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CARACTERISATIONS CHIMIQUES ET BIOLOGIQUES D'EXTRAITS DE PLANTES AROMATIQUES ET MEDICINALES OUBLIEES OU SOUS-UTILISEES DE MIDI-PYRENEES (FRANCE) ET CHONGQING (CHINE)

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Abstract

Medicinal and aromatic plants have been widely used as a rich source of drugs in several traditional medicine systems, food supplements, flavours, fragrances and cosmetic ingredients. However, some medicinal and aromatic plants, especially those which are not cultivated, have less and less uses over time and now seemed to be forgotten due to various reasons. These plants could be referred to as forgotten, medieval or underutilised medicinal and aromatic plants. Despite of limited studies and applications, the potentials of these plants for both aromatic and biological purposes remain to be quite high.

Essential oils are valuable natural molecules which are widely used in cosmetic, agricultural, and food industries. For most medicinal and aromatic plants, essential oil contents are only up to 1 %, which means that there will be 99 % of residues after extraction of essential oils. Containing rich second metabolites, the residues could be potential sources of bioactive compounds, e.g. natural antioxidants which, in recent years, have been increasingly sought after in food or cosmetic industries.

As a sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals and materials) and bioenergy (biofuels, power and heat), biorefinery seems to be really suitable for a global valorisation of different molecules in forgotten or underutilized medicinal and aromatic plants. Based on biorefinery, the concept of medicinal and aromatic plants-refinery or MAP-refinery was developed in this thesis. Plant materials were processed into four parts: essential oil (or volatile extract), aqueous extract, methanolic extract and final residue. In this way, the molecules in the plants can be sequentially extracted and used, thus avoiding a great waste of natural resources.

In the Midi-Pyrénées (France) and Chongqing (China) regions, there are rich and underutilized medicinal and aromatic plants. Based on extensive bibliographic studies, lists of forgotten or underutilized medicinal and aromatic plants in both regions were established. From the lists, six plants in the Midi-Pyrénées region (*Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Geranium robertianum* L., *Cytisus scoparius* L. and *Spartium junceum* L.) and three plants in the Chongqing region (*Tussilago farfara* L., *Citrus aurantium* L. and *Saussurea costus* (Falc.) Lipech.) were finally selected. *Tussilago farfara* L. was the common plant in two regions. Short reviews of scientific studies on these eight plants

were presented, including basic description, chemical components (especially essential oils and antioxidant compounds), biological activities and uses.

In order to realise the global valorisation of different molecules, the MAP-refinery was applied to the selected plants in two regions. Essential oils (or volatile extracts) were firstly obtained in the process and analyzed by GC-FID and GC-MS. Volatile extracts composition in the roots of *Tussilago farfara* L. and *Calendula arvensis* L., and flower buds of *Spartium junceum* L. were firstly identified. The main chemical compounds in volatile extract from *Tussilago farfara* L. roots were sesquiterpene hydrocarbons and aliphatic compounds while main chemical compounds in volatile extract from *Calendula arvensis* L. roots were oxygenated sesquiterpenes, oxygenated monoterpenes and oxygenated diterpenes. The volatile extract from flower buds of *Spartium junceum* L. was mainly composed of aliphatic compounds. Antioxidant capacity of aqueous extracts was evaluated by DPPH radical scavenging assay while antioxidant capacity of methanolic extracts was evaluated by several high-throughput screening assays on the microplate (DPPH, ABTS, FRAC, ORAC and TPC assays). Results showed that several plant samples like *Cytisus scoparius* L., *Tussilago farfara* L., *Citrus aurantium* L. and *Robinia pseudoacacia* L. could be potential sources of natural antioxidants.

To overcome the shortcomings in conventional methods of extraction, several water-based green extraction technologies of natural products were investigated, e.g. hydrodistillation (HD), steam distillation (SD) and subcritical water extraction (SWE). The emphasis was put on their effects on essential oil composition and antioxidants recovery from selected plants. In the comparison of four combined extraction techniques of essential oil (Hydrodistillation-Clevenger apparatus, Hydrodistillation-Deryng apparatus, Steam Distillation-Clevenger apparatus and Steam Distillation-Deryng apparatus), essential oils obtained by these four techniques were qualitatively similar but the relative percentages of the components varied greatly. Deryng apparatus and Clevenger apparatus had very limited effects on the recovery of antioxidants from extraction residues, in contrast with the processes of hydrodistillation and steam distillation. Hydrodistillation seemed to be a better method for recovery of water-soluble antioxidant compounds but steam distillation appeared to be better than hydrodistillation in recovering antioxidants from solid residue.

Another water-based technique, subcritical water extraction were also used for a comparison with HD and SD. Aerial parts of *Calendula arvensis* L. were chosen for examining their effects on essential oil composition and antioxidants recovery. Results showed that HD and SD exerted limited influence on essential oil composition. But HD, SD

and SWE were found to have significantly different impacts on the recovery of antioxidants. HD showed higher extraction yields than SD and SWE. At the conditions chosen for subcritical water extraction (110°C), total phenolic content in aqueous extract obtained by SWE was slightly lower than aqueous extract obtained by HD and methanolic extract after SD. However, the time for SWE was much shorter than HD and SD processes.

Subsequently, subcritical water extraction of phenolic compounds from aerial parts of *Calendula arvensis* L. and *Geranium robertianum* L. were optimized. Extraction temperature and extraction time were found to have significant effects on extracts yields, antioxidant capacity of extracts, total phenolic contents of extracts and extracted quantity of phenolic compounds. At optimized experimental conditions, SWE showed higher recovery of phenolic compounds from these two plants than boiling water extraction, especially for *Calendula arvensis* L. In conclusion, SWE was a very efficient method for the direct extraction of phenolic compounds from *Calendula arvensis* L. and *Geranium robertinum* L.

In addition, the effects of mineral contents in water on antioxidants recovery from residues of *Geranium robertianum* L. after hydrodistillation were studied using five mineralized waters. Mineral contents in water were found to have very limited effects on yields of aqueous extracts and methanolic extracts. However, mineral contents, especially concentrations of calcium and bicarbonate, had strong decreasing effects on antioxidant capacity and total phenolic content of both aqueous and methanolic extracts.

Finally, based on the subcritical water extraction results in our work, an improved MAP-refinery was developed. Subcritical water was used for further extraction of antioxidant compounds from residues in original MAP-refinery. Aerial parts of *Geranium robertianum* L. were subjected to the process of the improved MAP-refinery to obtain volatile extract, aqueous extract, methanolic extract, subcritical water extract and final residue. The results showed that the improved MAP-refinery significantly increased the recovery of antioxidants compared with original MAP-refinery. This promising process will also allow a better valorisation of the final solid residue due to the lower content of residual water.

Key words: medicinal and aromatic plants, MAP-refinery, hydrodistillation (HD), steam distillation (SD), simultaneous distillation extraction (SDE), antioxidant capacity (AOC), essential oil, subcritical water extraction (SWE), phenolic compounds, Deryng apparatus, Clevenger apparatus, volatile extract, aqueous extract, methanolic extract, improved MAP-refinery

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List of Abbreviations

AAPH	2,2’-azobis (2-amidino-propane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid
AOC	antioxidant capacity
ASE	accelerated solvent extraction
AUC	area under the curve
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CFD	crushing finger device
DHS	dynamic headspace
DP	dry plant
DPPH$^{\bullet}$	2,2-diphenyl-2-picrylhydrazyl radical
EO	essential oil
ET	electron transfer
FAD	flash aroma dispenser
F-C assay	Folin-Ciocalteu assay
FRAP	ferric reducing/antioxidant power
GAE	gallic acid equivalent
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography-olfactometry
GENP	green extraction of natural products
HAT	hydrogen atom transfer
HD	hydrodistillation
HD-CA	hydrodistillation with Clevenger apparatus
HD-DA	hydrodistillation with Deryng apparatus
MAP	medicinal and aromatic plants
MAP-refinery	medicinal and aromatic plants-refinery
MWAE	microwave assisted extraction
nd	not detected
ORAC	oxygen radical absorbance assay
PEFAE	pulsed electric field assisted extraction
PHWE	pressurized hot water extraction
PLE	pressurized liquid extraction
RI	retention index
RIL	retention index in literature

RP	relative percentage
SD	steam distillation
SD-CA	steam distillation with Clevenger apparatus
SD-DA	steam distillation with Deryng apparatus
SDE	simultaneous distillation extraction
SE	Soxhlet extraction
SFE	supercritical fluid extraction
SPME	solid phase microextraction
SWE	subcritical water extraction
t	trace (< 0.1 %)
TBHQ	*tert*-butylhydroquinone
TCM	traditional Chinese medicine
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TIC	total ion chromatogram
TLC	thin layer chromatography
TPC	total phenolic content
TPTZ	2,4,6-tripyridyl-*s*-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UAE	ultrasonic assisted extraction
VE	volatile extract

Contents

General introduction

In both Midi-Pyrénées region (France) and Chongqing region (China), there are rich and underutilized medicinal and aromatic plants (MAP). Aiming at exploiting different molecules in these plants, the thesis titled 'Chemical and biological characterisation of extracts from forgotten or underutilised medicinal and aromatic plants from Midi-Pyrénées (France) and Chongqing (China) regions' was set up.

The thesis have several aims: (1) based on extensive bibliographic studies, a list of forgotten or underutilized medicinal and aromatic plants from Midi-Pyrénées and Chongqing regions will be established according to several rules of selection; (2) Global valorisation approaches for sequential utilisation of plant molecules will be developed and applied to the selected plants from these two regions; (3) Several water-based green extraction technology of natural products will be investigated, especially to look at their effects on essential oils extraction and antioxidant capacity recovery from selected plants.

The thesis contains four chapters. The main work of each chapter is summarized as follows:

Chapter 1 provides a general introduction to the thesis. Thesis background was firstly presented, including definition of forgotten plants, problems existing in plants utilisation in two regions and possible valorisation of plant molecules. Based on concept of biorefinery, MAP-refinery was proposed. The extraction and analytical methods for essential oils (or volatile extracts) and antioxidants were also reviewed.

Chapter 2 deals with the selection of underutilized medicinal and aromatic plants in two regions. Finally, six plants from the Midi-Pyrénées region and three plants (one common plant with Midi-Pyrénées region: *Tussilago farfara* L.) from the Chongqing region were selected. Short reviews of these eight plants were given, including basic description, chemical components in plants, biological activities and applications.

Chapter 3 is devoted to the application of MAP-refinery to the selected plants in two regions. Plant materials were processed into four parts: essential oil (or volatile extract), aqueous extract, methanolic extract and final solid residues. Essential oils (or volatile extracts) were analyzed by GC-FID and GC-MS. Antioxidant capacities of aqueous and methanolic extracts were evaluated by different methods. The possible applications of final solid residues were proposed.

Chapter 4 is composed of five separate parts which focus on the effects of several water-based green extraction technologies on essential oils composition and antioxidants recovery from selected plants. Part 1 compared four essential oil extraction techniques. Part 2 looked at the effects of mineral contents in water on antioxidants recovery after hydrodistillation. Part 3 (a) examined the effects of three waters of different physical states on essential oil composition and antioxidants recovery. Part 3 (b) studied the subcritical water extraction of natural antioxidants from *Calendula arvensis* L. and *Geranium robertianum* L. Part 4 examines the feasibility of improved MAP-refinery. Based on the subcritical water extraction results, an improved MAP-refinery was developed. Subcritical water was used for further extraction of antioxidant compounds from residues in original MAP-refinery. *Geranium robertianum* L. was chosen as model plant.

Finally, the conclusions were summarized and some prospects were proposed.

Chapter 1 Bibliographic studies

1.1 General Background

Since ancient times Medicinal and Aromatic Plants (MAP) have been widely used as a rich source of drugs in several traditional medicine systems, food supplements, flavours, fragrances and cosmetic ingredients. However, some medicinal and aromatic plants, especially those which are not cultivated, have less and less uses over time and now seemed to be forgotten due to various reasons. These plants could be referred to as forgotten, medieval or underutilised medicinal and aromatic plants. As it is known, plants contain various molecules including primary metabolites (e.g., cellulose, hemicellulose, lignin, lipids, proteins and nuclear acids) and secondary metabolites (e.g., terpenoids, alkaloids and phenolics). Secondary metabolites are generally very valuable molecules which can find a lot of applications in food, cosmetics and pharmaceutical industries. From these forgotten or underutilized plants, new aromas or bioactive compounds could be found. Therefore the potentials of forgotten medicinal and aromatic plants for both aromatic and biological purposes seem to be quite high.

In the Midi-Pyrénées (France) region, there are rich medicinal and aromatic plants. However, most of these plants are not fully valorised. Even for some cultivated plants, only essential oils or some major active compounds are extracted, leaving large quantity of residues unattended. While in the Chongqing (China) region, there are also extremely rich plant resources. More than 6000 kinds of plants can be found in the area, but less than 15% of these plants have been widely studied and utilized. With its plentiful medicinal plants, Chongqing is a major producer of traditional Chinese medicinal plants in China. However, the utilisation of these medicinal plants is far from satisfactory. For a long time, many cultivated medicinal plants are often employed for producing only one or two target molecules, e.g., artemisinin from *Artemisia annua* L. The extraction residues are generally discarded. In Traditional Chinese Medicine (TCM), essential oils are not regarded as important molecules and often removed from plants in the pre-treatment process, leading to the loss of valuable products. Therefore more attention should be paid to the global valorisation of different

molecules in these plants, thus increasing the added values of plants and sustainability of plant resources.

In the extraction of natural products from plants, some organic solvents like hexane, petroleum ether and methanol, are often used in conventional methods. This causes increasing concerns on safety of plant extracts because of possible solvent residues. At the same time, conventional methods are also harmful to environments and require high energy consumption. These problems strongly require technological innovations in extraction and separation methods of natural products from plants.

In fact, the boundaries between medicinal plants and aromatic plants are not precisely defined. Some medicinal plants could contain high content of essential oils while some aromatic plants also contain molecules which could be used for medicinal purposes. Essential oils are valuable natural molecules which are widely used in cosmetic, sanitary, agricultural, aromatherapy and food industries. Due to its unique characteristics, they are easily separated from plants. For most medicinal and aromatic plants, essential oil contents are only up to 1%, which means that there will be 99% of residues after extraction of essential oils. From perspective of a full utilization of plants, some other by-products should be produced from the residues instead of leaving them as wastes. Containing rich secondary metabolites, the residues are potential sources of bioactive molecules, e.g. antioxidant molecules or antimicrobial molecules. After separation of different molecules, the final residue can still be used for making biomaterials, agromaterials or directly for fuels.

In the extraction of different molecules from residues, application as antioxidant additives appears to be promising in recent year since natural antioxidants are strongly sought after in food or cosmetic industries. An antioxidant may be defined as: ‘‘*any substance that when present at relatively low concentrations, compared with those of the oxidisable substrate significantly delays or inhibits oxidation of that substrate*’’ (Gutteridge, 1994). It has been known that antioxidants are very important in the human body by protecting the integrity of cellular structures and macromolecules from damage due to free radicals (Shahidi and Ho, 2007). Antioxidants are also essential to foods because auto-oxidation in fats, oils and lipid-containing foods can cause food quality deterioration with loss of nutrients, generation of unpleasant odours and even toxic substances. Therefore antioxidants are frequently used to retard oxidation processes in the food industry. However, at present, more antioxidants have been used in foods because of obvious health benefits (Finley *et al.*, 2011).

By their nature, antioxidants could be classified into natural and synthetic ones. The best known synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene

(BHT), gallates and *tert*-butylhydroquinone (TBHQ) (Miliauskas, 2006). However, the use of synthetic antioxidants in food products is strictly regulated because of concern on their safety. At the same time, an increasing interest in natural antioxidants applied in food industry has been observed on the market. In fact, a lot of medicinal and aromatic plants have been found to contain chemical compounds with antioxidant capacity, and a lot of research work has been done on new natural antioxidant formulation development and application in food and cosmetic industries, e.g. the application of plant extracts from rosemary and sage. For the underutilized medicinal and aromatic plants, after extraction of essential oil, some interesting natural antioxidants could possibly be obtained from the residues.

For a better and sustainable utilization of different molecules in underutilized medicinal and aromatic plants, research and development should go to two directions: (a) to develop a global valorisation approach for sequentially extracting essential oils, antioxidants and other molecules from plants; (b) to apply green and high-efficiency extraction technique in the valorisation approach. To solve the problems, we could turn to the concept and principles of biorefinery and green chemistry.

1.2 The development of the concept of MAP-refinery

Petroleum or oil refinery is a well known industrial process of transforming crude oil to transportation fuels, heat, power, chemicals, and materials. Biorefinery is similar to petroleum refinery except that it uses biomass for various ends. According to the definition by IEA Bioenergy Task 42, biorefinery or biorefining is the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals and materials) and bioenergy (biofuels, power and heat). At present, several different biorefinery concepts have been developed, e.g., conventional biorefineries, green biorefineries, whole crop biorefineries, lignocellulosic feedstock biorefineries, two platform concept biorefineries, thermo chemical biorefineries and marine biorefineries (Diep *et al.*, 2012). Because biomass is a renewable resource, the use of industrial biorefinery has been identified as one potential solution that may meet increasing demand for energy, fuel, chemicals and materials (King *et al.*, 2010).

Medicinal and aromatic plants are renewable biomass. As the separation process of biomass into distinct components which could be individually utilized, biorefinery is really suitable for a global valorisation of different molecules in these plants. Therefore, based on the biorefinery, the concept of medicinal and aromatic plants-refinery or MAP-refinery was

developed, i.e. valorization of the entire plant by sequential extractions of molecules of interest. As shown in Figure 1, essential oils or volatile extract will be firstly obtained by use of hydrodistillation (HD) or simultaneous distillation extraction (SDE) which is used when essential oil yield is too low. Then, aqueous extract will be obtained by removing water from aqueous residue. For solid residue, Soxhlet extraction using methanol as solvent will be carried out to get methanolic extract. And the final solid residue can be proposed for fabricating agromaterials and biomaterials or directly for fuels. The aqueous and methanolic extracts will be evaluated for their antioxidant activity. The plants with high antioxidant activity will be potential sources of natural antioxidants. Essential oil obtained can be also proposed for application in food industry and cosmetics industry. It should be pointed out, that the MAP-refinery proposed here is only a preliminary valorization process in which plant materials are firstly processed into several parts. For the final application, further separation and purification would be necessary for chemical compounds in each part. In this way, the molecules in the plants can be fully used, thus avoiding a great waste of natural resources.

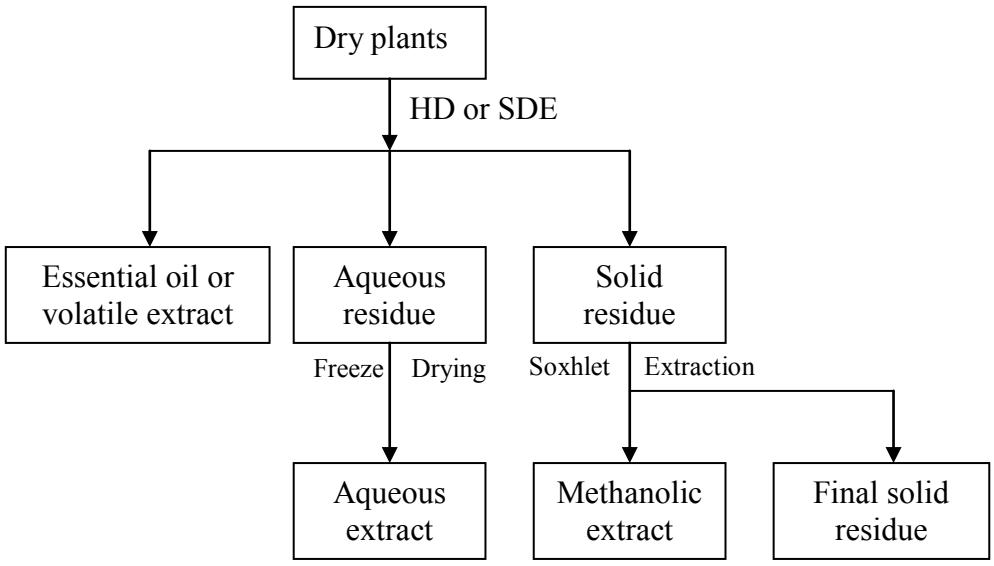


Figure 1 Scheme of the concept of MAP-refinery

MAP-refinery is developed on the basis of biorefinery, but it is different from the latter in several aspects: (1) the biorefinery process relies a lot on biological techniques but MAP-refinery uses more physical and chemical processes; (2) the main purpose of biorefinery process is to produce bioenergy and bio-based chemicals while MAP-refinery focuses on the production of highly valuble but low-content compounds for food and cosmetic industries, such as essential oil and antioxidants. MAP-refinery is also different from the agro-refinery

which is a concept of industrial processing of agricultural products into different products for food and non-food applications.

When MAP-refinery is applied to medicinal and aromatic plants for a sustainable and environmentally-friendly valorisation of different molecules, extraction and separation are the key steps. In other words, green and high-efficiency extraction methods should be employed to overcome shortcomings caused by conventional methods. Aiming at problems present in extraction of natural products, Chemat and co-workers proposed the concept and 6 principles of green extraction of natural products (Chemat *et al.*, 2012). Based on the concepts of green chemistry, the definition of green extraction of natural products (GENP) was given ''*Green extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product*''. In the development of green extraction technologies of natural products, use of alternative solvents (principally water or agro-solvents) has attracted increasing attention from researchers.

In our work, besides MAP-refinery applied to selected medicinal and aromatic plants, more emphasis will be put on several water-based extraction techniques, e.g. hydrodistillation, steam distillation and subcritical water extraction. Especially, their effects on essential oils and antioxidants recovery will be investigated.

