Analysis of Coffee-Herbal Beverages
For Potential Benefits Against
Dementia Diseases

A thesis submitted in fulfilment of the requirements for the degree of
Master of Science by Research

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To my wife Mao YE

For her on-going support and sacrifices,

I dedicate my research to you
DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the candidate alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged and ethics procedures and guidelines have been followed.

Tao Yu

1st May 2017
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**ABSTRACT**

The research strategy is focused on the potential prevention and treatment of cognitive impairment by recommending the consumption of herbal-coffee beverages as early as possible in life. General methods applicable for the analysis and extraction that are suitable for the preparation of a range of herbal-coffee beverages have been found. The herb-bioactive compounds are effectively extracted simply by using a coffee machine or microwave heating. These herbal-coffee beverages may provide significant neuro-protection against dementia and Alzheimer’s disease, including the development of the first examples containing ginger, liquorice and ginseng in coffee brews. Caffeine, caffeic acid and Vitamin B1 combined with HEWL to strongly inhibit ThT fluorescence. A small study of the perception and sensory responses to the new beverages was undertaken.
JOURNAL PUBLICATIONS ARISING FROM THIS THESIS

Hügel HM, Yu T and Jackson N, The Effects of Coffee Consumption on Cognition and Dementia Diseases, Gerontology & Geriatric Research 2015(4): 1-6

PRESENTATIONS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid β peptides</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholine cholinesterase,</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyryl-cholinesterase</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical Ionisation</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>GA</td>
<td>Glycyrrhizic acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Rb1</td>
<td>Ginsenoside Rb1</td>
</tr>
<tr>
<td>Rg1</td>
<td>Ginsenoside Rg1</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionization</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization deection</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>KI</td>
<td>Kováts index</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>MAE</td>
<td>Microwave-assisted extraction.</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MQ water</td>
<td>Milli-Q water</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NMDA Receptor</td>
<td>N-methyl D-aspartate Receptor</td>
</tr>
<tr>
<td>NPD</td>
<td>Nitrogen–phosphorus</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PMAE</td>
<td>Pressurized microwave-assisted extraction</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>SFMAE</td>
<td>Solvent-free microwave-assisted extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>RPC</td>
<td>Reversed-phase chromatography</td>
</tr>
<tr>
<td>RSD%</td>
<td>The percent of Relative standard deviation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to Nosie ratio</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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1

Introduction

“The more important reason is that the research itself provides an important long-run perspective on the issues that we face on a day-to-day basis.”

Ben Bernanke

1.1 Project Background

By 2050 it is estimated that the number of people over the age of 80 will triple globally and cognitive decline and dementia could become the greatest challenge of our times. According to the 2003 World Health Estimates Report, dementia inflicts 11.2% elderly people (>60 years) more than stroke, cardiovascular disease and various cancers [1]. Dementia is a collection of different disorders that influence brain function such as progressive memory loss, judgment decline and other symptoms; Alzheimer’s disease (AD) is the most common type of memory disorder and contributes to between 50% and 70% of all types of dementia [2]. From neuroimaging, AD advances in three stages: an early and preclinical stage with no symptoms; a middle stage of mild cognitive impairment (MCI); and a final stage of AD. [3] [Fig.1.1 (a)] The pathological process of AD can have a long development time even to 40-50 years (including the early and MCI periods) [4].

MCI is defined as the clinical condition of memory between normal aging and AD; in other words, patients with MCI incur more memory loss problems than healthy people of a similar age, however they do not yet have the cognitive function issues and criteria for AD [5]. According to epidemiological studies, elderly people have been classified as the highest risk of population to suffer from AD [6]. For example, the many memory complaints elderly people have include, having trouble remembering friends names they meet often, having a greater probability to misplace things
yet have normal judgment and thinking skills. These people can suffer from MCI for extended periods of time. They are at high risk (approximately 80%) of accelerating to a dementia disorder such as AD (mostly in 6 years); however their dementia may be slowed even stabilised after satisfactory therapeutic and lifestyle interventions [7]. Therefore, aging associated memory impairment is a major risk factor for the general degradation of memory that can progress from MCI [8,9] symptoms to AD [10]. Although it is difficult to determine whether a person’s memory loss reflects MCI symptoms. Aging is the highest risk factor that necessitates medical treatment for AD for which there is no known cure.

**Fig.1.1** (a) The AD development stages (left). (b) Neuroimaging from a healthy person (the left of picture b) and a patient with Alzheimer’s disease (right) [Reproduced from references 3, 14]

MCI symptoms and dementia is most likely caused by amyloid fibrils neurodegeneration that affect brain cells to communicate neural information, resulting in memory loss and behaviour and feeling changes. Damage in the memory and judgement functional regions such as the temporal lobe and corpus callosum [Fig.1.1 (b)] is the cause of memory loss and one of the earliest symptoms of AD. Amyloid fibrils are aggregated by normally soluble amyloid protein and are found in a wide range of misfolded β-sheet amyloid protein resistant to neuro-degradation [11]. Research studies indicate the structure of fibrils are mainly composed of β-sheet structure in a characteristic cross-β conformation [12]. Therefore, amyloid β peptides (Aβ) are the main components of the amyloid plaques found in the brains of Alzheimer patients where it is deposited abnormally in senile plaques containing both
Aβ40 and Aβ42. Aβ is produced from the amyloid precursor protein (APP) by the sequential cleavage via β- and γ-secretases [13]. Currently there are no medications that can remove or affect the dissolution of toxic Aβ from neurons to improve cognitive ability.

Symptomatic treatments have been widely applied for AD since the mid-1990s. There are two types of medications, cholinesterase inhibitors (Donepezil, Rivastigmine, Galantamine) and NMDA receptor antagonist (Memantine), that are approved by the USA Food and Drug Administration to relieve the cognitive symptoms (such as memory loss, confusion) of mild, moderate and severe AD [14]. These drugs are based on sustaining the acetylcholine (ACh) neurotransmitter levels (galantamine and the physostigmine-derivative rivastigmine) [15] in AD patients with cognitive degeneration have cholinergic neurons deficits in due to a decline in the levels of ACh in the hippocampus, temporal and parietal neocortex. ACh is hydrolytically destroyed by two cholinesterases, AChE and butyryl-cholinesterase (BuChE). As AD progresses, AChE activity can decrease by up to 67% of normal levels in the temporal lobe and hippocampus [16]. Thus, Cholinesterase inhibitors are commonly used for the treatment of symptoms of AD cognitive decline, but does not stop/prevent neuronal damage. NMDA glutamate receptors are composed of abundant glutamate-gated cation channels with high calcium permeability, which are distributed throughout the central nervous system (CNS). They have important roles in the process that learning, memory, and neuroplasticity. Some neurological disorders including cognitive disorders are caused by receptor over activation (excitotoxicity) and subsequent neurodegeneration [17,18]. Many AD patient’s memory loss and cognitive loss is due to prolonged Ca²⁺ excess and results in loss of synaptic function, followed by synaptotoxicity and ultimately cell death [19]. It is known that NMDA receptor inhibition and overactivation, leads to impairment of neuronal plasticity (learning) or cell death, and dysfunctional cognition [20]. Memantine is a low affinity, voltage-dependent and noncompetitive NMDA receptor antagonist, which has a higher affinity for Mg²⁺ and blocks Ca²⁺ influx. It has fast off-rate kinetics in the receptor channel, and so is only of benefit for dysfunctional synapses found in AD patients without affecting the physiological stimuli of the NMDA receptor [21].

Many AD patients have been recorded to have personality changes, behaviour and sleep difficulties even at the early stage. For example, the major behaviour alteration was apathy (72%), agitation (60%) and anxiety (48%), which significantly correlates to the cognitive deterioration caused by neuron cell damage in the brain [22]. Additionally, some medications may worsen these symptoms. Therefore, atypical antipsychotics such as risperidone, quetiapine, olanzapine, aripiprazole were utilized to treat the symptoms of short-term severe aggression in AD. However, these drugs have limited cognitive benefits, particularly risperidone that poses a significant risk for cerebrovascular
events [23, 24]. Currently, all antidepressants such as citalopram, sertraline are not licenced for AD treatment.

In summary, many researchers acknowledge that current Alzheimer’s drugs cannot cure AD. These drugs and non-drug clinical conditions may ameliorate cognitive and behavioural symptoms for improving the quality of life for some patients with AD.

A better strategy is to prevent AD onset or delay aging through the reduction of dementia risk factors; as the medications used to treat mild cognitive or dementia do not reverse the formation of ‘neurofibrillary tangles’. There are seven risk factors that have been associated with one in three Alzheimer’s disease cases. These include diabetes, midlife hypertension, midlife obesity, physical inactivity, smoking, depression, or low educational attainment [25,26]. These risk factors could promote AD with aging. The current lifestyle interventions preventing AD include better diets, brain games (Sudoku puzzles) and regular exercise. For example, research suggests that daily intake of fish with docosahexaenoic acid (DHA), and omega-3 fatty acid decreases the risk for AD [27]. Neurons and synapses are composed of long chain unsaturated fatty acids similar to omega-3; also lowering the dementia risk factors is related to the protection against cardiovascular disease [28].

Scientific research demonstrates that strategies such as exercise and diet, significantly delay or prevent aging cognitive decline or memory loss, many of which are based on small group and short-term studies. Obviously the dietary nutrition brain benefits are variable and it is common for people to take different kinds of foods containing vitamins, antioxidants and their effects are difficult to quantify. There is room for improvement, especially in generating personalised treatments, however the greatest advantage of dietary modifications is that they generally do not raise human safety issues.

The health claims of traditional herbal remedies, their effectiveness and safe use are growing rapidly. Functional foods are also popular for consumers who are seeking specific health effects to promote physical fitness. Recent research found that many herbal compounds can affect the mind, mood, cognitive function or health. For example, ginseng has been reported to improve short-term memory performance when consumed as a dietary supplement. *S. lavandulaefolia* Vahl. (Spanish sage) essential oil composition includes α-pinene (4%-7%), β-pinene (5%-12%), 1,8-cineole (15%-30%) and camphor (20%-30%), and it has been reported to improve accuracy for cognitive mental status examination after 6 week of use [29]. Certain herbs may be potentially important sources of
drug candidates against the onset of AD. For example, Zingiber officinale (ginger), Ginkgo biloba (ginkgo), Cinnamum cassia (Chinese cinnamon) extracts effectively protect primary neuronal cells against Aβ1-42 insult [30]. Huperzine A, originally isolated from Huperzia serrata, a type of moss used in traditional Indian and Chinese medicine and its derivative, ZT-1, is being developed as a new anti-AD drug for AD treatment. Yokukansan is composed of 7 herbs and is a useful formulation for the effective treatment of dementia symptoms in 106 patients diagnosed with AD that was safe to use [31].

The production of a functional beverage using safety tractional herbs and to encourage its long term consumption would provide a sound strategy for neuroprotection against dementia and be of general benefits for society. Firstly, these herbal effective compounds can be extracted into water via a convenient procedure; moreover, these ingredients can be analytically measured the amount consumed can be quality controlled, allowing the opportunity to monitor/measure the benefits over several years; the last but not the least factor is the functional beverage can be modified according to personal sensory perceptions and blended into daily consumption similar to coffee or tea intake.

1.2 Natural compound extraction methods and quality control

In order to quantify the herbal benefits for human health, the first important step is herb constituent extraction, separation, identification and quantitation/concentration of the herbal ingredients, as the bioactive compound concentration are product quality-related and also to herbal efficiency. These herbal bioactive substances can be classified into families of compounds including essential oils, alkaloids, steroids, saponins. Various classes of compounds have known methods for extraction for optimal pharmaceutical application [32]. For example, some bioactives found in plants are water soluble whilst others are hydrophobic/insoluble. Modern and traditional extraction and analysis techniques play an important role in food industry [33,34]. These techniques include decoction, percolation, Soxhlet extraction, solid phase micro-extraction, and microwave-assisted extraction, that are available in the lab or in domestic use. Chromatographic techniques have proven to be the most powerful and effective technology available for herbal analysis [35].

1.2.1 Decoction extraction

The usage of green extraction technology (also traditional extraction techniques) is able to protect the natural environment and minimizes harmful chemical affects on human and environmental
health. Traditional Chinese herbal teas and Turkish coffee are commonly prepared in this way. For decoction methods, the extractive yield is determined by time/temperature and the chemical nature of the herb. Botanic herbs such as stem and root from licorice, ginseng and ginger, are finely ground to enhance maximum herb extraction [36]. Water is biocompatible and an eco-friendly medium, which can be used to extract many bioactive constituents including alkaloids, flavonoids, saponins, vitamins and others [37]. The herbs to water ratio used ranges from 1:4 to 1:10. Depending on the consistency of the parts to be extracted, the decoction times of herbs generally decreases in the order: roots > stems > leaves and flowers, when boiled in water for 5 to 60 minutes [38,39]. During this time the evaporated water must be replaced. Essential oils are isolated by distillation of the volatile organic compounds.

1.2.2 Percolation extraction

Percolation is an extraction process whereby the slow passage of a solvent through fine substrate particles extracts constituents that pass through the filter into the collector. A coffee percolator is a good example of the percolation process, which was invented by Hanson Goodrich in 1889 [40]. Water is heated in the percolator chamber, passes through the vertical tube as steam-powered vacuum and is sprayed over the coffee grounds (that is continuously recycled through the grounds until complete) [Fig. 1.2(a)]. Extracted coffee was filtered into the bottom chamber. Percolation is a short and efficient extraction procedure that can be applied to the extraction of certain herbs [41]. As required, the coffee or herbs should be ground into powder form with a coffee blender or commercial grinder. Some procedures require a preparatory step, to allow crude herbal particles to swell to their maximum size as dense dry fine particles may block the filter mesh after swelling and resist solvent flow, giving rise to inefficient extraction [42].
1.2.3 Soxhlet Extraction

Soxhlet extraction was first described in 1879 by Franz von Soxhlet, who applied the apparatus to quantify fat in milk. It was then widely used for lipid extraction in agricultural chemistry [43]. The apparatus facilitates the separation of soluble compounds of interest from the insoluble solids with a solvent. The solvent reticulation enables continuous sample extraction from a few hours to days. Figure [Fig.1.2 (b)] illustrates the Soxhlet components of condenser, extractor and distillation flask. When the distillation flask is heated, the solvent vapour travels via the side-arm, liquefies on the condenser jacket and flow back through the thimble in the extraction chamber containing the crushed sample; the extractor chamber refills with the warm solvent slowly and extracts/dissolves the desired compounds from the crude material. The concentrated solvent returns to the distillation flask as the full chamber is emptied through the siphon arm. The fill-empty cycle is repeated until the extraction is deemed satisfactory [44]. The Soxhlet extractor has the advantage that it allows fresh recycled solvent to continuously extract the solid sample. The soluble compounds constantly dissolve in freshly condensed solvent and return to the extraction flask until all the soluble constituents are exhaustively extracted. The procedure operates automatically and is a gentle extraction process without agitation. The concentrated constituents are readily collected when completed [45]. This method was used for extraction of organic compounds from medicinal plant extracts including isoflavonoids from ginger [46], caffeine[47] from coffee and glycyrrhizin from liquorice [48]. Soxhlet extraction has been less used, as microwave-assisted extractions (MAE), supercritical fluid extraction (SFE), and pressurized solvent extraction (PSE) techniques are preferred. However, modified Soxhlet extractor systems such as high-pressure Soxhlet extraction, ultrasound-assisted Soxhlet extraction, microwave-assisted Soxhlet extraction have been developed [50]. These new systems employ auxiliary apparatus to decrease leaching time and improve the extraction yield.

