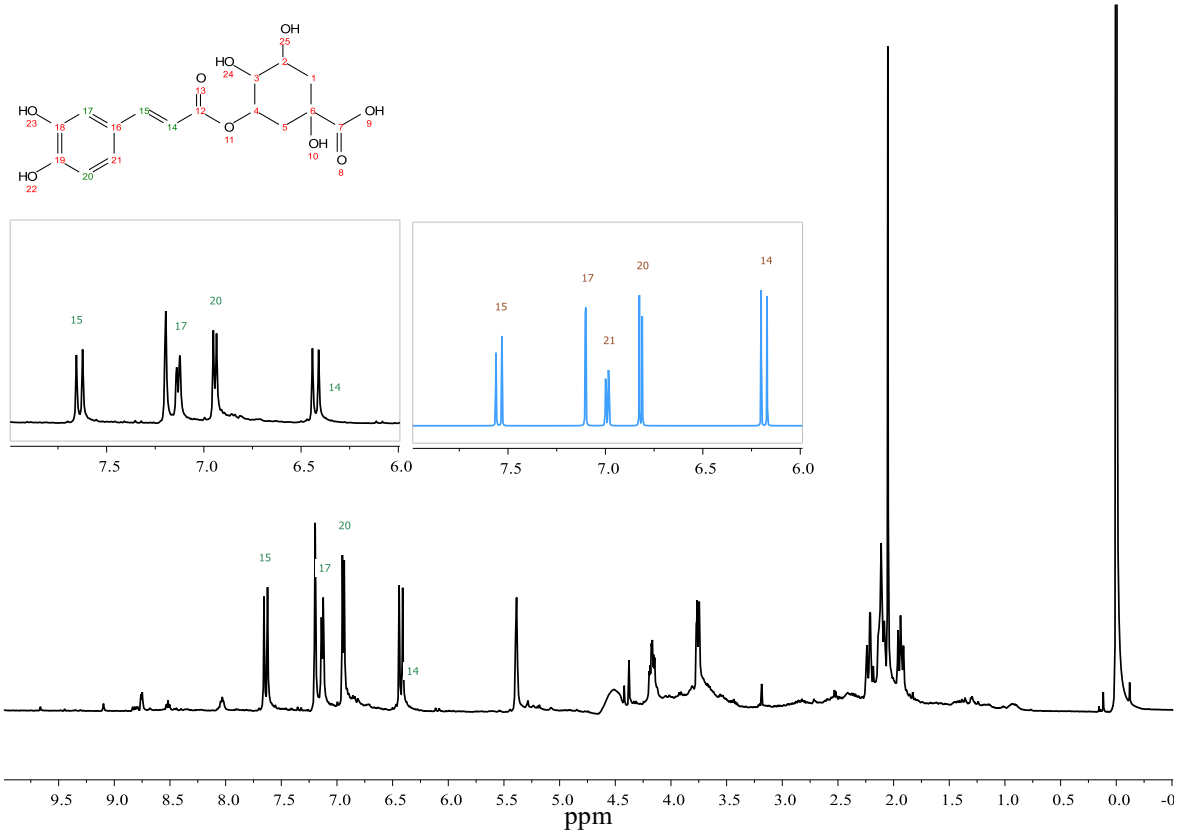
The obtained spectra of both LR and DR SPE 2 (Figure 20) shows similar peaks in the same regions which leads to the conclusion that the same compound was isolated in the two samples. By observing the predicted 5-CQA spectrum[89] and comparing with SPE 2 obtained spectra, protons with numbers 14, 15, 17 and 20, present in 5-CQA structure are also possible to identify in SPE 2. However, these peaks, situated in 6.0 – 8.0 ppm region, are common to every CGA and therefore it is not possible to identify specifically what type of CGA is present in the sample. Since CGAs are present in SPE 2, through the area of the identified peaks, its amount in this fraction can be calculated. For this, the known concentration of TSP and its area peak, whose corresponds to nine protons in its molecule, is used (Table 8). In the SPE 2 purified from LR, the CGAs concentration obtained was of 10.22 mM. In DR, CGA concentration was 3.75 mM. Since they are thermolabile, CGAs undergo a number of changes during roasting, such as degradation and incorporation into melanoidins,[3,22,33] and their content should decrease the harsher the roasting degree (as verified in Figure 19 of section 3.3.2.2.). Thus SPE 2 is in accordance with previous results, as the concentration of CGA is lower in DR when compared to LR.

Table 8: Concentration of CGAs in SPE 2. The calculation is made with a known concentration of TSP and its area peak that corresponds to nine protons. (LR – Light roast; DR – Dark roast)

	Name/ Proton number	**Area**	**Number of protons**	**Concentration (mM)**
LR	TSP	1236008.693	9	10.0
	15 (CH)	140380.18	1	10.2

DR	TSP	46306.654	9	0.50
	15 (CH)	38622.531	1	3.75

The spectrum also shows a high intensity peak around 2.0 ppm that can be from acetic acid since this fraction was eluted with acidic water (2 % acetic acid). The predicted spectrum of acetic acid is presented in Figure 5 in Annexes.



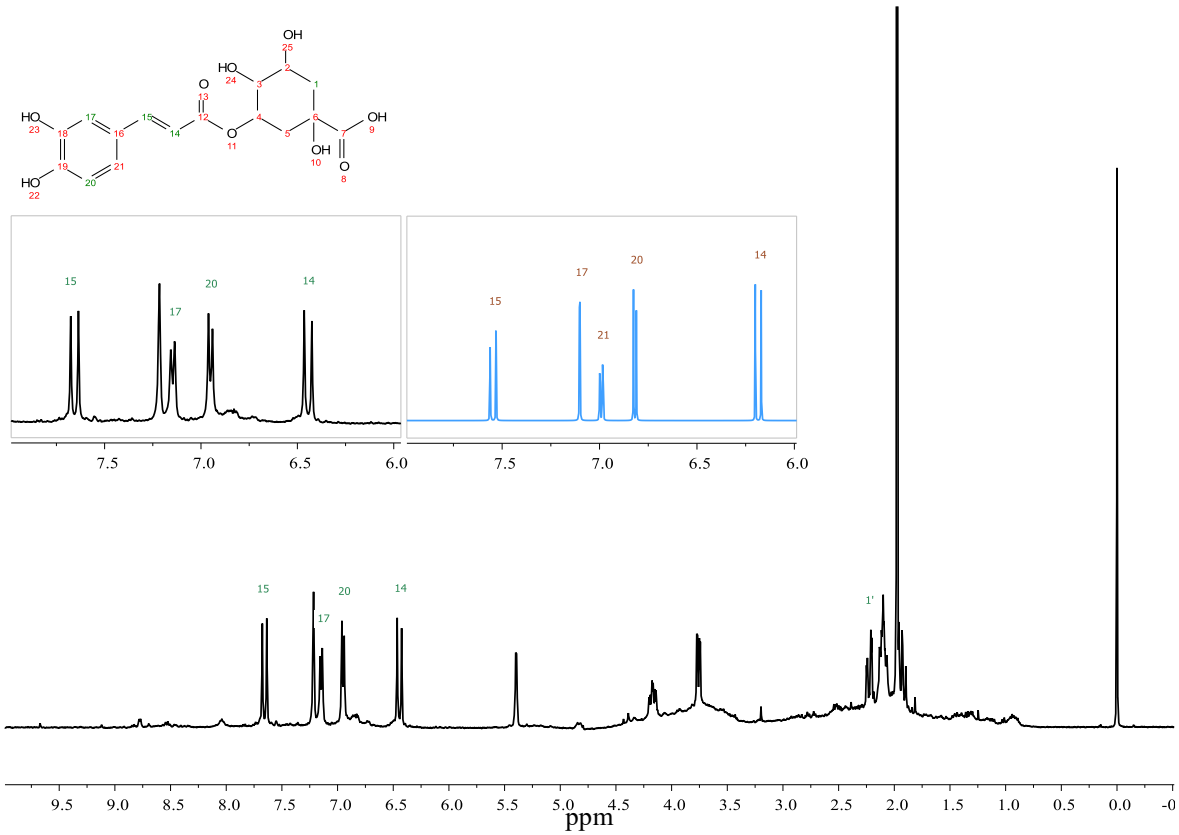


Figure 20: ^{1}H NMR spectrum of LR and DR fraction 2 obtained by solid phase extraction. Spectra have been corrected to baseline. The first spectra corresponds to light roasted coffee and the second to dark roasted coffee. Blue spectra corresponds to 6.0 – 8.0 ppm region of 5-CQA ^{1}H NMR predicted spectrum.

The obtained spectra of LR and DR SPE 4 also shows similar peaks in the same regions. DR peaks have a higher intensity than LR. The similarity between spectra indicates that the same compound is present in both samples. SPE 4 spectra have the same peaks as the ones observed in SPE 2 around 6.0 – 8.0 ppm, identified with numbers 14, 15, 17 and 20 in 5-CQA structure, which means that there were still CGAs being eluted in fractions 3 and 4. However in this sample there are two more peaks (numbers 27 and 28) that were not observed in SPE 2. CGAs result from the esterification of cinnamic acids with a quinic acid. The protons labeled as 14 and 15 are characteristic of cinnamic acids, in the case of 5-CQA, caffeic acid, and are present in all CGAs. However, there is a subclass of CGAs, diCQAs, that have two caffeic acids in their structure, and so in their NMR spectrum there is two doublets in the 6.0 – 6.5 ppm and 7.5 ppm regions, as shown in Figure 21.

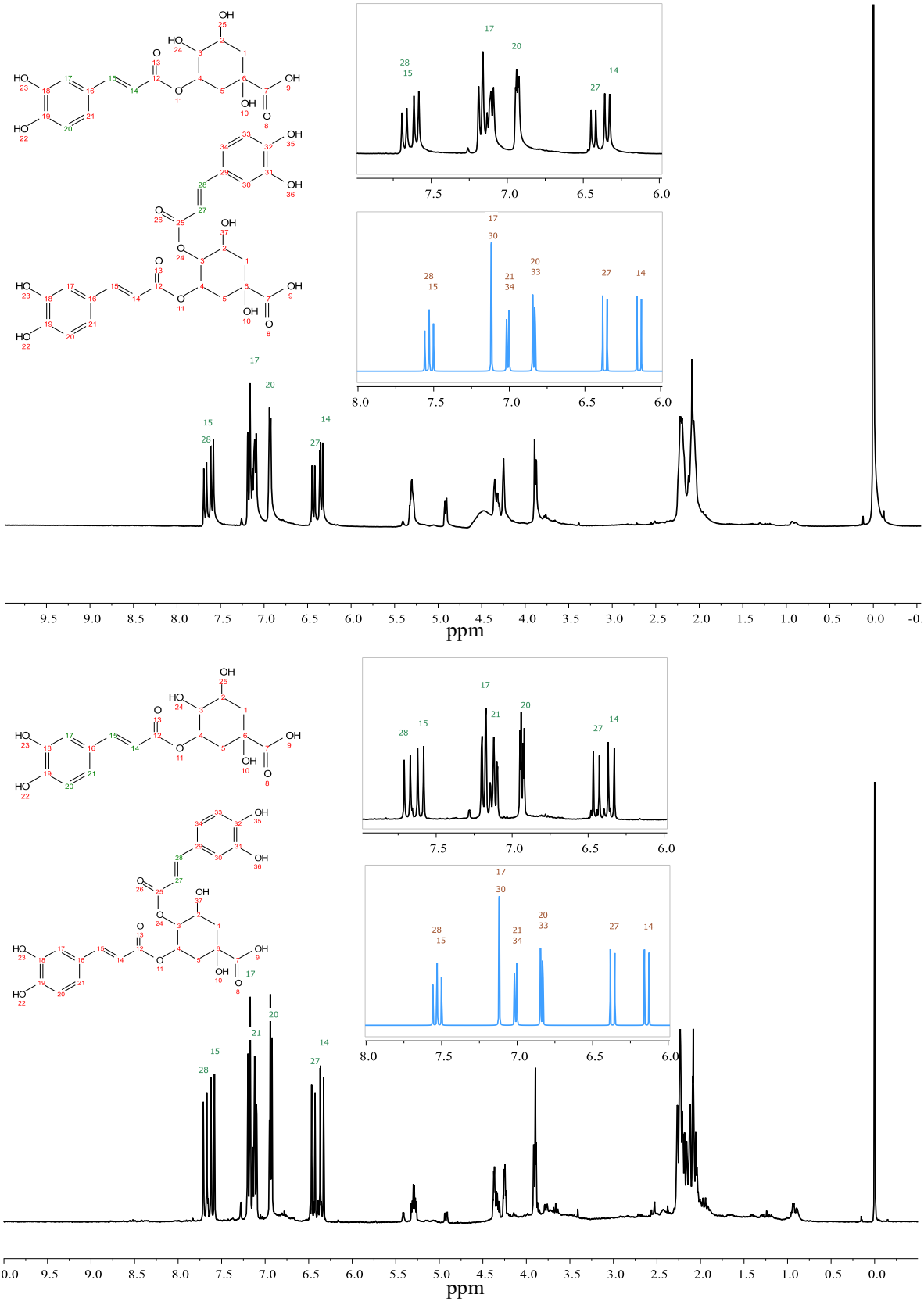


Figure 21: [1]H NMR spectrum of LR and DR fraction 4 obtained by solid phase extraction. Spectra have been corrected to baseline. The first spectra corresponds to light roasted coffee and the second