1.3 Extraction and analytic methods

1.3.1 Extraction of essential oils (or volatile extracts)

Essential oils are volatile, natural and hydrophobic liquids containing aroma compounds extracted from plants, characterized by a strong odor and generally obtained by hydrodistillation or steam distillation. They are also known as volatile oils or simply as oil of the plant from which they are extracted. The chemical constitutes of essential oils are very complex. These compounds could be divided into several categories: terpene hydrocarbons (e.g., monoterpene hydrocarbons, sesquiterpene hydrocarbons and diterpene hydrocarbons), oxygenated terpenes (e.g., oxygenated monoterpenes, oxygenated sesquiterpenes and oxygenated diterpenes), aromatic compounds and aliphatic compounds. In terms of chemical classification, these compounds could be alkenes, alkanes, esters, alcohols, phenols, ethers, aldehydes and acids.

Essential oils are soluble in organic solvents with a generally lower density than that of water. Since the middle ages, essential oils have been found to possess many activities, such as bactericidal, virucidal, fungicidal, antiparasitical, insecticidal and antioxidant activities (Bakkali *et al.*, 2008). Due to their various properties and activities, essential oils are very valuable natural products which are widely used in many fields, such as pharmaceutical, cosmetic, sanitary, agricultural, aromatherapy and food industries. However, essential oils also showed some negative effects including cytotoxicity, phototoxicity, nuclear mutagenicity, cytoplasmic mutagenicity and carcinogenicity (Bakkali *et al.*, 2008).

Essential oils are formed in medicinal or aromatic plants as secondary metabolites. The oil yield and chemical compositions are influenced by many different factors including physiological variations, environmental conditions, geographic variations, genetic factors and evolutions, political/social conditions and amount of plant material/space and manual labor needs (Figueiredo *et al.*, 2008). Besides these factors, essential oil yields and chemical compositions are also influenced by extraction methods.

Plant materials consisting of flowers, leaves, roots, peels, wood, seeds, fruits, stems, rhizomes, bark or aerial parts, could be extracted by different methods to obtain essential oils. Essential oils are generally extracted by distillation. In the distillation of plant materials, three physicochemical processes are involved: hydrodiffusion, hydrolysis and decomposition by heat. In the hydrodiffusion process, essential oil and water will become vapours and then be condensed in the essential oil separator. As plant materials make contact with water for long time, some compounds in essential oil will be hydrolyzed. At the high temperature of water boiling point, some thermally unstable compounds in essential oils will also decompose. Because hydrolysis and decomposition by heat could influence chemical compounds of essential oils, the choice of extraction method is an essential element to the yield and quality of essential oils. Other methods include expression and solvent extraction.

1.3.1.1 Hydrodistillation (HD)

Hydrodistillation is a widely used method for the extraction of essential oils on the lab scale. In this method, plant materials are completely immersed in water, which is boiled by heating. At present Clevenger apparatus seems to be the most commonly-used essential oil separator, while in Poland Deryng apparatus is widely used (Polish Pharmacopoeia VI, 2002). The biggest difference between two apparatus is the cooling part (Figure 1). The cooling part of Deryng apparatus is believed to be more efficient. Deryng apparatus has been reported to

obtain more essential oil compounds than Clevenger apparatus (Sajewicz *et al.*, 2009; Rzepa *et al.*, 2012). In hydrodistillation, the plant materials are kept in motion with boiling water, which permits a complete distillation of the finely powdered plant materials while in steam distillation finely powdered plant materials will form lumps from which steam could not penetrate. Another advantage of hydrodistillation is its simplicity and easy construction. The main disadvantage of hydrodistillation is that, some sensitive esters in essential oil will be hydrolyzed and some compounds will decompose at high temperature. To minimize the decrease of essential oil quality, overheating should be avoided in the hydrodistillation process. Generally, hydrodistillation takes several hours or even longer time, which needs a lot of energy.

(a) (b) (c)

Figure 2 Clevenger apparatus (a); Deryng apparatus (b) and its cooling part (c)

1.3.1.2 Steam Distillation (SD)

Steam distillation is the distillation process of plant materials with steam generated outside the essential oil separator and it is the mostly accepted method for the large-scale production of essential oils. An advantage of steam distillation is that the amount of steam could be easily controlled. As it is the steam which passes through the plant materials, long-time contact with water and overheating could be avoided, thus effectively minimizing hydrolysis and thermal degradation of compounds in essential oils. However, commercial steam distillation apparatus is more expensive than hydrodistillation apparatus, limiting the wide application of steam distillation.

1.3.1.3 Simultaneous Distillation Extraction (SDE)

The essential oil yields of some plant materials are very low and it is rather difficult to collect real essential oil using hydrodistillation or steam distillation. In this case, it is only possible to collect volatile extract using another distillation-based technique, i.e. simultaneous distillation extraction (SDE). In fact, SDE is a combination of hydrodistillation and solvent extraction. The classic Likens-Nickerson apparatus is often used. It is one popular method for preparation of volatile compounds from samples and has found wide application in the fields of environment, food, flavour and fragrance. Since its creation in 1964, many research works including the optimal experimental conditions, the improvement of SDE apparatus and different applications have been done (Chaintreau, 2001). The mostly often used organic solvents are dichloromethane and pentane.

Other water-related methods include solvent-free microwave extraction in which microwave was used to heat the in-situ water in the plant materials and essential oils were evaporated from the plant materials with water (Lucchesi *et al.*, 2004), microwave hydrodiffusion and gravity method which is a combination of microwave for hydrodiffusion of essential oils from the inside to the exterior of biological material and earth gravity to collect and separate (Vian *et al.*, 2008), and instant controlled pressure drop extraction (Besombes *et al.*, 2011).

1.3.1.4 Subcritical Water Extraction

Besides, another water-based extraction method, subcritical water extraction, has attracted increasing interests from researchers. Subcritical water is liquid water under pressure at temperature between the usual boiling point 100°C and the critical temperature 374°C. With the increase of temperature, the polarity of water decreases dramatically. Subcritical water extraction is believed to be able to extract essential oils. At present there have some uses of this technique for extraction of essential oils or volatile compounds from some aromatic or medicinal plants, such as marjoram leaves (Jimenez-Carmona *et al.*, 1999), *Thymbra spicata* (Ozel *et al.*, 2003), *Rosa canina* (Ozel and Clifford, 2004), coriander seeds (Eikani *et al.*, 2007), *Lavandula latifolia* Medik (Eikani *et al.*, 2008), *Thymus vulgaris* L. (Dawidowicz *et al.*, 2009), *Bunium persicum* Boiss. (Mortazavi *et al.*, 2010) and *Cinnamomum zeylanicum* (Jayawardena and Smith, 2010). Different parameters like temperature, pressure, mean particle size and water flow rate, were investigated and optimized. In fact, pressure is not an

important parameter and its main role is to main the water in liquid state at high temperature. The most important parameter is temperature which has great impact on polarity of water. In the studies mentioned above, subcritical water extractions were operated from 100°C to 200°C and the optimal temperatures ranged from 100°C to 150°C. However, in these studies, even if essential oil yield of plant materials is high, no real essential oils could be observed or directly collected. Always obtained is the aqueous solution of extract. The volatile compounds were finally obtained in organic solvent solution, by use of subcritical water extraction followed by a liquid-liquid extraction.

It is noted that, after collecting essential oils by applying water-based techniques, the residual water could be collected and concentrated. This water could be referred to as hydrosol, aromatic water or herbal distillate which may be used as another fragrant product. Popular hydrosols include rose water and lavender water. Especially, the residual water after hydrodistillation also contains many water-soluble compounds which could be one source of bioactive compounds.

1.3.1.5 Expression or cold-pressing

Expression or cold-pressing is only applied to separate essential oils from orange or citrus peel which contains relatively large quantity of essential oil.

1.3.1.6 Solvent extraction

Organic solvents, especially low-polarity solvents like hexane, are often used to extract oils from plant materials. However, these extract are not pure essential oils. Extract from hexane and other hydrophobic solvents are often called concretes which are a mixture of essential oils, waxes, resins and other lipophilic compounds. Because of large quantity of non-fragrant waxes and resins, concretes are not widely used in perfumery but are generally converted into an alcohol-soluble volatile concentrate known as an absolute, i.e. they will be extracted with alcohol (Handa *et al.*, 2008).

Supercritical fluid extraction (SFE) is a promising green method for the extraction of aromatic extracts from plant materials. Supercritical carbon dioxide is often used as a solvent in SFE. This method avoids the solvent residues in the product. The relatively lower operating temperature prevents the decomposition of some thermally sensitive compounds. The natural odours of aromatic plants will be more retained in the essential oil products. However, SFE

device is very expensive and application in essential oil extraction is limited. Meanwhile, supercritical carbon dioxide also extracts some other compounds besides essential oils.

1.3.1.7 Other extraction methods for volatile extracts

For plant materials with very low yields of essential oil, the extract can only be called volatile extract. Extraction of these volatile extracts is generally for analytical purpose. SDE mentioned above is one method to obtain the volatile extract from plant materials. Other methods include solid phase microextraction (SPME) and headspace trapping extraction (Handa *et al.*, 2008). For a rapid evaluation of natural volatiles in aromatic plant leaves, some techniques have been developed, such as artificial crushing finger device (CFD) and flash aroma dispenser (FAD). The former could crush the aromatic leaves and make volatiles be released. These released volatiles will then be concentrated by controlled dynamic headspace (DHS) and analyzed by GC-MS (EI Kalamouni *et al.*, 2010). The latter used abrupt pressure change to release the volatiles in aromatic plant leaves (Dobravalskyte, 2013).

1.3.2 Analysis of essential oils

Essential oil is a mixture of chemical compounds. Therefore the first step in essential oil analysis is to achieve the best possible separation by using some effective technologies. By the nature of compounds in essential oils, they will range from volatile compounds (about up to a mass unit of 220) to semi-volatile compounds (about up to a mass unit of 400). This range is particularly suitable for gas chromatography analysis. In fact, most commonly-used analytical techniques for essential oils are based on gas chromatography. Generally GC-FID and GC-MS are used together for identification of chemical compounds in essential oils. For essential oils with complex components, comprehensive two-dimensional gas chromatography GC/GC-MS could be used for a better resolution of chemical compounds. Gas chromatography olfactometry is often used for the identifications of characteristic odorants in essential oils.

1.3.2.1 Gas Chromatography and GC-Mass Spectrometry

GC-MS is a hyphenated technique with a combination of separation and identification. Gas chromatography separates the chemical compounds in essential oils and mass spectrometry

plays the role of identification. With the help of powerful software for mass spectra matching, GC-MS is a widely-used method for identification of chemical compounds in essential oils. However, one important feature of mass spectrometry for essential oils is that mass spectra are not unique in many cases. Within the broad class of terpenoids, a large number of isomers with the same molecular but with different structure exist and their mass spectra may be very similar (Marriott *et al.*, 2001). Only from mass spectrometry, it is not easy to give accurate identification for some chemical compounds in essential oils. Therefore other techniques are needed for the complementary identification. Gas chromatography with flame ionization detector is often used. Essential oils are subjected to GC analysis. At the same time, a series of normal alkanes will be analyzed under the same conditions. Retention index will be calculated based on the retention times of normal alkanes and those of each component in essential oils. The comparison of calculated retention index with published data can aid the identification. In some cases, authentic compounds will be analyzed by GC for the identification. Another important role of gas chromatography is to give more accurate quantification results. Generally, GC-MS are not used for the quantification because there is great variability in response factor of each component, based on fragmentation by mass spectrometry.

1.3.2.2 Gas Chromatography Olfactometry

Some popular applications of essential oils are due to their distinctive odours. However, the relationship between concentration and odour intensity may vary greatly between compounds. Due to the large variation between these two properties, the response of FID or MS is not equivalent to the intensity of odours (Eyres, *et al*., 2007). The odour thresholds of volatile compounds can differ by many orders of magnitudes. It is possible that the compound with distinctive odour may not have apparent peak on the chromatogram. The role of gas chromatography olfactometry is to identify the active compounds which are responsible for the distinctive odour of samples. In the GC-O analysis, more often, when the chemical compounds in essential oil are separated and elute from a GC column, well-trained technicians use their noses to detect and evaluate the odour of these compounds. GC-O analysis is particularly interesting to perfumery and flavour industries.

1.3.3 Extraction of natural antioxidants

As there is an increasing interest in natural antioxidants, more research works have focused on antioxidants from plants and other natural resources, in search for an alternative to synthetic antioxidants. Natural antioxidants include many different categories of compounds, such as phenolics, carotenoids, vitamins, some hormones and some enzymes. However, phenolics are the most abundant and widely studied natural antioxidants in the plants. Phenolic compounds are so closely connected with antioxidant capacity of plant extract that total phenolic content is often determined along with antioxidant capacity evaluation assays. As compounds possessing aromatic rings with one or more hydroxyl groups, phenolics are the most abundant secondary metabolites in the plants, with more than 8000 phenolic structures currently known (Dai and Mumper, 2010). Phenolic compounds can be divided into extractable phenolic compounds (flavonoids, phenolic acids, stibenes, lignins and other polyphenols) and non-extractable phenolic compounds (proanthocyanidins, hydrolysable tannins and phenolic acid oligomers) (Perez-Jimenez and Torres, 2011). Because extractable phenolic compounds are more widely studied in the antioxidant capacity evaluation of plants or foods, the real antioxidant capacity of these samples may be underestimated due to abundant existence of non-extractable phenolic compounds.

The natural antioxidants mentioned in this thesis will be mainly limited to extractible phenolic compounds. For the extraction of these compounds, many methods have been used, including conventional methods and modern high-efficiency methods.

1.3.3.1 Conventional methods

Over the years, conventional methods have been used to extract phenolic compounds from plant materials. Soxhlet extraction (SE) and direct solid-liquid extraction techniques are examples of conventional methods. For extracting solvents, methanol, ethanol or mixture solvents of water and alcohols are commonly used. Generally pure water is not used as an extraction solvent for herbal plants because it can’t dissolve most organic compounds in the plants due to its high polarity and strong hydrogen bonds. However, for the extraction of polyphenols, sometimes water is directly used, as in the extraction of rutin using hot water. However, these techniques have some drawbacks: long extraction time, low selectivity, low extraction yield and large use of explosive and sometimes toxic organic solvents.

1.3.3.2 Modern high-efficiency methods

To overcome the shortcomings of conventional methods, many high-efficiency extraction methods have been applied for extraction of antioxidants (especially phenolic compounds), such as ultrasonic assisted extraction (UAE) (Vilkhu *et al.*, 2008; Ghafoor *et al.*, 2009), microwave assisted extraction (MWAE) (Proestos and Komaitis, 2008), pulsed electric field assisted extraction (PEFAE) (Corrales *et al.*, 2008), accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) (Ju and Howard, 2003), supercritical fluid extraction (SFE-carbon dioxide) (Tena *et al.*, 1997) and subcritical water extraction (SWE). These extraction methods permit shorter extraction time, higher extraction yields and selectivity for target molecules.

Among these methods, subcritical water extraction, also referred to as pressurized hot water extraction (PHWE), has become an interesting alternative for extraction of phenolic compounds because it offers many advantages: simplicity, short extraction time, high quality of the extract, low cost of extracting solvent and being environmentally friendly (Herrero *et al.*, 2006). When the temperature is raised between 100°C and 374°C and high pressure is applied to maintain water in liquid state, the water will be in subcritical state. With the increase of temperature, the polarity of water decreases dramatically and subcritical water could extract more valuable compounds from plant materials. Some recent studies have reported the use of subcritical water as a very efficient extracting solvent for phenolic compounds: carnosic acid and flavonoids from rosemary (Ibanez *et al.*, 2003), flavonoids from aspen knotwood (Hartonen *et al.*, 2007), phenolic compounds from bitter melon (Budrat and Shotipruk, 2009), phenolic compounds from *Terminalia chebula* Retz. fruits (Rangsriwong *et al.*, 2009), phenolic compounds from potato peel (Singh and Saldana, 2011), flavonol quercetin from onion skin (Ko *et al.*, 2011), hesperidin and narirutin from *Citrus unshiu* peel (Cheigh *et al.*, 2012) and phenolic compounds from different varieties of *Mangifera indica* leaves (Fernández-Ponce *et al.*, 2012). In the subcritical water extraction of flavonol quercetin from onion skin, the quercetin yield by SWE was 8-, 6- and 4-fold higher than those obtained using ethanol, methanol and boiling water as solvent (Ko *et al.*, 2011).

Besides the extraction of essential oils and antioxidants, subcritical water extraction has also found a lot of applications in extraction of other natural products from different natural sources like plants, food-by-products, algae and microalgae (Herrero *et al.*, 2006).

1.3.4 Antioxidant capacity (AOC) evaluation methods

For antioxidant capacity measurement, many *in vitro* evaluation methods have been

developed. At present, there have been several review articles on these methods, presenting the measurement principles, experimental approaches, results expression, advantages and shortcomings, comparisons between methods, and recommendations from authors (Prior *et al.*, 2005; Huang *et al.*, 2005; Chanda and Dave, 2009; Apak *et al.*, 2013). According to the reaction mechanisms, these assays could be classified into two types: hydrogen atom transfer (HAT)-based assays and electron transfer (ET)-based assays. For the selection of AOC evaluation methods, in 2005, Prior and co-workers proposed that three methods (Oxygen radical absorbance capacity assay, Folin-Ciocalteu assay and Trolox equivalent antioxidant capacity assay) should be standardized for use in the routine quality control and measurements of AOC of dietary supplements and other botanicals (Prior *et al.*, 2005). According to this proposal, AOC of extract from different plants in our work will be evaluated by ORAC assay, Folin-Ciocalteu assay and $ABTS^{\bullet+}$-based TEAC assay. In addition, another two commonly-used methods, $DPPH^{\bullet}$ radical scavenging assay and Ferric reducing/antioxidant power (FRAP) assay, will also be employed for a comprehensive evaluation. At present more and more methods have been adapted for analysis on the microplate so that high-throughput screening of antioxidants could be made.

1.3.4.1 $DPPH^{\bullet}$ radical scavenging assay

$DPPH^{\bullet}$ (2,2-diphenyl-2-picrylhydrazyl) is a stable organic nitrogen radical with a deep purple colour absorbing at 515 nm in methanol solution. $DPPH^{\bullet}$ radical scavenging assays is based on the reducing ability of antioxidants towards $DPPH^{\bullet}$. This ability could be evaluated by measuring the decrease of its absorbance. The widely used method was firstly reported by Brand-Williams and co-workers (Brand-Williams *et al.*, 1995). When $DPPH^{\bullet}$ reacts with an antioxidant, it will accept an electron or hydrogen atom and loses its absorbance at 515 nm. The reaction can be monitored by a spectrometer. The radical scavenging capacity of antioxidants or samples could be expressed as EC_{50} which is defined as the concentration causing a decrease in the initial $DPPH^{\bullet}$ concentration by 50%, or expressed relative to Trolox (Trolox equivalent). $DPPH^{\bullet}$ test is widely used in antioxidants screening because of its simplicity and rapidity, but some disadvantages also exist. When compounds in samples have absorbance at 515 nm, interference occurs. Sometimes $DPPH^{\bullet}$ can not reflect the real antioxidant capacity of some compounds or samples due to complex reaction kinetics or steric effects of $DPPH^{\bullet}$ (Apak *et al.*, 2013)

1.3.4.2 $ABTS^{\bullet+}$ cation radical decolourization assay

$ABTS^{\bullet+}$ cation radical decolorization was firstly used in Trolox equivalent antioxidant capacity (TEAC) assay (Miller *et al.*, 1993). The later improved assay used potassium persulfate for oxidation of 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS) to produce $ABTS^{\bullet+}$(Re *et al.*, 1999). The prepared stock solution of concentrated $ABTS^{\bullet+}$ can be stored for a long time. For testing, the stock solution can be diluted with buffer to a workable absorbance, for example, 0.7 at 734 nm. When $ABTS^{\bullet+}$ reacts with an antioxidant, its absorbance will decrease at 734 nm. The antioxidant capacity of samples will be reflected by the absorbance decrease and the results are expressed as Trolox equivalents. $ABTS^{\bullet+}$ cation radical decolourization assay has widespread popularity because of simple operation and rapid reaction process. $ABTS^{\bullet+}$ cation radical is soluble in both aqueous and organic solvents is not affected by ionic strength, so it has been used in multiple media to determine both hydrophilic and lipophilic antioxidant capacity (Apak *et al.*, 2013).

1.3.4.3 Ferric reducing/antioxidant power (FRAP) assay

FRAP assay was originally developed to measure reducing power in plasma (Benzie and Strain, 1996), but the assay has been subsequently adapted for antioxidant capacity assay of plant extract (Benzie and Szeto, 1999). The assay measures the ability of samples in reducing ferric/2,4,6-tripyridyl-s-triazine (Fe^{3+}/TPTZ) to a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. The antioxidant capacity measured by FRAP could be expressed as Trolox equivalent or ferrous equivalent. The redox potential of ferric salt (about 0.70 V) is comparable to that of $ABTS^{\bullet+}$ (0.68 V), so there is not much difference between $ABTS^{\bullet+}$ cation radical decolourization assay and FRAP assay except two assays are carried out at different pH (Huang *et al.*, 2005). One problem with FRAP assay is that plant extract may also contain some compounds which can complex with ferric ion. Some polyphenols could be the chelating compounds, thus make the final results inaccurate.

1.3.4.4 Oxygen radical absorbance capacity assay

On the basis of previous work by several researchers, the oxygen radical absorbance capacity (ORAC) assay was developed by Cao *et al.* (1993). The ORAC assays in our work will be performed according to the methods by Prior *et al.* (2003), Ganske and Dell (2006).

The assay uses fluorescein as the fluorescent probe. Reactive oxygen species (ROS) generated by the thermal decomposition of AAPH [2,2'-azobis (2-amidino-propane) dihydrochloride] can quench the fluorescent signal emitted by fluorescein. The addition of antioxidant substances will inhibit the quenching and produce a more stable fluorescent signal which can reflect their antioxidant capacity. The ORAC assay is one of the standardized methods for determining the antioxidant capacity of substance and has been accepted by some nutraceutical producers who have put ORAC values on products labels. ORAC values are often reported as Trolox equivalents. The assay is readily automated and can be adapted for detecting both hydrophilic and hydrophobic antioxidants.