1.2.4 Microwave-assisted extraction

Microwave-assisted extraction (MAE) [Fig.2 (c)] is an efficient extraction technique, which applies microwave energy to heat the solvent/sample matrix containing plant tissue or herbs, allowing these natural compounds to be rapidly extracted from raw materials [51]. The technique was first used in
the laboratory in 1975 by Abu-Samra, who conducted the trace analysis of metals in biological samples and has become a very cost effective method [52]. The technique utilizes microwaves, electromagnetic energy waves are formed from electric and magnetic fields, with the spectral window between X-rays and infrared frequency from 0.3 to 300 GHz [53]. In general, the alternating current of electromagnetic energy interacts/aligns with the dipole moment of polar samples and/or polar solvents and this constant electric field-sample dipole rotation/realignment generates molecular friction converted to heat energy [54]. Only dielectric materials (such as porcelain (ceramic) glass and plastics) or solvents with permanent dipoles such as water are microwave active. A ceramic cup containing coffee or herbal tea can be heated in a domestic microwave. These herbal products contain minute traces of moisture that when heated by microwaves generate energy/pressure to break the plant cell walls so that many secondary metabolite bioactive compounds or aroma molecules can be dissolved in water [55]. MAE has many advantages, most significantly it reduces the extraction time compared to traditional methods such as Soxhlet extraction, also increases extraction efficiency and yields. There are several advanced MAE instruments commercially available including pressurized microwave-assisted extraction (PMAE) and solvent-free microwave-assisted extraction (SFMAE) technologies.

1.2.5 SPME extraction

'Headspace' refers to volatile extraction techniques of the gas above the sample of liquid or solid phase in a sealed chromatography vial, that started to be utilized in the late of 1950s. Headspace gas components are usually collected by a SPME fiber. Its advantage is simplicity of use, effectiveness and is a very clean method, which delivers volatile analytes into a gas chromatograph for analysis.

Solid-phase microextraction (SPME) fibers were invented by Janusz Pawliszyn and patented at the University of Waterloo in Ontario, Canada [56]. At present Supelco Analytical (a division of Sigma-Aldrich Co.) maintains the distribution of the materials for SPME. Many disciplines are using this technique, including the analysis of food, aroma and pharmaceutical samples. When volatile sample constituents distribute into the gas phase and reach equilibrium between solid/liquid phase and gas phase, the volatiles are adsorbed (or absorbed) in the headspace over liquid/solid samples, then are dispensed to a GC column [49,57]. Thus, there are absorption and adsorption characteristic SPME types fiber coating. The most widely applied coating, polydimethylsiloxane (PDMS), consists of absorption via a high viscosity rubbery liquid. However, it appears as a solid and converts to liquid under desorption temperature. The other adsorption materials PDMS–DVB (divinylbenzene),
Carbowax–DVB and Carboxen are commercially available, that are mixed coatings via a porous extracting phase [58]. In this project, different kinds of fiber were used according to the molecular weight and polarity of the analytes to be studied. For instance 100 µM PDMS are used for the most volatile compounds with molecular weights from 60 to 275 [59].

The SPME fiber is assembled in a modified barrel, the manual SPME holder [Fig. 1.3(a)]. In the standby position, the fiber is situated in a protective septum-piercing needle. Prior to sampling, the liquid or solid samples are settled in the sealed GC vials, leaving enough headspace (more than the fiber exposed including the needle) over the sample; the fiber needle is forced into the vial and pushes the plunger retaining screw in the middle of the “Z” slot. The SPME fiber, fused silica rod (typically 1 cm long and 0.11 µm i.d.) coated such as PDMS, is immersed the headspace contains the volatile compounds from the sample. After a time-interval (2-15 min), these compounds are subsequently absorbed and reach equilibrium. The fiber is withdrawn after the needle is taken out of the sampling vial. The needle is then inserted into the GC injector, and the volatile analytes are immediately thermally desorbed in 1-2 minutes and enter the GC column. The fiber still needs to be kept in the needle when removed from the GC injector [60] [Fig. 1.3(b)]. The SPME fiber assembly is disposable and may be used for approximately 100 injections.

![Fig.1.3](image)

**Fig.1.3** (a) Manual Solid-phase micro-extraction (SPME) Holder (left) (b) SPME extraction process of absorption and release (right) [Reproduced from reference 60].

**Table 1** A brief summary of the experimental conditions for various methods of extraction for herbal products
<table>
<thead>
<tr>
<th></th>
<th>Decoction Extraction</th>
<th>Percolation Extraction</th>
<th>Soxhlet Extraction</th>
<th>Microwave-assisted Extraction</th>
<th>SPME Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Solvents used</td>
<td>Mainly water</td>
<td>Mainly water</td>
<td>Methanol, ethanol, or mixture of alcohol and water</td>
<td>Water, ethanol, or mixture of alcohol and water</td>
<td>Any solvent</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>100</td>
<td>90-100</td>
<td>Depending on solvent used</td>
<td>Depending on solvent used</td>
<td>Low/high</td>
</tr>
<tr>
<td>Time required</td>
<td>≥30 min</td>
<td>≤20 min</td>
<td>3–18 hr</td>
<td>≤10 min</td>
<td>2-30min</td>
</tr>
<tr>
<td>Volume of solvent required (ml)</td>
<td>Depending on herbal weight</td>
<td>100-500</td>
<td>150–200</td>
<td>100-300</td>
<td>2-10</td>
</tr>
<tr>
<td>Types of herbs</td>
<td>Stem, root, seeds</td>
<td>Ground: leaves, flower, seed, roots, stem</td>
<td>Stem, root, seeds</td>
<td>Any parts of herbs</td>
<td>Herbal oil</td>
</tr>
</tbody>
</table>

### 1.3 Natural compound quality control [QC] via chromatographic analysis

Chromatography technology was first developed by Mikhail Tswett in 1901, separates the components in a mixture according to the different partitioning behaviours between a mobile phase and a stationary phase. The most common techniques are HPLC (High Performance Liquid Chromatography) and GC (Gas Chromatography). The stationary phase is packed into the column, through which the mobile phase (solvent or gas) continually passes. The sample is injected into the column. The individual components elute from the column as determined by their partition coefficient in the mobile phase, and are collected and measured by the detector measuring UV adsorption (diode array detector, DAD), molecular weight (Mass spectrometry detector, MS) [61]. [Fig.1.4(a, b)]
Resolution (R) is utilized to describe the extent of the separation of compounds A and B. The resolution of two species (A and B), is defined as:

\[ R = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( 1 + \frac{\kappa}{\kappa} \right) \]

where N expresses the number of theoretical plates, by lengthening the column leads to an increase in retention time, but in practice HPLC standard columns are fixed at about 15, 25 and 30cm; \( \alpha \), the selectivity factor, is usually described by the ratio of the retention (capacity) factors (\( \kappa \)) between the two compounds A and B peaks distances and determined by chemical characteristics; thus it is a priority to adjust retention (capacity) factors (\( \kappa \)) to improve the separation. When the amount of R achieved is 1.5, it is evaluated as good separation between the two compounds. The most useful optimization method is changing the solvent strength, for example the use of acetonitrile instead of methanol to increase the separation in reverse phase HPLC. Others include mobile phase solvent pH and column temperature adjustment. The column length and stationary phase composition can be changed when there are no improvements made by changing other variables [62].

**1.3.1 High Performance Liquid Chromatography (HPLC)**

High Performance Liquid Chromatography (HPLC) is a liquid chromatography system with a pump, sampler injector, column compartment (column and oven), detector (such as diode array detector, fluorescence detector or MS detector) and computer control system, some of which have a vacuum degasser. HPLC is an important technique for the qualitative and quantitative analysis for non-volatile compounds, for instance natural product extracts, proteins, salts, polymers and pharmaceuticals. HPLC separation procedure [Fig. 1.5 (a) and (b)] involves injection of a liquid sample (5ul-10ul) into the column packed with fine particles (3-5 \( \mu \text{m} \)) in diameter and high pressure from a pump onto individual components of the sample through the column for separation. Finally, when these constituents are eluted/separated from the column, the detector measures the eluted amount (peak area) converted into an electrical signal output.
1966 was the start of HPLC, when Piel published a method describing slurry-packed finely ground silica as a high liquid pressure (3500 psi) column to separate the samples [63]. Since the 1970s, the development of HPLC in column material and instrumentation in the hardware—pumps, detectors evolved. The octadecylsilane, [also called C18] was used as the stationary phase [64]. In the 1990s, smaller particles (less than 2 μm) and higher pressures (as high as 100,000 psi) were applied in the separation.

Most compounds can be separated by utilization of the four separation modes including reverse-phase chromatography, normal-phase and adsorption chromatography, ion exchange chromatography and size exclusion chromatography. The method of choice is determined by the characteristics of chemical compounds to be separated: polarity, electrical charge and molecular size. Many herbal compounds [65] and biological samples (involving protein and nucleotides) [66,67] have been separated by reverse-phase chromatography (RPC). Regarding RPC, the column is packed...
with porous particles of silica gel in various shapes (spherics or irregular) of various diameters (1.8, 3, 5, 7, 10 µM etc.) with various pore sizes (such as 60, 100, 120, 300), and the surface are covered with long covalently bonded alkyl chains (e.g. C18, C8, C3, phenyl, etc.) having a strong affinity for hydrophobic compounds (non-polar). The mobile phase is composed of water and organic solvent (methanol, acetonitrile). Generally, the water solvent strength is the highest and an initial gradient run allows the organic solvent percentage to be lower, as a function of the time the organic solvent content increases, thereby raising the elution strength with polar components eluting faster than the less polar components [68].

The HPLC solvent delivery programme options include isocratic or gradient conditions. Regarding the former conditions [69], the mixture of mobile phase is the optimized constant composition. The delivery system remains at equilibrium with the column during the separation procedure since the chemical constituents do not change. This method of chromatography has ideal reproducibility and accuracy for some sensitive and routine sample analysis such as caffeine and garlic constituents [70]. Many herbal compounds are complex mixtures including both very hydrophilic and hydrophobic compounds; but the HPLC system separates/resolves the individual constituents in reasonable separation times. Obviously, the composition of the mobile phase determines the retention times and resolution of the components. Therefore, the majority of chromatographic runs have been based on linear gradient conditions in the mobile phase in which the solvent strength is increased with time using a gradient elution separation. The retention factor (k or capacity) is usually improved by altering the amount of organic solvent (modifier) composition.

The Isocratic separation is suitable for quality control studies because the elution data reproducibility (e.g. retention time) by the separation conditions is stable. In contrast, the gradient elution separation is suitable for the analysis of complex samples, applied to unknown mixtures and linear gradients that are most commonly used. During the gradient elution the measured baseline is less stable than in isocratic separations due to the frequent equilibrium changes between the mobile and stationary phases. Therefore, it is advisable to choose high purity solvents as the mobile phase to avoid contamination effects, run the pre-blank baseline at the start before any sequences of injections to ensure the baseline and system is stable. It is also essential to perform blank sample checks, performed by using the sample or standard solvent, to check the solvent purity. Some pH modifiers are always added to the mobile phase, such as acetic acid, formic acid or ammonium salts, and it is recommended to ensure that in blank samples there is a solvent UV absorption free to target analyst interference before sample or standard injections are made [71]. When preparing
samples, some analytes may be less soluble in the mobile phase, which is detrimental to the column and decreases separation reproducibility. The mobile phase solvents are suggested also for dissolving the sample and standards. All samples need to be filtered (e.g. 0.45um filter) prior to injection into the flow stream.

The most common UV detection technique used is HPLC-DAD (diode-array detection), which measures the absorbance of UV wavelength against the concentration of the analyst as shown with the instrument [Fig.1.5 (c)].

Natural product analytes may contain nonbonding π* and σ* orbitals in the unsaturated atom bonds, aromatic groups, or functional groups. The electrons absorb the incident energy and rise to the higher energy state. The analyte UV energy absorbance is determined after the light passes through the sample. Analyte concentrations can be measured by the Beer-Lambert law equation: \( A = \varepsilon \cdot c \cdot l \), where \( A \) is the absorbance, \( \varepsilon \) is the absorptivity coefficient, \( c \) is the analyst concentration, and \( l \) is the length of the incident light through the flow cell containing the compound elution from the column. The absorptivity coefficient can be measured via standard calibration curves. So the same or similar substance concentrations in the unknown sample can be determined from the instrument response (absorbance) [72].

In the DAD the tungsten lamp (visible range) and the deuterium lamp (UV range) radiation is transmitted through the flow cell. The emission light is dispersed to individual wavelengths then reaches the diode-array for detection. In this way, the analyte absorbance signal can be measured for each component identification and quantitative analysis [73]. The detector sensitivity can be determined by the lamp and the manufacture design factors and the measures range from 190nm to 700nm approximately. Concerns for sample detection involves the solvent cut-off value that is the analyte approximate wavelengths (nm) when they are specified below the absorbance that may be masked in the solvent. The solvents themselves absorb UV light in the lower wavelength range, such as the UV cut off value (nm) of water, acetonitrile values are 190 nm and methanol for 205 nm. Especially, under the condition of reverse-phase HPLC, the increased solvent in the mobile phase might increase its UV absorbance that may mask the low wavelength sample elution absorbance.

1.3.2 Gas chromatography
The technique of gas chromatography (GC) was invented by James and Martin in 1952 and became the standard analytical method in many industries [74]. Separation of the components results from the distribution (partitioning) of each component between the mobile phase (carrier gas) and the stationary phase, but the system separation parameter control is mainly via the column temperature programme and gas flows. The basic requirement of GC analysis is sample and solvent volatility. Volatile samples generate gaseous mixtures containing the vapours of carrier gas, sample and solvent prior to passing on to the column. In general, the required amount sample is small for introduction onto the column and can be delivered by SPME fiber or microsyringe (about 1-20µl). After the sample is injected into the inlet, there are two injection modes available, split and splitless. The purpose of the two modes is to optimize the column separation. In the split mode, only a percentage of the gas mixture (from 1:1 to 1:500) is directed into the column, the remainder is expelled through the split outlet. This avoids column overloading and results in non-sharp and fronting peaks. When the split outlet is blocked, all gaseous are exposed to the column, this is the splitless mode and is mainly used in trace analysis due to sensitivity improvements. The carrier gas most commonly used is helium. Capillary column gives better performance efficiency, high resolution and are widely used [75]. The capillary column for GC consists of narrow tube coated on the interior with the stationary phase such as polysiloxanes or polyethylene glycols (i.e., 0.25 µm film in a 0.32 mm tube). Under the same column length condition (almost 30m), the separation efficiency can be determined by the column temperature (ranging from about 50°C to 250°C) and the gas flow rate. Overall, temperature increases result in reduced retention time however the resolution quality decreases. The priority separations method usually chooses temperature gradients starting with the column relatively cool (about 40-50°C), and then gradually and constantly increasing the temperature stepwise (temperature programming); as the volatile component can interact with the stationary phase coating determined by polarity and boiling points. High gas flow rates reduce the component retention time also. There are many different detectors used for GC, for example flame ionization (FID), nitrogen–phosphorus (NPD) detection and others.
1.3.3 **Mass spectrometry (MS)** [76,77]

The basic configuration of the gas chromatograph and mass spectrometer (GC/MS), for which is used to separate the components in an unknown sample and MS as GC detector is illustrated in Figure 1.6. Mass spectrometry is the most commonly used instrumental technique, for the identification and quantification of components in the mixture. The computer controlled GC and MS parameters such as separation programmes, injector temperature, MS scan mode and identification library match is available for two or more hyphenated GC-MS systems, for example GCxGC-MS and GC-MS-MS systems.