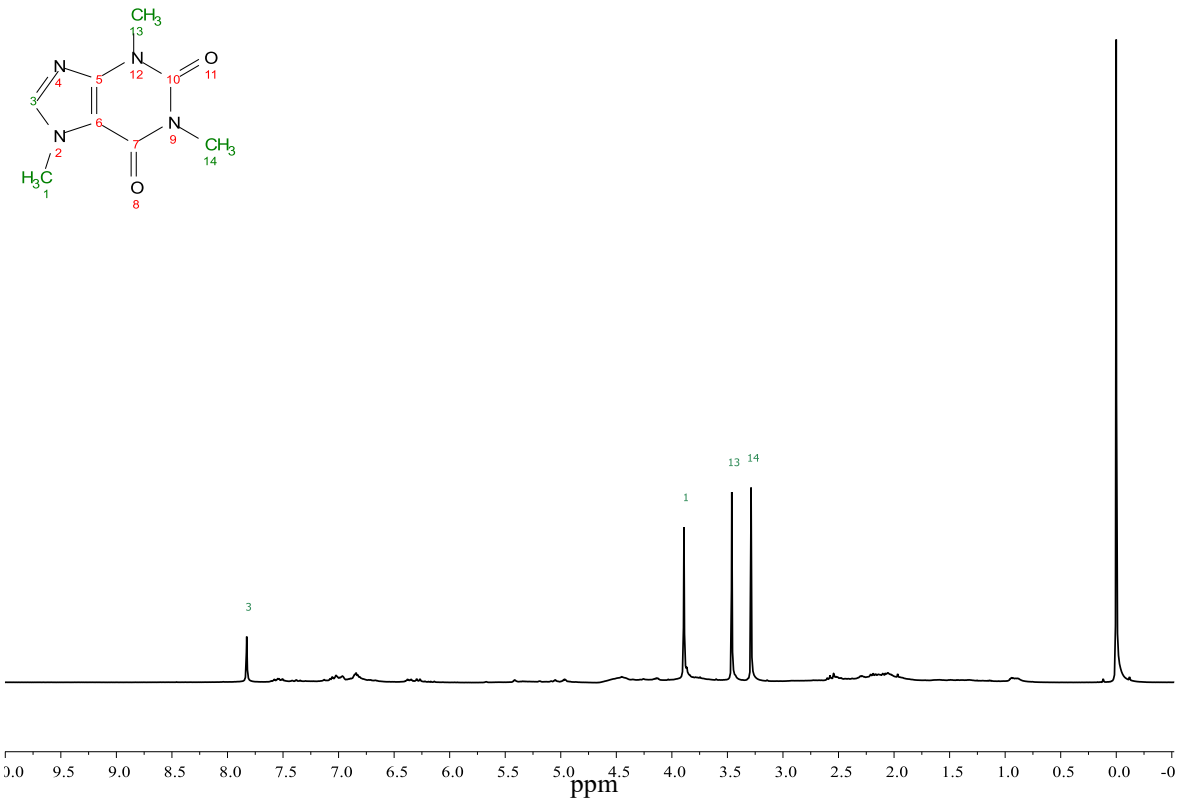
to dark roasted coffee. Blue spectra corresponds to 6.0 – 8.0 ppm region of 3,4-diCQA ^{1}H NMR predicted spectrum.

These two doublets, confirmed by predicted 3,4-diCQA spectrum, are seen in LR and DR spectra. These peaks are found in the SPE 4 spectrum, meaning that in addition to CGAs, diCQAs are also present in the samples. Again, through the area of the peaks it is possible to calculate the amount of each of these compounds in each sample (Table 9). In SPE 4 purified from LR there is a concentration of CGAs of 14.21 mM and diCQAs of 6.94 mM. In DR, CGAs concentration is 4.46 mM and diCQAs concentration is 3.73 mM, lower than that obtained for LR, as expected.

Table 9: Concentration of CGAs and diCQAs in SPE 4. The calculation is made with a known concentration of TSP and its area peak that corresponds to nine protons. (LR – Light roast; DR – Dark roast)

	Name/ Proton number	**Area**	**Number of protons**	**Concentration (mM)**
LR	TSP	1121161.418	9	10.0
	15 (CH) (CGAs)	177067.218	1	14.2
	28 (CH) (diCQAs)	86435.034	1	6.94
DR	TSP	48538.384	9	0.50
	15 (CH) (CGAs)	48105.257	1	4.46
	28 (CH) (diCQAs)	39922.779	1	3.73

The obtained spectra of both LR and DR SPE 6 (Figure 22) shows the same number of peaks in the same regions, 3.0 – 4.0 ppm and 8.0 ppm, that correspond to caffeine. Therefore, caffeine was eluted in fractions 5 and 6 of both roasting degrees. The predicted spectrum of caffeine sustains this conclusion (Figure 6 of Annexes).


CH3
13
N
12
O
11
10
N
4
5
3
N
2
6
7
N
9
CH3
14
H3C
1
O
8
3
1
13
14
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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4.5
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3.0
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2.0
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ppm

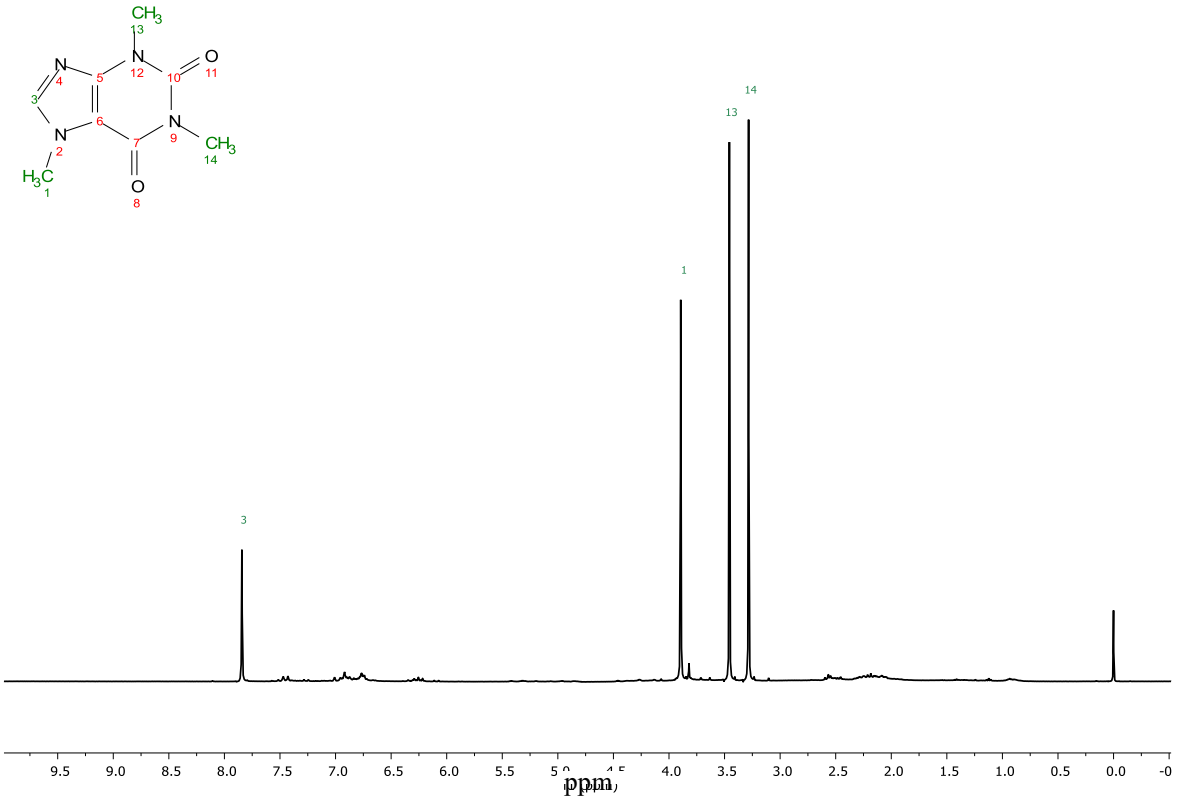

Figure 22: [1]H NMR spectrum of LR and DR fraction 6 obtained by solid phase extraction. Spectra have been corrected to baseline. The first spectra corresponds to light roasted coffee and the second to dark roasted coffee.

The amount of caffeine in this sample was calculated as mentioned before (Table 10). In SPE 6 purified from LR there is a concentration of caffeine of 13.2 mM and in DR caffeine is in a concentration of 15.0 mM.

Table 10: Concentration of caffeine in SPE 6. The calculation is made with a known concentration of TSP and its area peak that corresponds to nine protons. (LR – Light roast; DR – Dark roast)

	Name/ Proton number	Area	Number of protons	Concentration (mM)
LR	TSP	1041905.26	9	10.0
	3 (CH)	152666.478	1	13.2
DR	TSP	40758.313	9	0.50
	3 (CH)	136236.882	1	15.0

3.2.4. FTIR analysis of coffee and LMWM

FTIR is a rapid and non-destructive analysis that provides an infrared fingerprint of samples composition.[90] FTIR spectra have general band assignments that correspond to different coffee compounds, carbohydrate region is 800-1200 cm^{-1}, aromatic acids region is 1700-1680 cm^{-1}, CGAs appear at an absorption of 1150-1300 cm^{-1} and caffeine at 1600-1650 cm^{-1}, for example.[90,91] Spectra of coffee and LMW presented in Figure 23 shows higher absorbance in such regions confirming chemical analysis already performed. There are no differences observed between spectra of different coffee roasted samples and LMW obtained from coffee, which means samples are chemically close.

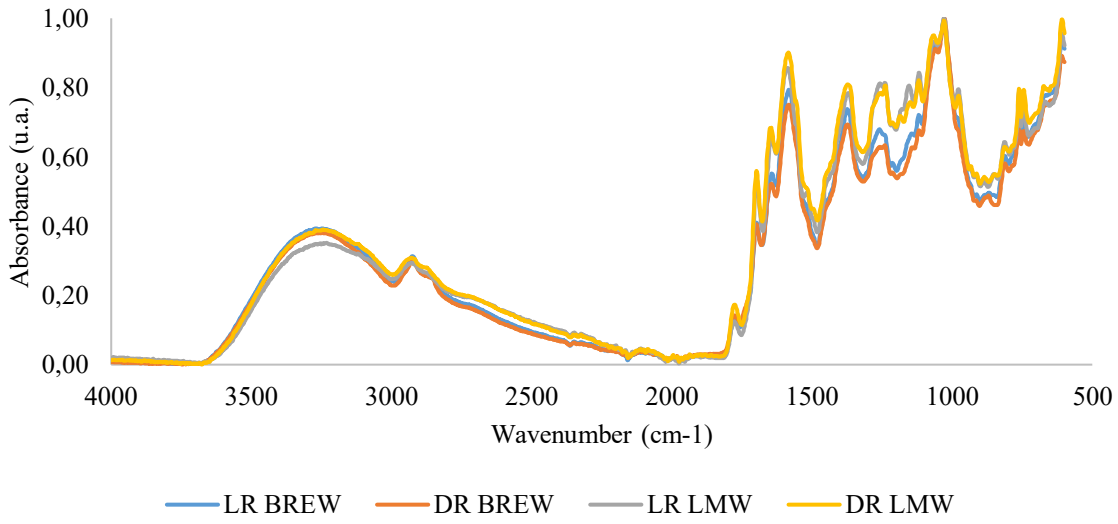

Figure 23: FTIR spectra of LR and DR coffee and LMW in the 500-4000 cm^{-1} region. (LR – Light roast; DR – Dark roast)

Figure 24 also shows higher intensity in the regions of absorption of CGAs and caffeine. These fractions are enriched with those compounds as confirmed by previous chemical

analysis. There are slight differences in intensity of absorption between LR and DR SPE 6 however they still fall in the same regions and so these samples are also chemically close.