1.3.4.5 Folin-Ciocalteu (F-C) assay

Folin-Ciocalteu assay is a widely used assay for determining total phenolic content (TPC) in samples. It was firstly developed in 1927 originated from chemical reagents used for tyrosine analysis (Folin, 1927). The content of total phenolic compounds of extract in this work will be determined according to a modified method (Medina, 2011). A mixture of phosphomolybdate and phosphotungstate are used to oxidize the phenolic compounds. The formed blue product has a maximum absorbance at 765 nm. Gallic acid is generally used as reference standard phenol. Total phenolic content is expressed as gallic acid equivalent (GAE). The method is simple, sensitive and precise. The total phenolic content in plant extracts usually has a good correlation with their antioxidant capacity.

1.3.5 Thin layer chromatography (TLC) bioautography

TLC bioautography is a method combining thin layer chromatographic separation and in situ biological activity evaluation. The samples are firstly separated on the TLC plate with a suitable developing solvent, and then separated compounds are tested for various biological activities. Antifungal assays, antibacterial assays, enzyme inhibition assays, antioxidant capacity assays and free radical scavenging assays could be performed using TLC bioautography (Marston, 2011). This method is mainly used for preliminary screening of natural products with biological activities and for the bioactivity-directed fractionation and isolation of active components from complex extract (Cheng and Wu, 2013).

The antioxidant capacity evaluation assays which could be performed on TLC plate include inhibition assay of bleaching of β-carotene (Pratt and Miller, 1984), inhibition assay of

bleaching of β-carotene induced by autooxidation of linoleic acid (Whittern *et al.*, 1984), ABTS assay (Miller and Rice-Evans, 1997) and DPPH assay (Zhao *et al.*, 2010; Ciesla *et al.*, 2012; Kowalska *et al.*, 2013). Because DPPH assay on the TLC plate could give more clear and stable spots and DPPH solution is easy to prepare, TLC-DPPH assay is more widely used for chemical or biological screening of plant extracts. In the TLC-DPPH assay, TLC plate with samples is developed with the elution solvents and dried. It is then sprayed with a DPPH solution in methanol of 0.05%. After drying, bands or spots on the TLC plate showing antioxidant capacity could be observed to be yellow on a purple background. For the identification of antioxidant compounds, the well-separated spots showing antioxidant capacity could be scraped from the plate and dissolved in suitable solvents. The antioxidant compounds could be further analyzed for their chemical structure, using mass spectrometry, infrared spectroscopy or nuclear magnetic resonance spectroscopy.

Despite there exist some sophisticated online HPLC bioassay methods, like HPLC-DPPH assay (Koleva *et al.*, 2000) or HPLC-ABTS assay (Koleva *et al.*, 2001), TLC-DPPH assay is still proving its promising potential as a simple, inexpensive and robust method for preliminary screening of antioxidant compounds in plant extract.

Chapter 2 Selection of candidate plants

Initially, several rules have been set up for the selection of underutilized medicinal and aromatic plants in the Midi-Pyrénées and Chongqing regions: (1) forgotten or underutilized plants on which there are not many scientific studies, especially about essential oils and antioxidant capacity; (2) selected plants should contain essential oils; (3) plants are not cultivated (generally, cultivated medicinal and aromatic plants have been widely studied.) (4) plants are not toxic. Toxic plants pose harm to health and application of MAP-refinery seems difficult. (5) plants could be collected in the fields or in the mountains, with an easy availability. However, in the selection process of candidate plants, these rules appeared to be too difficult to follow. The biggest problem encountered is the availability of the selected plants. Many seemingly perfect underutilized plants had to be ruled out from our lists because they could not be identified and collected in the Midi-Pyrénées region. Secondly, many medicinal and aromatic plants containing large quantity of essential oil have been widely studied. It is really difficult to find plants which are less studied but contains high content of essential oils. Due to these reason, the finally selected plants in two regions didn't strictly respect these rules.

2.1 Selection of candidate plants in the Midi-Pyrénées region

The selection of candidate plants in the Midi-Pyrénées region started from a list including 20 forgotten medicinal and aromatic plants (El Kalamouni, 2010). From this list, one plant was selected: *Tussilago farfara* L. The selection of this plant was due to its easy collection, relatively fewer scientific studies and purpose of comparison. This plant could also be found in the Chongqing region. Therefore *Tussilago farfara* L. was added into the list of candidate plants in the Chongqing region. After extensive bibliographic studies and discussions with the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse, other five medicinal and aromatic plants were finally selected, i.e. *Calendula arvensis* L., *Geranium robertianum* L., *Robinia pseudoacacia* L., *Cytisus scoparius* L. and *Spartium junceum* L. These six plants and their different parts were collected with the help of botanist Isabelle

FOURASTE in the fields near Toulouse or Midi-Pyrénées mountains during the periods from February to June in 2011 and 2012.

2.1.1 *Tussilago farfara* L.

2.1.1.1 Description

Tussilago farfara L., is a perennial herbaceous plant in the family of Asteraceae, commonly known as Coltsfoot. It is native to several places in Asia and Europe, and now has been introduced and cultivated in many places around the world. It can be found in almost all regions in France (Tela Botanica). It can grow up to 10-30 cm in height, producing yellow flowers which resemble dandelions in early spring. This species is also often found in waste places and along roadsides. In some places in Europe, it has been used as a food plant in the past while in Asia it was used as traditional medicine for curing lots of diseases. Its dry flower buds, called '*Kuandonghua*' in Chinese, have been used as Traditional Chinese Medicine (TCM) for thousands of years and have been recorded in many ancient medicinal books. It is widely used for the suppression of coughs and removal of phlegm.

Figure 1 *Tussilago farfara* L.

Because of its wide application in traditional medicine, since 1970s, there has been increasing interests in *Tussilago farfara* L. However, most of these investigations were about its dry flower buds on which Chinese researchers, especially, have done a lot of studies. In fact, at present, there have been many publications about its flower buds, including varieties of aspects: systematic chemical composition investigation, cultivation, pharmaceutical effects, biological activities, extraction, detection and purification of some active compounds, quality control, fingerprinting and application of some components. In this work, more emphasis will be put on aerial parts of *Tussilago farfara* L., the more commonly used parts in Europe (with

less studies on them) while dry flower buds from China is mainly used for comparison. Therefore, the information on its flower buds will only be selectively presented in literature review of *Tussilago farfara* L.

2.1.1.2 Chemical components

The systematic chemical components investigation on flower buds of *Tussilago farfara* L. have revealed several types of compounds: essential oil, sesquiterpenes, triterpenoids, flavonoids, steroids, polysaccharides, alkaloids, organic acids, amino acids and some inorganic elements (Liu *et al.*, 2006). Its aerial parts (flowers, leaves and stems) also contain some similar type of compounds but with different contents.

Essential oil

Essential oil content in flower buds of *Tussilago farfara* L. is relatively low, less than 0.1%. Essential oils from flower buds in different locations in China have been extracted and analyzed. Liu and co-workers extracted and analyzed the essential oil from flower buds of *Tussilago farfara* L. which was cultivated in GAP bases for Traditional Chinese Medicine (Liu *et al.*, 2006). 65 components were identified and main components were β-bisabolene (13.93%), *(E)*-cycloundecene (8.49%), 1-pentadecene (4.57%) and 1-undecene (4.83%).

Essential oil from flowers and stems of *Tussilago farfara* L. has also been investigated. Asta and Jurga used hydrodistillation to extract the essential oil from its flowers and stems growing in Lithuania and analyzed its composition using GC-FID and GC-MS (Asta and Jurga, 2011). Some aliphatic compounds like tricosane and pentacosane were found to be the major compounds, which was significantly different from the results of flower buds oil of Asian origin.

Other components

For the chemical components in the flower buds of *Tussilago farfara* L., sesquiterpenoids (e.g. tussilagone), total flavonoids and polysaccharides have attracted more attention, in terms of extraction and application. Some processes have been developed to optimize the extraction of these compounds, by use of some efficient extraction technologies such as SFE-CO_2 (Zheng *et al.*, 2009) and microwave assisted extraction (Li and Liu, 2010). From the perspective of new compounds identification, sesquiterpenoid compounds seem to be of

greater interest to researchers. Several recent publications concerned the identification of new sesquiterpenoid compounds (Park *et al.*, 2008; Liu, *et al.*, 2011; Li *et al.*, 2012). Other new compounds have also been isolated, e.g. a new phenolic compounds identified in 2008 (Liu, *et al.*, 2008).

There are much less studies on chemical compositions of flowers, leaves, stems and roots of *Tussilago farfara* L. In 1980, the phenolic compounds in aerial parts of *Tussilago farfara* L. were firstly investigated by French researchers (Didry *et al.*, 1980). In 2011, Dobravalskyte *et al.* studied the antioxidant activity and determined the phenolic compounds in the residue extracts (after hydrodistillation) from aerial parts of *Tussilago farfara* L. which was cultivated in Lithuania and in France (Dobravalskyte *et al.*, 2011). The results showed that this plant contained valuable antioxidant compounds. In the subsequent studies, dicaffeoylquinic acids and quercetin pentoside were identified as the major antioxidant compounds in extracts from *Tussilago farfara* L. (Dobravalskyte *et al.*, 2013).

There are also some alkaloids in this plant species. In 2005, five hepatotoxic pyrrolizidine alkaloids (HPAs) in *Tussilago farfara* L. of different origins were detected using thin layer chromatography and LC-MS (Pu *et al*, 2005), of which two known alkaloids (senecionine and senkirkine) have very strong mutagenetic activity.

2.1.1.3 Uses and biological activities

In the traditional medicine, *Tussilago farfara* L. has found a lot of uses, such as suppression of cough and removal of phlegm. In some European countries, it was used for cooking in the past. With increasing research work on this plant, other biological activities have been confirmed or discovered. Its antioxidant activity is one of the targets of interest to researchers. In 1997, extracts from its flowers were tested for inhibitory activities of tyrosinase and DOPA auto-oxidation to evaluate its use in cosmetics and showed inhibition of DOPA auto-oxidation activity (Lee *et al.*, 1997). In 2010, 56 Chinese plants were evaluated for their antioxidant activity in which extracts from flower buds of *Tussilago farfara* L. showed good activities and it was proposed as potential rich source of natural antioxidants (Song *et al.*, 2010). However, in the antioxidant activity evaluation of methanol extracts from its flowers growing in Bulgaria (Nikolova *et al.*, 2011), only moderate antioxidant activity was observed compared to other species. For *Tussilago farfara* L., some biological activities showed some degree of decrease when its flower buds turn to flowers during the growing period. Because

of this, for some medicinal purpose, flower buds of *Tussilago farfara* L. are preferred than flowers.

Other biological activities of *Tussilago farfara* L. include anti-inflammatory effect due to tussilagone (Hwangbo *et al.*, 2009), strong antimicrobial activity of extracts from its aerial parts and rhizome (Kokoska *et al.*, 2002; Dagmar *et al.*, 2003), moderate antimicrobial activity of extracts from its leaves (Dulger and Gonuz, 2004), anti-tumour effect (Akihisa, 2005), α-glucosidase inhibitory effect (Gao *et al.*, 2008), antiviral activity against avian influenza virus H1N1 (Lee *et al.*, 2010) and antigenotoxic effect (Karamova *et al.*, 2011).

It must be pointed out that, as *Tussilago farfara* L. contains senecionine and senkirkine which have very strong mutagenetic and hepatotoxic activity, the governments from many countries have formulated very strict standards for the use of *Tussilago farfara* L. and its extracts. Actually there were documented cases of liver problems in an infant after taking coltsfoot tea. In response, the Germany government has banned the sale of coltsfoot. Therefore, for the potential application and global valorisations of *Tussilago farfara* L., a highly efficient detection method must be firstly developed to determine the content of HPAs like senecionine and senkirkine in all extracts from this plant. Some extraction methods should also be developed to selectively extract the compounds of interest and even to eliminate the toxicity of these alkaloids in the process.

2.1.2 *Calendula arvensis* L.

2.1.2.1 Description

Calendula arvensis L. is an annual herbaceous plant in the family of Asteraceae, known by the common name field marigold which is growing wildly, opposed to garden marigold '*Calendula officinalis* L.' which is cultivated and has gained more attention because its wide application in phytotherapy and cosmetic industry. In French, *Calendula arvensis* L. is called 'souci des champs' and can be found in the fields of most regions in metropolitan France (Tela Botanica). It is native to central and southern Europe and also introduced in some parts of Asia and North Africa. It can grow from 10-30 cm, producing bright yellow to yellow-orange flowers from March to April. The leaves are lance-shaped and borne on petioles from the slender, hairy stem.

Figure 2 *Calendula arvensis* L.

2.1.2.2 Chemical components

Most of the phytochemical investigations of aerial parts of *Calendula arvensis* L. were concentrated on sesquiterpene glycosides and triterpenoid saponins which are abundant in this species and are responsible for various biological activities. *Calendula arvensis* L. also contains other varieties of compounds, such as essential oil, flavonoids, polyphenols and amino acids (Chemli *et al*., 1986).

Essential oil

Since 2004, several investigations have been done to study aroma compounds or essential oils in aerial parts of *Calendula arvensis* L. Carpino *et al*. used steam distillation to extract the volatile constituents from aerial parts of *Calendula arvensis* L. which grew in the Hyblean pasture in the south-east of Sicily (Carpino *et al*., 2004). GC-Olfactometry and GC-MS were employed to detect the odours and to identify the compounds, respectively. 17 different odours were found and several compounds were identified, e.g, β-bisabolene and methional. Al-Mazroa *et al.* also extracted and analyzed the essential oil of *Calendula arvensis* L. (Al-Mazroa *et al.*, 2006). In another study by Paolini and co-workers, the chemical composition in essential oil of *Calendula arvensis* L. growing in Corsica was established by GC-FID and GC-MS (Paolini *et al*., 2010). 85 components were identified, with α-cadinol and δ-cadinene as the major compounds. The more recent publication concerned the comparison of essential oils extracted by hydrodistillation (HD) and microwave distillation (MD) (Tosun *et al.*, 2012). It was found that these two methods gave similar results in the amount of total volatiles and identified constituents, and that terpenoid content was higher in essential oil extracted by HD

than that extracted by MD. At present there is no study on the volatiles from the roots of *Calendula arvensis* L.

Other components

The investigations on chemical components of *Calendula arvensis* L. started more than 20 years ago by research groups from Italy and France. Since then, dozens of sesquiterpene glycosides and triterpenoid saponins have been isolated and their structures have been elucidated, e.g. arvoside A (Pizza and Tommasi, 1987), arvensoside-A and arvensoside-B (Chemli *et al*, 1987), arvoside B (Pizza and Tommasi, 1988). In 1993, another four new sesquiterpene glycosides were isolated (Ahmed *et al.*, 1993). In 2006, Kirmizibekmez and co-workers isolated a new triterpenoid saponin named arvensoside C and three known flavonol glycosides (Kirmizibekmez *et al*., 2006).

Besides sesquiterpene glycosides and triterpenoid saponins, in recent years, phenolic compounds have attracted more attention of researchers. Li *et al* determined the polyphenols and flavones content in extracts from flowers of *Calendula arvensis* L. and results showed that its flower was a good source of polyphenols, with the content above 70 mg/g (Li *et al*., 2010).

2.1.2.3 Uses and biological activities

This herbaceous species has been used in Italian folk medicine as an anti-inflammatory, anti-cancer and antipyretic reagent (Tommasi and Pizza, 1990). In a study performed by Elias and co-workers, four saponins from *Calendula arvensis* L. showed antimutagenic activity with a dose-response relationship (Elias *et al.*, 1990). In the work of Chemli and co-workers, saponins isolated were found to have the hemolytic effect (Chemli *et al.*, 1990). Other biological activities include antiviral acitivity (Tommasi *et al.*, 1991), cytotoxic activity (Quetin-Leclercq *et al.*, 1992), antimicrobial activity (Ikram *et al*., 1980), and antibacterial activity (Dumenil *et al*., 1980).

As there are some polyphenols in this plant, the extracts of *Calendula arvensis* L. showed antioxidant activity. Cetkovic *et al.* compared the antioxidant activity of extracts from flowers of *Calendula arvensis* L. and *Calendula officinalis* L., using three different radical species: DPPH radical, hydroxyl radical and lipid peroxyl radical (Cetkovic *et al*., 2004). Extracts from *Calendula officinalis* L. were found to have higher antioxidant capacity than those from

Calendula arvensis L. and water was found to a better solvent than methanol to extract antioxidant compounds from these two plants. In another study in 2012, Ercetin *et al.* compared the antioxidant capacity and cholinesterase inhibitory effect of extracts from leaf and flowers of these two plants (Ercetin *et al*., 2012*)*. Antioxidant capacity was evaluated by DPPH radical scavenging assay, ferric-ion chelating capacity assay and FRAP assay. However, different results were obtained compared to those in the previous study. Methanol and ethyl acetate extracts obtained from flowers of *Calendula arvensis* L. showed higher DPPH radical scavenging capacity. This difference may result from the different plant parts studied in these two publications, flowers in the former study while leaf and flowers in latter one.

2.1.3 *Geranium robertianum* L.

2.1.3.1 Description

Geranium robertianum L. is an annual or biennial plant in the family of Geraniaceae, commonly known as Herb Robert. It can be found in all parts of metropolitan France (Tela Botanica), widely in Europe, Asia, North America and North Africa. It can grow at altitudes of up to 1500 m, producing small, pink, five-petal flowers from April until the autumn. Its stems are about 10-50 cm and often reddish. The newly picked leaves have a strongly disagreeable odour. Because of this, it is said that it can be put on the body to repel mosquitoes.

Figure 3 *Geranium robertianum* L.

2.1.3.2 Chemical components

At present, some studies have been on the phytochemical compositions of *Geranium robertianum* L., especially phenolic compounds. The active components in this plant include essential oil, flavonoids, tannins and other polyphenols.

Essential oil

Essential oil content of *Geranium robertianum* L. is relatively low, less than 0.05% in several locations. Its aerial parts and underground parts have been subjected to hydrodistillation and essential oils obtained have been analyzed by GC-MS and GC-FID. The essential oil composition of *Geranium robertianum* L. from Netherlands was firstly studied by Pedro and co-workers and the results showed that its major compounds were linalool (22.9%), γ-terpinene (13.9%), germacrene-D (7.8%), limonene (5.3%), geraniol (4.4%), α-terpineol (3.8%) and phytol (3.8%) (Pedro *et al.*, 1992). However, in another study performed by Radulovic and co-workers, essential oils from its aerial parts and roots from Serbia showed noticeably different compounds, with hexadecanoic acid (16.6% and 45.5%, respectively), pentacosane (28.5% in roots oil), hexahydrofarnesyl acetone (6.5% in aerial parts oil) and caryophyllene oxide (5.4% in aerial parts oil) as the most abundant ones (Radulovic *et al*., 2012). It is easily understandable that essential oil composition from the same species could be quite different because the secondary metabolites in the plants, including essential oil, are influenced by many factors, such as climate, soil, collection season and even extraction methods.

Other compounds

Previous phytochemical investigations of this plant were mainly on phenolic compounds, especially flavonoids. Kobakhidze and Alaniya isolated several phenolic acids and flavonoids from aerial parts of *Geranium robertianum* L. which was growing in Georgia (2004). Fodorea *et al.* identified and measured 7 compounds from its dry aerial parts (2005): hyperoside (3.64 μg/100 mg), ellagic acid (7599.76 μg/100 mg), isoquercetin (49.49 μg/100 mg), quercetin (83.92 μg/100 mg), kaempferols (143.43 μg/100 mg), caftaric acid (166.92 μg/100 mg), and rutoside (72.23 μg/100 mg). Because of good antioxidant activity shown by its extracts, some technologies have been used to concentrate the polyphenols in the extracts for the further medicinal applications, such as ultrafiltration process (Neagu *et al*., 2010) and nanofiltration process (Paun *et al*., 2011).

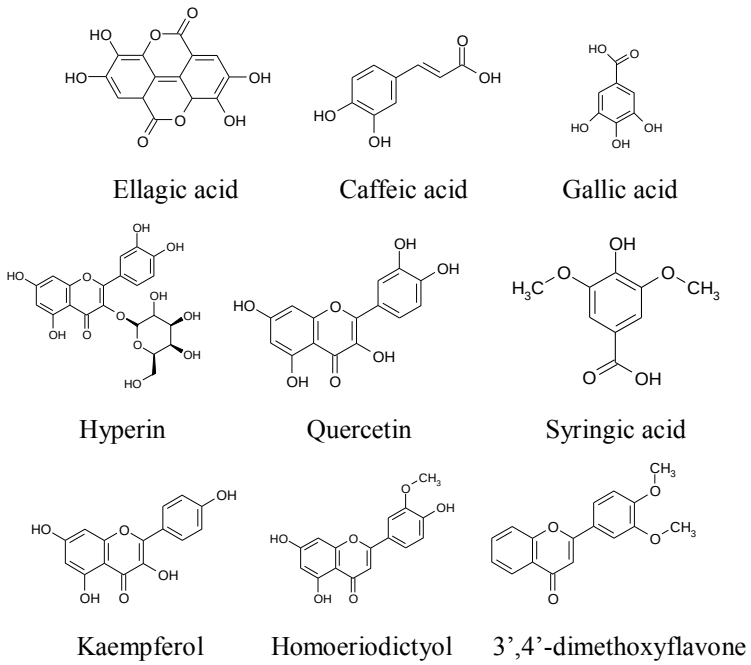


Figure 4 Some polyphenols in *Geranium robertianum* L. (Kobakhidze and Alaniya, 2004; Fodorea *et al*., 2005)

2.1.3.3 Uses and biological activities

Since ancient times, this plant has been used to cure toothache and nosebleeds and an infusion made from its aerial parts has been used for its diuretic and tonic effect. It was traditionally used for antihelmintic, antiparasitic and repellent purposes in central Italy (Guarrera, 1999). Because its reddish colour of stems, it is also used for dyeing reagent (Guinot *et al*., 2006). Its essential oil showed a variety of biological activities. The antimicrobial studies of its essential oil showed a good activity against *Escherichia coli* and *Aspergillus fumigatus* (Radulovic *et al*., 2012). Other activities included antibacterial effect (Hersch-Martinez *et al*., 2005), antiplasmid effect (Schelz *et al*., 2006), antifungal and antioxidant activities (Lis-Balchin *et al*., 1996**)**.