Mass spectrometers detect the ionized chemical species according to their mass to charge ratio (m/z), which include ionization source, mass analyser and ion detection system. First, sample needs to be ionized by various methods such as electron impact (EI), chemical ionisation (CI). EI is used in this research project at the standard ionization energy of 70 eV. Once the electrons collide with the molecular substrate (M), the loss one electron results in the molecular ion (M⁺) or it breaks into various fragments that contain smaller ions or it involves neutral fragment losses (eg. H₂O, NH₃).

Then the ions are focused by a mass analyser for separation and resolution via their m/z value. The ion process includes quadrupole, time-of-flight (TOF) analysers, magnetic sectors and so on. For the Agilent HP 5973 Mass Spectrometer in the RMIT laboratory, quadrupole mass filters were used [Fig.1.7]. This consists of four parallel metal rods, of which two opposite rods have an applied potential of radio frequency(RF) voltage and the other two rods have a potential of direct current (DC) offset voltage. The two pair applied voltage rods can select ions via m/z ratio. Only certain m/z ratio ions can travel down the quadrupole filter and reach the detector. After signal amplification,
the detector sends information to a computer recording all of the data produced and converts the electrical impulses into visual displays (mass spectrum). The mass spectrum expresses the fragment (ions) mass weight from the molecular with its characteristic relative abundances. The computer operations allow data acquisition with two scan modes, total ion chromatogram (TIC) and selected ion monitoring (SIM). The SIM mode utilizes selective monitoring of particular ions for example, a unique ion of the compound (e.g. intact molecular fragment) and high abundance especially higher mass. At present, data analysis programs run integration of chromatograms, and then compares the obtained spectra to reference spectral databases in library search to generate reports including identification and quality matches with reference library compounds.

![Configuration of quadrupole mass filter](image)

**Fig.1.7** Configuration of quadrupole mass filter [reproduced from reference 76]

Therefore, GC/MS can be used to analyse many low molecular weight compounds, applications that require compounds to be chemically stable at high temperatures and volatile. Many samples from complex matrices (e.g. soils, herbal mixture, tissues etc.) contain many non-targeting ingredients that may interfere with the data acquisition and analysis of the compounds of interest. It is essential to treat these samples with solvent extraction or SPME techniques before GC/MS analysis.

### 1.4 Thioflavin T (ThT) fluorescence assays

The present methods to assay amyloid fibrils is to utilize thioflavin T (ThT) fluorescence, congo red binding, transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR); ThT dye is the most commonly used fluorescence detector for the identification and quantification of amyloid fibrils in vitro.

The German physician scientist Rudolph Virchow coined ‘amyloid’ and utilized iodine-sulphuric acid treatment in 1854, as the first amyloid dyes to stain abnormal organs [78]. During the 20th century, the amyloid fibrils were detected using congo red dye observed under polarized light microscopy,
because the dye binding produces a birefringence pattern that differentiates amyloid fibrils from other fibril proteins such as collagen and non-amyloid deposits [79]. In 1959, the benzothiazole dye ThT was first used as a fluorescent marker to study amyloid detection in the kidneys [80]. The thiazole quaternary nitrogen of ThT selectively interacts with the hydroxyl groups of amyloid deposits to form hydrogen bonds and produce the specific binding within the grooves between solvent-exposed amino acid side chains of the amyloid fibril [81]. The fluorescence intensity increases are recorded [Fig. 8 (a), (b) and (c)] [82]. ThT assays are routinely applied to study amyloid fibril formation and the fluorescence intensity from the amyloid–ThT complex relates to the amount of amyloid fibril present. ThT can avoid many issues compared with congo red birefringence staining because amyloid deposits could be miscalculated due to sample thickness or orientation [83]. Also, the congo red dye can result in low reproducibility due to the staining background after washing. With the ThT assays, a phosphate buffer (10mM potassium phosphate, 150mM NaCl, or 50mM phosphate; pH: 5.8–7.0) are used to prepare ThT stock solutions (0.8mg/mL) in the phosphate buffer, needed to be covered with aluminium foil and kept at 4 °C refrigerator for less than 1 week; the working solution should be diluted 50 times and made fresh daily [84].

1.4.1 Fluorescence spectrometry measurements

Fluorescence spectrometry is mostly used to identify or quantify an analyte in solution using a beam of light with a wavelength between 180 and 800 nm being transmitted through the sample solution in a cuvette. In fluorescence spectrometry [85,86] the light from an excitation lamp source that passes through a filter or monochromator for selective specific spectral band (excitation spectrum), is absorbed by the sample and measured. Some of this fluorescent light emitted by the sample passes through a second filter or monochromator (emission spectrum) from an angle at 90° and the detector measures the intensity of the emission, which is directly proportional to the analyte sample [Fig. 1.8 (d)]. The samples include amyloid fibrils such as β-sheet-rich deposits after ThT was added, which fluoresces brightly with excitation and emission maxima at approximately 440-450 and 482-490 nm, respectively; however other non-binding ThT in the aqueous solution, exhibit a weak fluorescence intensity and lower (blue-shifted) excitation and emission maxima at 350 and 438-440 nm, respectively [87]. Therefore, after ThT addition to the samples containing amyloid and measurement of the fluorescence excitation at 440-450 nm the emission is detected at 482-490 nm. There are two common variations to this assay, (i) is a common measure with single time-point test, that is, the sample containing fibrils are diluted in ThT buffered solutions and then recorded at various time point readings (e.g. 24, 48, 72 hours); the other is fibrillation kinetics in real-time measurement. The fibrils formation can be tracked immediately.
Fig.1.8 (a) The structure of Thioflavin T; (b) Magnified view of the ThT–binding β-sheet of β-sheet-rich deposits. The site near ThT-binding are shown as red sticks; (c) Essential component of a fluorescence spectrometer; (d) Characteristic increase in ThT fluorescence upon binding to amyloid fibrils. [reproduced from references 82,85,86]

1.5 Coffee herbal functional beverages

Coffee and herbs have a long human use history and have many benefits for human health according to research studies. They are a combination of natural products to produce a functional beverage for chronic consumption. In aim of this project is the development of an analytical method to identify and quantify the major beverage ingredients and to explore the human taste perceptions of a selection of coffee-herbal beverages.

1.5.1 Coffee aroma induces/invites functional beverage usage

Coffee is one of the most popular beverages in the world, that provides a stimulating effect on human sensory organs. Progress in coffee chemical analysis utilizing GC-MS, has resulted in more than eight hundred volatile compounds [88] being identified, many of which have been
demonstrated to contribute to coffee aroma. The aroma profile of coffee is composed of the following notes: sweet/caramel-like, earthy, roasty, smoky, fruity and spicy. The volatiles are from the classes of compounds including furans, sulphur compounds, pyrazine, ketones, phenols, pyroles and comprise more than 60% of the total compounds. Kahweofuran, 2-furfurylthiol (roasty), 4-vinylguaiacol (smoky), three alkylpyrazines (earthy), four furanones (sweet/caramel-like, spicy) and five aliphatic aldehydes (fruity), are important determinants of coffee odour [88,89].

1.5.2 Herbs are widely used ingredients/nutrients to improve health

The documented benefits of liquorice and pharmacological studies indicate it is used to relieve cough, pain, and antiviral activity, oxidative stress, inflammation and has neuro-protective properties [90]. In the food industry, liquorice flavour is widely applied in candies or sweets and has long been used to flavour some dishes, drinks, and candies, as liquorice-like flavour in Western cuisines. Ginseng has a leading position in the best-selling natural supplementary food products in the world. Ginseng and its extracts have been traditionally used to revitalize the body and mind, increase physical strength, memory and prevent aging, particularly in China, Korea and Japan. Ginger has a long and well document history consumed as a spice herb in cooking. Therapeutically it is widely used to control fever, pain, indigestion and infection diseases.

1.5.3 Functional beverage sensory measuring

The sensory properties are a major determinant for the coffee-herbal functional beverage people select and how much/often they are consumed. The functional beverage familiarity evaluations apply a hedonic scale to predict the sensory acceptance, consumer behaviour and eventually modify the beverage recipe further. The 9-point hedonic scales are applied to evaluate the aroma and flavour of the functional beverage and investigate their contributing factors for the long term functional beverage consumption. This is the one of most used methods to quantify consumer acceptance, which was first published by Peryam and Pilgrim [91] and has been used in academic and industrial consumer research in America and Europe. This hedonic scale describes an equal number of positive and negative categories with intervals of equal size. For example, 1=not at all liked, 3=slightly liked, 5=moderate liked, 7= like very much liked and 9=extremely likeable.

1.6 Project hypothesis and scope
Dementia including Alzheimer’s disease is a slow progressive neurodegenerative disease commencing in midlife and it is difficult to reverse the process of loss of cognitive ability. However, we propose that if an effective treatment to reduce the rate of cognitive decline without any side effects could be found to delay and protect the brain from the dementia process, this would be highly desirable. Based on previous research, selected herbs and coffee to produce coffee herbal functional beverages against dementia were chosen for this project. The selected herbal compounds will be measured by analytical methods such as GC/MS and HPLC/UV in the coffee herbal beverage [Fig.1.9]. Also, we will compare various herbal forms to ensure the highest effective/best available sources of the herbs are used in our research. It is important to determine if the herbal bioactive compounds can be extracted by safe and convenient methods (such as decoction, percolation and microwave heating). A small group of volunteers will be invited to provide their personal/subjective estimate of the coffee-herbal beverage sensory perception via hedonic scales as feedback to improve the beverage recipe.

1.6.1 The selection criteria of the herbs used in functional beverages

Firstly, the herbs used should have a long history of consumption without any health side effects and also be available as food supplements. Secondly, their selection is based on research evidence that these herbal compounds can cross blood brain barrier (BBB) into the brain. Also that they are effective in improving memory, are able to inhibit β-amyloid fibril formation or have anti-inflammatory activity in the brain. Thirdly, the analysis of the main constituents present in the herbs will be studied to determine whether they can be maximally extracted into the coffee herbal beverage. Last but not the least, the herbs can produce favourable flavour and sensory perception, which will encourage continuous consumption.

1.6.2 Key project questions

Although the core task of the project is to analyse the bioactive compounds in coffee and in the herbs, the key questions addressed are not limited to their analysis. This is a multi-disciplinary project, which involves the medicinal knowledge of Alzheimer’s disease pathology, herbal pharmaceutical chemistry related to AD, natural product analysis (identification and quantitation) and human research ethics.
1. Why may coffee herbal beverages be chemopreventive/neuroprotective against dementia?
2. What kinds of herbal compounds can be selected for preventing cognitive decline and what are the herbal compound selection criteria?
3. What types of methods would be applied to measure the amount of target compounds in coffee herbal beverages, including instrumentation, procedure and data analysis?
4. Can these effective bioactive compounds be extracted by a simple procedure such as a coffee machine or microwave oven?
5. How can we measure the customers’ sensory responses to the new beverage?

![Diagram of typical sample preparation and separation steps](image)

**Fig.1.9** Typical sample preparation and separation steps involved in GC/MS and HPLC
1.7 References


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Chapter 2 is concerned with the cause and mechanism of Alzheimer’s disease and herbal bioactivities against the disease, of the compounds selected/utilised for this project. This chapter describes the coffee herbal beverage compounds investigated.

2.1 Multiple targets against Alzheimer’s disease by natural products

The German physician Alois Alzheimer first recorded the disease symptoms of Alzheimer’s disease in 1907, when he described the clinical case of Auguste Deter, in middle-age who had suffered serious memory loss that worsened. He demonstrated the shrinking of the outer layer and dramatic abnormal deposits around nerve cells in the patient’s brain. The accumulation of amyloid oligomers/plaques and neurofibrillary tangles are hypothesised to contribute to the neurodegradation of neurons (nerve cells) in the brain and amyloidosis is hypothesised as the central causative factor of Alzheimer’s disease [1]. A framework divides the disease into four stages: early dementia, mild dementia, moderate dementia and severe dementia. Recent research evidence has indicated that soluble Aβ oligomers are more toxic than mature fibrils [2,3] suggesting that oligomers should be a high priority target for designing potential antiamyloidogenic agents for AD.
Other AD hypotheses have been proposed including cholinergic, inflammatory /oxidative and adenosine hypotheses. However, the most widely accepted theory is the amyloid hypothesis [4]. These hypotheses are also the targets for natural products to prevent neurodegeneration.

2.1.1 Target 1: Amyloid β peptide is produced by proteases

Although the mechanism of AD is not fully understood, currently the amyloid β peptide (Aβ) is the main component of the amyloid plaques found in the brains of Alzheimer patients. Senile plaques contain both Aβ40 and Aβ42 [5] Fig.2.1. In particular, the increase of Aβ42 or Aβ42/Aβ40 ratio leads to amyloid oligomer formation, that causes subtle and then increasingly severe and permanent changes of synaptic function [6]. Aβ is cleaved from the amyloid precursor protein (APP) by three proteases: α-secretase (the non-amyloidogenic pathway shown in Figure 2.2a), β-secretase (cleaves at the β-site of APP by the transmembrane enzyme aspartic protease1 (BACE1)) and γ-secretase as illustrated in Figure 2.2b. APP is a single transmembrane polypeptide and can undergo a variety of proteolytic cleavages to secrete derivatives into the extracellular space [7]. β-Amyloid precursor protein (BAPP) is cleaved by β-secretase [8]. The γ-secretase enzyme cleaves the remaining 99 amino acid C-terminal fragment of APP (C99) to Aβ40 or Aβ42 [7] and a C59 residue. The APP is cleaved by α-secretase, releasing the soluble APPα peptide fragment and C-terminal fragment further cleaved by γ-secretase, producing p3 nontoxic fragments [10]. Therefore, increasing α-secretase activity can decrease the level of βAPP and enhance neuroprotective effects, while β-secretase and γ-secretase, endoproteolyze APP to liberate the Aβ peptide Fig.2.2. There is evidence that the increased Aβ production and enhanced deposition of β-sheet amyloid is linked to β-secretase upregulation/activity in sporadic AD patients [11].
2.1.2 **Target 2: Neurodegeneration is accelerated by inflammation and oxidative stress**

It has long been known that the damage of brain tissue and the deposition of highly insoluble abnormal materials (like damaged neurons, neuritis, amyloid β peptide deposits and neurofibrillary tangles) are powerful microglia activators [14]. Microglia are the immune cells of the brain where local inflammatory responses (microgliosis and astrocytosis) are observed. As microglial activation in particular, are core features of AD and secrete increased levels of multiple inflammatory mediators[15], Aβ induces the expression of proinflammatory cytokines including IL-1β, IL-6, TNF, oxygen free radicals and nitric oxide; also the induction of inflammatory enzyme systems such as the nitric oxide synthase (iNOS) and the cyclooxygenase enzyme (markers of inflammation COX-2). Overexpression of interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF-α) lead to dysfunction and neuronal death [16,17]. After the conversion of a ‘resting’ microglia into an ‘activated’ phenotype, it can be observed that the increase of expression of amyloid precursor protein and its β-secretase cleaved products (sAPPβs) as well as Aβ oligomers lead to accumulation of Aβ. However, normally this is reduced by the α-secretase cleaved products (sAPPαs) in human neuronal cell lines [18]. There is a correlation between diminished cognitive status and microglial activation in mild cognitive impaired patients [19]. Astrocytes, also known collectively as astroglia, are comprised of a highly abundant population of glial cells and serve an array of important functions including the regulation of extracellular ion concentrations, synaptic remodelling (addition and removal of synapses), and the
maintenance of protective barriers like the blood-brain barrier [20]. Senescent astrocytes were found accumulated in brain tissue from aged individuals and AD patients [21]. Anti-inflammatory approaches have been developed that may not cure AD directly but slow the progression or delay the onset of this devastating disorder. More than 50% of the benefit from taking non-steroidal anti-inflammatory drugs (NSAIDs) is to prevent the onset of dementia with long-term usage against AD [22].