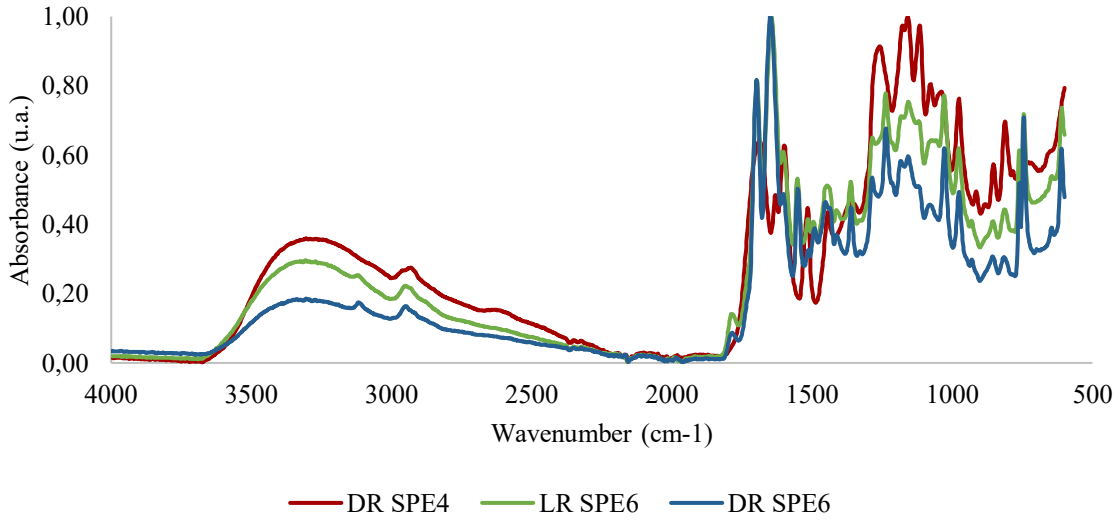

Figure 24: FTIR spectra of LR SPE 4 and LR and DR SPE 6 in the 500 - 4000 cm^{-1} region. (LR – Light roast; DR – Dark roast)

3.3. Properties of coffee and its compounds

3.3.1. Antioxidant activity of coffee, HMWM and LMWM

In neurodegenerative diseases the brain undergoes oxidative changes increasing the risk of ROS damaging neurons.[33] To understand the antioxidant potential of fractionated samples, antioxidant activity was measured by DPPH assay. The antioxidant activity of different roasting degrees of coffee brews presented was similar (EC50 0.24 mg/mL) (Figure 25). HMWM showed the lowest antioxidant activity. Arabinogalactans showed a higher antioxidant activity (EC50 0.50 mg/mL) than galactomannans (EC50 0.61 mg/mL), in every roasting degree. Polysaccharides activity was found to decrease from light to dark roast. Melanoidins showed a good antioxidant potential (EC50 0.21 mg/mL), similar to coffee and free LMW compounds. Roasting did not affect the antioxidant activity of the melanoidins. LMWM also presented a good antioxidant potential overall, analogous to melanoidins. Since CGA is known as having antioxidant activity it was used as control. This property results from CGAs ability to donate hydrogen atoms, decreasing presence of free radicals and hindering reactions of oxidation. At a structural level their antioxidant capacity is possible because CGAs have one or two aromatic rings linked to hydroxyl groups.[18] The great

antioxidant activity demonstrated by coffee, EtSn and LMWM must in part be attributed to CGAs, present in all these samples.

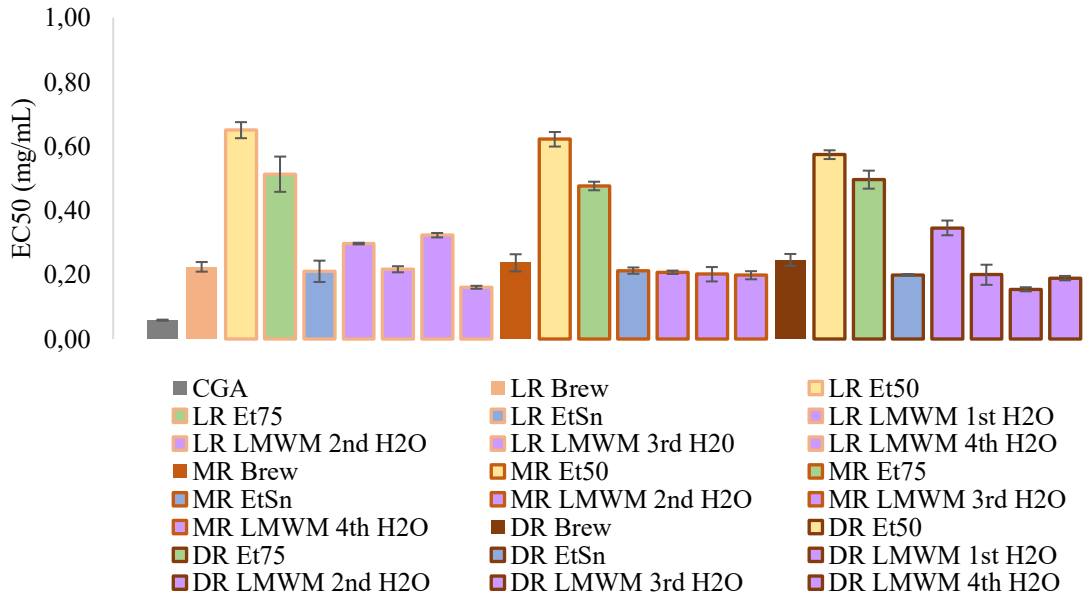

Figure 25: Antioxidant activity expressed in EC50. This parameter represents the concentration of antioxidant required to reduce the initial DPPH concentration by 50 %.[14] The lower EC50 value corresponds to the higher antioxidant activity. (LR – Light roast; DR – Dark roast)

3.3.2. Aggregation behavior of Aβ 1-42 followed by light scattering assay

To understand how Aβ aggregation varies over time, light scattering assay was performed with two different buffers, phosphate buffer[67] and Tris-HCl. As Tris-HCl is composed by NaCl two buffers were used to assess if presence of salt would alter Aβ aggregation curve. The absorbance was measured at different time points, namely when Aβ was added to the buffer Tris-HCl (0 h), and then after 14, 24 and 38 h (Figure 26). After that time, there were no changes in absorption contrary to what was expected. Therefore, and in an attempt to "force" the aggregation, the samples were maintained at a 37°C heated for different periods, since temperature induces aggregation in various proteins[92], and again read the absorption in a range of 200 to 700 nm as previously. Even so there were no alterations in the absorbances.

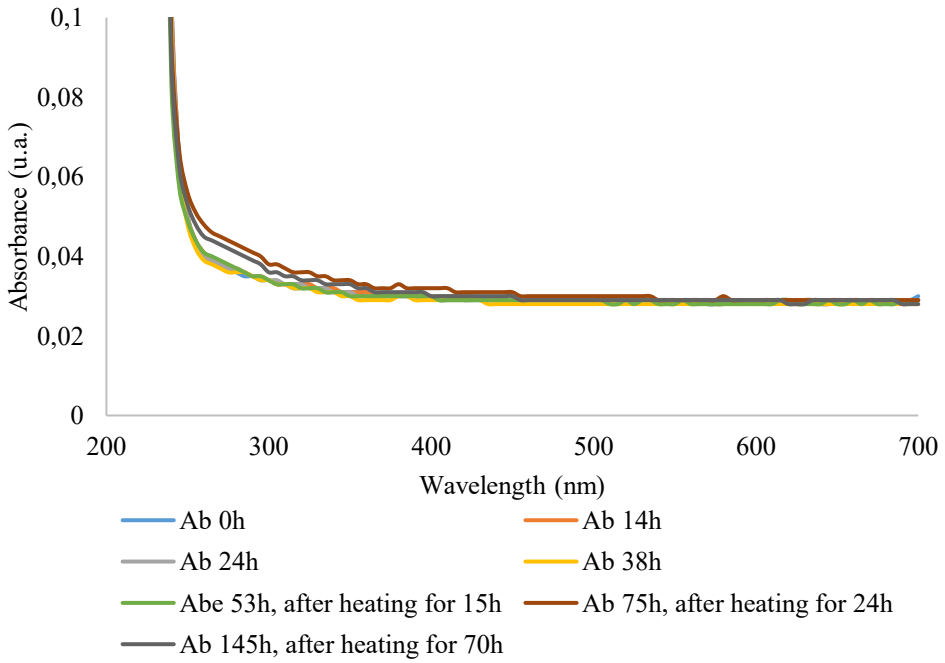

Figure 26: Curve of Aβ aggregation in the presence of Tris-HCl buffer obtained by light scattering assay.

Aβ was then tested with phosphate buffer at 0, 15, 37 and 107 h, with heating for long periods (Figure 27). Even so, no difference in absorption was observed.

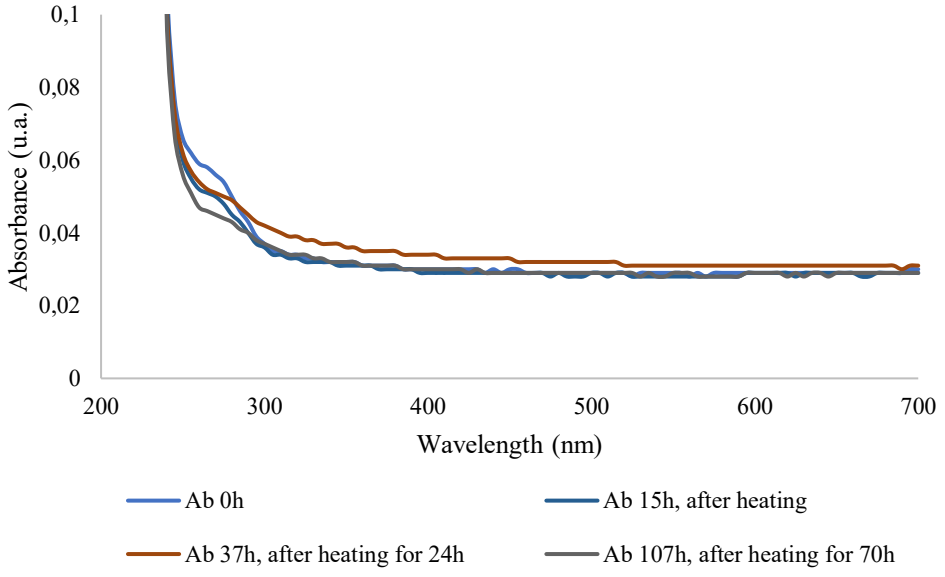

Figure 27: Curve of Aβ aggregation in the presence of phosphate buffer obtained by light scattering assay.

It was not possible to follow the expected aggregation pattern of Aβ, by light scattering, even changing the conditions. To study morphological changes in Aβ images of the surface of the samples were taken by SEM (Figures 28 and 29).

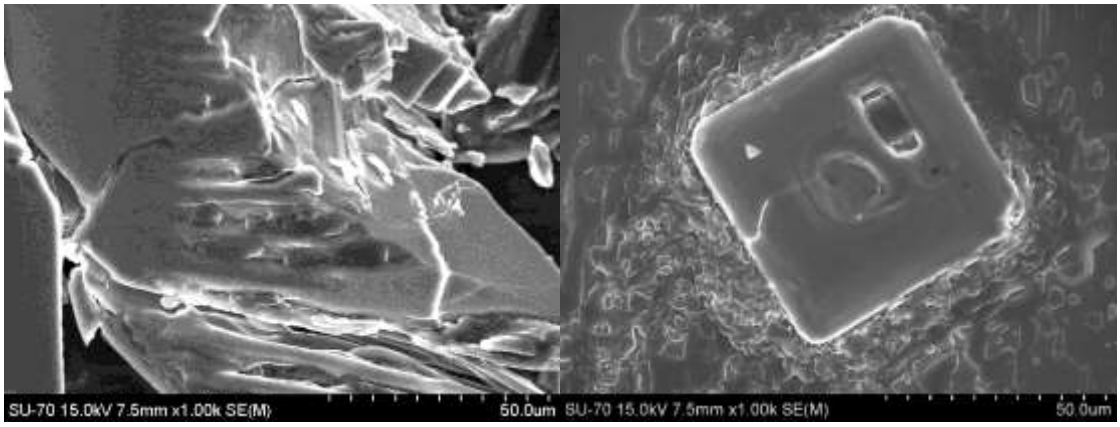

Figure 28: Images of the surface of the samples taken by SEM. Left image corresponds to Tris-HCl buffer and the right image is of Aβ in Tris-HCl buffer.

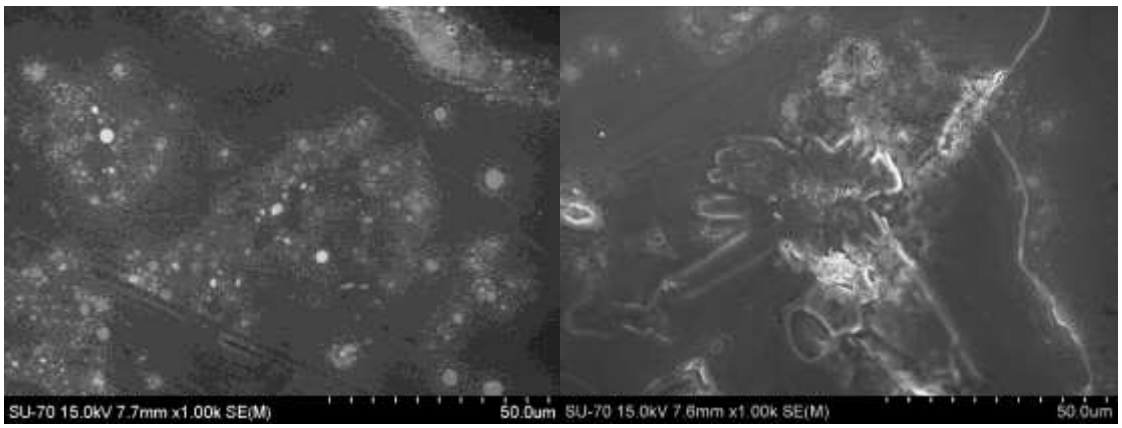

Figure 29: Images of the surface of the samples taken by SEM. Left image corresponds to phosphate buffer and the right image is of Aβ in phosphate buffer.

Through SEM was not possible to clearly identify Aβ fibres, a characteristic of the protein aggregation already described in literature.[67] In the image of Aβ with Tris-HCl buffer (Figure 28) it is observable a cube-like structure that most likely corresponds to salt from the buffer. Images of Aβ with coffee and CGA at different resolutions are presented in Figures 7, 8 and 9 in Annexes.