Of all biological activities of *Geranium robertianum* L., antioxidant activities are the most studied, especially those of extracts obtained from aerial parts using methanol, ethanol or their aqueous solution as extracting solvent. Different antioxidant capacity testing methods were employed, such as DPPH radical scavenging assay (Nikolova *et al*., 2010; Neagu *et al*., 2010), ABTS cation radical scavenging assay (Neagu *et al*, 2010), ferric reducing/antioxidant capacity assay (Katalinic *et al*, 2006), β-carotene/linoleic acid and reducing power and metal

chelating activity assays (Jemia *et al.*, 2013). *Geranium robertianum* L. seems to be one promising source of natural antioxidants.

From the basis of bibliographic work, it can be seen that present studies on *Geranium robertianum* L. are mainly centred about the chemical composition and some biological activities. Though *Geranium robertianum* L. showed good antioxidant activity, large-scale extraction of these active compounds from this plant and their application has not been put into practice. This probably resulted from the fact that it is not cultivated yet and it may not be obtained in big quantities. Actually, for some underutilised plants which are only growing wildly, collection in big quantity always poses a big problem for some in-depth investigation. As a result, cultivation in big area of this plant is an important basis for its further investigation and application.

2.1.4 *Robinia pseudoacacia* L.

2.1.4.1 Description

Robinia pseudoacacia L., a tall tree in the family of Fabaceae, is commonly known as black locust, common acacia or lotus tree. It has several varieties and some synonyms, such as *Robinia pseudoacacia* var. inermis, *Robinia pseudoacacia* var. crispa, *Robinia umbraculifera*, *Robinia tortuosa*, *Robinia stricta*, *Robinia spectabilis*, and so on (Tela Botanica). It is native to United States and now has been widely cultivated in North America, Europe and Asia. In some places, it is regarded as an invasive species. This plant could be found in almost all regions in metropolitan France. It can grow quite tall (exceptionally up to 52 m), with thick trunk up to 1.6 m in very old tree. In May or June, it can produce nectar-containing and intensely fragrant flowers which are a source of famous acacia monofloral honey. The flowers are white, borne in pendulous racemes of 8-20 cm long and are edible, with high nutrients and functional values. Its leaves are 10-25 cm long, pinnate with 9-19 leaflets, 2-5 cm long and 1.5-3 cm broad. The leaves are toxic due to the presence of toxic plant proteins called toxalbumins.

Figure 5 *Robinia pseudoacacia* L.

2.1.4.2 Chemical components

Chemical composition investigations on *Robinia pseudoacacia* L. have revealed many kinds of components. Its flowers contain volatile compounds, flavonoids, proteins, robinin, polysaccharide and some microelements while its leaves contain flavonoids, monoterpenes, tannins, proteins, and so on. Lectins, which belong to proteins or glycoproteins, existed in all tissues (roots, root nodules, bark, phloem and leaves) of this plant (Giet and Ziegler, 1980). In 1997, two lectins were purified from extracts of its root-tips using ion-exchange and affinity chromatography (Duverger and Delmotte, 1997).

Volatile compounds

Volatile compounds from flowers of *Robinia pseudoacacia* L. have been studied by several researchers, as well as aroma profiles in honey made from these flowers. In 1994, Kandem *et al.* used Tenax tube cartridges to trap the floral fragrance of living *Robinia pseudoacacia* L. and analyzed these volatile compounds using GC-MS (Kandem *et al.*, 1994). δ-3-carene (54.6%), linalool (21%), (*Z*)-β-farnesene (3.0%) and anthranilate aldehyde (3.9%) were found to be the major components. Xie *et al.* analyzed the chemical constituents of top fragrance from fresh flowers of *Robinia pseudoacacia* L. growing in China using solid phase microextraction followed by GC-MS analysis (Xie *et al.*, 2006). The main components identified were linalool (33.1%), *(E)*-β-ocimene (26.6%), (*E*)-α-bergamotene (8.9%) and formanilide (7.4%). In a study focusing on aroma profiles from *Robinia* honey, very low levels of aromatic compounds were detected and no characteristic compounds could be observed in the honey from this floral source (Bonvehi and Coll, 2003).

Other components

The chemical components investigation showed that flowers of *Robinia pseudoacacia* L. were rich in proteins and microelements which could be used for additives in food (Song *et al.*, 1992). Its flowers also contained an important bioactive compound called robinin which has found a lot of medicinal uses.

Flavonoids in *Robinia pseudoacacia* L. are also of increasing interests to researchers. In 2000, five flavonoids including acacetin, secundiflorol I, mucronulatol, isomucronulatol and isovestitol were isolated from ethanolic extracts of *Robinia pseudoacacia* L., following an activity-guided fractionation (Tian and McLaughlin, 2000). From its leaves, four flavone glycosides were isolated, such as luteolin and diosmetin (Veitch *et al.*, 2010). Recently, a new geranyl flavonol named robipseudin A has been isolated from its leaves, along with another known geranyl flavone called kuwanon S (Zhang *et al.*, 2013). These two compounds showed moderate antioxidant activity in the DPPH radical scavenging assay.

Chemical components in acacia leaves also include condensed tannins which have protein-precipitating capacity (Kumar and Horigome, 1986). In 1999, α-glucosidase was purified from proteins extracts of *Robinia pseudoacacia* L. (Berthelot and Delmotte, 1999). In 2001, a novel bioactive homo-monoterpene named robinlin was isolated (Tian *et al.*, 2001).

2.1.4.3 Uses and biological activities

Different parts of *Robinia pseudoacacia* L. have been put to many uses. Its flowers can be eaten and are generally used for making honey. In some places, they are used to make cakes. Its wood is extremely hard and rot-resistant, making it suitable for furniture. Its barks and leaves, however, contain some toxic compounds. Therefore, horses that consume this plant in excess will show signs of anorexia, depression, weakness, and cardiac arrhythmia. It seeds and young pods can also be eaten when being cooked. Heating is believed to be able to decompose the toxic compounds in the seeds and pods.

Some compounds from *Robinia pseudoacacia* L. showed several biological activities, such as antibacterial activity by its seed proteins (Talas-Ogras et al., 2005) and improvement of immune functions possessed by polysaccharides (Liang *et al.*, 2013), anthelmintic effect and antioxidant activity possessed by condensed tannins (Katiki *et al.*, 2013) and antioxidant activity shown by flavonoids. Because of their obvious biological activity, lectins from

Robinia pseudoacacia L. have been proposed for the manufacture of a medicament for the control of mucosal cell proliferation, for the reduction and/or treatment of damage caused by a cell-damaging agent (Pusztai *et al.*, 2002).

2.1.5 *Cytisus scoparius* L.

2.1.5.1 Description

Cytisus scoparius L., commonly known as Scotch Broom or Common Broom, is a perennial shrub in the family of Fabaceae. It has several synonyms, such as *Spartium scoparium* Linn, *Genista scoparius* Lam, *Sarothamnus scoparius* Koch and *Genista scoparia*. This plant is native to central and Western Europe and can be found in all regions of metropolitan France (Tela Botanica). It has been introduced to other places and now widely cultivated as an ornamental plant. However, in North America, especially in US, it is regarded as an invasive species and some measures have been taken to limit its invasion. Generally *Cytisus scoparius* L. can grow to 1-3 m tall with green angled branches which are different from another broom named *Spartium junceum* L. which has green round branches. From April to June, this plant can produce bright yellow flowers and its leaves are very small.

Figure 6 *Cytisus scoparius* L.

2.1.5.2 Chemical components

The chemical components in *Cytisus scoparius* L. include essential oil, flavonoids, alkaloids, tannins, polysaccharides, wax, and so on. Of these compounds, flavonoids and alkaloids have received more attention because they are responsible for many biological activities of this species.

Essential oil or volatile compounds

The first study on chemical components of essential oil from flowers of *Cytisus scoparius* L. was done in 1980 (Kurihara and Kikuchi, 1980). *(E)*-3-hexen-1-ol, 1-octen-3-ol, benzyl alcohol, phenylethyl alcohol, phenol, cresols, guaiacol, eugenol, isovaleric acid, benzoic acid, palmitic acid, eight fatty acids, and *n*-paraffins were found to be the major compounds. Besides, some other compounds were also isolated from the residue after essential oil extraction, such as orobol, genistein, quercetin, kaempferol, aesculetin, *p*-coumaric acid and caffeic acid. One study in 1997 focused on one aroma compound from stems of this plant because it released one characteristic odour into the air (Tominaga and Dubourdieu, 1997). This aroma compound was identified as one kind of thiol called 4-mercapto-4-methylpentan-2-one and its maximum concentration could reach up to 16 ng/g of fresh stems.

Other components

Cytisus scoparius L. contains many kinds of alkaloids. Wink and co-workers analyzed the alkaloid composition in different organs of this plant (Wink *et al.*, 1981). *Cytisus scoparius* L. was found to contain some known alkaloids such as isosparteine, sparteine, 17-oxo-sparteine, lupanine and other quinolizidine alkaloids. Two new alkaloids were also identified. These alkaloids had significantly different distribution in different organs and the stems were found to have the highest content of total alkaloids. Sparteine was reported as the major compound in these alkaloids. In 1994, Saito *et al.* isolated a new ester alkaloid along with other six known alkaloids (Saito *et al.*, 1994). Gresser *et al.* investigated the distribution and content of quinolizidine alkaloids and phenylethylamine tyramine in various organs of *Cytisus scoparius* L. collected from different locations from Germany, Russia, Italy and France (Gresser *et al.*, 1996). Sparteine and sparteine-like derivatives were mainly found in flowers and shoots.

Some flavonoids were also reported to be in *Cytisus scoparius* L. These flavonoids include rutin, quercetin, quercitrin, isorhamnetin and kaempferol (Brum-Bousquet and Paris, 1974), 6"-*O*-acetyl scoparin (Brum-Bousquet *et al.*, 1977) and an isoflavone glycoside named sarothamnoside (Brum-Bousquet *et al.*, 1981).

In 2004, Ammar *et al.* analyzed the content of total phenolic compounds, extractable tannins and condensed tannins in flowers and leaves of *Cytisus scoparius* L. (2004).

Besides the compounds mentioned above, therephthalic acid dimethyl ester was found in the petals of this plant (Hörhammer *et al.*, 1966).

2.1.5.3 Uses and biological activities

Cytisus scoparius L. has been put to many uses. When planted on the side of steep banks or in the soil with a lot of sand, it can hold the earth and sand together. Its twigs and branches are used not only for making brooms, but also for making baskets in some places. The stem of this plant also contains fibres which are not as strong as those in Spanish broom, but these fibres can still be used in the manufacture of paper. The abandoned residues from *Cytisus scoparius* L. were reported to have high calorific value, with the potential to be additional energy source (Anon *et al.*, 1994).

This plant has also been employed medicinally. Pharmacological studies have revealed some biological activities, such as anti-spasmodic activity, diuretic activity and hypotensive activity (Bhakuni *et al.*, 1969), antimicrobial activity (Gowthamarajan *et al.*, 2002), antioxidant activity, and so on.

There are increasing interests in antioxidant activity of this plant. In 2006, extracts from aerial parts of *Cytisus scoparius* L. were tested for their DPPH radical scavenging capacity, nitric oxide radical scavenging capacity, superoxide anion radical scavenging capacity, hydroxyl radical scavenging capacity, antilipid peroxidation capacity, reducing power and total phenol content (Sundararajan *et al.*, 2006). Results showed that hydroalcoholic extracts from aerial parts of this species could be potential source of natural antioxidants as obvious antioxidant activity was observed. In 2007, extracts of its seeds from Scotland were tested for the antioxidant activity (Kumarasamy *et al.*, 2007). Moderate antioxidant activity was observed in hexane extracts. Besides *in vitro* antioxidant activity tests, some *in vivo* tests have been done (Raja *et al.*, 2007). Results also showed that hydroalcoholic extracts from *Cytisus scoparius* L. had significant antioxidant activity. In another study, Raja *et al.* evaluated the antioxidant activity of extracts from this plant on oxidative stress in liver of rats induced by carbon tetrachloride (Raja *et al.*, 2007). It was observed that extracts from *Cytisus scoparius* L. protected rat liver from oxidative stress. In 2009, Luis *et al.* determined the antioxidant activity of extracts from this species by DPPH test and beta-carotene bleaching test (Luis *et al.*, 2009). The contents of total phenolic compounds and total flavonoids were also determined. Antioxidant capacity of extracts was found to have high correlation with total

phenolic content, which means that phenolic compounds like flavonoids in this plant are responsible for the antioxidant activity of extracts.

2.1.6 *Spartium junceum* L.

2.1.6.1 Description

Spartium junceum L. is a perennial shrub in the family of Fabaceae, commonly known as Spanish Broom, Weaver's Broom and Yellow Spanish Broom. It has several synonyms such as *Spartium odoratum* Dulac, *Spartianthus junceus* (L.) Link, *Genista juncea* (L.) Scop., *Cytisus junceus* (L.) Vuk.(Tela Botanica).

It is native to Mediterranean region in southern Europe, southwest Asia and northwest Africa. But now it has been introduced into other areas and is regarded as a noxious invasive species in some places. This plant is often found in sunny sites and grows in many regions in France except several places in the north and in the centre. It can grow from 1 m to 3 m and produces yellow flowers from April to May. Its leaves are quite small, with the length in 1-3 cm and width up to 4 mm. The more important parts of this plant are flowers and stems which have attracted increasing attention from researchers. In Turkish folk medicine, its flowers are used for the treatment of gastric ulcers. Because of its fibre-containing stems, for centuries, this plant has been used to make brooms to clean houses, ovens, fireplaces, yards, streets as well as for some special functions in several countries of Europe (Nedelcheva *et al.*, 2007).

Figure 7 *Spartium junceum* L. in flowering season

2.1.6.2 Chemical components

Chemical components investigations in flowers, flower buds, leaves and stems of *Spartium junceum* L. showed several kinds of compounds, including volatile compounds, flavonoids, alkaloids, saponins and polyphenols. The contents of cellulose, lignin, pentosan and ash in the fibre of *Spartium junceum* L. have also been determined for the evaluation of its use in composite materials (Katovic *et al.*, 2011).

Essential oil (Volatile compounds)

In 1997 and 2001, Owen *et al.* investigated the volatile organic compounds (VOCs) emitted by *Spartium junceum* L. using bag-enclosure sampling method followed by a GC analysis (Owen *et al.*, 1997; Owen *et al.*, 2001). Their results showed that *Spartium junceum* L. could emit some molecules like isoprene, camphene, β-pinene, myrcene, *(z)*-ocimene, linalool, limonene, nonanal and decanal. In 2004, Miraldia *et al.* extracted the essential oil from fresh flowers of *Spartium junceum* L. using hydrodistillation and analyzed it using GC-MS and GC-FID (Miraldia *et al.*, 2004). 24 main constituents were identified and the major compounds were tricosane (22.9%), tetracosane (8.9%) and pentacosane (16.1%). In another study by Mancini and co-workers, comparison of compositions of volatile compounds from flowers of healthy and diseased *Spartium junceum* L., was carried out (Mancini *et al.*, 2010). The results appeared to be different, quantitatively and qualitatively. *n*-alkanes and aliphatic acids contents in healthy plant (55.2% and 18.7%, respectively) were higher than those in the diseased one (38.8% and 4.7%). Sesquiterpenes could only be detected in the diseased plant. One recent publication concerned the chemical compositions and anti-tumour activity of aromatic water of flowers of *Spartium junceum* L. (Cerchiara *et al.*, 2012). Results showed that this aromatic water was cytotoxic towards tested tumour cells.

Other components

Because its anti-ulcerogenic effect known in folk medicine, some studies have been done to ascertain the responsible molecules. In 1993, one saponin along with two new flavonoids was isolated (Billa *et al.*, 1993). In 1999, a new oleanene-type saponin with potent anti-ulcerogenic effect was isolated from flowers of *Spartium junceum* L., named as spartitrioside (Yesilada and Takaishi, 1999).

There are also increasing interest in flavonoids, flavonoid glycosides and other phenolic compounds in flowers of *Spartium junceum* L., which accounted for its antioxidant activity.

The first quantitative determination of its flavonoids was done by researcher from USSR (Osimina, 1985). In 2000, Yesilada *et al*. isolated 5 flavonoid glycosides from its flowers by activity-guided fractionation, of which quercetin 3,4'-diglucoside and quercetin 4'β-glucoside showed the highest *in vitro* antioxidant activities (Yesilada *et al*., 2000). In another study, flavonoids and phenolic compounds in flowers of *Spartium junceum* L. were analyzed (Proestos *et al.*, 2006). Some flavonoids (e.g., quercetin and luteolin) and phenolic compounds (e.g., gentisic acid, caffeic acid, *p*-coumaric acid and vanillic acid) were quantitatively determined. The compounds with relatively high content were caffeic acid (37.5 mg / 100 g dry sample) and *p*-coumaric acid (22.5 mg / 100 g dry sample).

Alkaloids are also important molecules existing in leaves, stems and flowers of *Spartium junceum* L. In 1990, its alkaloids were investigated by Greinwald and co-workers. Cystisine, N-methylcytisine, rhombifoline and epi-baptifoline were found to be the major alkaloids in this plant (Greinwald *et al.*, 1990). A significantly quantitative difference of alkaloids was found in various parts of this plant. Another study was on alkaloid content variations in four *Spartium junceum* L. populations in different dynamic stages (Barboni *et al.*, 1994). The highest alkaloid content was observed in young plants, especially in seeds and twigs, which could be interpreted as a defensive strategy against predators. In 2008, Belsito *et al.* used basic ethanolic conditions to extract the alkaloids (Belsito *et al.*, 2008). This method was proved to be efficient and selective: four alkaloids including N-formylcytisine, N-methylcytisine, sytisine and anagyrine were obtained in the extracts. In 2009, some researchers from Georgia studied the accumulation dynamics of alkaloids in various parts of *Spartium junceum* L. and isolated four known alkaloids: cytisine, N-methylcytisine, α-sophoridine and anagyrine (Vachnadze *et al.*, 2009).

3.1.6.3 Uses and biological activities

Besides the anti-ulcerogenic effect, antioxidant activity and anti-tumour effect mentioned above, extracts from *Spartium junceum* L. also showed some other biological activities. In 1990, essential oil from flowers of this plant was tested against six blastomycetes, six Gram-positive and seven Gram-negative bacteria and showed some degree of anti-microbiological activity (Bonsignore *et al.*, 1990). In 2006, hexane and methanol extracts obtained from its flowers were tested for their anti-inflammatory and analgesic effects (Menghini *et al.*, 2006). One fractioned part of hexane extracts showed anti-inflammatory effect while all extracts showed marked peripheral and central analgesic activity.

It is noteworthy that a good fibre could be obtained from twigs or stems of *Spartium junceum* L., with many good properties, e.g, tensile strength and elastic moduli. As it is cultivated in large scale in some European countries, recently there has been a revival interest in this species as a possible source of composite materials for automobile applications (Angelini *et al*., 2000). In 2010, a novel and efficient physical-chemical process was developed for the production of cellulose fibre from this species (Gabriele *et al*., 2010) and this process could produce fibres with many excellent physical-chemical properties, such as high mechanical resistance and high elasticity.

In conclusion, the existing studies on *Spartium junceum* L. are mainly about the chemical composition investigation and biological activity assay of extracts from its flowers. There are much less studies on the extracts from other parts of this species, such as leaves and stems. For stems, the biorefinery concept is quite suitable for the global utilization of this part. After some useful molecules are extracted from stems, the residue can also be used for producing composite materials.

2.2 Selection of candidate plants in the Chongqing region

As it is difficult to find botanists to identify and collect wild plants in the fields or mountains in the Chongqing region, the candidate plants could only be selected from the cultivated ones. Extensive bibliographic studies on a list of 48 medicinal plants cultivated in the Chongqing region were carried out. The main focuses are essential oil yields, extraction and separation techniques, analytical techniques and main components of essential oils (seen in Appendix A: Bibliographic studies of 48 medicinal plants in the Chongqing region). According to the bibliographic results, these 48 plants were classified into three groups: (1) 17 medicinal plants with many scientific studies on essential oils; (2) 14 medicinal plants with not many scientific studies on essential oils; (3) 17 medicinal plants with almost no scientific studies on essential oils. The plants in group **1** were ruled out due to too many studies on essential oils. Interesting ones are group **2** and group **3**. For group **3**, the reason that there were no scientific studies on essential oils may be that these plants didn't contain any essential oil. More importantly, availability of candidate plants should be considered. Finally three candidate plants were selected from group **2**: *Tussilago farfara* L. (one common plant with Midi-Pyrénées region), *Citrus aurantium* L. and *Saussurea costus* (Falc.) Lipech. These three plant samples were kindly provided by Professor from Chongqing University and they were from plantation bases of traditional Chinese medicinal plants in the Chongqing region.

2.2.1 *Citrus aurantium* L.

2.2.1.1 Description

Citrus aurantium L., commonly known as 'bitter orange', is a small tree in the family of Rutaceae. In China, there are several varieties including *C. aurantium* var. Daidai, *C. aurantium* var. Goutou cheng, *C. aurantium* var. Hutou Gan, *C. aurantium* var. Xiaohong cheng, *C. aurantium* var. Zhulan, *C. aurantium* var. Taiwanica and *C. aurantium* var. Natsudaidai (Zeng, 1997). It has been widely cultivated in the south of China and it can also be found growing wildly in some places. It is flowering from April to May.

Fructus aurantii, commonly known as '*Zhiqiao*' in Chinese, is the dried, closely matured fruit of *Citrus aurantium* L. and its cultivated varieties, and has been widely used in Traditional Chinese Medicine. Another well-known drug in TCM which resembles *Fructus aurantii* in appearance, *Fructus aurantii immaturus* (*Zhishi*), is young fruit of *Citrus aurantium* L. or its cultivated varieties, and *Citrus sinensis* (L.) Osbeck which is commonly known as sweet orange. These two drugs have been used for regulating *Qi* circulation and strengthening spleen and stomach in China since ancient times (Pharmacopoeia of P.R.C, 2010). *Fructus aurantii* will generally be collected in July when the peel is still green. The collected fruits will be cut into two parts and dried in the sun. At present, some of these fruits will also be freeze-dried to avoid the loss or change of some compounds.