Oxidative stress is produced by the imbalance between production and removal of free radicals and reactive oxygen species (ROS), such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^•$); the important sources of these are from glia cells inflammation and aging in the brain. Proteins and lipids are targets for oxidative attack increase the risk of neurodegenerative diseases occurring and result in protein misfolding [23]. During early dementia stage, it can be observed that oxidative damage precedes Aβ deposition due to mitochondrial metabolism dysfunction and apoptosis [24]. Many natural products that can eliminate free radicals and reactive oxygen species (ROS), are antioxidants [25].

2.1.3 Target 3: Cholinesterase inhibition against Alzheimer’s disease

Cholinesterase inhibitor drugs are widely applied for treating the symptoms of AD and are licenced AD drugs. Generally speaking, acetylcholine is an important neurotransmitter for cognition and memory, and patients with dementia, AD have low levels of acetylcholine. If natural compounds can sustain acetylcholine levels via cholinesterase inhibition, this sustains neurotransmission between nerve cells, which in turn, may temporarily ameliorate or stabilise the symptoms of dementia.

2.1.4 Target 4: Adenosine receptor inhibition against Alzheimer’s disease

Adenosine, an endogenous nucleoside, plays important roles in synaptic transmission and neuronal excitability in glia and neurons, which activate adenosine receptor including A1, A2A, A2B and A3 G-protein coupled receptors to have effect on cognition, memory and neuronal degeneration [26]. Some research has suggested that the receptors of A1 and A2 were inhibited to improve cognitive function or decrease cognitive impairment [27]. To prevent neuron damage, A1 adenosine receptors activation tends to restrain glutamate release that hyperpolarize neurons by a predominantly presynaptic action, while A2A adenosine receptors inhibition are more likely to
decrease transmitter release and postsynaptic depolarization and promote synaptic plasticity [28]. These two receptors are composed of dimers with different metabotropic receptors operated by other neurotransmitters such as glutamate, NMDA and nitric oxide [29]. Therefore, adenosine receptors could promote the inflammatory process regulation and pro-inflammatory cytokine release such as interleukins and TNF and promote cognitive impairment during brain damage. It has been observed that A1Rs expression is reduced in cortical and hippocampal regions but A2ARs increase in limbic regions in aged animals. It is proposed that a new target for the development of new therapies that A1 adenosine receptor (AR) agonists, A2AR antagonists and A3AR antagonists for neuroprotection can be based on the adenosine neuromodulatory system. In the context of this project, coffee is neuroprotective, as caffeine is a non-selective inhibitor of adenosine receptors and is a protective agent against AD [30,31].

2.2 Coffee protects against dementia

There are over 25 varieties of coffee plants known, although only two are exploited in economically significant quantities: Arabica (Coffee Arabica) and Robusta (Coffee by the canephora plant). Coffee has long been demonstrated to possess various mechanisms against AD. We have focused on coffee protection against dementia and Aβ inhibition as caffeine can cross the blood-brain barrier (BBB) and reduce the symptoms of inflammation and oxidative stress in brain.

2.2.1 Caffeine and coffee compounds exert protective effects against AD

Caffeine is rapidly absorbed from the gastrointestinal tract in the stomach and small intestine, and distributes rapidly in all tissues including the brain. In rodent studies aged cognitively-impaired AD mice exhibited memory restoration and lower plasma and brain Aβ levels following 1–2 months of caffeine treatment (the human equivalent of 500 mg caffeine or 5 cups of coffee per day). It suppresses both β- and γ-secretase levels [32,33]. Caffeine can increase brain Aβ clearance at the BBB and decrease the inflammatory cytokine levels in the hippocampus [34,35]. It is a non-specific adenosine antagonist, its neuroprotective effect is likely to be the blockade of adenosine A2A, which play a role in the control of neuroinflammation [36,37]. Trigonelline, a coffee constituent and a component of the popular Indian curry spice, [38]. Both caffeine and trigonelline are investigated as anti-AD drug candidates [39]. Trigonelline docking with Aβ42 interacts with key residues (His6, Tyr10, His13 and His14) and alters its structure to inhibit its aggregation [40]. After 15 days of orally
administered trigonelline (500 mg/kg) there was improved memory in mice induced by Aβ (25–35) injection [41]. Chlorogenic acid (9mg/kg) a major polyphenolic component in coffee, significantly improved the cognitive impairment of experimental models of AD induced by scopolamine (0.5mg/kg) in mice and involved anti-AChE and anti-oxidative mechanisms [42]. The investigation indicated that these coffee compounds including chlorogenic acid, quercetin, caffeine, can reduce the release of TNF-α and IL-6 from the activated microglia and astrocytes [43].

Fig.2.3 Structures of caffeine, trigonelline and caffeic acid

2.2.2 Human studies of neuroprotection of coffee against dementia

According to the Cardiovascular Risk Factors, Aging and Dementia (CAIDE) study data after an average follow-up of 21 years (midlife visit), a total of 61 cases were identified as demented (48 with AD) of 1409 individuals. Moderate coffee drinkers (3-5 cups/day) at mild life had a 65–70% decreased risk of dementia and AD compared with those drinking no or only little coffee (0-2 cups/day) [44]. Aβ levels were not seen in human blood following acute caffeine administration. Other survey analysis also concluded that coffee consumption is associated with a reduced risk of AD [45]. Interestingly, Caffeine intake was associated with a better cognitive response to acetylcholinesterase inhibitors (AChEIs) in AD women, according to northern Taiwan clinical trial that included 234 mild to moderate AD patients studied from 2007 to 2010 [46].

2.3 Liquorice protects against dementia

Glycyrrhiza is a genus of about 18 accepted species in the legume family, of which Glycyrrhiza glabra is a species native to the Mediterranean region called Licorice (liquorices); G. Echinata is a Russian licorice and G. Uralensis are known as Chinese Licorice. More than 400 compounds have been identified from Glycyrrhizin species; including flavonoids, glycyrrhizic acid (GA) and glabridin. All are utilized for major medicinal purposes [47]. Glycyrrhizic acid contains both hydrophilic (glucuronic
acid) and hydrophobic (glycyrrhetic acid) regions, which can form stable inclusion complexes with various organic molecules because GA in solution can create cyclic structures [Fig.2.4] [48]. It may be reasonable to propose that this molecular characteristic provides not only high stability but may reduce side effects or improve the therapeutic efficiency.

![Fig.2.4 β-Glycyrrhizic acid converts to a stable inclusion complex with other molecules (GA “donut” ring) [reproduced from reference 48]](image)

### 2.3.1 Liquorice compounds that may affect Alzheimer’s Disease

Liquorice intake (150 mg/kg for 7 successive days) [49] inhibited acetylcholinesterase (AChE) in brains of mice and prevents Aβ25-35-induced (25μM) apoptosis in PC12 cells under 1mg/ml [50,51]. Glycyrrhizinic acid (GA) (5,10 mg/kg) [Fig.2.5], which is the major active component of liquorice suppressed the inductions of TNF-α, IL-1β, COX-2, and iNOS to have the anti-inflammatory effects [52] and protect neurons via anti-oxidative [53]. Some reports show that GA can be metabolized into 18β-glycyrrhetinic acid (18βGA), another major active compound in liquorice by β-D-glucuronidase in intestinal bacteria [54]. 18βGA can bind to mineralocorticoid receptors (MRs) and 11β-hydroxysteroid dehydrogenase type-1 (11β-HSD1) distributed mainly in neurons, moderately in astrocytes of the hippocampus [55]. Diammonium glycyrrhizinate (DG) (0.01μg/μl) attenuates neuronal injury via decreasing ROS production induced by Aβ1-42(2uM) and improved cognitive impairment in Aβ1-42 induced AD mice with 10mg/kg for 14 days [56]. Liquiritigenin (50μM) effectively protects against glutamate-induced (5mM) hippocampus-derived neuronal cell line (HT22 cells) early apoptosis via inhibition of Ca^{2+} influx, intracellular reactive oxygen species (ROS) production, and lipid peroxidation in mice [57]. It is similar to isoliquiritigenin (1-20μM) that inhibited Aβ (25-35)-induced (10μM) neuronal apoptotic death in relation with Ca^{2+} and ROS [51]. Liquorice-derived dehydroglyasperin C (DGC) has effects on inflammation-mediated
neurodegeneration and also reduces cytotoxicity and ROS generation [58].

![Fig.2.5 Glycyrrhizic acid (GA) and Glabridin](image)

2.3.2 Human studies of liquorice protection against dementia

Liquorice of 260ml hot water extract taken for 8 weeks, decreased in 20 healthy male smokers (aged 20-60) blood sample plasma levels of conjugated dienes (a maker for lipid peroxidation), that might be ascribed to its antioxidant compounds [59]. According to the 2010 edition of the Chinese Pharmacopoeia, liquorice is recorded at position 320 of 1062 prescriptions as a principal or assistant drug [60]. It is reported that 10 mg glycyrrhizic acid intake daily is safe for most healthy adults [61].

2.4 Ginseng protects against dementia

Panax ginseng C. A. Meyer (Asian ginseng) and Panax quinquefolius L. (American ginseng) are the two most recognized ginseng type around the world [62]. The key active ingredients of ginseng are known as saponins or ginsenosides such as Ginsenoside-Rg1 (G-Rg1), Ginsenoside-Rg2 (G-Rg2) and Ginsenoside-Rb1 (G-Rb1), Re, Rd [Fig.2.6a]. These compounds are known to improve attention, processing, memory and preventing memory impairment [63]. Asian and American ginseng contain similar ginsenoside profiles but Rb1 is richer in American ginseng [64.]

2.4.1 Ginseng compounds that may affect Alzheimer’s disease

Red ginseng is produced by steaming raw ginseng, the extract (100mg/kg) significantly ameliorated young and aged rats with hippocampal lesions in the place-navigation deficits (PLT), because expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were reduced in the serum level
Ginsenoside composition from steamed ginseng also can inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities [66]. Ginsenoside Rg1 (10, 20 mg/kg) promoted spatial learning and memory on ovariectomized rat model of AD after treatment 6 weeks; It can reduce Aβ1-42 generation by increasing ADAM 10 expression (one of α-secretase) and decreasing BACE1 expression (one of β-secretase) [67,68]. Ginsenoside Rb1 can inhibit liver cells fibrosis via decrease plasma aminotransferase activities and inflammation [69]. The long-term pretreatment with ginsenoside Rb1 attenuated the aged mice brain impairment via extensive anti-oxidant activity. Ginsenoside Rb1 selectively inhibited the activity of L-type voltage-gated calcium channels to decline the damage in hippocampal neurons [70,71]. In vitro, ginsenoside Rb1 (0.1, 1mM) inhibited β-secretase activity [72] and protected against Aβ25-35 induced (25 or 50μM) cytotoxicity in PC12 cells, Ginsenoside Re (0.1-100uM) [73]. The inhibitory effect of ginsenoside Rg2 (0.2 mmol/L) against the formation of Aβ1–40 induced by glutamate in PC12 cells [74]. Rg2 (2.5, 5 and 10 mg/kg) also prevented memory impairment in a rat model with vascular dementia via modulating the expression of apoptotic related proteins [75].

Fig.2.6 (a) Ginsenosides Rb1, Rg1; (b) α-Pinene, β-Pinene and Camphene

2.4.2 Human studies of ginseng protection against dementia

Endogenous antioxidants in human plasma like glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) activities were significantly increased after Ginseng (6 g/day) supplement for 8 weeks because ginseng has effect on regulation of immune system to enhance
resistance to illness [76,77]. In AD patients, red ginseng including Rb1 (1.96%), Rb2 (2.18%) Rg1 (0.49%), and Rg2 (0.13%) treated with 9 g/day the dose lasting 12 weeks, significantly improved the Alzheimer’s Disease Assessment Scale (ADAS), Clinical Dementia Rating (CDR) scale [78]. Red Ginseng is a cognitive enhancing saponin used as complementary anti-dementia medication. Panax ginseng administration for 4 weeks was shown to be safe, tolerable, and free of any untoward toxic effect in healthy male and female volunteers [79].

2.5 Ginger protects against dementia

Ginger (ginger root) is the rhizome of the plant Zingiber officinale and contains many potentially bioactive compounds. Studies have shown that the volatile oil of ginger contains up to three percent of fragrant essential oil including monoterpenoid fraction (α-pinene, camphene, geraniol, geranial and zingiberene) [Fig.2.6b]. Overall, the in vitro evaluation of the ginger extract expressed high antioxidant activity, inhibited AChE and BChE and activity against the formation of Aβ oligomers with the ThT binding assay, which showed the neuroprotective functions [80].

2.5.1 Compounds in ginger that may affect Alzheimer’s disease

The ginger component such as 6-gingerol has anti-inflammatory activities [81]. Furthermore, ginger extract profoundly blocked expression of IL-1β and COX-2 by fibril Aβ (1-42) activation with THP-1 cell line preclinical studies, with properties similar to human microglial cells [82]. It should be possible to reduce or delay the progression the pathogenesis of AD through anti-inflammatory approaches. 6-Shogaol (10 mg/kg/day) for 5 consecutive days significantly decreased activation of microglia and astrocyte visualized by rat monoclonal anti-CD11b (Mac-1) compared to after stereotaxic injection of Aβ peptide 1–42 (10µM, 3µl). The same consequence was observed in the hilar region of dentate gyrus (DG) in hippocampus. Meanwhile, 6-shogaol reduced memory impairment with Aβ peptide 1–42 hippocampus lesion in mice through the passive avoidance test. This has also been confirmed in the scopolamine-induced memory impairment model by passive avoidance test. Neuron growth factor and synaptophysin levels were significantly elevated in the 6-shogaol treatment of mice hippocampal tissues [83]. In clinical trials, ginger is often advocated for use for its beneficial effects against nausea and vomiting.

2.6 References


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The Analysis of Herbal-Coffee Beverages

We utilized coffee and herbs, including liquorice, ginseng and ginger, to produce herbal coffee beverages. Quality control is one of the most important aspects for beverage usage and modification. According to the effective bioactive compounds for preventing dementia described in chapter 2, the bioactive compounds caffeine, trigonelline, glycyrrhizic acid ginsenoside Rb1 and camphene were set as the analysis compounds. A herbal quality valuation method choose one or two pharmacologically active components from the herbal mixtures [1]. The method has the advantage to ensure the active compound concentrations are known and are reproducible providing beverage quality assurance as the concentrations of the bioactive compounds determines the potential pharmacological effects.

Chromatographic techniques have been developed to assess qualitative and quantitative analysis of these herbal compounds over the past decades. For the analysis of the bioactive compounds in beverages several different techniques have been applied, including liquid chromatography with UV absorption detection (LC/UV), SPME-gas chromatography-mass spectrometry (SPME-GC/MS) as the most commonly validated methods. In this chapter the results of the analysis of the coffee-herbal beverages are described along with a preliminary test for natural product inhibition of amyloid fibril formation.

3.1 The validation parameters of the chromatographic method

3.1.1 Quantitation using external standards

The reason analytical standards are employed is to identify the retention time of the substance of interest and quantify its chemical peak area (or height), when compared with the pure reference
substance in an unknown matrix /solution, using chromatography. The standard analysis methods can be used in two ways, either as an internal standard or as an external standard. In the external standard method a series of the pure chemical, with the sample solution in varying ratios, then run the blank and the combination of standards and samples respectively. The standard absolute response is then plotted against the ratio of the substance concentration to provide the calibration curves [2]. Regression analysis is used to define the relationship between the variables, and the fit assessed by the coefficient of determination ($R^2$) of the amount of unknown samples can be calculated using the regression.