3.3.3. Aggregation behavior of Aβ 1-42 followed by thioflavin assay

As it was not possible to follow Aβ aggregation by light scattering, another strategy was adopted, and so intensity of fluorescence was measured by the thioflavin (ThT) assay. ThT assay is the protocol frequently used to evaluate aggregate formation, where an increase in fluorescence is related with β-sheet formation in the aggregate.[92] Expected aggregation curve for Aβ is a sigmoidal curve shown in Figure 30. Initially no fluorescence is detected since β-sheet formation did not yet occur and proteins are in an individual state. As time progresses, protein denaturing and aggregation of proteins into β-sheet fibrils increases, enhancing fluorescence until it reaches a plateau. This stage is reached when no new formation of β-sheet is detected and all fluorescent molecules are bound or cannot bind evolving β-sheets because the pathway is blocked.[92]

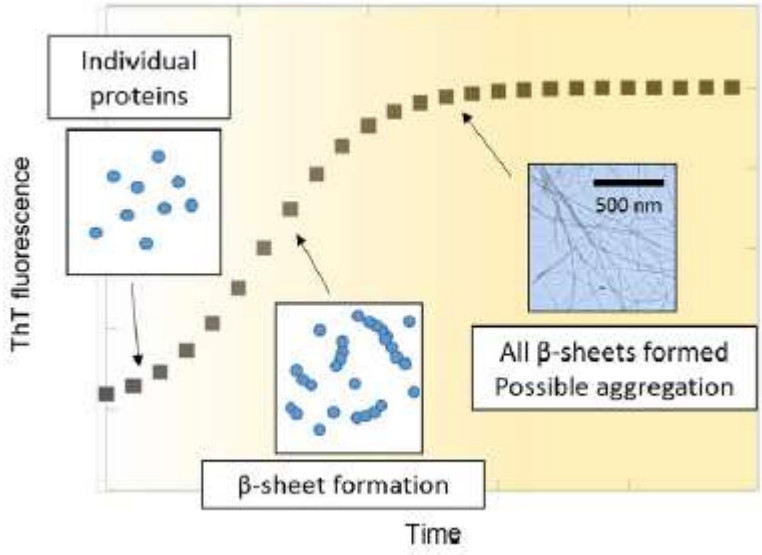

Figure 30: Typical curve of protein aggregation obtained by ThT fluorescence assay. Based on Batzli and Love. [92]

Firstly, Aβ affinity to different buffers, such as PB, HEPES and Tris, was tested, with and without NaCl (Figure 31). In the HEPES and Tris buffers, there was no formation of Aβ aggregates during the 22 h of assay, unlike the PB buffer. In PB, Aβ aggregation appears to plateau around 570 min (approximately 10 h). The reaching of the plateau and the general curve of aggregation were not affected by either the presence or absence of NaCl. Thus, the existence of salt does not significantly alter the Aβ aggregation pattern. In view of these

results, the PB buffer without NaCl was chosen for the continuation of the ThT assay with the coffee and LMW samples.

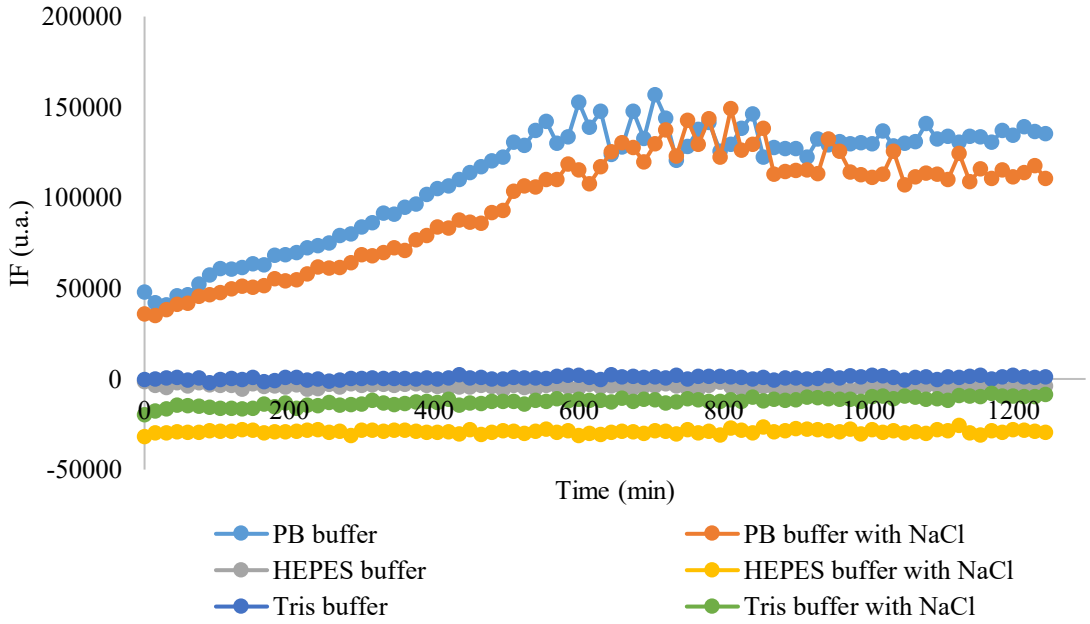

Figure 31: Aβ aggregation in the presence of different buffers (PB, HEPES and Tris) with and without NaCl.

The objective of this assay is to understand if the addition of these samples to Aβ causes any change in the expected pattern of aggregation. All results were normalized to start at the same fluorescence intensity value to better compare the data obtained on each sample with those of Aβ. Coffee was the first sample to be tested. The figure below (Figure 32) shows the aggregation of Aβ in the presence of coffee (LR and DR).

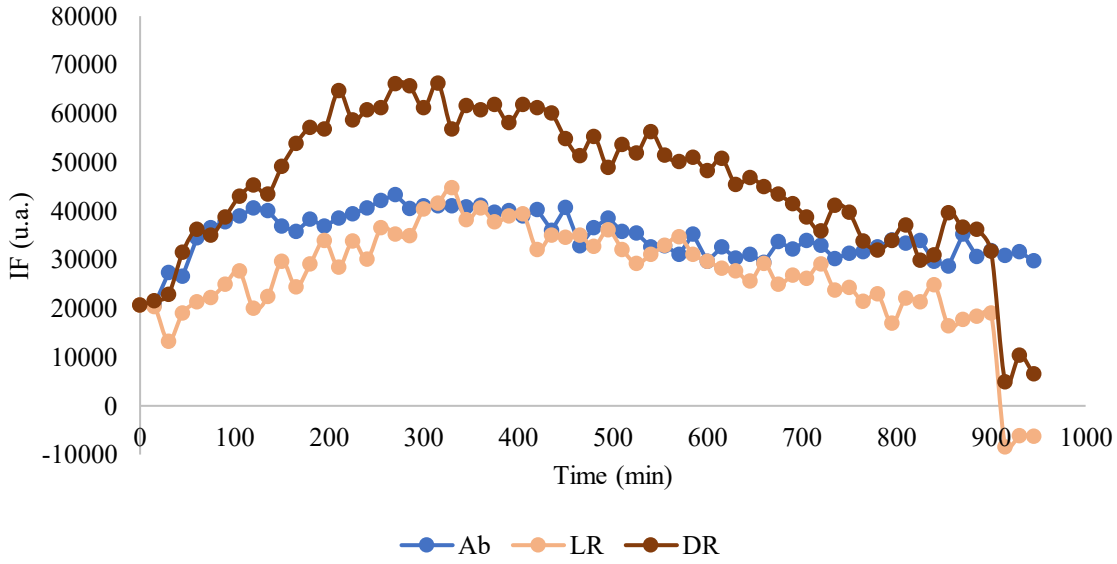

Figure 32: Aβ aggregation in the presence of light and dark roasted coffee. (LR – Light roast; DR – Dark roast)

Results showed that the aggregation profile of Aβ in the presence of coffee is similar to that of Aβ itself, until the 12 hours. At this time coffee, in both roasting degrees, did not affect Aβ aggregation. However, at higher times a tendency in reducing the aggregation was shown, being higher for the light roast coffee.

Then, LMW compounds isolated from coffee dialysis were tested, more precisely the 2nd dialysis water. In this sample, compounds with CGAs and caffeine, among others, are present (see section 3.2.3.). Due to sampling variability obtained, it was only possible to obtain results referring to DR. As observed in Figure 33 DR LMW seem to prevent Aβ aggregation. Comparing with coffee itself, LMW has a more pronounced effect.

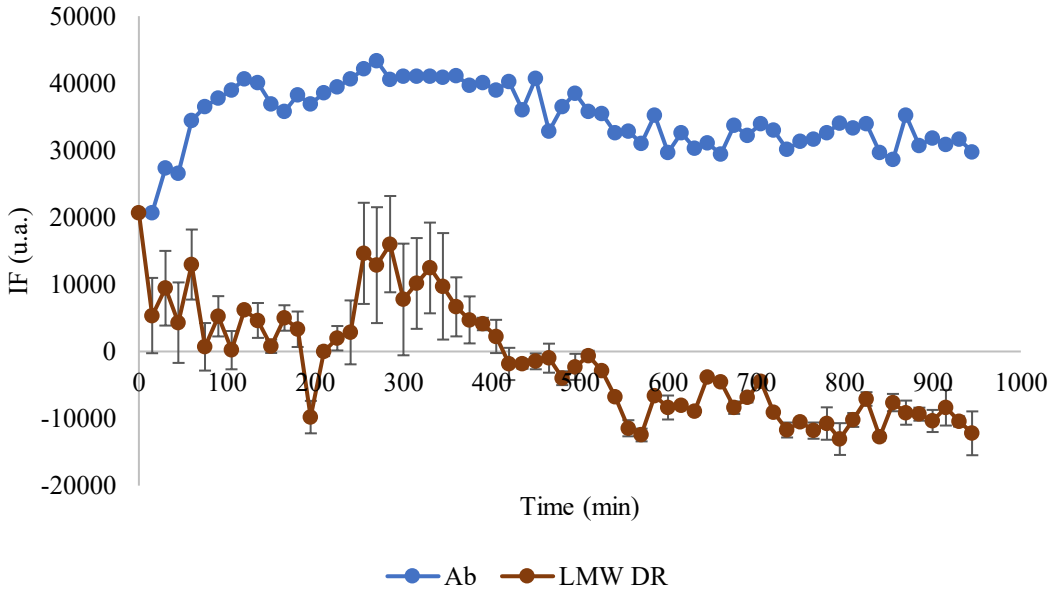

Figure 33: Aβ aggregation in the presence of LMW coffee compounds. (DR – Dark roast)

LR and DR fractions 4 and 6 separated through SPE were also tested. Standard CGA and caffeine were used as controls for each SPE purified fractions, since SPE 4 contains CGAs and SPE 6 contains caffeine (see section 3.2.3.). Standard CGA shows effect, preventing Aβ aggregation as expected. The isolated fractions that contain CGAs (SPE 4) show different behaviors. LR SPE 4 does not seem to have had any effect in preventing aggregation, unlike DR, whose effect is closer to what was expected (Figure 34).

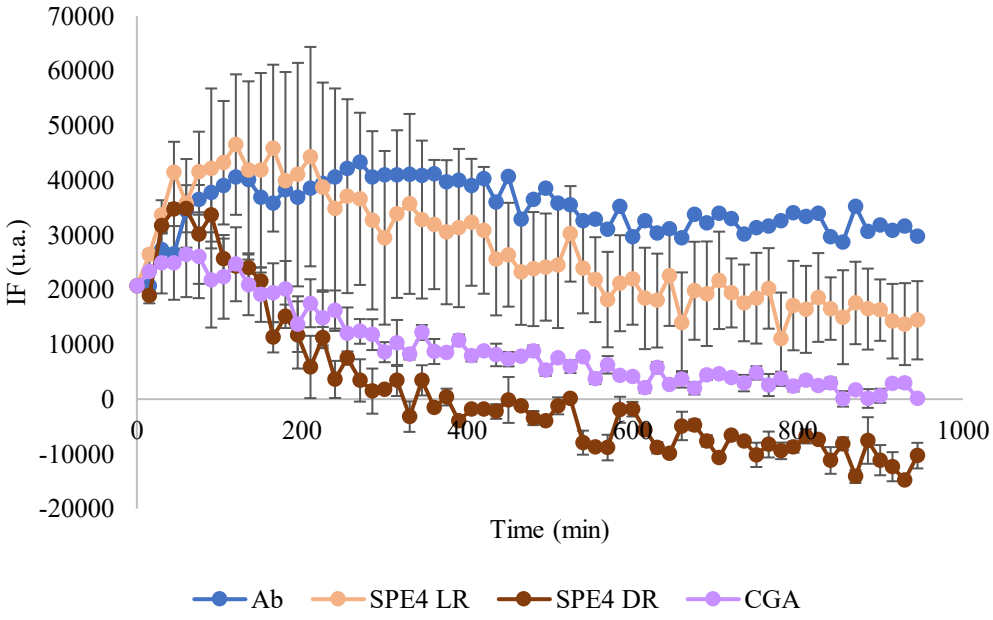

Figure 34: Aβ aggregation in the presence of solid phase extraction fraction 4 and CGA. (LR – Light roast; DR – Dark roast)

Standard caffeine did not prevent Aβ aggregation demonstrating a similar aggregation profile to Aβ alone. Fractions containing caffeine (SPE 6) follow the same tendency in both roasting degrees showing no effect in aggregation (Figure 35).