There have been numerous investigations on *Citrus aurantium* L., from its essential oils to various biological activities including antioxidant activity. Lots of applications of chemical compositions in this plant have been developed. Its essential oil from its peels, flowers and leaves have been widely studied and have been on the market as commercial essential oils. Therefore, literature review here will only be limited to *Fructus aurantii*, the dried unripe fruit of Chinese varieties of *Citrus aurantium* L., on which not so much investigations have been done. For comparison, chemical composition of essential oil from normal *Citrus aurantium* L. will also be presented.

Figure 8 *Citrus aurantium* L.

2.2.1.2 Chemical components

The chemical compounds in *Fructus aurantii* include essential oil, flavonoids, alkaloids, coumarin, and so on. Flavonoids are the most important components of *Fructus aurantii* and responsible for many pharmaceutical effects.

Essential oil

Chemical components in essential oils from *Fructus aurantii* have been investigated using GC-FID and GC-MS. The highest oil yield could reach up to 1%. The major component of its essential oil of different origins is always limonene. Liao *et al* analyzed the essential oils of *Fructus aurantii* and *Fructus aurantii immaturus* (Liao *et al.*, 2004). *Fructus aurantii* essential oil was found to contain more identified compounds (51) than *Fructus aurantii immaturus* oil (15). These two essential oils contained similar content of limonene (around 40%), but the content of linalool in *Fructus aurantii immaturus* oil (26%) was about two times that in *Fructus aurantii* oil (13%). Gong *et al.* analyzed essential oils of *Fructus aurantii* of different origins in China and also compared the oil chemical compositions before and after pre-treatment (Gong *et al.*, 2007). In essential oils of different origins, limonene was the major component (around 50%). After pre-treatment of raw *Fructus aurantii*, there was only slight increase of limonene content. However, a lot of new compounds up to 17 were observed and some compounds disappeared.

For *Citrus aurantium* L., essential oil from its fruits, flowers and leaves have similar chemical composition, but the content of each compound differs significantly. Dugo and co-

workers compared the essential oils from the fruits, flowers and leaves of this species from Egypt (Dugo *et al.*, 2011). The major component in essential oil from its fruits is limonene (around 95%), whose content is much higher than that (40-50%) in essential oil from *Dry fruits of Citrus aurantium L.*used in China. Its flower oil (along with flower oil of sweet orange), known as neroli oil, has been widely used in cosmetics. Linalool (around 50%), limonene (around 10%) and linalyl acetate (around 10%) were reported as its major compounds. While the essential oil extracted from the leaves and twigs of *Citrus aurantium* L. is commonly known as petitgrain whose major components were linalyl acetate (55%) and linalool (28%). However, in another study on chemical composition of petitgrain from Tunisia (Ellouze *et al.*, 2012), main compounds in different seasons were linalool (43.2% to 65.97%), linalyl acetate (0.77% to 24.77%), and α-terpineol (9.29% to 12.12%). It can be seen that essential oil composition are easily influenced by many factors.

Flavonoids

Flavonoids have attracted more attention from researchers because of their high contents in *Fructus aurantii* and various biological activities. Actually, two flavonoid glycosides (naringin and neohesperidin) have been selected as the standards to evaluate the quality of *Fructus aurantii*. According to Pharmacopoeia of People's Republic of China, the contents of naringin and neohesperidin in *Fructus aurantii* which are used medicinally shouldn't be less than 4% and 3%, respectively. Therefore, some technologies have been used for detection, isolation and purification of these two compounds for the quality control. Luan *et al.* used reversed-phase HPLC and spectrophotometry for quantification and fingerprinting analysis of total flavonoids in *Fructus aurantii*, in which naringin and neohesperidin were used as two important indices (Luan *et al.*, 2011). Han *et al.* employed macroporous resin column chromatography and high-speed counter-current chromatography for high efficient and large-scale purification of naringin (Han *et al.*, 2008). The purity of final naringin could reach up to 98.3% and total recovery of naringin was about 90.9%. Besides naringin and neohesperidin, other flavonoid glycosides including neoeriocitrin, isonaringin, hesperidin and neoponcirin have been identified (Zhou *et al.*, 2006).

As major lipid-soluble constituents in *Fructus aurantii*, polymethoxylated flavones are of increasing interests to researchers and have been quantitatively detected (Chen *et al.*, 2012). The contents of four polymethoxylated flavones (e.g. nobiletin and tangeretin) were

determined using HPLC-ESI-MS-MS. In this study, three coumarins including meranzin hydrate, marmin and auraptene were simultaneously detected.

Besides the fruits, flavonoids are also present in flowers, leaves and twigs of *Citrus aurantium* L.

2.2.1.3 Uses and biological activities

Being used in TCM for regulating *Qi* circulation and strengthening spleen and stomach, *Fructus aurantii* can be used as a single drug and also formulated with other drugs to make the complex drug with enhanced effects or different pharmaceutical effects. One famous complex drug containing *Fructus aurantii* is called *Chaihu-Shugan-San* which has been used for centuries to improve symptoms of depression. One recent study focused on the antidepressive effect of this complex drug and activity-guided isolation led to the isolation of a new antidepressive compound called meranzin hydrate (Fan *et al.*, 2012).

Pharmacological studies of extracts or the chemical components of *Fructus aurantii* have showed many biological activities, such as gastric mucosal protective effect (Takase *et al.*, 1994), anti-carcinogenic effect (Manthey and Guthrie, 2002; Li *et al.*, 2007), anti-ischemic effect (Kang *et al.*, 2007), neuroprotective activity (Nakajima *et al.*, 2007), anti-diabetes effect (Kim *et al.*, 2010), and so on.

As essential oils from sweet orange, lemon and bergamot have been found to inhibit a range of Gram-positive and Gram-negative bacteria and are generally recognised as safe natural product, Fisher and Phillips proposed that this group of essential oils may provide natural antimicrobial agents for food industry (Fisher and Fhillips, 2008). Essential oil from *Fructus aurantii* also contains similar chemical components with high content of limonene and can also be proposed for anti-microbial agent in food industry. Actually, some applications of essential oil from *Citrus aurantium* L. in food industry have been developed, such as aromatization of corn oil (Karoui et al., 2010).

Because of high content of flavonoids (especially the hydrophilic ones) in extracts of *Fructus aurantii*, antioxidant activity has been observed and some of pharmaceutical effects can be attributed to the antioxidant nature of extracts. In 2008, Su *et al.* determined total phenolic content, DPPH free radical scavenging activity, hydrogen peroxide scavenging activity, ferrous ion-chelating activity and ferric-reducing antioxidant power of methanolic extracts from four citrus herbal products including *Fructus aurantii* (Su *et al.*, 2008). The difference between results obtained by different methods was significant and *Fructus aurantii*

extracts showed the highest antioxidant activity among these four extracts. But its total phenolic content was not the highest one. In another study, an on-line method combining analytical techniques for simultaneous monitor and identification of antioxidants in *Fructus aurantii*, was developed (Lin *et al.*, 2012). 25 antioxidant compounds were identified by LC/MSn and ten of them were firstly identified from *Fructus aurantii*, such as naringenin-7-*O*-triglycoside, isovitexin, isosinensetin, auranetin, 3'-methoxy isovitexin, naringenin-7-*O*-sophorose, and so on.

2.2.2 *Saussurea costus* (Falc.) Lipech.

2.2.2.1 Description

Saussurea costus (Falc.) Lipech., a perennial herbaceous plant in the family of Asteraceae, is widely used in traditional medicine systems of China and India. It has several synonyms, such as *Aucklandia costus* Falc., *Aplotaxis lappa* Decne., *Saussurea costus* (Falc,) Sch.-Bip., *Aucklandia lappa* Decne, *Saussurea lappa* (Decne.) C. B. Clarke., *Theodorea costus* O. Kuntz (Shi et Jin, 1999). Its dry roots which are also called *Radix Aucklandia* or costus roots, have been used for the treatment of asthma, inflammatory diseases, ulcer and stomach problems in traditional medicines (Pandey *et al.*, 2007). *Saussurea costus* (Falc.) Lipech. is native to Kashmir and was later introduced into Yunnan province, China. In Traditional Chinese Medicine (TCM), it is called '*Muxiang*' and now it has been cultivated in many places in China, such as Sichuan, Chongqing, Guangxi and Guizhou provinces. This species can grow to 1.5-2 m and the diameter of its main roots can reach up to 5 cm. Its flowering period is from July to August and its roots can be harvested after three-year growing in the fields.

Figure 9 *Saussurea costus* and its dry roots

2.2.2.2 Chemical components

Because of various pharmaceutical effects of its roots, the majority of chemical components investigations in *Saussurea costus* (Falc.) Lipech. are centred on them. *Saussurea costus* roots contain many kinds of chemical components, such as essential oil, sesquiterpenes, sesquiterpene lactones, triterpenes, lignin, phenolic compounds, flavonoids, and so on. Of these compounds, sesquiterpene lactones were reported as the most important ones and have attracted more attention from researchers.

Essential oil

There have been some studies on chemical compositions and its application of essential oil from *Saussurea costus* roots. Essential oil composition of *Saussurea costus* roots and its larvicidal activity of against the mosquito were investigated (Liu *et al.*, 2012). Essential oil yield was 0.89% (v/w) and the density was very close to water, 0.99 g/mL. 39 compounds were identified and dehydrocostus lactone (46.75%), costunolide (9.26%), 8-cedren-13-ol (5.06%), α-curcumene (4.33%), α-selinene (2.79%), α-lonene (2.49%), α-pinene (2.47%) and spathulenol (2.31%) were found to be the major compounds. In recent publication, 35 chemical compounds were identified in the essential oil from *Saussurea costus* roots in Uttarakhand Himalayas (India) (Gwari *et al.*, 2013). The major compounds were found to be (7Z,10Z,13Z)-7,10,13-hexadecatrienal (25.5%), dehydrocostus lactone (16.7%), elemol (5.84%), valerenol (4.20%) and vulgarol B (3.14%). In 2002, Mao and Lu used orthogonal design to optimize the beta-cyclodextrin inclusion conditions of its essential oil for possible large-scale production of this conclusion compounds which could be easier for medical use (Mao and Lu, 2002). Aiming at the easy confusion of *Saussurea costus* roots with roots from similar plants, Shum *et al.* used the chemical profiles of its essential oil obtained by GC-MS to distinguish its roots from others (Shum *et al.*, 2007).

When talking about the chemical composition of essential oil from Traditional Chinese Medicine, some attention should be given to the origins of these plants. Some of essential oil chemical compositions may be ‘artefacts’, which are not native in the plants and formed in the subsequent processing after plant collection. The results are due to the fact that, in the traditional Chinese medicine, before coming onto the market, medicinal plants or their medicinal parts have subjected to some pre-treatment process in the purpose of reinforcing the pharmaceutical effects, eliminating the toxicity, removing the bad odours or extending the

storage time. These pre-treatment processes include frying, stir-heating with some ingredients, roasting, soaking with water or alcohol, and so on. As essential oil is quite sensitive to heating and oxygen, inevitably, chemical components in essential oil of these medicinal plants which are purchased from drug store or markets, have changed. Some compounds with lower molecular weight may have been lost in the pre-treatment process involving heating. In 2012, Wen *et al.* examined the influence of some processing methods on chemical compositions of essential oil from *Saussurea costus* roots (Wen *et al.*, 2012). Four different methods, frying without additional ingredients, stir-heating with bran, roasting surrounded with bran, and roasting wrapped in wet paper were used. Results showed significant decrease of essential oil content after processing except first method. In addition, chemical compositions have changed a lot, with the disappearance of some compounds and formation of some other compounds. Obviously, if for perfumery application, the modified essential oil may not be appreciated. As a result, processing methods must be considered to suit different applications of its essential oil.

In the chemical compositions of essential oil from *Saussurea costus* roots, dehydrocostus lactone and costunolide accounted for more than 50% (Liu *et al.*, 2012). These two compounds are the major components of sesquiterpene lactones in *Saussurea costus* roots, and sesquiterpene lactones are responsible for many pharmaceutical effects. The contents of dehydrocostus lactone and costunolide have been used as the standard index to evaluate the quality of *Saussurea costus* roots and its products. As a result, high-efficiency and rapid detection, isolation and purification of these two compounds are of increasing interest to researchers. Some recent detection methods include rapid densitometric TLC method for simultaneous detection of these two compounds (Vijayakannan *et al.*, 2006), and matrix solid-phase dispersion extraction coupled with HPLC-diode array detection method (Zhang *et al.*, 2011). For the high-purity preparative isolation of these two compounds, high-speed counter-current chromatography (HSCCC) has been used (Li *et al.*, 2005).

Other components

Besides dehydrocostus lactone and costunolide, some other sesquiterpene lactones have been isolated and investigated. For example, in 2008, a new sesquiterpene lactone named isodihydrocostunolide was isolated (Robinson *et al.*, 2008).

Chemical components in *Saussurea costus* roots also include amino acid-sesquiterpene adducts and lignin glycoside (Yoshikawa *et al.*, 1993), phenolic compounds like chlorogenic

acid (Pandey *et al.*, 2005), acylated flavone glycosides which showed moderate to high antifungal activity and antimicrobial activity (Rao *et al.*, 2007), sulfonated guaianolides (Wang *et al.*, 2008), guaiane sesquiterpenoids (Choi *et al.*, 2009), sesquiterpene including a new one which was isolated (10-α-hydroxyartemisinic acid) in 2010 (Duan *et al.*, 2010), glucofructans (Olennikov *et al.*, 2011), and so on.

2.2.2.3 Uses and biological activities

Saussurea costus roots are widely used in folk medicine. In Traditional Chinese Medicine, they are used for the treatment of Qi stagnation, gastro-intestinal distention, food retention, diarrhea, dysentery and abdominal pain. Pharmaceutical effects investigations of extracts from *Saussurea costus* roots have revealed many biological activities, such as anti-inflammatory effect (Cho *et al.*, 2000), anti-ulcer effect (Han *et al.*, 2005), antifungal activity and antimicrobial activity (Rao *et al.*, 2007), anticonvulsant activity (Ambavade *et al.*, 2009), would-healing effect (Patil et al., 2009), anti-hepatotoxic effect (Yaeesh *et al.*, 2010), anti-cancer effect (Kim *et al.*, 2012), and so on.

Extracts from *Saussurea costus* roots also possess antioxidant activity. Their antioxidant capacity has been evaluated by scavenging DPPH radical, nitric oxide, superoxide radicals along with the ability to inhibit lipid peroxidation and glutathione (GSH) oxidation (Pandey *et al.*, 2005). Extracts from *Saussurea costus* roots showed antioxidant activity in all these assays. Chlorogenic acid, whose content was 0.027% in the extracts, was proposed to be the possible molecule which was responsible for the antioxidant activity.

From the perspective of full utilization of plant resources, more attention should also be given to other parts of *Saussurea costus*, such as its flowers, leaves and stems. Some valuable molecules could also be extracted from these parts and some possible application could be proposed to maximize the value of this plant species.

Apendix A: Bibliographic studies of 48 medicinal plants in the Chongqing region

Abbreviations:

HD (Hydro distillation); SD (Steam Distillation); SDE (Simultaneous Distillation Extraction); SE (Solvent Extraction); SFE (Supercritical Fluid Extraction); SPME (Solid Phase Micro Extraction); HS (Head Space); UAHD (Ultrasonic Assisted Hydro Distillation); MAHD (Microwave Assisted Hydro Distillation); Column C (Column Chromatography); GP-MSE (Gas Purge Micro Syringe Extraction); MD (Molecular Distillation); GC-FID (Gas Chromatography-Flame Ionization Detector); NPLC-GC (Normal Phase Liquid Chromatography-Gas Chromatography); TLC (Thin Layer Chromatography); MAE (Microwave Assisted Extraction); UVS (Ultraviolet Spectrometry);

Table 1 Medicinal plants in the Chongqing region (A lot of scientific studies on essential oil or volatile compounds)

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
1	*Qinghao*	All parts	*Artemisia annua* L.	Compositae	0.2%-0.7%	HD; SD; SFE; SE; MD. etc	GC-MS; GC/GC-MS; GC-FID	Artemisia ketone, camphor, borneol. etc
2	*Zisu*	Stem, leaves and seeds	*Perilla frutescens*	Lamiaceae	Leaves (0.40%-5.1%); Seeds (0.26%);	MAHD; UAHD; HD; SFE; HS-SPME; etc	GC-MS	Perilla ketone, thymol, carvacrol, limonene. etc
3	*Baisu*	Rhizome *Rhizoma Atractylodes macrocephalae*	*Atractylodes macrocephala* Koidz	Compositae	Rhizome (1.01%-2.32%)	HD; UAHD; SFE; HS-SPME;	GC/MS; HPLC; TLC;	Atractylon, elemene, etc
4	*Zhizi*	Fruits	*Gardenia jasminoides* Ellis	Rubiaceae	Fruits (0.033%); Flowers (0.04%-1.78%); Roots (0.85%-3.76%)	HD; SFE; SPME; SD	GC-MS	Fruits (stearic acid); Flower (linalool, jasmine lactone); Roots (--)

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
5	*Baizhi*	Roots	*Angelica dahurica*	Umbelliferae	Roots (0.04%-1%)	MAHD; HD; SFE;	GC-FID; GC-MS;	Cyclododecane, aristolone, carvacrol.
6	*Qianhu*	Roots	*Peucedanum praeruptorum* Dunn.	Umbelliferae	Aerial parts (0.4%); Roots (0.5%)	SFE; HD	GC-MS; GC-FID; GC/FTIR;	Pinene, myrcene, caryophyllene, careen.
7	*Foshou*	Fruits	*Citrus medica* var. sarcodactylis	Rutaceae	Fruits (about 1.6%)	HD; SD; SE; SFE; Cold-pressing; SDE; etc	GC-MS; TLC; HPLC; GC/GC-MS; GC-FID; GC-sniffing;	Limonene, terpinene, etc
8	*Duhuo*	Roots	*Angelica pubescens*	Umbelliferae	About 0.3%	HD; SD; GP-MSE; SFE; HS-SPME	GC-FID; GC-MS;	Pinene, elemol, eudesmol. etc
9	*Houpu*	Barks	*Magnolia officinalis*	Magnoliaceae	0.09%-0.50%	HD; SD; SPME;	GC-MS;	Eudesmol, caryophyllene oxide, cadinene. etc
10	*Xiaohuixiang*	Seeds, leaves and stem	*Foeniculum vulgare*	Umbelliferae	3%-8%	UAHD; SFE; HD; SD; MAHD; etc	GC-MS; GC-FID;	Trans-anethole, estragole, fenchone, etc
11	*Jinyinhua*	Flower buds and flower	*Lonicera Japonica*	Caprifoliaceae	0.16%-0.56%	Homogenate Extraction; SFE-MD; NPLC-GC; HD; SFE; HS-SPME; HD-SE; SE	GC-MS; GC-FID;	Linalool, epoxylinalool, terpineol, etc
12	*Qianghuo*	Rhizome *Radix Notopterygii*	*Notopterygium incisum*	Umbelliferae	1.09%-7.76%	HD; SFE; SD;	GC-MS; GC/GC-FID; GC/GC-MS;	Pinene, cadindienol, limonene, linoleic acid. etc
13	*Sanqi*	Stalks, leaves, flowers and roots	*Panax pseudo-ginseng* var. notoginseng or *Panax notoginseng* (Burk.) F.H.Chen	Araliaceae	Stalks (about 1.3%)	SFE; SDE; HD;	GC-FID; GC-MS;TLC	Spathulenol, germacrene D, bicyclogermacrene

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
14	*Chuanxiong*	Rhizome	*Ligusticum chuanxiong* Hort.	Umbelliferae	Not clear	HD; HS-SPME; SD; SFE	GC-MS	Ligustilide, fenipentol, 3-butylidene phthalide. etc
15	*Wuzhuyu*	Fruits *Fructus Evodiae*	*Evodia rutaecarpa*	Rutaceae	Fruits (0.2%-0.75%)	HS-SPME; SFE; HD;	GC-MS	Limonene, elemene, linalool, myrcene. etc
16	*Yuxingcao*	Aerial parts	*Houttuynia cordata* Thunb.	Saururaceae	0.04%-0.12%	HD; SFE; SE;	GC-MS; GC-FID; UVS	Pinene, myrcene, limonene, sabinene, bornyl acetate. etc
17	*Xinyi*	Flower buds	*Magnolia biondii* Pamp. *Magnolia denudata* Desr. *Magnolia sprengeri* Pamp.	Magnoliaceae	Flower buds (2.3%-4.4%)	SFE; HD; MAE; MAHD; UAHD; Water reflux	GC-MS	Pinene, eucalyptol, limonene, camphor. etc

Table 2 Medicinal plants in the Chongqing region (Not many studies on essential oil or volatile compounds)

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
1	*Zhiqiao*	Fruits *Fructus aurantii*	*Citrus aurantium* L.	Rutaceae	Fruits (about 1%)	HD;	GC-FID; GC-MS;	Limonene, linalool, terpinene. etc
2	*Gegen*	Roots *Radix puerariae*	*Pueraria lobata* (Willd.) Ohwi	Fabaceae	Not given	HD	GC-MS	Eicosanoic acid, 9,12-octadecadienenoic aicd, etc
3	*Dansen*	Rhizome	*Salvia miltiorrhiza*	Lamiaceae	Flower (not given); Rhizome (not given)	HD-SE; HD	GC-MS	Flower (Bourbonene, caryophyllene, cadindiene.etc); Rhizome (palmitic acid, oleic acid)
4	*Muxiang*	Rhizome or roots	*Saussurea costus*	Compositae	Roots (0..89%)	HD;	GC-MS;	Dehydrocostulactone, costunolide , etc