With the external standard method, the reference standard (or standards) must have the same retention time as the substance in the sample using the same chromatographic conditions. In addition, the substance accuracy (recovery rate) should be assessed and be in acceptable range (100±2%) because errors could be induced by the standard concentrations preparing or injection-to-injection variation in each test. The detector is assumed to supply the same response to the standard and the samples and provide a linear response for the range of the standards measured. The equation used to calculate target analysis is, $C(u)=\frac{A(u)}{A(std)}\times C(std)$ Assuming, the response index of the detector is unity, where $A(u)$ is the peak area of target analyte in unknown sample, $A(std)$ the peak area of standard and $C(u)$ and $C(std)$ are the concentration of target analyte in the unknown sample and standard solution respectively [3]. However, it is necessary to understand the behavior of the response factor as the concentration or amount of target compound and external standard are variable. The behavior of the response factor allows one to set limits on acceptance range (similar to confidence interval) of the concentrations of the standards batch. These parameters include identity confirmation, accuracy, precision, linearity and detection limit.

3.1.2 Peak identification [4,5]

In chromatography, the evidence provided for peak identification is that that the retention time of the unknown peaks are the same as the known standard peaks. However some other compounds could have similar retention times. Spectrometric methods are applied to confirm the detected compound is the same as the analyte. For example, DAD detectors are classical spectrometric HPLC detectors, which supply information about structural features of compounds from their UV spectra. Mass spectrometry is also another powerful method for identity confirmation, which provides mass-to-charge (m/z) values of ions fragment from the analyst molecules and the intact molecular masses.
with little fragmentation can be observed as well. The mass spectrum is mass-to-charge (m/z) values
(x-axis) against intensity of the ions (y-axis) [6].

Many GC/MS instruments utilize electron ionization (EI) mode to form a radical cation via collision-
induced dissociation with standard ionization energy of 70 eV. The intensity ratio of ions derived
from the same compound should be consistent under the same EI, which result in fragmentation in a
characteristic and reproducible way. These fragments are able to be scanned via full scan mode or
selected ion monitoring (SIM) mode. The MS spectrum can then be compared to large reliable
reference libraries obtained with different instruments because of standardized EI, and the GC/MS
analysis programme can provide a with the reference substance [7]. Additionally, The MS spectra of
samples can compare the presence of these ions and their relative intensities with standard peaks.
Some guidelines recommend 2–4 ions be selected for compound identification, and the ions
intensity should be in the acceptable range. The intact molecular fragment or high m/z value ions
are selected as specific, but the low abundant ions (relative intensity <5%) are not usually used for
identification. The normal neutral losses (e.g. H₂O, NH₃) do not have a characteristic diagnostic value
and are also not used for identification [8].

### 3.1.3 Accuracy (recovery) [9,10]

The accuracy analysis is an analytical method in the matrix in which the targets compounds test
results obtained are compared to the the true value. The spike recovery analysis is the most used
method for determining in herbal compounds studies, in which the recovery is based on the known
amount of the analyte working in the linear range of detection. The evaluated constituent of interest
is placed in the blank matrix and then as a percentage of the true value increase in the matrix
(spiked). The assay analyses are performed over a range of 50-150% of the target concentration
calculation under the chromatographic conditions. Accuracy criteria for drug substances and drug
products are recommended to be performed in 100 ± 2% and at least in triplicate as stated in FDA
Guideline for Submitting Samples and Analytical Data for Methods Validation.

In the present analysis, the accuracy is measured by spike recovery with the ratio of standards
concentrations at the 50, 75 and 100% levels (the concentrations are 40ppm, 60ppm and 80ppm
each). A series of different mixed standard solutions (caffeine, trigonelline, GA and Rb1 in HPLC)
were prepared with known added amounts (20ppm) and injected in triplicate. The herbal target
compound peak area will increase by approximately 50, 75 and 100%, and correspond to the ratio of concentration levels rising respectively. Regarding the semi-quantitative camphene analysis by SPME-GC/MS for ginger, the higher standard range is also set at the 50, 75 and 100% levels. The purity of standards can also affect accuracy. In the HPLC and GC/MS investigation, the five major standards were purchased from a commercial supplier, Sigma-Aldrich at >97% except for GA (>70%).

### 3.1.4 Precision [11,12]

The precision of an analytical method assess the closeness of agreement between a set of replicate measurements performed under the separation conditions. Precision may be discussed at the two areas: one is repeatability (method precision), which carry out the analysis at the three levels (50%-150%) and performed in triplicate; the other is intermediate precision which examines the analysis on multiple days and maybe with a different operator, equipment or column. The agreement is measured in terms of relative standard deviation (RSD%) and the calculation equation: $\text{RSD}\% = \frac{\sigma}{\mu} \times 100\%$, where the $\sigma$ is the analysis standard deviation (SD) and $\mu$ express the mean. In general, the RSD% accepted criteria are less than 10% in the combined assay, which is contributed to by variation in days of sample preparation, column or operator.

### 3.1.5 Linearity [13]

The Calibration curve is defined by the model equation. $R^2$ measures how well the model fits the data. The linearity of the model is determined by a ‘lack-of-fit’ test which determines whether a linear equation is the best fit for the data. In chromatography, the detector linearity represents the range of concentrations of substance that will give a linear response. This is also defined as the specific concentration range.

In the project, the DAD standards concentration range was set between 20ppm to 100ppm. The method chosen ensured that the output of the plot of peak area is proportional to the concentration (or mass of solute passing though it per unit time) and that is the chemical standards injection (such as caffeine, trigonelline, GA and Rb1) were eluted from the column in a reasonable time. The calibration curves for HPLC utilized a five concentrations batch and represent the standard individual performance to the DAD and measurement of goodness of fit (coefficient of determination ($R^2$)). Generally, these responses from each analytical method were matched to the linear regression
model, \( y = mx + b \), where \( y \) refers to DAD response (peak area), \( x \) represents standard concentrations batch and \( m \) and \( b \) relate to the best fit slope and \( y \) axis intercept for each calibration curve respectively. Strictly, the quantitative method accepted a good \( R^2 \) criteria of 0.99 or greater, but in some semi-quantitative analysis, \( R^2 > 0.97 \) can also be acceptance to be linear. The measurement accurately should be validated by other analysis methods for comparison.

### 3.1.6 Limits of detection [14, 15]

The term ‘Limit of Detection’ [LOD] refers to the lowest concentration of the analyte that can be reliably distinguished from the noise (baseline). In chromatographic analysis, the common approach to estimate LOD, via linear regression method calculation, assumes that the instrument response \( y \) is linear against the standard concentration \( x \) over the range. The linear equation expressed as: \( y = mx+b \), where \( m \) is the slope of the calculation curve. LOD calculations relate to Signal to Noise ratio (S/N), which is the analyte peak against the baseline noise and should be at least 3 times higher than baseline as the threshold measurement caused by instrument parameters such as lamp aging, the column, the mode of detection and even manufacture. The valuation analysis of LOD can be defined by the equation: \( \text{LOD} = 3\sigma / m \); where \( \sigma \) represents the standard deviation of the analyst response and \( m \) also expresses the slope of the calculation curve. The SD of response is accepted to utilize the lowest concentration in the standards range, which is the closest to the zero during the assessment. Therefore, LOD is important to evaluate whether the analytical method is suitable for analytical purposes.

### 3.1.7 Kováts index (retention index) [16,17,18]

Kováts index or Kováts retention index refers to normalization of the component retention times in GC via logarithmic calculation with n-alkanes as standards. Retention time is essential for the identification parameters of the component and should always be reproducible under the same analysis conditions. However, the retention time is determined by various factors including the characteristic component, column (e.g. length, diameter, stationary phase and thickness of film) flow rate gas carrier and oven temperature programme. It is a widely accepted method to use relative retention times to reduce the influence of these factors. The equation is \( \text{RRT} = \text{standard RT} / \text{sample RT} \), where the standard is one of the same/similar to the sample. The disadvantage is the standard variability of different samples.
Swiss chemist Ervin Kováts who introduced this concept of the retention index system concept during the 1950s. This indicates the retention behaviour of the various substance determined by a uniform scale of a series related closely standards. In other words, the well-behaved series of standards (such as n-alkanes) are measured on several instruments and the standards as reference describe the relation with the elution sample in one instrument via system-independent constants. The similar constant can then be produced according to the association between the reference (the standards e.g. n-alkanes) and the samples on another instrument. Therefore, the Kováts index uses two standards (n-alkanes) elution times to adjust the elution behaviour of the component in the unknown sample and create system-independent constants, which locate before and after the component. The constant (Kováts index) has the advantage that the same component should have a close index values on different GC instruments whatever the various samples or separation conditions and overcomes the instrument parameters influences mentioned above. It is important to choose the right n-alkanes as reference standards; it should be an increasing homologous series for instance alkane (C₈-C₂₀) and in high purity (analytical grade).

The Kováts index can be calculated using the equation below, based on the log-linear relationship between number of carbons (n) in an n-alkane homologous series and retention time.

\[
I = 100 \left[ n + \frac{\log t'_{NN} - \log t'_{NN+1}}{\log t'_{N(n+1)} - \log t'_{NN}} \right]
\]

Where: I is the Kováts index, n is the number of carbon atoms in the alkane (before the component), \( t'_{NN} \) is the lower carbon atoms retention time (before the component) in the alkane, \( t'_{N(n+1)} \) is the higher carbon atoms retention time in the alkane (after the component), and the component elutes between \( C_n \) and \( C_{n+1} \). There are software programs available that then generate I from the retention times.

### 3.2 Introduction to the coffee and herbal compound analysis method

The heterocyclic components of coffee (trigonelline, nicotinic acid and caffeine) concentrations were determined by RP-HPLC- diode-array detection of the brewed beverage sample [19,20]. The mobile phase used was methanol or acetonitrile, timeline limit in one hour, the flow rates between 1.0 and 2 mL min⁻¹ and thermostatically controlled columns normally kept at ambient or slightly above room temperatures; trigonelline analysed at 266nm and caffeine at 275nm [21,22].
Triterpene saponins and flavonoids are the major components in liquorice; the concentrations of which are determined by plant growth, harvesting time and time of processing [23]. The main bioactive constituent GA is a triterpene saponins, which can be separated on reversed-phase C18 eluted with isocratic methanol: MQ water: (0.2 M ammonium acetate or acetic acid) (67:33:1) and detected at 254nm. Because of the acidity of saponins, modifiers (such as ammonium acetate or acetic acid) were usually used to suppress their ionization and to achieve optimal separation [24,25].

The concentrations of seven major ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) were analysed by reversed-phase HPLC with standard C18 columns (150 or 250 mm × 4.6 mm; 5µm) and the mobile phase of acetonitrile and water with buffer was used with an optimized gradient elution program. Diode-array detection (DAD) is set at 198–205 nm for weak UV absorption for ginseng saponin analysis [26, 27].

In the present study polydimethylsiloxane (PDMS) 100uM fibre was used to separate and identify essential oil volatile components, such as the five main components in ginger consisting of camphene, α-phellandrene, β-phellandrene, camphor and zingiberene, by SPME–GC–MS analysis [28,29].

### 3.3 Coffee herbal compounds extraction procedure

#### 3.3.1 Information on instruments and reagents

1. HPLC: HP Agilent series 1100 DAD system
2. GC: HP 6890 series GC system
3. MS: HP 5973 Mass Selective Detector
4. Fluorimetry: HORIBA Floromax-4 Spectrofluorometer
5. All coffee and herbs were purchased as food samples as commercial products, which means that these companies are responsible for quality control, evaluating the safety and their products ingredients are stable. [Table 3.1]

<table>
<thead>
<tr>
<th>Product</th>
<th>Content</th>
<th>Species</th>
<th>Storage condition</th>
<th>Expiry date</th>
<th>Brand</th>
<th>Product code (Lot #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roast Coffee</td>
<td>250g (per bag)</td>
<td>Arabica</td>
<td>≤30°C</td>
<td>08/2017</td>
<td>Giancarlo coffee</td>
<td>-</td>
</tr>
<tr>
<td>Liquorice</td>
<td>425mg</td>
<td>Glycyrrhiza</td>
<td>≤30°C</td>
<td>11/2017</td>
<td>Natures</td>
<td>17036</td>
</tr>
</tbody>
</table>
3.3.2 Mode 1: Comparison of beverages with varying ratios of herbs, via HPLC and GC/MS

All herbs were stored separately in well-closed containers with protection from light, the laboratory temperature was around 20°C with low humidity. All herbal materials, coffee bean, liquorice and ginseng were ground into powder form (size <2mm) and fresh ginger root was prepared by cutting into small pieces (size <4mm). The coffee herbal (100mg) mixtures were transferred to a vial with 2 mL of MQ water, with the coffee and herbal ratios as shown in Table 3.2. The mixtures were well shaken and allowed to stand for 1 hour in a heat block at 90-100°C in sealed vials. All samples were passed through a filtration membrane (0.45um) and the content of caffeine, trigonelline, Rb1 and GA were determined by HPLC-DAD. For camphene, the SPME fiber was inserted into the vials to obtain coffee herbal beverage aroma for 15min.

<table>
<thead>
<tr>
<th>Sample (100mg)</th>
<th>Coffee</th>
<th>Ginseng</th>
<th>Ginger</th>
<th>Liquorice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10%(10mg)</td>
<td>20% (20mg)</td>
<td>30%(30mg)</td>
<td>40% (40mg)</td>
</tr>
<tr>
<td>2</td>
<td>20%(20mg)</td>
<td>10% (10mg)</td>
<td>40%(40mg)</td>
<td>30% (30mg)</td>
</tr>
<tr>
<td>3</td>
<td>30%(30mg)</td>
<td>40% (40mg)</td>
<td>10%(10mg)</td>
<td>20% (20mg)</td>
</tr>
<tr>
<td>4</td>
<td>40%(40mg)</td>
<td>30% (30mg)</td>
<td>20%(20mg)</td>
<td>10% (10mg)</td>
</tr>
</tbody>
</table>

Table 3.2 Coffee and Herbal Mixture Ingredients and Ratio lists
3.3.3 Mode 2: Comparison of different extraction methods for the coffee herbal beverage via HPLC

For the microwave oven extraction procedure, the total mixtures of coffee and herb weight was 2g and made up to a volume of 200 ml, which covered the sample powder thoroughly in the ceramic cups. The series of beverage samples were put into the microwave cavity, and then heated by microwave irradiation (0.5 min, 1 min and 1.5 min). The sample solutions were then transferred to the 15 ml flasks every 30 second and all samples were stored at −20 °C until HPLC analysis was performed.

For Soxhlet extraction, the ratios of coffee herbal samples (2 g) were weighed into the thimble. The water solvent was made up to a volume of 200 ml. The Soxhlet extraction was performed by gentle boiling for 8 hours, then the sample solution was collected in a 15 ml volumetric flask. All samples were stored at −20 °C until HPLC analysis.

For the percolation extraction, coffee and herbal sample were prepared from a 2g ratio mixture made up to a volume of 200 mL, using a filter coffee machine. The extraction took around 5 min at 90 °C. The coffee herbal beverage samples were collected and transferred to 15 ml volumetric flask and stored at −20 °C till HPLC-DAD analysis.

After all collections, the samples were well shaken in the flask. After shaking, the supernatant was passed through a filtration membrane (0.45um) and the concentrations of caffeine, trigonelline and Rb1 and GA were determined by HPLC-DAD.

3.4 The analysis methods of coffee-herbal beverage

3.4.1 HPLC protocol for coffee and Liquorice [30]

The HPLC protocol utilized measures the concentration of key coffee and liquorice constituents in coffee-herbal beverage including caffeine, trigonelline and glycyrrhizic acid (GA). The mobile phases
constituted 4.1% Acetic Acid MQ water and methanol. Agilent C18 reverse-phase column (150 × 4.6 mm; 5μm) was used and isocratic separation of 28% methanol within 1.5 min, then a linear gradient of 28–95% methanol for next 10 min, post run 3 min at last. Separations were carried out at room temperature with a flow rate of 2mL/min. The PAD detector was used in the range 200–320nm, and quantitative analysis was performed at 254nm (GA), 266nm (trigonelline) and 275nm (caffeine).