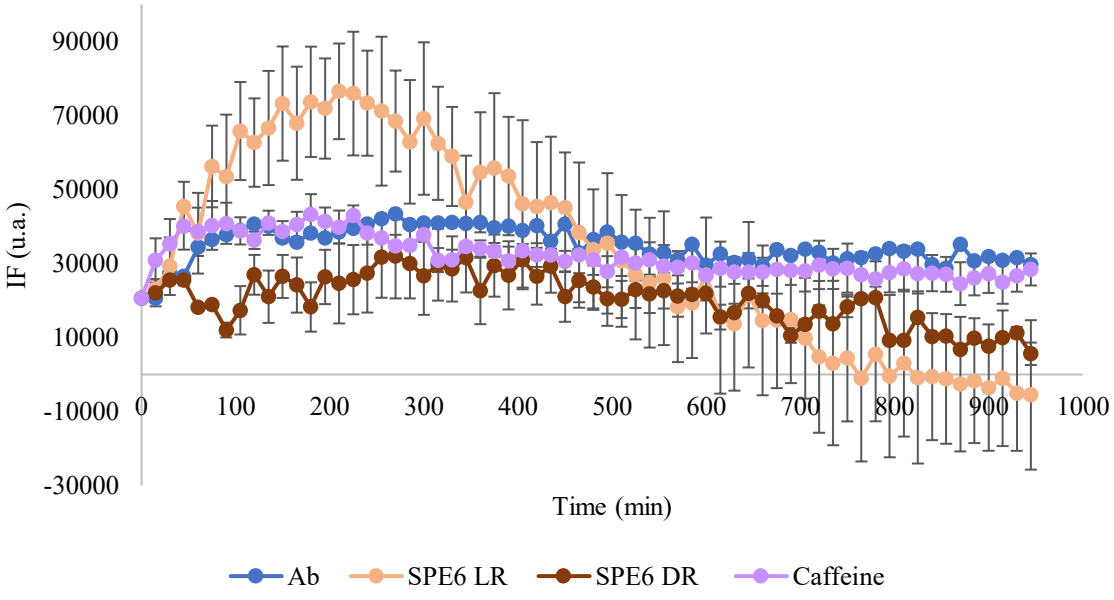

Figure 35: Aβ aggregation in the presence of solid phase extraction fraction 6 and caffeine. (LR – Light roast; DR – Dark roast)

Overall, the samples that showed the greatest potential to prevent Aβ aggregation were LMW and SPE 4 from dark roast. The results show a wide variability within results mostly when food extracts are used and further assays are needed to better corroborate the preliminary described effects.

3.3.4. Neurotoxicity assays

To assess the effect of CGAs and LMW, cellular viability of neurons was tested in the presence of these compounds. Thus resazurin assay was firstly performed using monomeric and oligomeric forms of Aβ to establish optimal conditions, such as concentration, to use in a further assay with CGA and LMW. Through the results (Figure 36) it is possible to observe that there is extensive cell death in ABO and vehicle treated cells at different concentrations. Aβ monomers are dissolved in DMSO, because neurotoxicity of the controls is similar to that of the monomers, the death of the neurons may be due to the effect of DMSO, which may be toxic to cells causing their death. Cell treatment with CGA, with and without Aβ, resulted in non-viable neuronal cells. However, the wells where CGA was added changed its colour to dark green, compromising this assay.

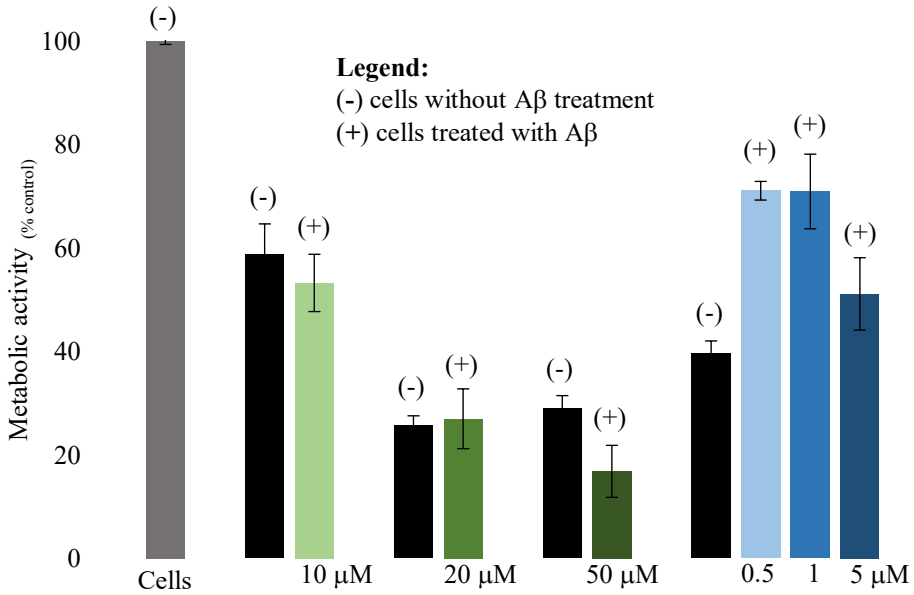

Figure 36: Metabolic activity of neuronal cells treated with different concentrations of Aβ monomers (green) and oligomers (blue), measured by resazurin assay. A control (black) was made for Aβ

monomers and oligomers, where only the vehicle in which Aβ was dissolved was added. Metabolic activity was normalized to the cells without treatment (grey).

Considering these results, it was decided to exclude the monomers from the following trials and continue only with Aβ oligomers (AβO). AβO oligomers were tested again in the same concentrations and with the corresponding control. To better understand what may have caused the colour change reaction, CGA was tested once more but without AβO stimulus. LR LMW without AβO were also tested (Figure 37).

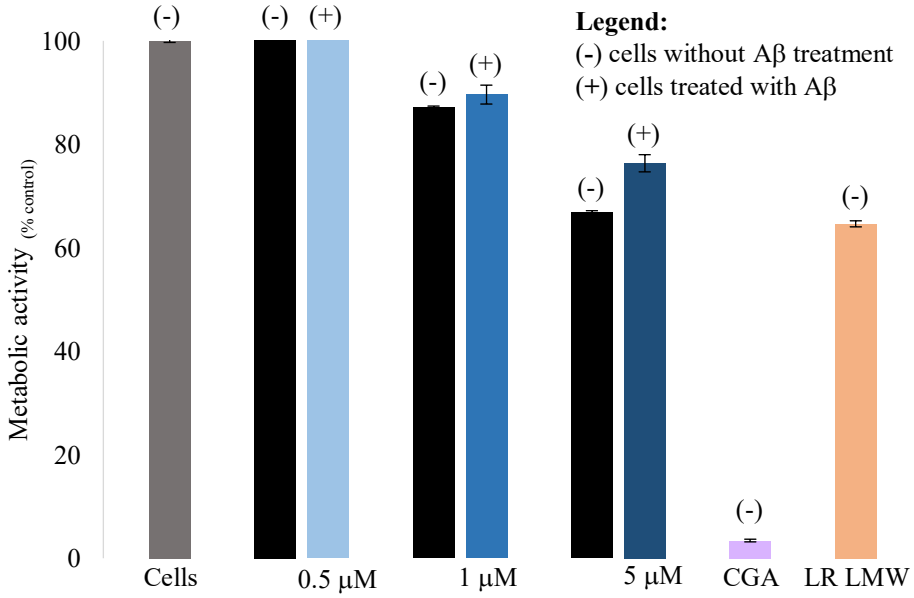

Figure 37: Metabolic activity of neuronal cells with different concentrations of Aβ oligomers and different treatments (Aβ, CGA and LR LMW), measured by resazurin assay. A control (black) was made for each of the tested Aβ concentrations, where only the vehicle in which the oligomers were dissolved was added. Metabolic activity was normalized to the cells without treatment (grey). (LR – Light roast)

When comparing cells treated with AβO, at different concentrations, and their respective control the results are very similar and close to the ones obtained for cells without treatment, which leads to the conclusion that, contrary to what was expected, AβO did not induce cell death in any of the tested concentration.

Once again, while carrying out the resazurin test, and before the addition of resazurin, it was possible to observe that the wells that contained CGA acquired a dark green colour and the assay revealed extensive cell death. The acquired green colour may be due to the interaction of CGA with amino acids present in the culture medium, which, when together, react giving rise to another compound of such colour.[93] Even though LMW contains

compounds such as CGAs, there was not a colour change and LR LMW only slightly declined cell viability.

To overcome the issue of colour formation of chlorogenic acids with medium rich in amino acids, another strategy was designed using Hoechst known to stain the DNA in the cell nucleus, which allows to count living and dead cells. Cells strictly control their number not just trough regulation of cell division but also by regulation of cell death rate. Cells that are no longer required start an intracellular death programme, called programmed cell death or apoptosis. Injury-related cell death usually manifests as swelling and bursting, a process known as cell necrosis, which might result in an adverse inflammatory reaction. However, an apoptotic cell suffers condensation and shrinking. The nuclear envelope disintegrates and the nuclear DNA fragments.[94] Thus, in the images obtained from the Hoechst assay, cell death by apoptosis can be identified by their smaller size or fragmented nucleus and a higher fluorescence intensity (Figure 38). Living cells are larger in size and with a more diffuse fluorescence in the nucleus.

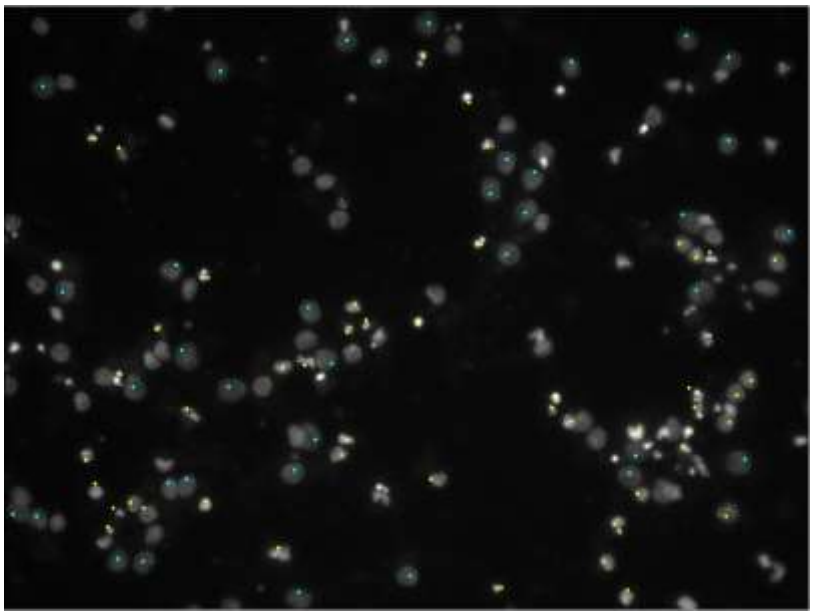

Figure 38: Cells without treatment obtained by microscopy. Live cells are identified with blue markers and dead cells are identified with yellow markers.

This analysis was used in cells treated with different compounds (Aβ, CGA or LMW) and results are presented in Figure 39. Results were normalized to vehicle-treated cells (control AβO). The treatment with only AβO decreased cell viability to 70 %, similar results occurred in cells treated with CGA + AβO. These results indicate that CGA did not attenuate AβO-induce cell death. Treatment with CGA alone showed higher viability (85 %) than when in

presence of AβO alone. LR LMW reduced cell viability in 50 % alone and with LR LMW + AβO the decrease in viability was 38 %. These results indicate that LR LMW did not have a neuroprotective effect.

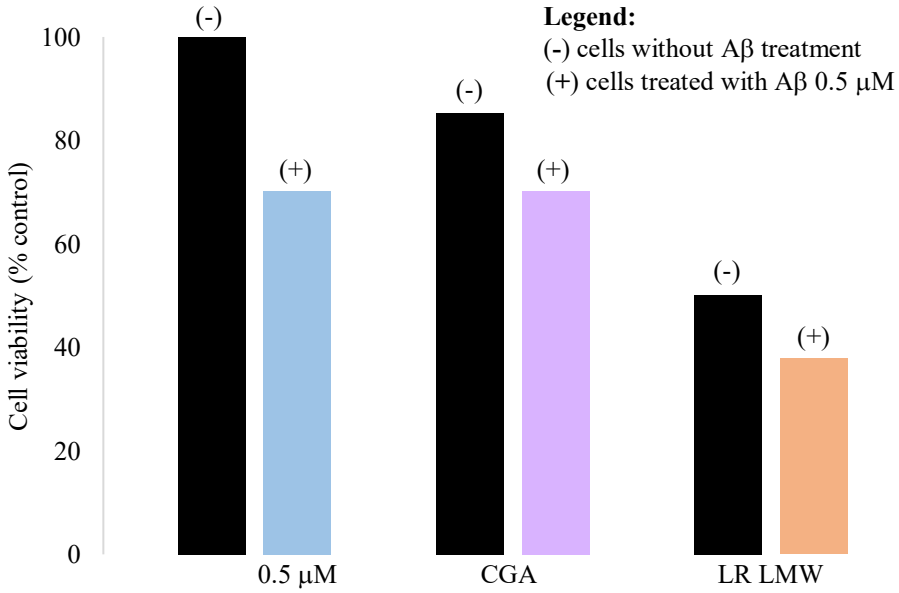

Figure 39: Cell viability of neuronal cells with different treatments (Aβ, CGA and LR LMW). Cell viability is given by the ratio between live and total cells and normalized to the control of Aβ 0.5 µM (vehicle-treated cells). (LR – Light roast)

4. Conclusions and future work

The objective of this work was to understand if CGAs adsorbed to melanoidins could prevent the aggregation of Aβ 1-42, that represents a hallmark of AD, and if different roasting degrees could lead to different neuroprotective activities. For such, compounds that constitute the coffee brew were fractionated through dialysis, obtaining HMWM and LMWM. HPLC analysis confirmed that the 2nd and 3rd dialysis water are the richest in CGAs (10 % w/w) and so LMW 2nd water was further fractionated by SPE to obtain fractions rich in CGAs. Antioxidant potential of the different coffee fractions was done using DPPH assay. Moreover, to evaluate neuroprotective potential a number of *in vitro* assays were performed, including Aβ peptide and neuronal cells assays.