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
5	*Huzhang*	Rhizome *Rhizoma polygoni Cuspidati*	*Polygonum cuspidatum or Reynoutria japonica*	Polygonaceae	0.66 %	SDE; SE; HD	GC-MS	2-hexenal, 3-hexen-1-ol, etc
6	*Kuandonghua*	Flower buds	*Tussilago farfara* L.	Compositae	Flower buds (about 0.1%)	HD; HD-SE; SDE	GC-MS	Bisabolene, cadinene, undecene, etc
7	*Banxia*	Rhizome	*Pinellia ternata* (Thunb.) Breit.	Araceae	Not given	SDE;	GC-MS	Anethole, etc
8	*Huangbo*	Barks *Cortex phellodendri Chinensis*	*Phellodendri chinensis*	Rutaceae	Fruits (0.95%)	HD;	GC-MS	Limonene, limonene oxide, carvone. etc
9	*Duzhong*	Barks	*Eucommia ulmoides*	Eucommiaceae	Leaves (not given)	HD-SE	GC-MS	2-furanmethyl acetate, 1H-Imidazole-2-methanol.
10	*Yujin*	Roots *Radix Curcumae Aromaticae*	*Curcuma aromatica* Salisb.	Zingiberaceae	Not given	SD; HD-SE;	GC-MS; TLC	Germacrene D, germacrone, camphor, elemicin.etc
11	*Tianma*	Rhizome	*Gastrodia elata* Blume	Orchidaceae	About 0.35%	HD; SE; MAE	GC-MS	Palmitic acid. etc
12	*Yinxing*	Fruits, leaves	*Ginkgo biloba* L.	Ginkgoaceae	Leaves (about 0.15%)	HD; SDE;	GC-MS	Stearic acid, phytol, tetradecanoic acid. etc
13	*Buguzhi*	Fruits Malaytea scurfpea Fruit	*Psoralea corylifolia* L.	Fabaceae	Fruits (0.2%)	HS-SPME; HD	GC-MS	Caryophyllene, linalool, caryophyllene oxide.etc
14	*Shijunzi*	Fruits roots and leaves	*Quisqualis indica*	Combretaceae	Leaves (0.12%)	SD	GC-MS	Palmitic acid, etc

Table 3 Medicinal plants in the Chongqing region (Almost no scientific studies on essential oil or volatile compounds)

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
1	*Xuansen*	Roots *Radix Scrophulariae*	*Scrophularia ningpoensis* Hemsl.	Scrophuariaceae	unknown	--	--	--
2	*Huanglian*	Roots and Rhizome	*Coptis chinensis* Franch.	Renunculaceae	unknown	--	--	--
3	*Heshouwu*	Rhizome	*Fallopia multiflora* (Thunb.) Harald or *Polygonum multiflorum* Thunb.	Polygonaceae	unknown	--	--	--
4	*Huinianmaorendong*	Flowers	*Lonicera maeranthoides* Hand-Mazz	Caprifoliaceae	unknown	--	--	--
5	*Jinqiancao*	All parts	*Lysimachia christinae* Hance	Primulaceae	Unknown	--	--	--
6	*Dianqie*	All parts	*Atropa belladonna*	Solanaceae	Unknown	--	--	--
7	*Mugua*	Fruits	*Chaenomeles sinensis*	Rosaceae	Unknown	--	--	--
8	*Chuanbeimu*	Rhizome	*Fritillaria cirrhosa*	Liliaceae	Unknown	--	--	--
9	*Gualou*	Fruits, seeds and rhizome *Fructus Trichosanthis*	*Trichosanthes kirilowii* Maxim.	Cucurbitaceae	Unknown	--	--	--
10	*Baiji*	Rhizome Rhizoma Bletillae	*Bletilla striata*	Orchidaceae	Unknown	--	--	--
11	*Wubeizi*	Gall in the tree *Galla chinensis*	*Rhus chinensis* Mill.	Anacardiaceae	--	--	--	--
12	*Yuanhu*	Roots and rhizome	*Corydalis turtschaninovii Corydalis yanhusuo*	Papaveraceae	Unknown	--	--	--

No.	Chinese Name	Parts used in TCM and the name	Latin Name of Plant	Family	Essential oil yield	Extraction and separation techniques	Analytical techniques	Main components (only 1-5 components listed)
13	*Chuankehu*	Stalks	*Dendrobium denneanum* Kerr. *Dendrobium aurantiacum* var. denneanum	Orchidaceae	Unknown	--	--	--
14	*Chuandansen*	Roots	*Codonopsis tangshen*	Campanulaceae	Unknown	--	--	--
15	*Fuzi*	Roots	*Aconitum carmichaelii* Debx.	Renunculaceae	Unknown	--	--	--
16	*Tiandong*	Roots *Radix Asparagi*	*Asparagus cochinchinensis*	Liliaceae	Unknown	--	--	--
17	*Chuanniuqi*	Roots	---	Amaranthaceae	unknown	--	--	--

Chapter 3 MAP-refinery applied to selected plants

3.1 Introduction

In both Midi-Pyrénées region (France) and Chongqing region (China), there are rich and underutilized medicinal and aromatic plants (MAP). Aiming at fully exploiting different molecules in these plants, MAP-refinery developed in first chapter was applied to several selected plants from two regions: 5 plants from the Midi-Pyrénées region including *Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Spartium junceum* L. and *Cytisus scoparius* L., 3 plants from the Chongqing region including *Tussilago farfara* L., *Citrus aurantium* L. and *Saussurea costus*. In the MAP-refinery process, plant materials will be processed into four parts, essential oil (or volatile extract), aqueous extract, methanolic extract and final residue. Essential oils will be analyzed by GC-MS and GC-FID. Aqueous and methanolic extracts will be tested for their antioxidant capacity using different evaluation methods. Possible applications of final residues will also be proposed, based on bibliographic studies. For stems of *Spartium junceum* L. and *Cytisus scoparius* L., because there are almost no essential oils in them, Soxhlet extraction is directly carried out to obtain the methanolic extracts.

3.2 Materials and methods

3.2.1 Plant materials

Tussilago farfara L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Spartium junceum* L. and *Cytisus scoparius* L. were collected in the fields along the road near mountain Midi-Pyrénées from March to June in 2011 and identified by the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse. These samples were then placed in the shady room to be naturally dried. The fully-dried samples were cut into pieces before distillation. Flower buds and stalks of *Tussilago farfara* L. from Chongqing, dry fruits of *Citrus aurantium* L. and dry roots of *Saussurea costus* were from plantation bases of Traditional Chinese Medicines in Chongqing and kindly provided by Prof. Xu from Chongqing

University in China. Different parts of these plants and extraction methods for essential oil (or volatile extract) were shown in Table 1.

Table 1 Selected plants and extraction methods of essential oil (or volatile extract)

N°	Plant name	Plant parts	Extraction method for EO or VE
1	*Tussilago farfara* L. (CN)	Flower buds	SDE
2	*Tussilago farfara* L. (CN)	Stalks	SDE
3	*Tussilago farfara* L. (FR)	Flower buds	SDE
4	*Tussilago farfara* L. (FR)	Roots	SDE
5	*Calendula arvensis* L.	Roots	SDE
6	*Robinia pseudoacacia* L.	Flowers	SDE
7	*Robinia pseudoacacia* L.	Leaves	SDE
8	*Spartium junceum* L.	Flowers	SDE
9	*Spartium junceum* L.	Flower buds	SDE
10	*Spartium junceum* L.	Stems	-
11	*Cytisus scoparius* L.	Flowers	SDE
12	*Cytisus scoparius* L.	Stem	-
13	*Saussurea costus*	Roots	HD
14	*Citrus aurantium* L.	Fruits	HD

CN: plants from Chongqing (China); **FR**: plants from Midi-Pyrénées (France); **EO**: essential oil; **VE**: volatile extract; **HD**: hydrodistillation; **SDE**: Simultaneous Distillation Extraction

3.2.2 Chemicals

n-pentane (HPLC grade, Scharlau, Spain), anhydrous sodium carbonate (99.7%, VMR International, Belgium), deionized water, sodium acetate (Reachim, Riga, Latvia), KH_2PO_4•12 H_2O (Jansen Chimica, Belgium), acetic acid (Lachema, Czech Republic), TPTZ (2,4,6-tripyridyl-*s*-triazine) and fluorescein (Fluka Chemicals, Germany); methanol (HPLC grade), DPPH• (2,2-Diphenyl-1-picrylhydrazyl hydrate, 95%), Folin-Ciocalteu reagent, Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%), gallic acid, AAPH [2,2'-azobis (2-amidino-propane) dihydrochloride] were purchased from Sigma-Aldrich Chemie (Steinheim, Germany); ABTS [2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid)], NaCl, Na_2HPO_4, KCl and $K_2S_2O_8$ were from Merck (Darmstadt, Germany).

3.2.3 Extraction of essential oil (or volatile extract)

3.2.3.1 Simultaneous Distillation Extraction (SDE)

Simultaneous Distillation Extraction (SDE) of volatile extracts from plant samples (Table 1) were carried out using classic Likens-Nickerson apparatus. Plant samples arranging from 5 g to 30 g were put into one flask for distillation, followed by addition of 300 mL of water. Then 50 mL of pentane was put into the other flask for extraction of volatile extracts. The SDE process lasted for 3 h. After that, solvents containing volatile extracts in the Likens-Nickerson apparatus were combined and separated from water by separatory funnel. The solvent obtained was then concentrated to about 10 mL using rotary evaporator at reduced pressure and finally concentrated to about 1 mL under the gentle flow of nitrogen gas. The obtained solvent with volatile extract was put into a bottle and stored in the refrigerator for the following analysis.

3.2.3.2 Hydrodistillation (HD)

Hydrodistillation of essential oils from dry fruits of *Citrus aurantium L.*and roots of *Saussurea costus* was carried out using Clevenger apparatus. About 300 g of samples were first soaped in water overnight and then cut into small pieces before distillation. 3 L of water was put into the flask to mix with the samples. Distillation process lasted for 3 h and three repetitions were made.

3.2.4 Essential oil (or volatile extract) analysis by GC-FID

GC-FID analysis was carried out on a VARIAN CP-3380 Gas Chromatograph equipped with Flame Ionization Detector and fused-silica capillary columns HP-5 MS (0.25 μm × 0.25 mm × 30 m) and Carbowax 20M (0.25 μm × 0.25 mm × 30 m). GC parameters with HP-5 MS column: oven temperature programming: rising from 60℃ to 200℃ at 3℃ /min and then held at 200℃ for 14 min. Carrier gas: nitrogen (flow rate: 0.8 mL/min); injector temperature: 200°C, detector temperature: 200°C. Injection volume: 2 μL; split ratio: 1/100. A mixture of a series of normal alkanes (C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18} and C_{20}) was analyzed for the calculation of the retention indices (RI).

GC parameters with Carbowax 20M column: oven temperature programming: rising from 50℃ to 200℃ at 5℃ /min and then held at 200℃ for 10 min. Carrier gas: nitrogen (flow rate: 0.8 mL/min); injector temperature: 200°C, detector temperature: 200°C. Injection volume: 2 μL; split: 1/100. A mixture of a series of normal alkanes (C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{20}, C_{22} and C_{24}) was analyzed for the calculation of the retention indices (RI).

3.2.5 Essential oil (or volatile extract) analysis by GC-MS

Essential oil samples were analyzed on a Hewlett Packard 5890 series Π Gas Chromatograph coupled with a 5970 mass spectrometer and equipped with fused-silica capillary columns HP-5 MS (0.25 μm × 0.25 mm × 30 m) and Carbowax (0.25 μm × 0.25 mm × 30 m).

GC-MS parameters with a HP-5 MS column: carrier gas: helium (flow rate: 0.6 mL/min), oven temperature programming: rising from 60℃ to 220℃ at 3℃ /min and then held at 220℃ for 12 min. Injector temperature: 250℃ , ion source temperature: 280℃ . Electron ionization: 70 eV; mass spectra range: 30-300 amu and 2.77 scan/s; split ratio: 1/100; injection volume: 1 μL, pentane solution.

GC-MS parameters with a carbowax column: carrier gas: helium (flow rate: 0.6 mL/min), oven temperature programming: at 70℃ for 2 min, rising to 220℃ at 5℃ /min and then held at 220℃ for 8 min. Injector temperature: 250℃ , ion source temperature: 280℃ . Electron ionization: 70 eV; mass spectra range: 30-300 amu and 2.77 scan/s; split ratio: 1/100; injection volume: 1 μL, pentane solution.

Identification of individual components in the essential oils or volatile extracts was based on the comparison of their retention indices calculated with reference to a series of *n*-alkanes, with those found in the literature (Adams, 2007). Further identification was made by comparing their mass spectra with those in the mass spectra library of data process software (NBS75K database, Wiley 7th NIST 98 EPA/NIH Mass Spectral Library, Mass finder 3/Hochmuth and FFNSC2/Mondello, 2nd Edition, 2011 Nov.), and also those found in published data. The relative percentage of each component in the essential oil was given according to the normalisation results of peaks in GC chromatograms.

3.2.6 Preparation of aqueous and methanolic extracts from residues

After essential oils (or volatile extracts) were collected, the mixtures of solid residue and aqueous residue were filtered. The solid residues were collected and dried by using air-drying machine at around 60℃ while liquid residues were collected and freeze-dried to obtain the aqueous extracts. The Soxhlet extraction using methanol as solvent was carried out for all the solid residues to obtain the methanolic extracts. The extraction lasted for 5 h.

3.2.7 Antioxidant capacity evaluation of aqueous extracts

3.2.7.1 DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH$^{\bullet}$ was determined by a slightly modified method of Brand-Williams and co-workers (Brand-Williams *et al.*, 1995). Extract solution of 10 mg/mL was prepared by dissolving 10 mg of each extract in 1.00 mL of methanol/water solution (50% v/v). Ultrasonic bath for 10 min was used to accelerate the dissolution. For further DPPH scavenging assessment, the more effective plant extracts were diluted 2-, 4- and 8-fold (5 mg/mL, 2.5 mg/mL and 1.25 mg/mL, respectively). The solution of DPPH in methanol (6×10^{-5} M) was prepared daily, before measurements. 2 mL of this solution was mixed with 50 µL of extract solution and the mixed solution was put in the darkness for 30 min. The absorption was then read at 515 nm on the UV-vis spectrophotometer (UV-1800, SHIMADZU). Each assay was repeated three times and measurement was carried out at room temperature in 30 min. Radical Scavenging Activity (RSA) was calculated by the following formula:

% Inhibition = $[(A_B\text{-}A_A)/A_B] \times 100$

in which: A_B = absorbance of DPPH solution (t = 30 min); A_A = absorbance of tested extract solution (t = 30min). The methanol solutions of Trolox with known concentrations ranging from 100 to 750 µmol/L were used for calibration. The antioxidant capacity (AOC) of extract was expressed in µmol of Trolox equivalents (TEs) / g of plant extract.

3.2.7.2 Analysis of total phenolic contents (TPC)

Total phenolic contents (TPC) in extracts were determined with Folin-Ciocalteu reagent. The assays were performed according to a modified method of Medina (Medina, 2011). The gallic acid solutions with known concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.25 mg/mL) were used for calibration. The TPC in extract was expressed as mg of gallic acid equivalents (GAE) / g of extract. 100 µL of extract solution was mixed with 1 mL of Folin-Ciocalteu reagent (10%), followed by the addition of 400 µL of deionized water and 1 mL of 7.5% sodium carbonate solution. The mixed solution was then put in the thermostat for 30 min at 40℃. The absorption was read after 30 min at 765 nm on the spectrophotometer (UV-1800, SHIMADZU). Each assay was repeated three times.

3.2.8 Antioxidant capacity evaluation of methanolic extracts

3.2.8.1 DPPH radical scavenging assay

The original method of Brand-Williams and co-workers (Brand-Williams *et al.*, 1995) was adapted for the radical scavenging activity evaluation of plant extracts against stable DPPH$^{\bullet}$ on the microplate. The experiments were carried out by using FLUOstar Omega microplate reader (BMG LABTECH, Germany). When DPPH$^{\bullet}$ reacts with an antioxidant compound, it donates hydrogen atom and it is reduced. The changes in colour from deep violet to light yellow were measured at 515 nm.

Extract solution was prepared by dissolving 50 mg of each extract in 5.00 mL of methanol (10 mg/mL). For extracts which were not fully soluble, the solutions were treated in an ultrasonic bath for 10 min and centrifuged, the supernatants were further analyzed. For further DPPH scavenging assessment, the more effective plant extracts were diluted 2- and 4-fold (5 mg/mL and 2.5 mg/mL, respectively). The solution of DPPH in methanol (5.8×10^{-5} M) was prepared daily, before measurements. On a 96-well plate, 320 µL of this solution was mixed with 8 µL of extract solution. 320 µL of methanol was used as blank and also 320 µL of DPPH$^{\bullet}$ solution was mixed with 8 µL of methanol. Each assay was repeated four times and measurements were carried out at room temperature, for 45 min. Radical Scavenging Activity (RSA) was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

in which: A_B = absorbance of DPPH solution (t = 45 min); A_A = absorbance of tested extract solution (t = 45 min).

The methanol solutions of Trolox with known concentrations ranging from 100 to 750 µmol/L were used for calibration. The antioxidant capacity (AOC) of extract was expressed in µmol of Trolox equivalents (TEs) / g of plant extract.

3.2.8.2 *ABTS*$^{\bullet+}$ radical cation decolourisation assay

ABTS$^{\bullet+}$ radical cation was produced by reacting ABTS (2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) with potassium persulfate (Re *et al.*, 1999). Firstly PBS (Phosphate buffered saline) solution was prepared by dissolving 8.18 g NaCl, 0.27 g $KH_2PO_4 \bullet 12\ H_2O$, 3.58 g Na_2HPO_4 and 0.15 g KCl in 1 L of deionized water. The pH value of PBS was determined and adjusted to 7.4. The stock solution of ABTS (2.13 mM) was then prepared by dissolving 0.0549 g ABTS in 50 mL of PBS. Deionized water was used to

prepare 70.29 mM solution of $K_2S_2O_8$. $ABTS^{\bullet+}$ radical cation was produced by reacting 50 mL of ABTS stock solution with 200 µL of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. For the assessment of extracts, the $ABTS^{\bullet+}$ was diluted with PBS solution to obtain the absorbance of 0.7 ± 0.02 at 734 nm. The absorbance readings were carried out using FLUOstar Omega microplate reader (BMG LABTECH, Germany). On a 96-well plate, 300 µL of $ABTS^{\bullet+}$ solution was mixed with 5 µL of extract solution. 300 µL of PBS solution was used as blank and also 300 µL of $ABTS^{\bullet+}$ solution was mixed with 5 µL of PBS solution. Each assay was repeated four times and measurements were carried out at room temperature, for 30 min. Radical Scavenging Activity (RSA) was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

in which : A_B = absorbance of $ABTS^{\bullet+}$ solution (t = 30 min); A_A = absorbance of tested extract solution (t = 30 min).

The PBS solutions of Trolox with known concentrations ranging from 100 to 1500 µmol/L were used for calibration. The antioxidant capacity of extract was expressed in µmol of Trolox equivalents (TEs) / g of plant extract.

3.2.8.3 Ferric Reducing/Antioxidant Power (FRAP) assay

The ability of plant extracts to reduce ferric ion to ferrous ion, ferric reducing/antioxidant power (FRAP) assay was performed according to microplate adaptation of testing method performed by Benzie and Szeto (1999). Ferrous ion produced in this assay forms a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. The acetate buffer solution (300 mmol/L, pH 3.6) was prepared by dissolving 0.9344 g CH_3COONa and 8 mL acetic acid in 500 mL of deionized water. 10 mmol/L of TPTZ (2,4,6-tripyridyl-s-triazine) solution was obtained by dissolving 0.3123 g of TPTZ in 100 mL HCl solution (40 mmol/L). 20 mmol/L ferric solution was prepared by dissolving 0.5406 g $FeCl_3 \bullet 6H_2O$ in 100 mL deionized water. Working FRAP reagent was prepared by mixing 50 mL acetate buffer, 5 mL TPTZ solution and 5 mL $FeCl_3 \bullet 6H_2O$ solution. Aqueous solutions of known ferrous ion with concentrations in the range of 100-1000 µmol/L and Trolox solutions (in methanol) with concentrations ranging from 100 to 1000 µmol/L were used for calibration.

The FRAP assays were performed by use of FLUOstar Omega microplate reader (BMG LABTECH, Germany). The working FRAP reagent was warmed to 37° C before use. On a 96-well plate, 10 µL of ferrous solution, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-

carboxylic acid) solution, sample solution and methanol for blank were added into the well, respectively. Then 30 μL of deionized water was added into all the tested wells, followed by 300 μL of FRAP reagent. Each assay was repeated four times and measurements were carried out at 37° C. The absorbance was recorded every minute up to 15 min. The change in the absorbance between the final reading and the absorbance of blank reagent was calculated for each sample, ferrous solution and Trolox solution. FRAP values of each sample were expressed as μmol of ferrous ion equivalent / g of plant extract and μmol of Trolox equivalent (TE) / g of plant extract.

3.2.8.4 Oxygen Radical Absorbance Capacity (ORAC) assay

Oxygen Radical Absorbance Capacity (ORAC) assays were performed according to the methods of Prior *et al.* (2003), Ganske and Dell (2006). It is one of the standardized methods for determining the antioxidant capacity of substance.

Firstly, PBS solution was prepared by dissolving 8.18 g NaCl, 0.27 g $KH_2PO_4 \bullet 12\ H_2O$, 3.58 g Na_2HPO_4 and 0.15 g KCl in 1 L of deionized water. The pH value of PBS solution was determined and adjusted to 7.4. The stock fluorescein solution (Stock #1) was then prepared by dissolving 0.0225 g of FL in 50 mL of PBS solution. A second stock solution #2 was prepared by diluting 50 μL of stock solution #1 in 10 mL of PBS solution. 800 μL of stock solution #2 was added to 50 mL of PBS to obtain the fluorescein solution of 95.68 nmol/L for the subsequent assays. 240 mM of AAPH solution was prepared by dissolving 1.6272 g of AAPH in 25 mL of PBS solution.