3.4.2 HPLC protocol for ginseng compounds [31]

The HPLC protocol was used to measure the concentration of Rb1 in the coffee herbal beverage. The mobile phases constituted 0.001% formic acid MQ water and acetonitrile (ACN). Agilent C18 reversed-phase column (150 × 4.6 mm; 5μm) was used and isocratic separation of 26% ACN within 8 min, then a linear gradient of 26-33% ACN at 10min, 33-45% ACN runs 35 min at last. Separations were carried out at 35 °C with a flow rate of 0.8 mL/min. The PAD detector was operated in the range 200–320nm, and quantitative analysis was performed at 203nm (Rb1).

3.4.3 GC/MS analysis of coffee-herbal beverage [32]

Extraction temperature and time are critical parameters in the SPME-GC/MS process. SPME of coffee herbal beverage was achieved with a 100uM polydimethylsiloxane (PDMS) coating fiber from Supelco at 90-100°C for 15 min. After the adsorption step, the fibre was transferred into GC injector in which the analysts were thermally desorbed at 250°C and kept in the hot GC injection spot for 5 min before removal to obtain complete desorption. GC/MS column oven temperature 40 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min, and finally ramped at a rate of 40 °C/min to 250 °C and kept for 2 min, He carrier gas at 1mL/min. Before use, the fiber was conditioned according to the supplier’s instructions.

3.4.4 Analytical standards information

Analytical Standards of sufficient purity, stability and homogeneity were utilized to prepare the calibration curve [Table3.3]. We obtained compounds from Sigma-Aldrich, who provided a certificate of analysis (ISO system) for all analytical standards [25]. These standards can achieve the accuracy and precision in the analytical results for qualitative and quantitative tests. Most standards are of analytical grade except caffeine (≥99.0%) and glycyrrhizinic acid (≥70%). During the preliminary stage the coffee herbal beverage contents were not fixed; these should be developed for the process using, for example, published data and the feedback from human sensory measurement. Therefore, this grade reached the requirements for evaluation at the beginning stage and in an economic way.
<table>
<thead>
<tr>
<th>Product code</th>
<th>Compound</th>
<th>Grade</th>
<th>Purity</th>
<th>Water</th>
<th>Storage condition</th>
<th>Expiry date</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>04070</td>
<td>Alkane (C₈-C₂₀)</td>
<td>Analytical standard</td>
<td>neat</td>
<td>None</td>
<td>4°C</td>
<td>2018</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>C0750</td>
<td>Caffeine Reagent Plus</td>
<td>≥99.0%</td>
<td>≤0.55</td>
<td>4°C</td>
<td>2018</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>T5509</td>
<td>Trigonelline hydrochloride</td>
<td>Analytical standard</td>
<td>neat</td>
<td>None</td>
<td>4°C</td>
<td>2018</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>G2137</td>
<td>Glycyrrhizic acid Non supply</td>
<td>≥70%</td>
<td>None</td>
<td>4°C</td>
<td>2018</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>442505</td>
<td>Camphene Analytical standard</td>
<td>≥96%</td>
<td>None</td>
<td>4°C</td>
<td>2017</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>Y0001347</td>
<td>Ginsenoside Rb1 European Pharmacopoeia (EP) Reference Standard</td>
<td>neat</td>
<td>None</td>
<td>4°C</td>
<td>2018</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Standards information list

Standard compounds were dissolved in methanol, glycyrrhizic acid (GA), caffeine and trigonelline at 2.0 mg/ml and camphene, Rb1 at 10mg/mL stock solutions then stored at -20°C until use. The mixtures of standard compounds including glycyrrhizic acid (GA), caffeine and trigonelline were then diluted to 1000ppm with 25% methanol solution, camphene diluted to 1000ppm and 10ppm respectively via pure ethanol and Rb1 by 25% ACN to 1000ppm for usage.

3.5 Thioflavin T (ThT) spectroscopic assay materials and methods [33,34]

Thioflavin T (ThT), is a benzothiazolium salt dye that was first utilized as a fluorescence microscopy probe to study amyloid fibril deposits in histological samples. Then, much later it was developed for the in vitro spectrophotometric quantification/assay of amyloid fibrils by the detection of the fluorescence emission of ThT because the emission intensity was found to correlate with the fibril concentration. ThT has become one of the most widely used methods for selectively identifying and analysing the formation of amyloid fibrils both in vivo and in vitro. When it binds to beta-sheet-rich structures such as amyloid fibrils, ThT displays enhanced fluorescence and a characteristic blue shift in the emission spectrum from approximately 510nm in the free state to 480nm when bound to amyloid fibrils. In protein-only solutions, the interaction of ThT with amyloid fibrils is highly specific. Neither amorphous aggregates nor soluble proteins in folded, unfolded, or partially folded states enhance ThT fluorescence. However, ThT in situ also interacts with other biomolecules such as DNA.
by intercalating between DNA base pairs and/or by binding to DNA grooves. Some polysaccharides, including proteins such as acetylcholinesterase also induce the characteristic ThT fluorescence, presumably through binding to structural cavities. These properties reduce its usefulness for the analysis of amyloid fibrils in diagnostic histology.

Hen egg white lysozyme was purchased from Sigma-Aldrich. The protein concentration was prepared at 50mg/ml then adjusted pH to 2 using aqueous HCl and an aliquot of 330ul was added to the 1.5ml tube. All test compounds were dissolved in the MQ water including caffeine (10mg/ml), trigonelline (10mg/ml), Vitamin B1 (10mg/ml) and caffeic acid (0.5mg/ml). These drugs (330ul) were mixed in the protein tube separately and change pH to 2. All tubes were heated in the heat block under 50 °C for 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours and 168 hours and the fluorescence intensity changes can be measured daily.

Thioflavin T was purchased from Sigma-Aldrich. ThT (1 mg), dissolved in 1.25 mL phosphate saline buffer (PBS, 10mM phosphate, 150mM NaCl, pH 7.0) and kept in the dark at 4 °C. The stock solution was then diluted 50 times in the PBS buffer before daily usage. The diluted ThT fluorescence intensity was measured at 440 nm (slit width 5 nm) excitation and emission measured at at 482 nm (slit width 10 nm). All test tubes were classified into blank (base line), negative control (untreated protein solution), positive control (treated protein solution), caffeine protein group (5mg/mL), trigonelline protein group (5mg/ml), Vitamin B1 protein group (5 mg/mL) and caffeic acid protein group (0.25 mg/ml). 5 µL of each solution was added to the ThT solution cuvette (about 1.5mL), stirred for 1 min then the fluorescence intensity was measured. The measured fluorescence intensity increase at 482 nm indicated the presence of aggregated protein accumulation in the solution.

3.6 The analysis of herbal-coffee beverages

3.6.1 Validation of the HPLC assay

The coffee herbal beverage analysis method was validated by the HPLC chromatogram of caffeine, trigonelline, GA and Rb1 at 275nm, 266nm, 254nm and 203nm. [Fig.3.1, Fig.3.2]. Calibration curves for all the components were obtained in 4-6 independent days using five batches of prepared calibration standards containing 20, 40, 60, 80 and 100ppm. The slopes of the standard calibration curves of the four compounds showed a good fit and for all least squares analyses; the R² values were greater than 0.999. The Limit of Detection (LOD) was estimated by considering a signal to noise
ratio (s/n) of 3:1. The the range of these LODs were around 3-4ppm except for GA of liquorice. The results, calibration curves and linear ranges, together with the LOD values, are summarized in Table 3.4 and Fig.3.3. The percentage relative standard deviations (RSD%) and Mean Recovery against 3 nominal concentrations (40ppm, 60ppm and 80ppm) were calculated in order to evaluate precision (repeatability) and demonstrate the accuracy of the method during days, which RSD% values of intra-day were less than 10% except GA of liquorice at the low concentration level (20ppm), and the corresponding Mean Recovery values range were between 99.2% and 102.5%. The RSD% and Mean Recovery are presented in Table 3.5. The results that the values noted above were acceptable precision and accuracy, although the repeatability intra-days of this measurement deteriorated at 20ppm of GA, the methods are still satisfactory.

Fig.3.1 HPLC chromatogram of a standard solution of Trigonelline, Caffeine and Glycyrrhizic acid (GA)
Fig. 3.2 HPLC chromatogram of standard solution of Ginsenoside Rb1

Table 3.4 Regression equations and detection limits of components determined for the presented HPLC assay

<table>
<thead>
<tr>
<th>Components</th>
<th>Calibration Curves</th>
<th>Linear Range (ppm)</th>
<th>$R^2$</th>
<th>Detection Limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>$y = 30.181x + 10.882$</td>
<td>20-100</td>
<td>0.9997</td>
<td>3.334</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>$y = 14.856x - 0.8158$</td>
<td>20-100</td>
<td>0.9994</td>
<td>4.116</td>
</tr>
<tr>
<td>GA</td>
<td>$y = 5.5154x + 16.704$</td>
<td>20-100</td>
<td>0.9998</td>
<td>12.313</td>
</tr>
<tr>
<td>Rb1</td>
<td>$y = 3.6325x + 3.6498$</td>
<td>20-100</td>
<td>0.9992</td>
<td>3.674</td>
</tr>
</tbody>
</table>

Detection of Limit is defined as the concentration where the signal-to-noise ratio is $\rho$; $\rho \geq 8$

Fig. 3.3 Calibration curves of Trigonelline, Caffeine and Glycyrrhizic acid (GA) and Ginsenoside Rb1 the regression equations of the four compounds with $R^2 > 0.999$, respectively. (n≥4)
Table 3.5 Intra-day precision and accuracy for the HPLC assay of four components

<table>
<thead>
<tr>
<th>Nominal Concentration (ppm)</th>
<th>Measured Concentration</th>
<th>R.S.D (%)</th>
<th>Mean Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caffeine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>39.609±0.494</td>
<td>1.247</td>
<td>99.023</td>
</tr>
<tr>
<td>60</td>
<td>60.122±0.633</td>
<td>1.206</td>
<td>100.202</td>
</tr>
<tr>
<td>80</td>
<td>79.208±1.247</td>
<td>1.574</td>
<td>99.01</td>
</tr>
<tr>
<td><strong>Trigonelline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>39.555±1.602</td>
<td>4.051</td>
<td>98.887</td>
</tr>
<tr>
<td>60</td>
<td>59.915±3.014</td>
<td>5.036</td>
<td>99.859</td>
</tr>
<tr>
<td>80</td>
<td>78.963±4.883</td>
<td>6.184</td>
<td>98.703</td>
</tr>
<tr>
<td><strong>GA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>39.709±5.056</td>
<td>12.733</td>
<td>99.273</td>
</tr>
<tr>
<td>60</td>
<td>60.695±6.021</td>
<td>9.449</td>
<td>101.158</td>
</tr>
<tr>
<td>80</td>
<td>79.565±7.028</td>
<td>8.832</td>
<td>99.456</td>
</tr>
<tr>
<td><strong>Rb1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>39.162±1.585</td>
<td>4.046</td>
<td>97.905</td>
</tr>
<tr>
<td>60</td>
<td>61.473±2.357</td>
<td>3.835</td>
<td>102.456</td>
</tr>
<tr>
<td>80</td>
<td>80.109±1.756</td>
<td>2.192</td>
<td>100.13</td>
</tr>
</tbody>
</table>

N=4; R.S.D: Relative Standard Derivation; Mean value (± standard deviation)

3.6.2 Validation of the GC/MS assay

3.6.2.1 Method validation by Kováts index (I).

Kovats indices are used to measure/characterize compounds under isothermal column condition, where NX is the unknown analyte, Nn and N(n+1) are the number of carbon atoms of the reference n-alkanes which bracket the retention time of the unknown analyts, t' are the retention time of the analysts or references of n-alkanes. Here the n-alkanes retention time NC8 was set as t’Nn, while another t’NC20 as t’N(n+1). Values were calculated via the Kováts index (I) calculator.

\[
I = 100 \left[ n + \frac{\log t'_{NX} - \log t'_{Nn}}{\log t'_{N(n+1)} - \log t'_{Nn}} \right]
\]

From Fig.3.4, n-alkanes elution peaks were free of noise interference. Under the same three day conditions, the n-alkanes (C8-C20) Kováts Indices relative standard deviation (RSD) values are <0.5%. The method precision and accuracy is considered to be acceptable. The n-alkanes (C8-C20) mass match quality received 95% on average [Table 3.6; Table 3.7]. The absorption amounts of
monoterpenoid from coffee-ginger beverages relate to the absorption period. The amounts of caffeine were obtained in 10min by the conditions, [Fig.3.5] however caffeine cannot be detected less than 2min or 5min. The essential oils from ginger and caffeine Kováts Indices relative standard deviation (RSD) values are also less than 0.5% after test three times and the analytes mass match quality were 95% on average.

Table 3.6. Comparison of three times n-alkanes(C8-C20) Kovats Indices and the percent of quality Mass Match.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Mass Match (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octane C8H18</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>96</td>
</tr>
<tr>
<td>Nonane C9H20</td>
<td>931</td>
<td>930</td>
<td>931</td>
<td>95</td>
</tr>
<tr>
<td>Decane C10H22</td>
<td>1047</td>
<td>1047</td>
<td>1048</td>
<td>94</td>
</tr>
<tr>
<td>Undecane C11H24</td>
<td>1152</td>
<td>1151</td>
<td>1152</td>
<td>95</td>
</tr>
<tr>
<td>Dodecane C12H26</td>
<td>1247</td>
<td>1246</td>
<td>1248</td>
<td>95</td>
</tr>
<tr>
<td>Tridecane C13H28</td>
<td>1335</td>
<td>1335</td>
<td>1336</td>
<td>95</td>
</tr>
<tr>
<td>Tetradecane C14H30</td>
<td>1418</td>
<td>1417</td>
<td>1419</td>
<td>93</td>
</tr>
<tr>
<td>Pentadecane C15H32</td>
<td>1506</td>
<td>1503</td>
<td>1505</td>
<td>95</td>
</tr>
<tr>
<td>Hexadecane C16H34</td>
<td>1619</td>
<td>1611</td>
<td>1613</td>
<td>97</td>
</tr>
<tr>
<td>Heptadecane C17H36</td>
<td>1768</td>
<td>1758</td>
<td>1758</td>
<td>97</td>
</tr>
<tr>
<td>Octadecane C18H38</td>
<td>1882</td>
<td>1880</td>
<td>1883</td>
<td>98</td>
</tr>
<tr>
<td>Nonadecane C19H40</td>
<td>1944</td>
<td>1943</td>
<td>1944</td>
<td>97</td>
</tr>
<tr>
<td>Eicosane C20H22</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>99</td>
</tr>
</tbody>
</table>

Fig. 3.4 GC chromatogram of standard of n-alkanes(C8-C20)
Table 3.7 Comparison of three times monoterpenoid and caffeine Kováts Indices and the percent of quality mass match. * was not detected