In general, all samples showed good antioxidant activity (EC50 0.20 mg/mL), with exception of Et50 and Et75 fractions. Through the ThT assay which showed to be quite dependent on the buffer, PB without NaCl was chosen as the buffer with the highest affinity with Aβ 1-42. Of the samples tested, LMW and SPE 4 (rich in CGAs) showed the greatest potential to decrease Aβ 1-42 aggregation. In the resazurin assay, CGAs could not be tested due to their reaction with the amino acids present in media used causing interference with the measurement method. Thus, neurons viability using nucleus DNA Hoechst dye assay was performed using CGAs and LMW fractions. The results indicated that the presence of such compounds could not prevent cell death and therefore exert no neuroprotective effect. Samples such as LMW, which showed good antioxidant activity and potential to attenuate Aβ 1-42 aggregation by the ThT assay, did not seem to have neuroprotective effect on neuronal cells.

Either way, it was possible to conclude that the degree of roasting affected some properties of the samples tested, such as antioxidant activity of Et50 an Et75 and Aβ 1-42 aggregation followed by ThT, pinpointing the importance of coffee processing on their bioactive properties. In this work it was very hard to have reproducible results using ThT assay, which were attributed to the complexity of coffee extracts. Future work, should further address the effect of coffee low molecular compounds on neuroprotective mechanisms addressing to clarify their role, as well as exploring other melanoidins-rich food matrices rich in phenolic compounds, such as bread and beer, which are consumed regularly and can have an impact on neurological health.

References

1. Saito, M. Sustainable Coffee Production. in *Coffee: Growing, Processing, Sustainable Production* 384–390 (Wiley-VCH Verlag GmbH, 2008). doi:10.1002/9783527619627.ch13.
2. Carneiro, S. M., Oliveira, M. B. P. P. & Alves, R. C. Neuroprotective properties of coffee: An update. *Trends Food Sci. Technol.* **113**, 167–179 (2021).
3. Farah, A. Coffee Consumption and Health Implications. in *Coffee* P001–P004 (Royal Society of Chemistry, 2019). doi:10.1039/9781788015028-FP001.
4. de Melo Pereira, G. V. *et al.* Chemical composition and health properties of coffee and coffee by-products. in *Advances in Food and Nutrition Research* vol. 91 65–96 (Elsevier Inc., 2020).
5. Lee, K. H., Cha, M. & Lee, B. H. Neuroprotective Effect of Antioxidants in the Brain. *Int. J. Mol. Sci.* **21**, 7152 (2020).
6. Fullan, M. *Chapter 5 - Chemical Constituents of Coffee*. (2010). doi:10.1007/978-3-030-54437-9.
7. Lopes, G. R. *et al.* Carbohydrate content, dietary fibre and melanoidins: Composition of espresso from single-dose coffee capsules. *Food Res. Int.* **89**, 989–996 (2016).
8. Silva, J. A., Borges, N., Santos, A. & Alves, A. Method Validation for Cafestol and Kahweol Quantification in Coffee Brews by HPLC-DAD. *Food Anal. Methods* **5**, 1404–1410 (2012).
9. Martins, V. D. C. *et al.* Fraud investigation in commercial coffee by chromatography. *Food Qual. Saf.* **2**, 121–133 (2018).
10. Moreira, A. S. P., Nunes, F. M., Domingues, M. R. & Coimbra, M. A. Coffee melanoidins: Structures, mechanisms of formation and potential health impacts. *Food Funct.* **3**, 903–915 (2012).
11. Zhang, Q., Ames, J. M., Smith, R. D., Baynes, J. W. & Metz, T. O. A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry: Probing the Pathogenesis of Chronic Disease. *J. Proteome Res.* **8**, 754–769 (2009).
12. Oosterveld, A., Voragen, A. G. J. & Schols, H. A. Effect of roasting on the carbohydrate composition of Coffea arabica beans. *Carbohydr. Polym.* **54**, 183–192 (2003).

13. Redgwell, R. J., Curti, D. & Fischer, M. Effect of roasting on degradation and structural features of polysaccharides in Arabica coffee beans. **337**, 421–431 (2002).
14. Lopes, G. R. Comprehensive study of coffee infusions composition targeting espresso properties. (2019).
15. Arya, M. & Rao, L. J. M. An Impression of Coffee Carbohydrates. *Crit. Rev. Food Sci. Nutr.* **47**, 51–67 (2007).
16. Vignoli, J. A., Viegas, M. C., Bassoli, D. G. & Benassi, M. de T. Roasting process affects differently the bioactive compounds and the antioxidant activity of arabica and robusta coffees. *Food Res. Int.* **61**, 279–285 (2014).
17. Mussatto, S. I., Machado, E. M. S., Martins, S. & Teixeira, J. A. Production, Composition, and Application of Coffee and Its Industrial Residues. *Food Bioprocess Technol.* **4**, 661–672 (2011).
18. Moreira, A. S. P. *et al.* Transglycosylation reactions, a main mechanism of phenolics incorporation in coffee melanoidins: Inhibition by Maillard reaction. *Food Chem.* **227**, 422–431 (2017).
19. Ripper, B., Kaiser, C. R. & Perrone, D. Use of NMR techniques to investigate the changes on the chemical composition of coffee melanoidins. *J. Food Compos. Anal.* **87**, 103399 (2020).
20. De Marco, L. M., Fischer, S. & Henle, T. High Molecular Weight Coffee Melanoidins Are Inhibitors for Matrix Metalloproteases. *J. Agric. Food Chem.* **59**, 11417–11423 (2011).
21. Wang, H., Qian, H. & Yao, W. Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chem.* **128**, 573–584 (2011).
22. Farah, A. *2 Coffee Constituents*. (Blackwell Publishing Ltd., 2012).
23. Coelho, C. *et al.* Nature of Phenolic Compounds in Coffee Melanoidins. *J. Agric. Food Chem.* **62**, 7843–7853 (2014).
24. Martins, S. I. F. S. *Unravelling the Maillard reaction network by multiresponse kinetic modelling*. *Hoogleraar in Productontwerpen en Kwaliteitskunde* vol. PhD (2003).
25. Augustine, D. A. & Bent, G.-A. Reducing Acrylamide Exposure: A Review of the Application of Sulfur-Containing Compounds - A Caribbean Outlook. *Eur. J. Nutr. Food Saf.* **9**, 192–209 (2019).

26. Pérez-Burillo, S., Rajakaruna, S., Pastoriza, S., Paliy, O. & Ángel Rufián-Henares, J. Bioactivity of food melanoidins is mediated by gut microbiota. *Food Chem.* **316**, 126309 (2020).
27. Tagliazucchi, D. & Bellesia, A. The gastro-intestinal tract as the major site of biological action of dietary melanoidins. *Amino Acids* **47**, 1077–1089 (2015).
28. Rufián-Henares, J. A. & Morales, F. J. Effect of in Vitro Enzymatic Digestion on Antioxidant Activity of Coffee Melanoidins and Fractions. *J. Agric. Food Chem.* **55**, 10016–10021 (2007).
29. Zhang, H., Zhang, H., Troise, A. D. & Fogliano, V. Melanoidins from Coffee, Cocoa, and Bread Are Able to Scavenge α-Dicarbonyl Compounds under Simulated Physiological Conditions. *J. Agric. Food Chem.* **67**, 10921–10929 (2019).
30. Nunes, Fernando M.; Reis, Ana; Domingues, M. Rosário M.; Coimbra, M. A. Characterization of Galactomannan Derivatives in Roasted Coffee Beverages. 3428–3439 (2006) doi:10.1021/jf052971+.
31. Nunes, F. M. & Coimbra, M. A. Chemical Characterization of the High Molecular Weight Material Extracted with Hot Water from Green and Roasted Arabica Coffee. *J. Agric. Food Chem.* **49**, 1773–1782 (2001).
32. Hwang, C., Chen, C. & Ho, C. Contribution of coffee proteins to roasted coffee volatiles in a model system. *Int. J. Food Sci. Technol.* **47**, 2117–2126 (2012).
33. Liang, N. & Kitts, D. Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions. *Nutrients* **8**, 16 (2015).
34. Monente, C., Ludwig, I. A., Irigoyen, A., De Peña, M. P. & Cid, C. Assessment of total (Free and Bound) phenolic compounds in spent coffee extracts. *J. Agric. Food Chem.* **63**, 4327–4334 (2015).
35. Moreira, A. S. P. *et al.* Data on coffee composition and mass spectrometry analysis of mixtures of coffee related carbohydrates, phenolic compounds and peptides. *Data Br.* **13**, 145–161 (2017).
36. Wu, L., Sun, D. & He, Y. Coffee intake and the incident risk of cognitive disorders: A dose–response meta-analysis of nine prospective cohort studies. *Clin. Nutr.* **36**, 730–736 (2017).
37. Gelber, R. P., Petrovitch, H., Masaki, K. H., Ross, G. W. & White, L. R. Coffee intake in midlife and risk of dementia and its neuropathologic correlates. *J.*

Alzheimer's Dis. **23**, 607–615 (2011).

38. Haller, S., Montandon, M. L., Rodriguez, C., Herrmann, F. R. & Giannakopoulos, P. Impact of coffee, wine, and chocolate consumption on cognitive outcome and MRI parameters in old age. *Nutrients* **10**, 1–13 (2018).
39. Ding, M. *et al.* Association of Coffee Consumption With Total and Cause-Specific Mortality in 3 Large Prospective Cohorts. *Circulation* **132**, 2305–2315 (2015).
40. Cho, B.-H., Choi, S.-M., Kim, J.-T. & Kim, B. C. Association of coffee consumption and non-motor symptoms in drug-naïve, early-stage Parkinson's disease. *Parkinsonism Relat. Disord.* **50**, 42–47 (2018).
41. Kim, J. W. *et al.* Coffee intake and decreased amyloid pathology in human brain. *Transl. Psychiatry* **9**, 270 (2019).
42. Beghi, E. *et al.* Coffee and Amyotrophic Lateral Sclerosis: A Possible Preventive Role. *Am. J. Epidemiol.* **174**, 1002–1008 (2011).
43. Kim, J. *et al.* Caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid up-regulate NQO1 expression and prevent H2O2-induced apoptosis in primary cortical neurons. *Neurochem. Int.* **60**, 466–474 (2012).
44. Twarda-Clapa, A., Olczak, A., Białkowska, A. M. & Koziołkiewicz, M. Advanced Glycation End-Products (AGEs): Formation, Chemistry, Classification, Receptors, and Diseases Related to AGEs. *Cells* **11**, 1312 (2022).
45. Rungratanawanich, W., Qu, Y., Wang, X., Essa, M. M. & Song, B.-J. Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. *Exp. Mol. Med.* **53**, 168–188 (2021).
46. Sirangelo, I. & Iannuzzi, C. Understanding the role of protein glycation in the amyloid aggregation process. *Int. J. Mol. Sci.* **22**, (2021).
47. Neurodegenerative Diseases. https://www.niehs.nih.gov/research/supported/health/neurodegenerative/index.cfm.
48. Perlikowska, R. Whether short peptides are good candidates for future neuroprotective therapeutics? *Peptides* **140**, 170528 (2021).
49. Kovacs, G. G. Concepts and classification of neurodegenerative diseases. in *Neuropathology* vol. 145 301–307 (Elsevier B.V., 2018).
50. Kim, G. H., Kim, J. E., Rhie, S. J. & Yoon, S. The Role of Oxidative Stress in Neurodegenerative Diseases. *Exp. Neurobiol.* **24**, 325–340 (2015).