The ORAC assays were performed by use of FLUOstar Omega microplate reader (BMG LABTECH, Germany). On a 96-well plate, 25 μL of samples, Trolox solutions and PBS solution for blank, were added into the well, followed by 150 μL of fluorescein solution. Each assay was performed in triplicate. After the gain adjustment, the microplate was covered and incubated in the microplate reader for 15 min at 37° C. Then fluorescence measurements (Ex. 485 nm, Em.520 nm) were taken every 66 s. After 3 cycles, 25 μL of AAPH was quickly injected into the well by use of multi-channel pipette and the test was resumed. The whole measurements will be finished in about 90 min (81 cycles).

The final $ORAC_{FL}$ values were calculated by using a regression equation ($Y = aX + b$) between Trolox concentration and the net area under the fluorescein decay curve. The PBS solutions of Trolox with known concentrations ranging from 50 to 200 μmol/L were used for

calibration. The antioxidant capacity of each sample is expressed as μmol of Trolox equivalent (TE) / g of plant extract. The area under the curve (AUC) was calculated as:

$$AUC = (0.5+f_5/f_4+f_6/f_4+f_7/f_4+\cdots+f_i/f_4) \times CT$$

Where f_4 = initial fluorescence reading at cycle 4, f_i = fluorescence reading at cycle i and CT = cycle time in minutes.

The net AUC was obtained by subtracting the AUC of the blank from that of each sample.

3.2.8.5 Analysis of total phenolic contents (TPC)

The total phenolic contents (TPC) in extracts were determined with Folin-Ciocalteu reagent. The assay was performed according to a slightly modified method by Medina (2011). The gallic acid (20% methanol) solutions with known concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.25, 0.4 mg/mL) were used for calibration. The TPC in extract was expressed as mg of gallic acid equivalents (GAE) / g of extract.

The assays were performed by use of FLUOstar Omega microplate reader (BMG LABTECH, Germany). On a 96-well plate, 10 μL of extract solution was added into the well, followed by 100 μL of Folin-Ciocalteu reagent (10%). 110 μL of deionized water was also added and used as the blank. Then the mixtures were shaken for 3 min on the microplate reader. After that, 40 μL of deionized water and 100 μL of Na_2CO_3 solution (7%) were added into the wells and the mixtures were shaken for 3 min before the measurements. The absorbance was measured at 760 nm up to 90 min.

3.3 Results and Discussion

3.3.1 Essential oil (or volatile extract) analysis

3.3.1.1 Extraction yields of essential oil (or volatile extract)

For selected medicinal and aromatic plants in the Midi-Pyrénées and Chongqing region, only essential oils from dry fruits of *Citrus aurantium* L. and dry roots of *Saussurea costus* could be directly collected and essential oil yields were 0.21% and 0.23%, respectively. Essential oil from dry fruits of *Citrus aurantium* L. was of light yellow colour while *Saussurea costus* oil was of yellow colour. For the other selected plants, as it was very difficult to directly collect essential oil by using hydrodistillation, simultaneous distillation

extraction was employed to obtain volatile extracts. In the follow-up analysis by GC-FID and GC-MS, no apparent chemical compounds could be detected from flower extracts of *Cytisus scoparius* L., flowers and leaves extracts of *Robinia pseudoacacia* L. and flower extracts of *Spartium junceum* L. Volatile extracts in flower buds of *Tussilago farfara* L. (FR) and stalks of *Tussilago farfara* L. (CN) were not analyzed because samples were destroyed and there were not enough samples for redoing experiments.

3.3.1.2 Chemical composition of volatile extract from flower buds of *Tussilago farfara* L. (CN)

GC-FID and GC-MS analyses of volatile extract from flower buds of *Tussilago farfara* L. from Chongqing allowed the identification of 28 chemical compounds, accounting for 84.6% of the total volatile extract. The results in Table 2 showed that the main components were 1-undecene (19.9%), 1-nonene (14.2%), 1,10-undecadiene (9.3%) and (*E*)-cycloundecene (5.5%) and β-bisabolene (4.8%). A previous study published by Liu *et al.* (2006) identified the same predominant components as our results but with different proportions: β-bisabolene (13.93%), *(E)*-cycloundecene (8.49%) and 1-undecene (4.83%).

Table 2 Main chemical compounds of volatile extract from flower buds of *Tussilago farfara* L. (CN)

N°	Components	RI[a]	RIL[a]	RP %	Identification
1	furfural	-	828	t	MS
2	1-nonene	882	886	14.2	RI, MS
3	1-decene	982	986	1.4	RI, MS
4	α-phellandrene	1001	1002	1.5	RI, MS
5	*para*-cymene	1019	1020	1.9	RI, MS
6	phenylacetaldehyde	1041	1042	t	RI, MS
7	1,10-undecadiene	1074	1078	9.3	RI, MS
8	2-butylidene-bicyclo[2.2.1]heptane	1079	-	2.5	MS
9	1-undecene	1083	1086	19.9	RI, MS
10	(*E*)-cycloundecene	1271	1279	5.5	MS
11	1-tridecene	1283	1290	1.3	RI, MS
12	carvacrol	1302	1298	t	RI, MS
13	α-copaene	1375	1374	1.2	RI, MS
14	β-cubebene	-	1387	t	MS
15	α-gurjunene	-	1409	t	MS
16	β-caryophyllene	1421	1417	0.7	RI, MS
17	*(E)*-β-farnesene	1450	1454	0.7	RI, MS
18	germacrene D	1482	1484	2.4	RI, MS
19	β-bisabolene	1504	1505	4.8	RI, MS

N°	Components	RI[a]	RIL[a]	RP %	Identification
20	δ-cadinene	1522	1522	1.6	RI, MS
21	spathulenol	1579	1577	4.3	RI, MS
22	caryophyllene oxide	1586	1582	1.5	RI, MS
23	β-oplopenone	1611	1607	4.7	RI, MS
24	α-muurolol	1642	1644	2.3	RI, MS
25	α-cadinol	1659	1652	2.2	RI, MS
26	myristic acid	1759	1762	t	RI, MS
27	palmitic acid	1951	1959	t	RI, MS
28	methyl linoleate	-	2095	0.7	MS
	Monoterpene hydrocarbons			**3.4**	
	Oxygenated monoterpenes			**t**	
	Sesquiterpene hydrocarbons			**11.4**	
	Oxygenated sesquiterpenes			**15.0**	
	Aromatic compounds			**t**	
	Aliphatic compounds			**54.8**	
	Total			**84.6**	

RI[a]: Retention index calculated with reference to a series of n-alkanes using an apolar column; **RIL**[a]: Retention index on apolar column reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

The chemical compounds classification of the volatile extract showed that aliphatic compounds and sesquiterpene compounds (including sesquiterpene hydrocarbons and oxygenated sesquiterpenes) constituted the main class with the relative percentages as 54.8% and 26.4%, respectively. However, monoterpene compounds only accounted for 3.4%.

3.3.1.3 Chemical composition of volatile extract from roots of *Tussilago farfara* L. (FR)

By GC-FID and GC/MS analyses, 21 chemical compounds were identified in volatile extract of *Tussilago farfara* L. roots, amounting to 76.1% of the total volatile extract. As far as the literature survey could ascertain, volatiles composition of *Tussilago farfara* L. roots has never been reported. As shown in Table 3, the main components were *(E)*-β-farnesene (22.9%), palmitic acid (17.8%), β-selinene (5.8%) and 1-nonene (5.7%), which were quite different from the results obtained with *Tussilago farfara* L. flower buds, but with 1-nonene as a common predominant component.

From the perspective of chemical classes, volatiles in *Tussilago farfara* L. roots were mainly composed of sesquiterpene hydrocarbons (35%) and aliphatic compounds (32.5%). The relative percentage of the oxygenated sesquiterpenes was 5.7% and the content of monoterpene compounds were also very low, only 1.5%.

Table 3 Main chemical compounds of volatile extract from roots of *Tussilago farfara* L. (FR)

N°	Components	RI[a]	RIL[a]	RP%	Identification
1	1-nonene	882	886	5.7	RI, MS
2	α-pinene	930	932	0.7	RI, MS
3	benzaldehyde	957	952	t	RI, MS
4	2-pentyl furan	985	984	0.7	RI, MS
5	α-phellandrene	1001	1002	0.9	RI, MS
6	*para*-cymene	1021	1020	0.6	RI, MS
7	1-undecene	1084	1086	1.5	RI, MS
8	(*E*)-cinnamaldehyde	1268	1267	0.6	RI, MS
9	7-*epi*-silphiperfol-5-ene	1345	1345	0.7	RI, MS
10	eugenol	1355	1356	0.8	RI, MS
11	β-caryophyllene	1422	1417	1.1	RI, MS
12	*(E)*-β-farnesene	1452	1454	22.9	RI, MS
13	β-selinene	1486	1489	5.8	RI, MS
14	β-bisabolene	1505	1505	4.5	RI, MS
15	spathulenol	1579	1577	1.5	RI, MS
16	β-oplopenone	1612	1607	1.9	RI, MS
17	silphiperfol-6-en-5-one	1627	1624	0.8	RI, MS
18	khusinol acetate	1825	1823	1.5	RI, MS
19	methyl palmitate	1916	1921	1.6	RI, MS
20	palmitic acid	1951	1959	17.8	RI, MS
21	methyl linoleate	-	2095	4.5	MS
	Monoterpene hydrocarbons			**1.5**	
	Sesquiterpene hydrocarbons			**35.0**	
	Oxygenated sesquiterpenes			**5.7**	
	Aromatic compounds			**1.4**	
	Aliphatic compounds			**32.5**	
	Total			**76.1**	

RI[a]: Retention index calculated with reference to a series of n-alkanes using an apolar column; **RIL[a]**: Retention index on apolar column reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

3.3.1.4 Chemical composition of volatile extract from roots of *Calendula arvensis* L.

In the analysis of volatile extract of *Calendula arvensis* L. roots, 32 chemical compounds were identified, constituting 68.1% of the total volatile extract. Spathulenol (27.4%), α-cadinol (6.9%), manool oxide (5.7%), dodecanol (4.4%) and spirolepechinene (4.0%) were found to be the main components (Table 4). Three unknown compounds (**B**, **C** and **D**) were also listed in the table (accounting for 20.8% in total) and their mass spectra fragmentations were given. α-cadinol was not well resolved and mixed with another unknown compound **A**

(MW = 206). The identified compounds were dominated by oxygenated sesquiterpenes (38.0%), oxygenated monoterpenes (9.1%) and oxygenated diterpenes (8.2%). Monoterpene hydrocarbons accounted for only 1.9%.

Table 4 Main chemical compounds of volatile extract from *Calendula arvensis* L. roots

N°	Components	RI[a]	RIL[a]	RP%	Identification
1	1-nonene	882	886	1.0	RI, MS
2	2-pentyl furan	982	984	0.4	RI, MS
3	δ-2-carene	999	1001	0.6	RI, MS
4	α-phellandrene	1001	1002	0.2	RI, MS
5	δ-3-carene	1007	1008	0.2	RI, MS
6	*para*-cymene	1019	1020	0.5	RI, MS
7	β-phellandrene	1027	1029	0.4	RI, MS
8	linalool	1093	1095	t	RI, MS
9	*cis*-*p*-menth-2-en-1-ol	1116	1118	1.9	RI, MS
10	*trans*-*p*-menth-2-en-1-ol	1134	1136	1.9	RI, MS
11	*p*-methyl acetophenone	1179	1179	0.6	RI, MS
12	*p*-cymen-8-ol	1179	1179	0.8	RI, MS
13	α-terpineol	1187	1186	0.8	RI, MS
14	*cis*-piperitol	1191	1195	0.6	RI, MS
15	*trans*-piperitol	1202	1207	1.2	RI, MS
16	piperitone	1252	1249	0.1	RI, MS
17	α-terpinen-7-al	1271	1287	1.8	RI, MS
18	dictamnol	1425	1429	1.3	RI, MS
19	(*Z*)-β-farnesene	1438	1440	0.5	RI, MS
20	spirolepechinene	1450	1452	4.0	RI, MS
21	dodecanol	1483	1469	4.4	RI, MS
22	α-farnesene + *epi*-cubebol	1490	1494	t	RI, MS
23	δ-cadinene	1522	1522	t	RI, MS
24	spathulenol	1578	1577	27.4	RI, MS
25	*epi*-α-muurolol	1638	1640	0.1	RI, MS
26	**A** (MW =206) + α-cadinol	1652	1654	6.9	RI-MS
27	eupatriochromene	-	-	t	MS
28	hydroxy calamenene	-	-	2.3	MS
29	manool oxide	1996	1987	5.7	RI, MS
30	**B** (MW = 304)	-	-	2.6	MS (b)
31	nezukol	-	2132	2.5	MS (c)
32	**C** (MW = 306)	-	-	4.6	MS (d)
33	**D** (MW = 288)	-	-	13.6	MS
34	n-tetracosane	2400	2400	t	MS
	Monoterpene hydrocarbons			**1.9**	
	Oxygenated monoterpenes			**9.1**	
	Sesquiterpene hydrocarbons			**4.5**	

Oxygenated sesquiterpenes	**38.0**
Oxygenated diterpene	**8.2**
Aromatic compounds	**1.0**
Aliphatic compounds	**5.4**
Total	**68.1**

RI[a]: Retention index calculated with reference to a series of n-alkanes using an apolar column; **RIL**[a]: Retention index on apolar column reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature; **MW**: molecular weight.

MS (b): m/z (%) = 43 (80), 55 (65), 69 (41), 81 (31), 95 (31), 109 (24), 123 (27), 135 (100), 151 (10), 165 (12), 177 (4), 201 (13), 271 (11), 289 (19)

MS (c): m/z (%) = 43 (100), 55 (70), 69 (68), 81 (52), 95 (41), 109 (28), 123 (25), 135 (13), 149 (15), 164 (23), 175 (7), 190 (12), 201 (8), 208 (6), 255 (10), 273 (7), 291 (33)

MS (d): m/z (%) = 43 (100), 55 (36), 69 (28), 81 (30), 95 (18), 109 (14), 119 (14), 135 (9), 147 (8), 153 (6), 163 (4), 175 (6), 190 (26), 201 (8), 218 (4), 243 (1), 255 (8), 273 (35)

To our knowledge, the volatile extract composition of *Calendula arvensis* L. roots has never been reported in the previous literature. Several previous studies focused on the volatiles and essential oil of aerial parts of this plant. The volatiles compositions investigation of aerial parts of *Calendula arvensis* L. which grew in the south-east of Sicily revealed several odour compounds like β-bisabolene and methional (Carpino *et al*., 2004). In another study, essential oil from aerial parts of *Calendula arvensis* L. growing in Corsica contained 85 identified components, with α-cadinol and δ-cadinene as the major compounds (Paolini *et al*., 2010). In comparison, the main compositions of volatile extract from *Calandula arvensis* L. roots were quite different from those of its aerial parts.

3.3.1.5 Chemical composition of volatile extract from flower buds of *Spartium junceum* L.

GC and GC/MS analyses of volatile extract from *Spartium junceum* L. flower buds led to the identification of 27 chemical compounds, amounting to 75.6% of the total volatile extract (Table 5). Three fatty acids, myristic acid (21.8%), palmitic acid (24.7%) and oleic acid (5.3%) accounted for more than half of total constituents. While some typical compounds existing in essential oil, like monoterpene and sesquiterpene compounds, were found to have a very low concentration in the *Spartium junceum* L. flower buds. Monoterpene compounds accounted for only 2.4%, with linalool (1.2%). Aliphatic compounds (66.7%) were dominant in this volatile extract, mainly fatty acids and their esters.

Table 5 Main chemical compounds of volatile extract from *Spartium junceum* L. flower buds

N°	Components	RI[a]	RIL[a]	RP%	Identification
1	*(E)*-2-hexenal	843	846	0.5	RI, MS
2	1-nonene	882	886	3.8	RI, MS
3	α-pinene	936	932	t	RI, MS
4	benzaldehyde	957	952	0.9	RI, MS
5	1-octen-3-ol	971	974	0.7	RI, MS
6	2-pentyl furan	986	984	0.8	RI, MS
7	α-phellandrene	1003	1002	0.6	RI, MS
8	*para*-cymene	1022	1020	0.6	RI, MS
9	phenylacetaldehyde	1041	1042	1.5	RI, MS
10	linalool	1095	1095	1.2	RI, MS
11	2-phenyl ethanol	1111	1116	1.4	RI, MS
12	α-terpineol	1185	1186	t	RI, MS
13	methyl salicylate	1189	1190	t	RI, MS
14	safranal	1196	1196	t	RI, MS
15	2,3-dihydrobenzofuran	1212	1223	0.9	RI, MS
16	indole	1292	1290	0.5	RI, MS
17	*p*-vinyl-guaiacol	1312	1309	0.5	RI, MS
18	γ-nonalactone	1357	1358	t	RI, MS
19	(*Z*)-β-damascenone	1361	1361	t	RI, MS
20	methyl laurate	1524	1524	1.6	RI, MS
21	lauric acid	1658	1659	0.7	RI, MS
22	methyl myristrate	1718	1722	2.6	RI, MS
23	myristic acid	1759	1762	21.8	RI, MS
24	methyl palmitate	1919	1921	2.9	RI, MS
25	palmitic acid	1957	1959	24.7	RI, MS
26	methyl linoleate	-	2095	2.1	MS
27	oleic acid	-	2141	5.3	MS
	Monoterpene hydrocarbons			**1.2**	
	Oxygenated monoterpenes			**1.2**	
	Aromatic compounds			**6.5**	
	Aliphatic compounds			**66.7**	
	Total			**75.6**	

RI[a]: Retention index calculated with reference to a series of n-alkanes using an apolar column; **RIL[a]**: Retention index on apolar column reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

To the best of our knowledge, volatile extract of *Spartium junceum* L. flower buds has not been studied yet. In our study, flower volatiles of *Spartium junceum* L. were also extracted and analyzed but almost no chemical compounds could be detected. However, there are several previous investigations on the volatile organic compounds or essential oil of its

flowers. The volatile organic compounds (VOCs) emitted by *Spartium junceum* L. were investigated and results showed that this plant could emit some monoterpene compounds like camphene, β-pinene, myrcene, *(Z)*-ocimene, linalool and limonene (Owen *et al.*, 1997; Owen *et al.*, 2001). In 2004, the major compounds of oil from fresh flowers of *Spartium junceum* L. were found to be tricosane (22.9%), tetracosane (8.9%) and pentacosane (16.1%) (Miraldia *et al.*, 2004). In another study, *n*-alkanes and aliphatic acids were reported as the major compounds in flowers of this plant (Mancini *et al.*, 2010). Therefore, compounds in flower or flower buds of *Spartium junceum L.* are mainly aliphatic ones, like fatty acids and alkanes.

3.3.1.6 Chemical composition of essential oil from dry roots of *Saussurea costus*

Chemical composition of essential oil from dry roots of *Saussurea costus* collected in Chongqing (China) is shown in Table 6. Forty five chemical compounds were identified, accounting for 64.7% of the total essential oil. Dehydrocostunolide (28.9%), hexadecatrienal (9.2%) and α-costol (7.4%) were found to be the major identified compounds. Besides dehydrocostunolide, *Saussurea costus* oil also contained high contents of other sesquiterpene lactones, such as the mixtures **B**, **C**, **D**, **E**, **F** and **G** whose molecular weights and mass spectra fragmentations were given after the table. Another unknown compound **A** was a sesquiterpene ketone. In total, these unidentified oxygenated sesquiterpenes accounted for 19.6%. The mass spectra of these unknown compounds were compared with those of possible compounds in the published data, but they couldn't be identified. However, these compounds were not saussurea lactone, dehydrocostus lactone, costus lactone, alantolactone, isoalantolactone, costunolide or 8-cedren-13-ol. The coexistence of two or three compounds in one peak (in the case of mixtures **B**, **C**, **D**, **E**, **F** and **G)** also limited the identification.