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Kováts Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
</tr>
<tr>
<td>Pinene&lt;Alpha-&gt; C_{10}H_{16}</td>
<td>975</td>
</tr>
<tr>
<td>Camphene C_{10}H_{16}</td>
<td>996</td>
</tr>
<tr>
<td>Myrcene C_{10}H_{16}</td>
<td>1036</td>
</tr>
<tr>
<td>Limonene C_{10}H_{16}</td>
<td>1083</td>
</tr>
<tr>
<td>Cineole &lt;1,8-&gt; C_{10}H_{18}O</td>
<td>1088</td>
</tr>
<tr>
<td>Linalool C_{10}H_{18}O</td>
<td>1152</td>
</tr>
<tr>
<td>Neral C_{10}H_{16}O</td>
<td>1283</td>
</tr>
<tr>
<td>Geranial C_{10}H_{16}O</td>
<td>1309</td>
</tr>
<tr>
<td>Zingiberene &lt;Alpha-&gt;C_{15}H_{26}</td>
<td>*</td>
</tr>
<tr>
<td>Caffeine C_{8}H_{10}N_{4}O_{2}</td>
<td>1910</td>
</tr>
</tbody>
</table>

3.6.2.2 Camphene calibration curves

The coffee herbal beverage quantitative analysis of camphene from ginger method was validated by SPME-GC/MS chromatography. The linear range of the calibration curves for the GC-MS analysis were 20-60 ppm ($R^2$ = 0.9686) and 0.2-5 ppm ($R^2$ = 0.9998) [Fig.3.6 and Fig.3.7]; The limit of detection (LOD) was measured using a a signal to noise ratio (s/n) of 3:1 and were 7.100 ppm and 0.055 ppm, using 20 ppm and 0.2 ppm camphene standard solution respectively. [Table 3.8] The results, shown in Table 3.9, are expressed as the percentage relative standard deviations (RSD%) and Mean Recovery against 3 nominal concentration (20, 40, 60 ppm; or 0.2, 1, 5 ppm) in both methods. The typical precision range (RSD%) lies between 2 and 17%, which is acceptable considering the very low concentrations of the analyte. The accuracy data, represented as mean recovery (%), suggested the recovery was 91–112% with a mean of 100.364% and a RSD% of 8.382%.
Table 3.8 Regression equations and detection limits of components determined for the presented GC/MS assay

<table>
<thead>
<tr>
<th>Components</th>
<th>Calibration Curves</th>
<th>Linear Ranger (ppm)</th>
<th>$R^2$</th>
<th>Detection Limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camphene</td>
<td>$y = 7 \times 10^6 x + 121186$</td>
<td>0.2-5</td>
<td>0.9998</td>
<td>0.055</td>
</tr>
<tr>
<td>Camphene</td>
<td>$y = 5 \times 10^6 x + 2 \times 10^7$</td>
<td>20-60</td>
<td>0.9686</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Detection of Limit is defined as the concentration when the signal-to-noise ratio is 3

![Calibration curves of Camphene](image)

Fig. 3.6 Calibration curves of Camphene in the both the concentration ranges with $R^2 > 0.96$
### Table 3.9 Intra-day precision for the GC/MS assay of ginger component (n=4)

<table>
<thead>
<tr>
<th>nominal concentration (ppm)</th>
<th>Measured concentration</th>
<th>R.S.D (%)</th>
<th>Mean Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camphene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.216±0.018</td>
<td>8.489</td>
<td>108.014</td>
</tr>
<tr>
<td>1</td>
<td>0.910±0.020</td>
<td>2.272</td>
<td>91.003</td>
</tr>
<tr>
<td>5</td>
<td>4.670±0.425</td>
<td>9.303</td>
<td>93.436</td>
</tr>
<tr>
<td>Camphene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>22.403±2.366</td>
<td>10.564</td>
<td>112.014</td>
</tr>
<tr>
<td>40</td>
<td>38.296±1.552</td>
<td>3.016</td>
<td>95.741</td>
</tr>
<tr>
<td>60</td>
<td>61.188±10.546</td>
<td>17.236</td>
<td>101.98</td>
</tr>
</tbody>
</table>

R.S.D: Relative Standard Derivation; Mean value (± standard deviation)

![GC/MS chromatogram of standards solution of Camphene and Caffeine](image)

**Fig.3.7** GC/MS chromatogram of standards solution of Camphene and Caffeine

### 3.6.3 Mode 1: Compounds concentration in coffee-herbal beverage

In order to evaluate the applicability of the developed method, coffee-herbal beverage samples were analysed, with ingredients content from 10% to 40%. Caffeine, trigonelline, GA and Rb1 peak areas correspond to the concentration by the use of external calibration curves. The contents of caffeine, trigonelline, GA and Rb1 in these samples are shown in Table 3.10, Table 3.11 and Table 3.12. Fig.3.8 and Fig.3.9 shows typical chromatograms that caffeine, trigonelline, GA and Rb1 were separated in the beverage. Caffeine values ranged from 60.90 to 211.05 mg in a 350mL coffee cup. Trigonelline contents in a 350mL cup are from 84.7mg to 170.8mg. Therefore, the contents of the GA and Rb1 can be determined as well.

Various herbal source and extract methods lead to the contents variability. Clearly, the GA peak areas in the chromatograms compare Liquorice Liquid extract (LL) with Liquorice Powder (LP), indicating the greater mass GA obtained in Liquorice Liquid extract (LL). The results of the GA analysis assays demonstrate that the amount is almost 2-3 fold greater than Liquorice Powder (LP) in
coffee herbal beverage (350mL). Additionally, the GA concentration is more reproducible in liquorice liquid extract (LL). The Rb1 assay results indicate that the amounts of the main ginsenosides Rb1 in the extracts of American ginseng (AG) appears to be greater than that present in undefined ginseng (UG), which are 3-4 times in coffee herbal beverage (350mL).

Table 3.10 Amounts peak area and the concentration of Caffeine, Trigonelline in coffee-herbal beverage (n=4)

<table>
<thead>
<tr>
<th>Beverage Compounds</th>
<th>10% Coffee</th>
<th>20% Coffee</th>
<th>30% Coffee</th>
<th>40% Coffee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(100mg mixture in 2mL)</td>
<td>Caffeine (mAU)</td>
<td>Trigonelline (mAU)</td>
<td>Caffeine (mAU)</td>
</tr>
<tr>
<td>Peak Area</td>
<td>539.012±57.564</td>
<td>973.390±36.507</td>
<td>1446.192±84.73</td>
<td>1830.223±105.92</td>
</tr>
<tr>
<td>(ppm)</td>
<td>17.499±6.284</td>
<td>36.169±9.910</td>
<td>47.556±3.6</td>
<td>60.281±3.510</td>
</tr>
<tr>
<td>Contents in 350ml cup(mg)</td>
<td>60.900</td>
<td>84.700</td>
<td>111.650</td>
<td>126.700</td>
</tr>
</tbody>
</table>

Mean ± standard deviation.

Table 3.11 Comparison of peak area and the concentration of GA from liquorice Powder(LP), GA from liquorice liquid extract(LL) in coffee-herbal beverage (n=3)

<table>
<thead>
<tr>
<th>Beverage Compounds</th>
<th>10% Liquorice</th>
<th>20% Liquorice</th>
<th>30% Liquorice</th>
<th>40% Liquorice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(100mg mixture in 2mL)</td>
<td>GA(LP)1:5 (ppm)</td>
<td>GA(LP)1:10 (ppm)</td>
<td>GA(LL)1:5 (ppm)</td>
</tr>
<tr>
<td>Peak Area</td>
<td>88.694±70.264</td>
<td>146.541±32.621</td>
<td>194.040±98.032</td>
<td>249.470±133.588</td>
</tr>
<tr>
<td>(ppm)</td>
<td>70.264±12.740</td>
<td>13.053±5.915</td>
<td>123.541±17.774</td>
<td>231.53±6.935</td>
</tr>
<tr>
<td>Contents in 350ml cup(mg)</td>
<td>22.750</td>
<td>82.250</td>
<td>56.000</td>
<td>147.700</td>
</tr>
</tbody>
</table>

Mean ± standard deviation.

Table 3.12 Comparison of peak area and the concentration of Rb1 from American ginseng(AG), Rb1 from unidentified ginseng source(UG) in coffee-herbal beverage (n=3)

<table>
<thead>
<tr>
<th>Beverage Compounds</th>
<th>10% Ginseng</th>
<th>20% Ginseng</th>
<th>30% Ginseng</th>
<th>40% Ginseng</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(100mg mixture in 2mL)</td>
<td>Rb1(AG) (ppm)</td>
<td>Rb1(AG) (ppm)</td>
<td>Rb1(AG) (ppm)</td>
</tr>
<tr>
<td>(ppm)</td>
<td>5.918±0.241</td>
<td>1.018±0.137</td>
<td>0.281±0.081</td>
<td>0.851±0.025</td>
</tr>
</tbody>
</table>

Mean ± standard deviation.
Fig. 3.8 Coffee herbal beverage sample HPLC chromatogram of trigonelline, caffeine and GA and dilution (1:10) with 25% methanol.

Fig. 3.9 Coffee herbal beverage sample HPLC chromatogram of Rb1(AG) and dilution (1:10) with 25% Acetonitrile.

3.6.4 Camphene concentration

Camphene concentrations in the coffee herbal beverage samples [Fig. 3.10] were calculated from the peak area with both standard calibration curves linear ranges defined. The camphene concentration in fresh ginger (FG) was much greater, especially (30% and 40% ratio) in coffee herbal beverage [Table 3.13] and were 16-26 fold higher than Ginger Capsule (GC). Therefore, Camphene is a highly volatile compound in Fresh Ginger (FG) and it has been mentioned that Ginger Capsule (GC) should be taken to account on rapid loss of the analyst.
Table 3.13 Comparison of peak area and the concentration of camphene from fresh ginger (FG), and from ginger capsule (GC) in coffee-herbal beverage (n=3)

<table>
<thead>
<tr>
<th>Beverage</th>
<th>10% ginger</th>
<th>20% ginger</th>
<th>30% ginger</th>
<th>40% ginger</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FG</td>
<td>GC</td>
<td>FG</td>
<td>GC</td>
</tr>
<tr>
<td>Peak Area (Abundance)</td>
<td>4.197×10^7</td>
<td>±1.650×10^7</td>
<td>2.490×10^7</td>
<td>±7.029×10^6</td>
</tr>
<tr>
<td>Con. In Analysis (ppm)</td>
<td>5.980</td>
<td>±2.358</td>
<td>0.339</td>
<td>±0.100</td>
</tr>
<tr>
<td>Contents in 350ml cup (mg)</td>
<td>2.093</td>
<td>±0.118</td>
<td>1.105</td>
<td>±0.155</td>
</tr>
</tbody>
</table>

Mean ± standard deviation.

Fig. 3.10 Coffee herbal beverage sample GC/MS chromatogram of camphene and caffeine

3.6.5 Mode 2: Herbal-Coffee beverage extraction methods

Obviously, these compounds including caffeine, trigonelline, Rb1 and GA from coffee herbal functional beverage, had a good yield obtained via pure water as the extraction solvent in microwave-assisted, percolation (coffee machine) and Soxhlet extraction [Fig.3.11]. The recipe of each component used a 25% ratio in the beverage, that is 0.5 each in 2g composites. Caffeine (alkaloids), trigonelline (hydrochloride salt), glycyrrhizic acid (saponin) and ginsenoside Rb1 (protopanaxadiol and dammarane family) are all extracted via the excellent polar solvent water. It is accepted coffee indigents are extracted via microwave heating and coffee machines and, especially, Rb1 and GA from Liquorice and Radix ginseng are released at low temperature (90-100°C) and in a short time (1min). However, there will be a shortage for Rb1 in the coffee machine extract in this case. The coffee machine requires slow passage of a solvent through fine particles. Therefore, the
sample mixture must be merged properly so that the solvent contacts these particles equally, which sometimes is determined by the machine extract design. As Rb1 extraction can be seen in the Soxhlet case, the longer the extracting time, the lower the yield. This phenomenon could be result from the hydrolysis of one or two sugar from ginseng saponin (containing 4 carbohydrates) with long heating time. On the other hand, because of water as the extract solvent, it carries along with lots of other plant components such as sugar, starches proteins with longer heating time, that could adsorb the effective constituents extracted. We considered that water as solvent and the microwave heating and percolation (coffee machine) extraction can be used to produce coffee herbal functional beverage. Hence Fig 3.11 shows that, except for Rb1, similar amounts of the compounds are extracted by each method (the mean value for each method is within confidence intervals of the other methods ie. they all overlap).

The microwave effect of coffee herbal functional beverage amount on the extraction efficiency was also studied. Samples with the ratio of 75% coffee and 25% herbs the total mass 2 g in 200 ml water were analysed by HPLC-DAD with these collected at 0.5 min, 1min and 1.5min. The above figure shows the concentration of the four compounds (caffeine, trigonelline, GA and Rb1) in the functional beverage. As seen from the above Figure 3.11, increasing the extraction time (from 0.5 min to 1.5 min) did not significantly enhance the extracted amount of the analysts in the beverage and the extraction balance was achieved. Therefore, the mid extraction time for coffee herbal compounds

**Fig.3.11** The coffee herbal beverage compounds concentration measurement via various extraction methods (with 95% confidence intervals)
with the microwave oven extraction set at 1 min. This extraction time shows that the maximum of compounds are extracted and when preparing a coffee beverage with microwave heating, 1 min would give a good temperature for herbal-coffee preparation [Fig.3.12].

**Fig.3.12** The amount of compounds extraction via microwave heating

### 3.7 ThT assay measurements

Hen egg white lysozyme amyloid protein can spontaneously assemble into protein fibrils under the experimental acid conditions of the in vitro studies. Green tea polyphenols [35], curcumin [36], myricetin [37], have been reported as effective inhibitors of the fibrillogenesis of hen egg white lysozyme (HEWL). A report demonstrated that curcumin can inhibit the aggregation and formation of amyloid fibrillation of HEWL at pH 2.0 in a dose dependent manner [36]. Importantly, evidence indicates that amyloid fibril inhibition of HEWL by phenolic compounds involves formation of quinoprotein interactions [38]. HEWL was incubated with or without these compounds and ThT fluorescence intensity increased in the presence of protein fibrils which were measured every 24 hours for each group. We monitored the temperature-dependence of ThT fluorescence intensity to examine the ability of natural compounds to inhibit the aggregation of protein such as caffeine, trigonelline and caffeic acid from coffee and Vitamin B1 as a resource from daily food. We will utilize semi-quantitative analysis to make the decision “yes or no” to confirm the possibility for natural compounds to inhibit protein fibrillation. Another purpose of the experiment was to ensure
measuring conditions including incubation temperature, test time point, drug concentration as optimal and the reproducibility of all these parameters is important for further quantitative assessment of more compounds at next stage. All ThT Fluorescence intensity normalized to the blank and was plotted as a function of time for 5mg/ml or 0.25 mg/mL at 50 °C in PBS (pH=2) without or with herbs. The normalization ThT fluorescence intensity incubated without herb constituents (positive control) increased rapidly after the equilibration period (72 hours), which demonstrated protein fibril formation. Caffeine, caffceic acid and vitamin B1, all led to a dramatically decreased ThT fluorescence intensity normalization [Fig. 3.13], which was individually compared to a positive control. This suggests that the compounds could affect the neuro-protection against protein aggregation formation and this requires further investigation of the efficient concentration-dependent inhibition. However, trigonelline, at concentrations up to 5mg/mL, had little effect on the ThT fluorescence response and only a small decrease and this also needs further confirmation. The negative control is untreated/blank protein solution as a measurement baseline, which the protein solution did not change the pH when heated. In addition, all test coefficient of determination ($R^2$) should improve further by more replicate test times during quantitative analysis.

![Caffeine Inhibition Curve for Fibrils Formation (caffeine n=3)](image-url)
Vitamin B1 Inhibition Curve for Fibril Formation (Vitamin B1 n=3)

Caffeic acid Inhibition Curve for Fibrils Formation (caffeic acid n=3)
Fig. 3.13 Semi-quantitative curve of protein fibrillation in the presence or absence of different nature compounds. The values are means ± SD, n = 3.