51. Bianchi, V. E., Herrera, P. F. & Laura, R. Effect of nutrition on neurodegenerative diseases. A systematic review. *Nutr. Neurosci.* **24**, 810–834 (2021).
52. Esang, M. & Gupta, M. Aducanumab as a Novel Treatment for Alzheimer's Disease: A Decade of Hope, Controversies, and the Future. *Cureus* **13**, 10–13 (2021).
53. Tiwari, S., Atluri, V., Kaushik, A., Yndart, A. & Nair, M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. *Int. J. Nanomedicine* **Volume 14**, 5541–5554 (2019).
54. Malafaia, D., Albuquerque, H. M. T. & Silva, A. M. S. Amyloid-β and tau aggregation dual-inhibitors: A synthetic and structure-activity relationship focused review. *Eur. J. Med. Chem.* **214**, 113209 (2021).
55. Coronel, R. *et al.* Role of Amyloid Precursor Protein (APP) and Its Derivatives in the Biology and Cell Fate Specification of Neural Stem Cells. *Mol. Neurobiol.* **55**, 7107–7117 (2018).
56. Milà-Alomà, M., Suárez-Calvet, M. & Molinuevo, J. L. Latest advances in cerebrospinal fluid and blood biomarkers of Alzheimer's disease. *Ther. Adv. Neurol. Disord.* **12**, 175628641988881 (2019).
57. Grodzicki, W. & Dziendzikowska, K. The Role of Selected Bioactive Compounds in the Prevention of Alzheimer's Disease. *Antioxidants* **9**, 229 (2020).
58. How Is Alzheimer's Disease Treated? | National Institute on Aging. https://www.nia.nih.gov/health/how-alzheimers-disease-treated.
59. Alzheimer's dementia | Alzheimer Europe. https://www.alzheimer-europe.org/dementia/alzheimers-dementia.
60. Li, R., Zhang, Y., Rasool, S., Geetha, T. & Babu, J. R. Effects and Underlying Mechanisms of Bioactive Compounds on Type 2 Diabetes Mellitus and Alzheimer's Disease. *Oxid. Med. Cell. Longev.* **2019**, 1–25 (2019).
61. Reinke, A. A. & Gestwicki, J. E. Structure-activity Relationships of Amyloid Beta-aggregation Inhibitors Based on Curcumin: Influence of Linker Length and Flexibility. *Chem. Biol. Drug Des.* **70**, 206–215 (2007).
62. Zhang, H. *et al.* Fabrication of genistein-loaded biodegradable TPGS-b-PCL nanoparticles for improved therapeutic effects in cervical cancer cells. *Int. J. Nanomedicine* **10**, 2461–2473 (2015).

63. Trollope, L. *et al.* Inclusion of trans -resveratrol in methylated cyclodextrins: synthesis and solid-state structures. *Beilstein J. Org. Chem.* **10**, 3136–3151 (2014).
64. Claude Elama, Mohammed Tarawa & Fuad Al-Rimawi. Oleuropein from Olive Leaf Extract as Natural Antioxidant of Frozen Hamburger. *J. Food Sci. Eng.* **7**, (2017).
65. Owais, M. *et al.* Chemotherapeutic potential of curcumin-bearing microcells against hepatocellular carcinoma in model animals. *Int. J. Nanomedicine* **9**, 1139 (2014).
66. Farias-Pereira, R., Young, L. & Park, Y. Neuroprotective effects of green coffee bean extract against Alzheimer's and Parkinson's disease: a mini review. *Food Life* (2020) doi:10.5851/fl.2020.e11.
67. Ciaramelli, C. *et al.* NMR-driven identification of anti-amyloidogenic compounds in green and roasted coffee extracts. *Food Chem.* **252**, 171–180 (2018).
68. Liu, Q. F. *et al.* Nardostachys jatamansi Ethanol Extract Ameliorates A β 42 Cytotoxicity. **41**, 470–477 (2018).
69. Gao, L. *et al.* Chlorogenic Acid Alleviates Aβ25-35-Induced Autophagy and Cognitive Impairment via the mTOR/TFEB Signaling Pathway. *Drug Des. Devel. Ther.* **Volume 14**, 1705–1716 (2020).
70. Wei, M. *et al.* Protective effects of a Chotosan Fraction and its active components on β-amyloid-induced neurotoxicity. *Neurosci. Lett.* **617**, 143–149 (2016).
71. Kwon, S. *et al.* Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. *Eur. J. Pharmacol.* **649**, 210–217 (2010).
72. Wang, X. *et al.* Chlorogenic acid protects against aluminium-induced cytotoxicity through chelation and antioxidant actions in primary hippocampal neuronal cells. *Food Funct.* **8**, 2924–2934 (2017).
73. Saitou, K. *et al.* Effect of Chlorogenic Acids on Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Trial. *Nutrients* **10**, 1337 (2018).
74. Kato, M., Ochiai, R., Kozuma, K., Sato, H. & Katsuragi, Y. Effect of Chlorogenic Acid Intake on Cognitive Function in the Elderly: A Pilot Study. *Evidence-Based Complement. Altern. Med.* **2018**, 1–8 (2018).
75. Ishida, K. *et al.* Coffee polyphenols prevent cognitive dysfunction and suppress amyloid β plaques in APP/PS2 transgenic mouse. *Neurosci. Res.* **154**, 35–44 (2020).

76. Goals & Benefits of Solid-Phase Extraction (SPE) | Waters. https://www.waters.com/nextgen/gb/en/education/primers/beginner-s-guide-to-spe/goals-and-benefits-of-spe.html.
77. What is HPLC (High Performance Liquid Chromatography) ? : SHIMADZU (Shimadzu Corporation). https://www.shimadzu.com/an/service-support/technical-support/analysis-basics/basic/what_is_hplc.html#1.
78. Hatzakis, E. Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **18**, 189–220 (2019).
79. What is FTIR Spectroscopy? https://www.sigmaaldrich.com/PT/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ftir-spectroscopy.
80. Xiao, F., Xu, T., Lu, B. & Liu, R. Guidelines for antioxidant assays for food components. *Food Front.* **1**, 60–69 (2020).
81. Scanning Electron Microscopy - SEM - Advancing Materials. https://www.thermofisher.com/blog/materials/what-is-sem-scanning-electron-microscopy-explained/.
82. Rovnyagina, N. R. *et al.* Fluorescence lifetime and intensity of thioflavin T as reporters of different fibrillation stages: Insights obtained from fluorescence up-conversion and particle size distribution measurements. *Int. J. Mol. Sci.* **21**, 1–16 (2020).
83. Almeida, R. D. *et al.* Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ.* **12**, 1329–1343 (2005).
84. Resazurin Assay Kit (Cell Viability) (ab129732) | Abcam. https://www.abcam.com/resazurin-assay-kit-cell-viability-ab129732.html.
85. Passos, C. P. *et al.* Role of coffee caffeine and chlorogenic acids adsorption to polysaccharides with impact on brew immunomodulation effects. *Foods* **10**, (2021).
86. Coreta-Gomes, F. M. *et al.* In Vitro Hypocholesterolemic Effect of Coffee Compounds. *Nutrients* **12**, 437 (2020).
87. Tajik, N., Tajik, M., Mack, I. & Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. *Eur. J. Nutr.* **56**, 2215–2244 (2017).

88. Bekedam, E. K., Roos, E., Schols, H. A., Van Boekel, M. A. J. S. & Smit, G. Low molecular weight melanoidins in coffee brew. *J. Agric. Food Chem.* **56**, 4060–4067 (2008).
89. Ripper, B., Kaiser, C. R. & Perrone, D. Use of NMR techniques to investigate the changes on the chemical composition of coffee melanoidins. *J. Food Compos. Anal.* **87**, 103399 (2020).
90. Barbin, D. F., Felicio, A. L. de S. M., Sun, D. W., Nixdorf, S. L. & Hirooka, E. Y. Application of infrared spectral techniques on quality and compositional attributes of coffee: An overview. *Food Res. Int.* **61**, 23–32 (2014).
91. Lyman, D. J., Benck, R., Dell, S., Merle, S. & Murray-Wijelath, J. FTIR-ATR analysis of brewed coffee: Effect of roasting conditions. *J. Agric. Food Chem.* **51**, 3268–3272 (2003).
92. Batzli, K. M. & Love, B. J. Agitation of amyloid proteins to speed aggregation measured by ThT fluorescence: A call for standardization. *Mater. Sci. Eng. C* **48**, 359–364 (2015).
93. Yabuta, Goro; Koizumi, Yukimichi; Namiki, Kazuko; Hida, Mitsuhiko; Namiki, M. Structure of Green Pigment Formed by the Reaction of Caffeic Acid Esters (or Chlorogenic acid) with a Primary Amino Compound. (2001).
94. Alberts, B. *et al. Molecular Biology of the Cell*. (Garland Science, 2002).

5. Annexes

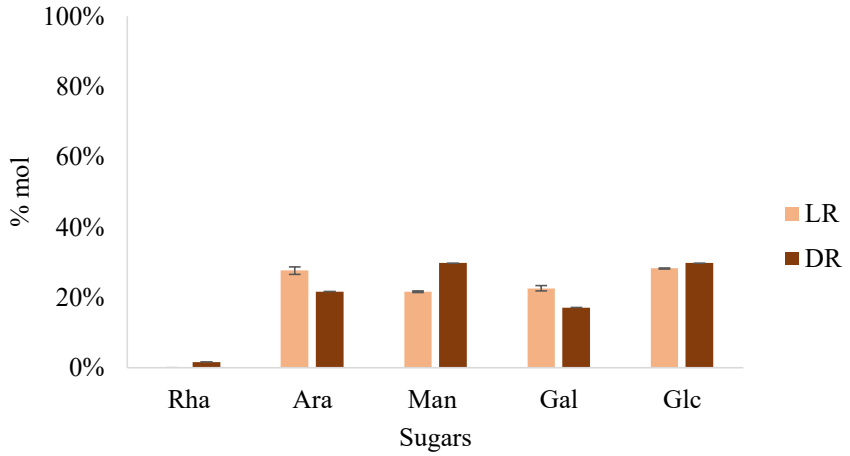

Figure 1: Sugar (% mol) and total sugars (% w/w) composition in LMW material collected from dialysis (first collected water). (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; DR – Dark roast)

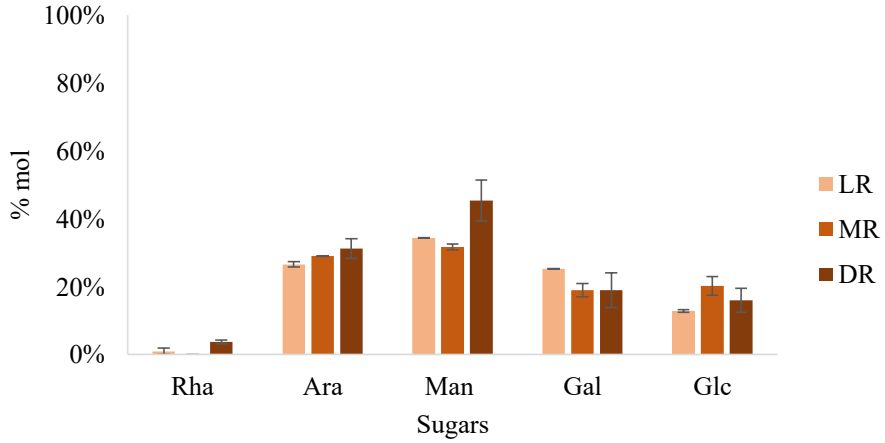

Figure 2: Sugar (% mol) and total sugars (% w/w) composition in LMW material collected from dialysis (first collected water). (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

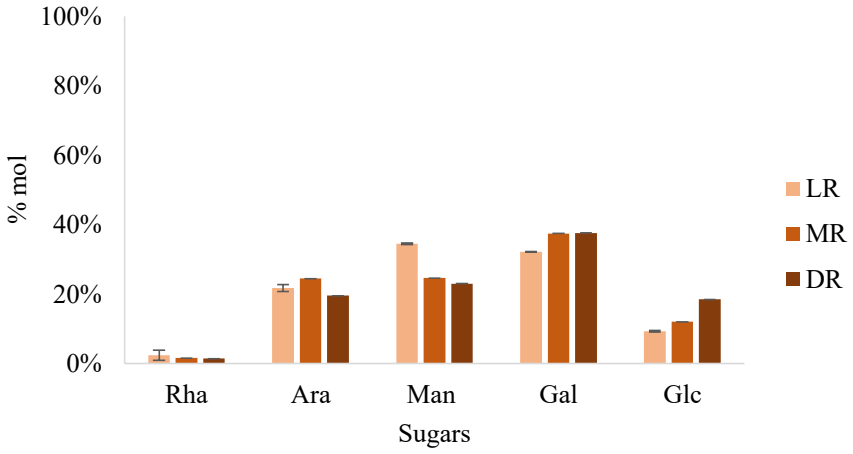

Figure 3: Sugar (% mol) and total sugars (% w/w) composition in LMW material collected from dialysis (first collected water). (Rha – Rhamnose; Ara – Arabinose; Man – Mannose; Gal – Galactose; Glc – Glucose; LR – Light roast; MR – Medium roast; DR – Dark roast)

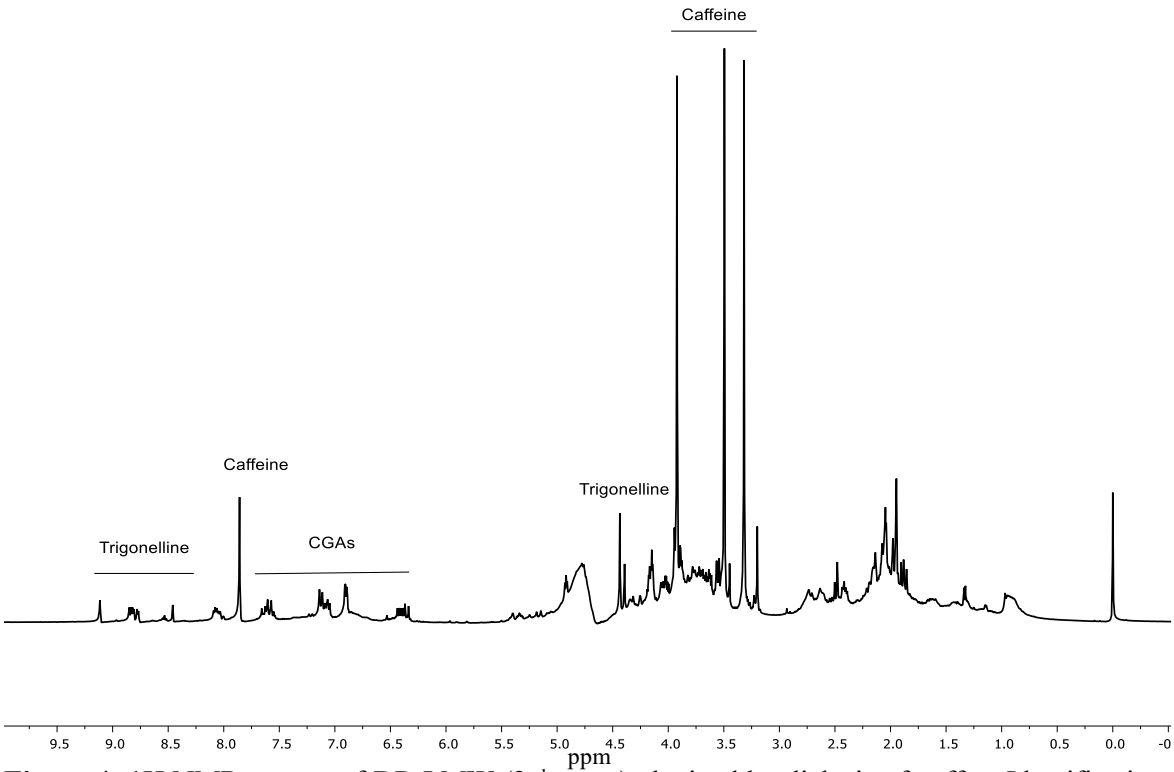

Figure 4: 1H NMR spectra of DR LMW (2nd water) obtained by dialysis of coffee. Identifications of compounds were made according to literature.[67]

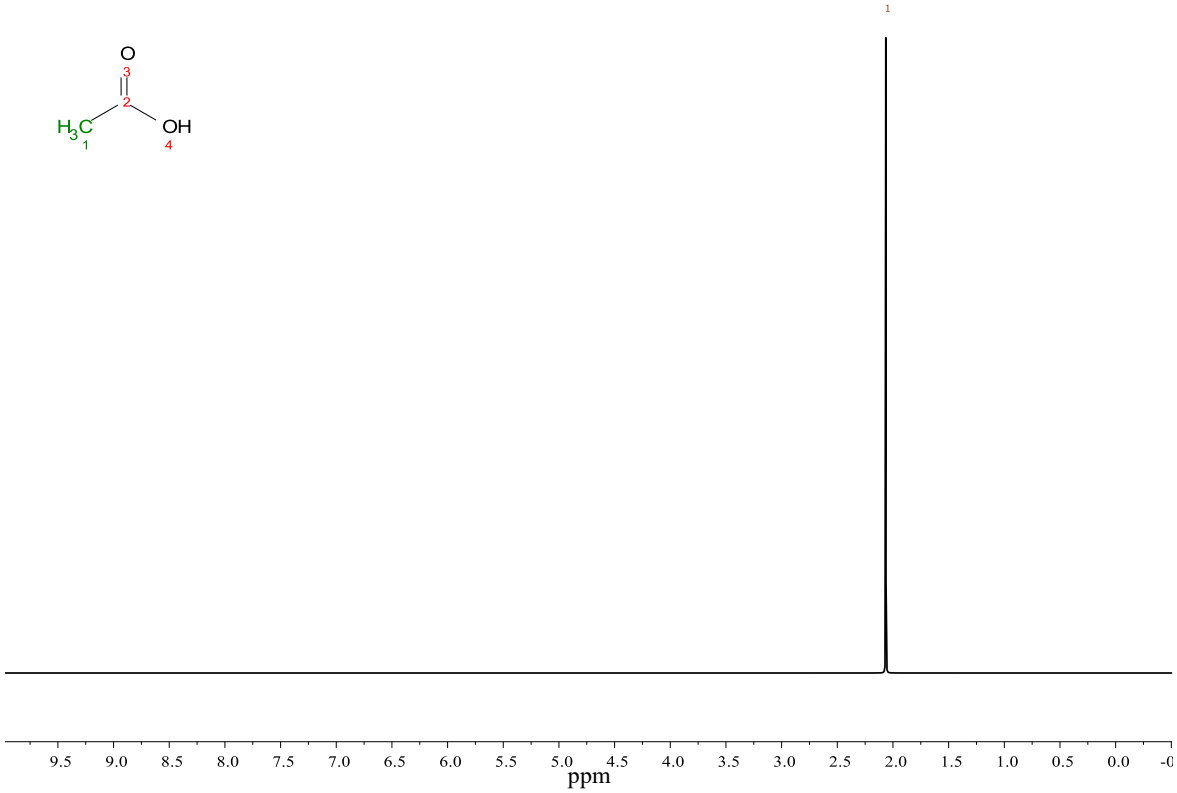

Figure 5: Predicted 1H NMR spectra of acetic acid.

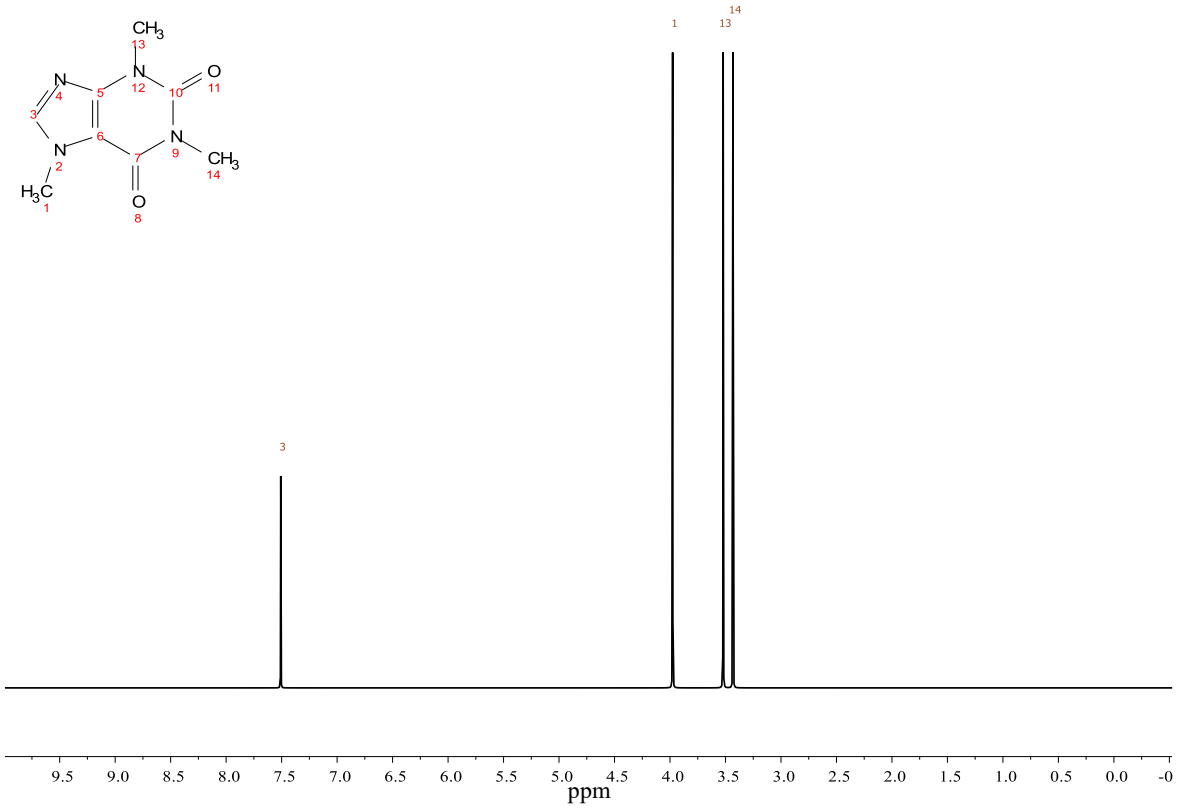

Figure 6: Predicted 1H NMR spectra of caffeine.

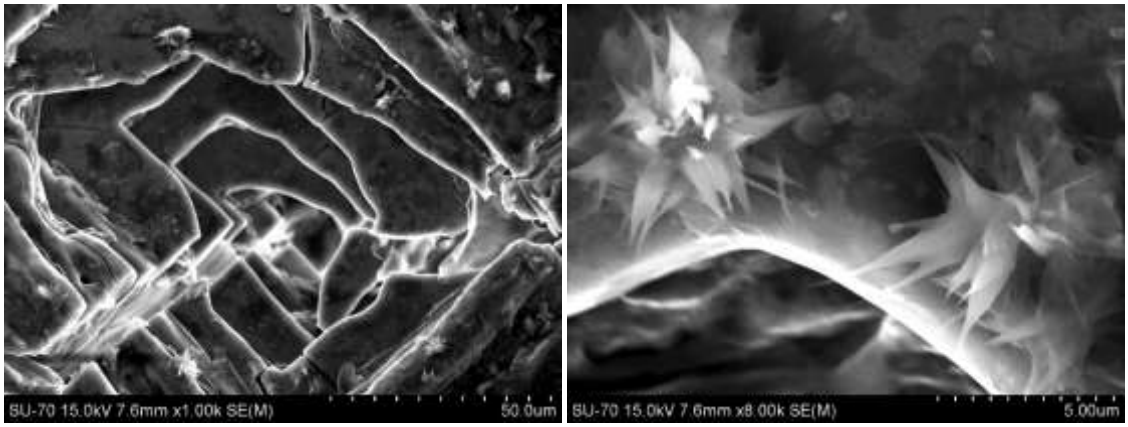

Figure 7: Images of the surface of Aβ in Tris-HCl buffer with LR coffee (40 μg/mL) taken by SEM.

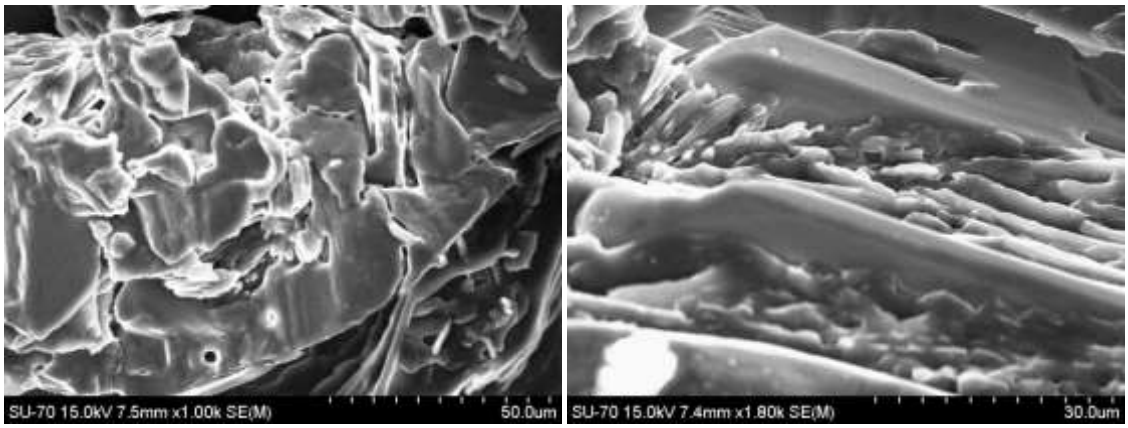

Figure 8: Images of the surface of Aβ in Tris-HCl buffer with DR coffee (40 μg/mL) taken by SEM.

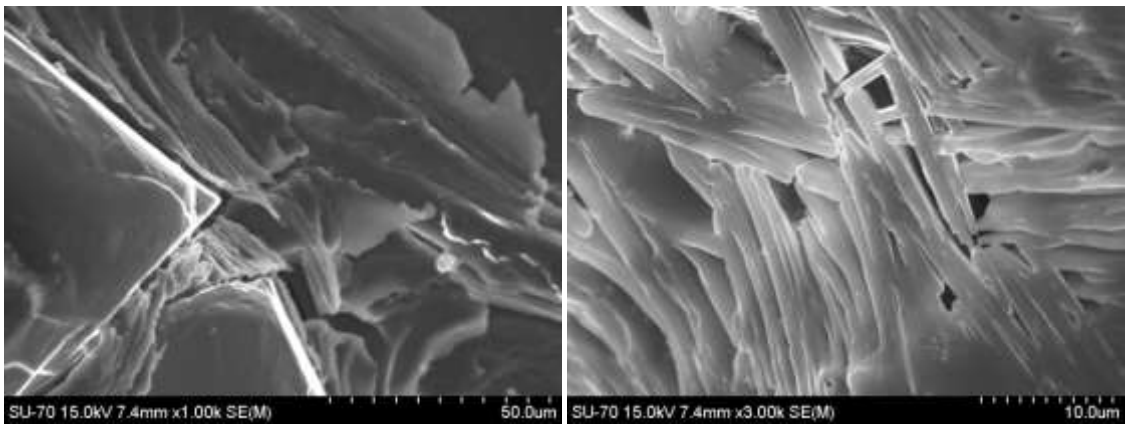

Figure 9: Images of the surface of Aβ in Tris-HCl buffer with CGA (40 μg/mL) taken by SEM.