3.6 References


[27]. Lian WQ, Chong ZW and Chun SY. Isolation and analysis of ginseng: advances and challenges. Natural Product Reports. 2011(28):467-495


[34]. Sean AH, Heath E. et al. The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. FEBS Letters, 2009(276):5960–5972


Herbal-coffee beverage sensory analysis

Chapters 2 and 3 described the herbal-coffee beverage targeted bioactive compounds selection and analysis. Chapter 4 describes the assessment of herbal-coffee sensory human perceptions of potential/desirable/continuous consumption to sustain effective prevention against dementia.

4.1 Functional beverage sensory measurement

The European Functional Food Science Commission defined functional foods (which include beverages) as: "...satisfactorily demonstrate to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either an improved state of health and well-being and/or reduction of risk of disease." Functional foods became popular for consumers who are seeking specific health effects to keep fit and feel good regardless as to whether the food has been modified in some fashion [1]. Several herbs claim medicinal benefits due to their preventative properties such as anti-inflammation, anti-oxidants that also includes the perception of taste, aroma and the oral perception of texture [2,3,4]. The sensory properties determine peoples’ selection and how much they consume [5]. The bioactives from natural herbs not only affect function beverage performance but also the drinking habits and long term consumption. However, undesirable flavours in functional beverages may decrease the sensory acceptance and eventually may discourage continued consumption if perceived as bitter, acrid, astringent or salty off flavours.

The human study is designed to review results from investigation of human preferences and the sensory perceptions to the new functional beverages that were sampled. Functional beverages of orange flavoured barely β-glucan was assessed by 105 members of a panel through sensory evaluation including sweetness, sourness, orange flavour and thickness to estimate overall market acceptability [6].

4.2 Effect of herbal-coffee beverage constituents on sensory perception
Aroma is also central to a pleasurable eating/drinking experience and is one of the most labile components of food, coffee is an outstanding example. There are more than eight hundred volatile compounds that have been identified in coffee, many of which have been demonstrated to contribute to its aroma. The aroma profile of coffee is composed of the following notes: sweet/caramel-like, earthy, roasty, smoky, fruity and spicy [7]. Liquorice has a sweet flavour and is a diet multi-therapy herb. Glycyrrhizinic acid, a chief sweet-tasting constituent extract from liquorice root is about 50 times as sweet as cane sugar [8]. This product has been approved in the United States for a wide range of applications [9]. Some applications claim liquorice root extract can be used to make foamy food, to enhance foam stability or to impart aroma. Ginseng functional products have been largely limited to functional beverages because of the undesirable sensory properties of ginseng such as earthy, wood, astringent and bitter [10]. Ginger has been used as a spice for over 2000 years and has pleasurable aromas such as sweety, warm and also a pungency flavour.

4.3 Sensory assessment (9-point hedonic scale) applications in food science

Taste provides important sensory information of beverages and it also impacts hedonics, pleasure and displeasure. Hedonics reflect the immediate experience or anticipation of pleasure from the sensory stimulation of eating a food including taste, smell, and texture, [11] and promotes behaviours of long term usage associated with the enhanced acceptance [12].

In human taste panel studies, subjects assess the taste quality and intensity such as overall intensity, sweet, sour, salty, bitter, metallic, cooling, spicy, anaesthetic, astringent, irritating and other tastes [13]. Twenty five assessors participated in sensory analysis of coffee colour, aroma and flavour evaluation using a hedonic scale to determine the degree of likeness or not of the coffee samples [14]. Sixty eight customers participated in a hedonic value characteristic of wild blueberry–soy beverages by 9-point hedonic scale comprising colour, flavour, viscosity, customer attitudes and overall acceptability [15]. Twelve persons using the sip-and-spit method, assessed the individual theaflavins for the overall astringency of black tea [16].

Therefore, the consumers’ sensory perception can be recorded through a 9-point hedonic scale (sensory preferable level). And the sensory assessment form can be used to qualify or quantify the participant’s hedonic factors or levels of distress.
4.4 Participant recruitment method

Any healthy males and females aged 18-70 years old, who like to drink coffee and are free of allergies to herbs were potential participants in a sensory evaluation of coffee-herbal beverages at RMIT University. The project was approved by the RMIT Human Research Ethics Committee. Eight people were invited to participate in the project.

In the future the herbal beverage will be modified to satisfy the general public. All potential participants were randomly selected. A possible way of random participant selection could be using a lottery method. In practice, a Random Number Generator produces 2 random number orders between the ranges 1 to 5 without duplicating numbers.

4.5 Herbal-coffee beverage preparation

The coffee-herbal samples (ginseng, ginger and liquorice) were boiled with hot water (90-100 degrees) for 45 min. The concentration used was 100mg of a mixture in 2 mL water.

The Testing Sample Ingredients are shown below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coffee</th>
<th>Ginseng</th>
<th>Ginger</th>
<th>Liquorice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% (10mg)</td>
<td>20% (20mg)</td>
<td>30% (30mg)</td>
<td>40% (40mg)</td>
</tr>
<tr>
<td>2</td>
<td>20% (20mg)</td>
<td>10% (10mg)</td>
<td>40% (40mg)</td>
<td>30% (30mg)</td>
</tr>
<tr>
<td>3</td>
<td>30% (30mg)</td>
<td>40% (40mg)</td>
<td>10% (10mg)</td>
<td>20% (20mg)</td>
</tr>
<tr>
<td>4</td>
<td>40% (40mg)</td>
<td>30% (30mg)</td>
<td>20% (20mg)</td>
<td>10% (10mg)</td>
</tr>
<tr>
<td>0</td>
<td>Water Blank</td>
<td>Blank</td>
<td>Blank</td>
<td>Blank</td>
</tr>
</tbody>
</table>

4.6 Herbal-coffee beverage sensory assessment procedure

Participants should follow a few steps when testing the samples. The whole procedure is expected to take 30min. The test procedure was a "one off". No photographs or videos were recorded during the assessment.
Testing procedure

1. Every participant to mouth rinse with water at the beginning of the study.
2. The participants receive 5 taste samples about 1-2 ml per cup in 1 to 5 sequence.
3. These samples are in the mouth for at least three seconds; then the participant can choose to swallow or expel sample.
4. The participants immediately record for each sample, their response to hedonic scale such as flavour, tasty, texture.
5. Provide voluntary feedback on the coffee-herbal beverage for future modification (optional).

4.7 Sensory measurement protocol

Previously no adverse consequences or clinical reports were found after drinking 1g of the herb-beverage. But undesirable flavours such as bitterness, astringent, earthy in some of the beverages may decrease the sensory perception. This hedonic scale describes an equal number of positive and negative categories with intervals of equal size; for example, 1=not at all like, 3=slight like, 5=moderate like, 7= like very much and 9=extremely likeable [Appendix 1].

4.8 Hedonic scale statistical analysis

Every participant tasted samples 1 to 5 once, so these sample items such as colour, sweetness and astringent etc., were repeated measurement 8 times by 8 individuals. Then we compared the difference of the items between the two sample groups via Paired Tests (the items of sample 1vs 2, 3, 4, 5; the items of sample 2vs 3, 4, 5; the items of sample 3vs 4, 5; and the items of sample 4vs 5). Thus the null and alternative hypotheses are:

H0: μD ≤ 0 (mean the item of Hedonic Scale in this sample is less than or equal to that sample)
H1: μD > 0 (mean the item of Hedonic Scale in this sample is greater than that sample)

Choosing a level of significance of α=0.05 and assuming that the differences are normally distributed, for an item of n=8, there are n-1=7 degrees of freedom.

The reasons for assuming that the differences normally distribution:

1. There are no literature reviews to provide this information with regard to herbal-coffee hedonic scale population distribution.
2. According to the Central Limit Theorem conclusion, if we do not know the specific shape of the population distribution more than 30 samples would be selected. However, we want to improve the
result accuracy as well as cost effectives of the procedure. This would be the best cost-effective method of choice for the small group selection before any valued results obtained.

The conclusion data will reflect what sensory participant is preferable.

4.9 Hedonic scale record discussion

A small group of 8 healthy participants (4 males and 4 females) were randomly selected and completed the *Sensory Analysis Evaluation Form* to evaluate the functional beverage flavor acceptance and consumption. Overall, there were no significant average or distribution differences in hedonic scale ratings between these samples, which could be induced by a small group selection as one of reasons. All participants who tasted the beverage perceived the samples (1-4) between strong and very strong, and the prepared herb concentrations were perceived as too high. The normalized ratings (negative control) refer to the code of “0”, produced similar functions except the sweetness tasty, which needs to be investigated further. The sample 3 and 4 (including 30% and 40% coffee), texture (colour) number averages were greater than 30% of sample 1, 2 and had a favourable colour of coffee brown [Fig.4.1]. Participants were more likely to enjoy spicy flavour, where scale numbers exceeded nearly 40% in sample 1 and 2 containing 30-40% ginger. However, ginseng root produced similar earthy scale ratings and modest equivalent associations with the content, which also included roast smell sensations [Fig.4.2]. In the oral sensory perceptions, participants were skewed toward higher intensities of astringent and bitterness, which indicates we must remove astringent compounds such as ginseng, and particularly bitter compounds in coffee because these decrease tasty acceptance and enjoyment and subtract from sweet sensory perceptions [Fig.4.3]. Through this small group analysis some valuable data was obtained; we consider that the elimination of the perceived negative sensory compounds that lead to earthy astringent taste perceptions will enhance the perceived acceptable beverage taste including the favored roasty and sweetness perceptions for future larger human taste perception studies.
Fig. 4.1. Texture of Hedonic Scales values in herbal-coffee beverage

Fig. 4.2. Flavors of Hedonic Scales values in herbal-coffee beverage

Fig. 4.3. Tasty of Hedonic Scales values in herbal-coffee beverage
4.10 References


[7]. Belitz H.-D, Grosch W, Schieberle P. *Food Chemistry*. 4th revised and extended ed.


Conclusion and suggestions for further work

The preceding chapters have illustrated the experimental design, instrumental analysis of coffee herbal functional beverages. Sample extraction and analysis can be achieved with convenient extraction methods that are compatible with existing HPLC-DAD and GC-MS detection. The participant sensory evaluations were measured by a small group (8 people invited) with a 9-point hedonic scale.

5.1 Conclusion

The strategy of this research project is focused on the potential prevention and treatment of cognitive impairment by recommending the consumption of coffee herbal beverages as early as possible in life.

Firstly, epidemiological studies suggest that dietary measures, physical exercise, and mental activity may reduce the risk of cognitive impairment and AD in elderly subjects. However, these preventative activities are not easy to qualify, quantify and to track their performance. As evidenced by the effective constituents of coffee and herbs against the cognitive impairment (presented in Chapter 2) they are an emerging area of interest. At the start of this project, we set the selection criteria and chose suitable herbs and their constituent bioactives including caffeine, trigonelline, glycyrrhizic acid (GA), Rb1 and camphene from coffee, liquorice, ginseng and ginger. These natural products are active against dementia and have an acceptable human safety profile related to the amyloid hypothesis, cholinergic hypothesis, inflammatory/oxidative hypothesis, $\text{Ca}^{2+}$ and adenosine receptor hypothesis, that contribute to the potential causes that lead to AD.
Secondly, the purpose of the project was the analysis of the functional beverage to confirm the concentration of the effective constituents that are conveniently extracted into the beverage, which enables the analysis of the ingredients to evaluate their protective effects over long term consumption (presented in Chapter 3). Water, a polar solvent, safe for the intake of the effective constituents in coffee-herbal beverages. The target compounds were measured after the various extraction methods such as decoction, percolation (coffee machine), microwave heating, Soxhlet and SPME to evaluate if the compounds were effectively extracted by these various techniques. After HPLC analysis, we concluded that the constituents (e.g. caffeine, trigonelline, GA and Rb1) were released completely at 90-100°C (coffee machine) or 1min (microwave oven) that were much better than the high temperature (100°C), or longer extraction times (≥8 hours) by Soxhlet extraction. In addition, we measured the increasing constituent ratios of the target compounds concentration range from 10% to 40% by HPLC and GC/MS from decoction extraction methods. The HPLC and GC/MS analysis methods were validated, by parameters including identification, accuracy, precision, linearity and acceptable limit of detection. Therefore, the different ratio of the components of these target compounds (caffeine, trigonelline, GA, Rb1 and camphene) is available for evaluation for future work. We also compared the contents of these compounds from different sources. The results indicated that American ginseng extracts contained the highest amount of Rb1 and fresh ginger containing camphene of ginger oil extracted the most GA. The result showed a promising prospect for quality control of extracting the effective herb constituents to produce a coffee herbal functional beverage.

We used *in vitro* Thioflavin T (ThT) fluorescence assays to confirm that the selected natural products have the potential to inhibit β-sheet amyloid protein fibrillation. *In vitro* measurements of the pure natural compounds including caffeine, caffeic acid and vitamin B1, inhibited the amyloid protein of HEWL from fibrillation over extended heating times (50°C for 144 hours) in an acidic environment (pH=2). Also, a small number of people (8 healthy adult participants) estimated the sensory perception of the herbal-coffee beverages. The positive feedback from the participants indicated that the sampled herbal-coffee beverages were too “strong”, implying that the effective constituent concentration were too high. In future a balance between neuroprotection and sensory acceptance needs to be determined. This is particularly relevant to ginseng, as it has a characteristic earthy astringent perception that strongly influences the taste of herbal-coffee beverages.

5.2 Suggestions for future work
We used the *in vitro* model amyloid protein of HEWL to investigate whether some natural binding effects were present in the coffee-herb constituents to inhibit amyloid fibril formation. Future work could utilize native or synthetic amyloid β protein or infected cells to assess the rate of inhibition of natural products *in vitro*. The small [8] number of participants in human sensory perception study was a limitation of this study and could be extended to a much larger cohort of panel members that would more reliably determine the best acceptable herbal-coffee formulations. Future work should include post-consumption/intake of the herbal beverages, the analysis of the detection, measurement of relevant and specific herbal biomarker metabolites as evidence for their benefits/presence, to encourage continuous consumer consumption whilst also providing/impacting benefits against dementia.

In addition, it is important to measure the functional beverage-brain affects *in vivo* with animal model studies in mice or rats, that can be used to monitor the beverage safety and efficiency. Also for coffee-herbal beverages safe intake studies, the use of healthy animals would allow the investigation and analysis of any affects on various organs extended over months or years. Additionally, AD transgenic mice can be used to evaluate the beverage brain protection performance and efficacy.
### Appendix 1

#### Table 1. Herbal Coffee Beverage Sensory Analysis Evaluation

This template was used for data collation to help assess the herbal coffee beverage acceptance.

<table>
<thead>
<tr>
<th>Code:</th>
<th>Gender:</th>
<th>Age ranges:</th>
<th>~40</th>
<th>40~60</th>
<th>60~</th>
</tr>
</thead>
<tbody>
<tr>
<td>0=none of above, 1=not at all like, 3=slight like, 5=moderate like, 7=like very much and 9=extremely likeable</td>
<td>Number the items in order of your taste</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Texture</td>
<td>0,1,3,5,7,9</td>
<td>0,1,3,5,7,9</td>
<td>0,1,3,5,7,9</td>
<td>0,1,3,5,7,9</td>
<td>0,1,3,5,7,9</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| B Odour Flavour | | | | | |
| Roasty | | | | | |
| Spicy | | | | | |
| Root/Earthy | | | | | |

<p>| C Tasty | | | | | |
| Sweetness | | | | | |
| Bitterness | | | | | |</p>
<table>
<thead>
<tr>
<th>Astringent</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D Expected cost (350ml/bottle)</strong></td>
<td>~$3</td>
<td>$3~$5</td>
<td>$5~</td>
</tr>
<tr>
<td><strong>E Opinion(optional)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>