Table 6 Main chemical compounds of essential oil from dry roots of *Saussurea costus*

NO.	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %	Identification
1	α–thujene	928	924	-	1021	t	RI, MS
2	α-pinene	936	932	1030	1032	0.1	RI, MS
3	camphene	951	946	1077	1076	t	RI, MS
4	sabinene	976	969	1126	1132	0.1	RI, MS
5	β-pinene	982	974	1117	1113	0.5	RI, MS
6	α-phellandrene	1008	1002	1167	1166	0.2	RI, MS
7	α-terpinene	1019	1014	1185	1177	0.1	RI, MS
8	*para*-cymene	1026	1020	1275	1280	0.3	RI, MS
9	limonene	1031	1024	1205	1203	0.1	RI, MS
10	β-phellandrene	1036	1025	1214	1209	t	RI, MS

NO.	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %	Identification
11	phenylacetaldehyde	1041	1036	-	1615	t	RI, MS
12	γ-terpinene	1060	1054	1248	1244	0.2	RI, MS
13	*p*-methyl-benzaldehyde	1082	1077	1649	1642	t	RI, MS
14	δ-terpinene	1091	1086	1286	1295	0.1	RI, MS
15	linalool	1100	1095	1545	1553	0.1	RI, MS
16	<*cis*-*p*>menth-2-en-1-ol	1123	1118	-	-	t	RI, MS
17	borneol	1171	1165	-	1677	0.1	RI, MS
18	terpinen-4-ol	1181	1174	1602	1611	0.7	RI, MS
19	α-terpineol	1187	1186	1695	1706	0.1	RI, MS
20	methyl salicylate	1193	1190	-	-	0.1	RI, MS
21	carvacrol	1302	1298	-	-	0.1	RI, MS
22	α-(*E*)-ionol	1381	1376	1899	1923	0.1	RI, MS
23	β-elemene	1397	1389	1594	1595	1.2	RI, MS
24	dihydro-α-ionone	1422	1411	1820	-	1.6	RI, MS
25	β-caryophyllene	1423	1417	1601	1594	t	RI, MS
26	α-ionone	1434	1428	1854	-	1.6	RI, MS
27	geranyl acetone	1452	1453	-	-	2.3	RI MS
28	paeonol	1458	1463	-	-	1.4	RI, MS
29	α-curcumene	1484	1479	-	1777	0.1	RI, MS
30	β-ionone	1487	1487	1943	-	0.4	RI, MS
31	α-selinene	1502	1498	1721	1711	1.5	RI, MS
32	italicene ether	1534	1536	-	1880	0.5	RI MS
33	elemol	1555	1546	-	-	0.6	RI, MS
34	trans-nerolidol	1567	1561	-	2009	t	RI, MS
35	caryophyllene oxide	1592	1582	1991	1962	1.7	RI, MS
36	globulol	1598	1590	-	2104	t	RI, MS
37	γ-eudesmol	1635	1630	-	2182	t	RI, MS
38	pogostol	1662	1651	-	-	2.3	RI, MS
39	hexadecatrienal	1681	-	1886	-	9.2	MS
40	Compound **A** (MW = 218)	-	-	-	-	5.1	MS (a)
41	<(*Z*)-α-trans> bergamotol	1698	1690	-	-	0.2	RI, MS
42	valerenal	1711	1706	-	-	0.4	RI, MS
43	lepidozenal	1748	1744	-	-	0.1	RI, MS
44	β-costol	1768	1778	-	-	0.3	RI, MS
45	α-costol	1784	1785	-	-	7.4	RI, MS
46	Mixture **B**	-	-	-	-	2.6	MS (b)
47	Mixture **C**	-	-	-	-	1.1	MS (c)
48	Mixture **D**	-	-	-	-	1.2	MS (d)
49	Mixture **E**	-	-	-	-	1.4	MS (e)
50	Mixture **F**	-	-	-	-	0.9	MS (f)
51	Mixture **G**	-	-	-	-	7.3	MS (g)
52	dehydrocostunolide	-	-	-	-	28.9	MS
	Monoterpene hydrocarbons					**1.7**	

Oxygenated monoterpenes	**7.1**
Sesquiterpene hydrocarbons	**2.8**
Oxygenated sesquiterpenes	**42.4**
Aromatic compounds	**1.5**
Aliphatic compounds	**9.2**
Total (Identified compounds)	**64.7**
Unidentified oxygenated sesquiterpenes	**19.6**
Total	**84.3**

RI^a and **RI^p**: Retention index calculated with reference to a series of n-alkanes using apolar and polar columns; **RIL^a** and **RIL^p**: Retention index on apolar and polar columns reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature; **MW**: molecular weight

Compound A (sesquiterpene ketone, MW = 218); **MS (a)**: m/z (%) = 41 (100), 53 (68), 67 (83), 81 (86), 91 (67), 107 (41), 119 (32), 133 (20), 147 (20), 161 (25), 175 (14), 185 (10), 203 (12), 218 (2)

Mixture B: mixture of two sesquiterpene lactones [MW = 232 (major) and 234] and very small quantity of sesquiterpenoid compound (MW = 262); **MS (b):** m/z (%) = 41 (100), 55 (71), 67 (56), 79 (70), 91 (81), 105 (50), 111 (46), 119 (63), 134 (50), 145 (20), 161 (13), 171 (12), 179 (9), 187 (20), 202 (6), 219 (9), 232 (21), 247 (1), 262 (1)

Mixture C: mixture of two sesquiterpene lactones [MW = 232 (major) and 234] and very small quantity of sesquiterpenoid compound (MW = 256); **MS (c)**: m/z (%) = 41 (76), 53 (48), 65 (31), 79 (70), 91 (96), 105 (66), 119 (100), 134 (94), 145 (27), 159 (17), 171 (24), 187 (22), 203 (8), 217 (15), 232 (55), 256 (1)

Mixture D: mixture of sesquiterpene lactone (MW = 234) and very small quantity of sesquiterpenoid compound (MW = 256); **MS (d)**: m/z (%) = 41 (100), 55 (64), 67 (54), 79 (87), 91 (96), 105 (58), 119 (53), 134 (28), 145 (29), 161 (25), 173 (14), 189 (15), 205 (9), 219 (78), 234 (33), 256 (1)

Mixture E: mixture of sesquiterpene lactone (MW = 232) and sesquiterpenoid compound (MW = 256); **MS (e)**: m/z (%) = 41 (97), 55 (72), 67 (30), 79 (76), 91 (80), 105 (47), 117 (28), 131 (26), 143 (20), 152 (71), 158 (100), 171 (10), 187 (6), 204 (4), 217 (23), 232 (20), 256 (10)

Mixture F: mixture of sesquiterpene lactone (isomer, MW = 232) and very small quantity of sesquiterpenoid compound (MW = 256); **MS (f)**: m/z (%) = 41 (62), 55 (47), 67 (23), 79 (69), 91 (74), 105 (43), 117 (26), 131 (25), 143 (17), 152 (72), 158 (100), 175 (8), 187 (5), 204 (4), 217 (12), 232 (16), 256 (1)

Mixture G: mixture of sesquiterpene lactone (another isomer, MW = 232) and very small quantity of sesquiterpenoid compound (MW = 256); **MS (g)**: m/z (%) = 41 (88), 53 (90), 67 (48), 79 (85), 91 (100), 105 (53), 117 (32), 131 (28), 150 (22), 163 (45), 175 (18), 189 (22), 204 (10), 217 (38), 232 (40), 256 (2)

There have been some studies on chemical compositions and application of essential oil from *Saussurea costus* roots originated from other places in China. More recently, Liu and co-workers studied essential oil composition of *Saussurea costus* roots and dehydrocostus lactone (46.75%), costunolide (9.26%), 8-cedren-13-ol (5.06%) and α-curcumene (4.33%) were found to be the major compounds (Liu *et al.*, 2012). In recent publication, 35 chemical compounds were identified in the essential oil from *Saussurea costus* roots in Uttarakhand

Himalayas (India) (Gwari *et al.*, 2013). The major compounds were found to be (7*Z*,10*Z*,13*Z*)-7,10,13-hexadecatrienal (25.5%), dehydrocostus lactone (16.7%), elemol (5.84%), valerenol (4.20%) and vulgarol B (3.14%). In comparison, our results seemed different from the previously published data. Dehydrocostus lactone, costunolide and 8-cedren-13-ol could not be identified in our *Saussurea costus* oil.

3.3.1.7 Chemical composition of essential oil from dry fruits of *Citrus aurantium* L.

Chemical composition of essential oil from dry fruits of *Citrus aurantium* L. is shown in Table 7. Thirty chemical compounds were identified, amounting to 96.5% of the total essential oil. The essential oil was characterised by a very high content of limonene (84.3%). Monoterpene compounds, including monoterpene hydrocarbons (90.8%) and oxygenated monoterpenes (3.6%), were found to be the main components. Besides limonene, only three compounds had the contents of more than 1%, β-pinene (2.7%), linalool (2%) and myrcene (1.5%).

Table 7 Main chemical compounds of essential oil from dry fruits of *Citrus aurantium* L.

N°	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP%	Identification
1	α-pinene	936	932	1028	1032	0.6	RI, MS
2	camphene	951	946	1071	1076	t	RI, MS
3	sabinene	976	969	1127	1132	0.1	RI, MS
4	β-pinene	982	974	1117	1113	2.7	RI, MS
5	myrcene	991	988	1166	1174	1.5	RI, MS
6	octanal	1003	998	1292	1300	0.3	RI, MS
7	α-phellandrene	1008	1002	1162	1166	0.1	RI, MS
8	α-terpinene	1019	1014	1169	1177	0.1	RI, MS
9	*para*-cymene	1026	1020	1275	1280	t	RI, MS
10	limonene	1036	1024	1218	1203	84.3	RI, MS
11	β-phellandrene	1036	1025	1221	1209	0.5	RI, MS
12	(*E*)-β-ocimene	1047	1044	1253	1242	0.4	RI, MS
13	γ-terpinene	1060	1054	1249	1244	0.3	RI, MS
14	δ-terpinene	1091	1086	1286	1295	0.2	RI, MS
15	linalool	1101	1095	1546	1553	2.0	RI, MS
16	nonanal	1105	1100	1395	1400	t	RI, MS
17	terpinen-4-ol	1180	1174	1602	1611	0.6	RI, MS
18	α-terpineol	1193	1186	1695	1706	0.8	RI, MS
19	decanal	1205	1201	1499	1510	0.2	RI, MS
20	perillaldehyde	1273	1269	1784	1777	t	RI, MS
21	δ-elemene	1341	1335	1465	1468	0.1	RI, MS
22	neryl acetate	1364	1359	1725	1730	0.2	RI, MS

N°	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP%	Identification
23	β-elemene	1395	1389	1590	1595	0.1	RI, MS
24	γ-elemene	1440	1434	1623	1642	t	RI, MS
25	germacrene D	1485	1484	1709	1705	0.7	RI, MS
26	valencene	1497	1496	1751	1726	t	RI, MS
27	δ-cadinene	1524	1522	1757	1749	0.1	RI, MS
28	α-cadinol	1657	1652	2242	2167	t	RI, MS
29	myristic acid	1766	1762	-	2690	t	MS
30	palmitic acid	1963	1959	-	2899	0.6	MS
	Monoterpene hydrocarbons					**90.8**	
	Oxygenated monoterpenes					**3.6**	
	Sesquiterpene hydrocarbons					**1.0**	
	Oxygenated sesquiterpenes					**t**	
	Aliphatic compounds					**1.1**	
	Total					**96.5**	

RI[a] and **RI[p]**: Retention index calculated with reference to a series of n-alkanes using apolar and polar columns; **RIL[a]** and **RIL[p]**: Retention index on apolar and polar columns reported in literature; **RP**: relative percentage; **t**: trace (<0.1); Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

As one Traditional Chinese Medicine, chemical components in dry fruits of *Citrus aurantium* L. essential oils have been previously investigated using GC-FID and GC-MS. The highest oil yield could reach up to 1%. The major component of its essential oil of different origins was always limonene. In one study, limonene (around 40%) and linalool (13%) were found to be major compounds in essential oils from dry fruits of *Citrus aurantium* L.(Liao *et al.*, 2004). In 2007, the essential oil compositions of dry fruits of *Citrus aurantium* L. before and after pre-treatment were investigated (Gong *et al.*, 2007). Limonene was the major component (around 50%). After pre-treatment of fruits of *Citrus aurantium* L., there was only a slight increase of limonene content. However, a lot of new compounds up to 17 were observed and some compounds disappeared, which means that chemical compounds of essential oil have been changed after pre-treatment. In comparison, our results showed a much higher content of limonene, reaching up to 84.3%.

3.3.2 Different extracts of selected plants in MAP-refinery

In the framework of MAP-refinery, it is very important to consider a global valorisation of all molecules in selected plants without putting away some of them. In the first step, knowledge about the contents of different extracts and residues in every selected plant should be provided for a better evaluation of their application. It can be seen in Table 8 that 12 plant

samples have been processed into four parts, i.e. essential oil (or volatile extract), aqueous extract, methanolic extract and final residue. Two stem samples have been processed into only two parts because it was not necessary to apply hydrodistillation to these two samples.

The quantities and percentages of different extracts and residues were interconnected by the following formulas:

$$M_{DP} = M_{EO} \text{ (or } M_{VE}) + M_{AE} + M_{SR}$$
$$100\ (\%) = P_{EO} \text{ (or } P_{VE}) + P_{AE} + P_{SR} \quad \text{(After hydrodistillation)}$$

$$M_{DP} = M_{EO} \text{ (or } M_{VE}) + M_{AE} + M_{ME} + M_{FR}$$
$$100\ (\%) = P_{EO} \text{ (or } P_{VE}) + P_{AE} + P_{ME} + P_{FR} \text{ (After Soxhlet extraction)}$$

M_{DP}: mass of dry plant

M_{EO} (P_{EO}) and M_{VE} (P_{VE}): mass and percentage of essential oil or volatile extracts

M_{AE} and P_{AE}: mass and percentage of aqueous extracts

M_{ME} and P_{ME}: mass and percentage of methanolic extracts

M_{SR} and P_{SR}: mass and percentage of solid residue

M_{FR} and P_{FR}: mass and percentage of final residue

For stems of *Cytisus scoparius* L. and *Spartium junceum* L., the formulas were simplified:

$$M_{DP} = M_{ME} + M_{FR} \quad \text{and} \quad 100\ (\%) = P_{ME} + P_{FR}$$

Table 8 Different extracts of selected plants in MAP-refinery

N°	Plant name	Plant parts	EO or VE % /DP	Aqueous extract % /DP	Methanolic extract % /DP	Final residue % /DP
1	*Tussilago farfara* L. (CN)	Flower buds	t	69.35	5.21	25.44
2	*Tussilago farfara* L. (CN)	Stalks	t	71.6	3.70	24.70
3	*Tussilago farfara* L. (FR)	Flower buds	t	77.25	2.10	20.65
4	*Tussilago farfara* L. (FR)	Roots	t	57.12	4.24	38.64
5	*Calendula arvensis* L.	Roots	t	39.54	3.48	56.98
6	*Robinia pseudoacacia* L.	Flower	t	54.1	2.87	43.03
7	*Robinia pseudoacacia* L.	Leaves	t	44	5.56	50.44
8	*Spartium junceum* L.	Flower	t	45.61	8.36	46.03
9	*Spartium junceum* L.	Flower buds	t	46.44	7.18	46.38
10	*Spartium junceum* L.	Stems	-	-	16.50	83.50
11	*Cytisus scoparius* L.	Flower	t	45.7	5.53	48.77
12	*Cytisus scoparius* L.	Stems	-	-	18.51	81.49
13	*Saussurea costus*	Roots	0.23	34.97	19.27	45.53
14	*Citrus aurantium* L.	Fruits	0.21	48.09	7.00	44.70

EO: essential oil; VE: volatile extract; DP: dry plant; t: trace (it was difficult to directly collect essential oil or volatile extract due to low concentration)

From perspective of valorisation, more attention should be paid to aqueous extracts whose contents in the selected plants ranged from 34.97% to 77.25%. It was demonstrated that many molecules in these plants could dissolve in water. In this way, hydrodistillation is a good extraction method which allowed the extraction of not only essential oil but also large-quantity water-soluble molecules from these plants. However, these selected plants were not very interesting in term of essential oil. Only from two plants originated in China (*Saussurea costus* and *Citrus aurantium* L.), easy-to-collect essential oils could be obtained. In the hydrodistillation of *Calendula arvensis* L., in fact, small drop of essential oil was observed but it was very difficult to collect. For other plants, only volatile extracts were obtained by using simultaneous distillation extraction.

Methanolic extracts accounted for 2.1% to 19.27%. For most selected plants, methanol extraction yields were relatively low. Except two stem samples of *Cytisus scoparius* L. and *Spartium junceum* L., Soxhlet extractions using methanol were applied to the plant residues after hydrodistillation or simultaneous distillation extraction. In these two processes, water has extracted most polar compounds before methanol extraction. However, methanol extraction provided a further extraction of active compounds from solid residue, and in some plants like *Saussurea costus*, methanol still extracted a high percentage of molecules from solid residue after hydrodistillation.

Final residues also had very high contents in selected plants, from 20.65% in flower buds of *Tussilago farfara* L. to 83.50% in stems of *Spartium junceum* L. After water and methanol extraction, the final residues were supposed to be rich in cellulose, hemi-cellulose and lignins. Further treatment process for the potential fabrication of agromaterials or biomaterials could be proposed. *Spartium junceum* L. is a promising plant in producing materials from residues in MAP-refinery because a good fibre could be obtained from its twigs or stems, with many good properties. Recently there has been a revival of interest in this species as a possible source of composite materials for automobile applications (Angelini *et al*., 2000). A novel and efficient physical-chemical process has been developed for the production of cellulose fibre from this species (Gabriele *et al*., 2010), with many excellent physical-chemical properties, such as high mechanical resistance and high elasticity.

3.3.3 AOC and TPC of aqueous extracts

Antioxidant capacity (AOC) and total phenolic contents (TPC) of aqueous extracts were determined by DPPH radical scavenging assay and Folin-Ciocalteu assay, respectively. Results are shown in Table 9. In fact, DPPH radical scavenging assay is the simplest and least expensive method to evaluate antioxidant capacity. What are only needed are the reagents, some cuvettes and a UV-vis spectrophotometer which can be easily found in laboratories (Apak *et al.*, 2013), which probably explains its popularity and extensive use in antioxidant screening. Folin-Ciocalteu assay is also a popular and accepted method to determine the total phenolic content. Both methods are electron transfer (ET) based assays. For DPPH assay, hydrogen atom transfer (HAT) mechanism is also possible but it is a marginal reaction pathway.

3.3.3.1 DPPH radical scavenging assay

The antioxidant capacity (AOC) of aqueous extracts by use of DPPH radical scavenging assay was expressed in μmol of Trolox equivalents (TEs) / g of plant extract. For 12 samples tested, the strongest antioxidant capacity was observed in aqueous extract from flower buds of *Tussilago farfara* L. (FR) (488.48 μmol TE / g extract**)** followed by leaves of *Robinia pseudoacacia* L. (403.64 μmol TE / g extract**)** and the weakest antioxidant capacity was observed in aqueous extract from roots of *Saussurea costus* (47.85 μmol TE / g extract**)**. It is noteworthy that antioxidant capacity in the aqueous extract from flower buds of *Tussilago farfara* L. from Chongqing (201.30 μmol TE / g extract**)** was less than half of the French one. The results may be due to the fact that, in the traditional Chinese medicine, before coming onto the market, medicinal plants or their medicinal parts have been subjected to some pre-treatment process in the purpose of reinforcing the pharmaceutical effects, eliminating the toxicity, removing the bad odours or extending the storage time. These pre-treatment processes include frying, stir-heating with some ingredients, roasting, soaking with water or alcohol, and so on. After the pre-treatment of *Tussilago farfara* L. flower buds, some antioxidant compounds may have been destroyed.

Table 9 AOCs and TPCs of aqueous extracts

N°	Plant name	Plant parts	AOC μmol TE/ g of extract	TPC mg GAE/ g of extract
1	*Tussilago farfara* L. (CN)	Flower buds	201.30 ± 1.66	41.54 ± 0.12
2	*Tussilago farfara* L. (CN)	Stalks	119.25 ± 1.33	24.75 ± 0.07
3	*Tussilago farfara* L. (FR)	Flower buds	488.48 ± 6.78	96.14 ± 0.91

4	*Tussilago farfara* L. (FR)	Roots	142.59 ±1.61	29.15 ± 0.15
5	*Calendula arvensis* L.	Roots	50.59 ± 0.95	19.50 ± 0.12
6	*Robinia pseudoacacia* L.	Flower	114.90 ± 0.96	34.43 ± 0.29
7	*Robinia pseudoacacia* L.	Leaves	403.64 ± 5.70	80.86 ± 0.29
8	*Spartium junceum* L.	Flower	142.41 ± 0.80	42.37 ± 0.27
9	*Spartium junceum* L.	Flower buds	219.03 ± 1.91	48.24 ± 0.29
10	*Cytisus scoparius* L.	Flower	369.67 ± 3.77	112.97 ± 0.77
11	*Saussurea costus*	Roots	47.85 ± 0.49	14.09 ± 0.10
12	*Citrus aurantium* L.	Fruits	83.07 ± 1.64	79.32 ± 0.10

TE: Trolox equivalent**; GAE:** Gallic acid equivalent; Results are expressed as a mean ± standard deviation (n = 3).

3.3.3.2 Total phenolic content (TPC)

The TPC in extract was expressed as mg of gallic acid equivalents (GAE) / g of extract. TPC results of these 12 samples presented a different tendency from antioxidant capacity results. The highest TPC was determined in aqueous extract from flowers of *Cytisus scoparius* L. (112.97 mg GAE / g extract) followed by flower buds of *Tussilago farfara* L. (FR) (96.14 mg GAE / g extract). Roots extract from *Saussurea costus* possessed the lowest TPC, which was in accordance with AOC results. It should be pointed out that, TPC of fruits extract from *Citrus aurantium* L. (79.32 mg GAE / g extract) was comparable to that (80.86 mg GAE/g extract) of leaves from *Robinia pasudoacacia* L., but antioxidant capacity of the former (83.07 μmol TE / g extract**)** was only one fifth of the latter (403.64 μmol TE / g extract**)**, which means that DPPH radical scavenging assay may not reflect the real antioxidant capacity of extract from dry fruits of *Citrus aurantium* L. However, these results are reasonable. Because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved in antioxidant assays, no single assay can accurately reflect all antioxidants in a mixed or complex system (Prior et al., 2005). For a comprehensive evaluation of antioxidant capacity, several evaluation methods are usually needed.

For all 12 samples, a relatively weak correlation (r = 0.6514) was found between TPC and AOC values. As only one antioxidant method was employed and different plants contain different antioxidant compounds which will react with DPPH radical differently, it is very difficult to give a clear relationship between antioxidant capacity and total phenolic contents for each of these samples. However, for 4 samples from *Tussilago farfara* L., a very high correlation (r = 0.9998) was observed between TPC and AOC values, which showed that

phenolic compounds are mainly responsible for antioxidant capacity in extracts from *Tussilago farfara* L.

3.3.4 AOC and TPC of methanolic extracts

There are many different antioxidant capacity evaluation methods which have their advantages and shortcomings. At present more and more methods have been adapted for analysis on the microplate so that high-throughput screening of antioxidants could be made. After critically reviewing most frequently used AOC evaluation methods, Prior *et al.* proposed that three methods (ORAC, Folin-Ciocalteu and TEAC) should be standardized for use in the routine quality control and measurements of AOC of dietary supplements and other botanicals (Prior *et al.*, 2005). According to this proposal, AOC of 14 methanolic extracts from different plants were evaluated by ORAC assay, Folin-Ciocalteu assay and ABTS-based TEAC assay. In addition, other two commonly used methods, DPPH radical scavenging assay and ferric reducing/antioxidant power (FRAP) assay, were also employed for a comprehensive evaluation. All these assays were performed on the microplate and the results are shown in Table 10.

Table 10 AOC and TPC of methanolic extracts from residues after HD or SDE (Samples 10 and 12 were directly extracted from stems)

N°	Plant name	Plant parts	DPPH	FRAP		ABTS	$ORAC_{FL}$	TPC
			µmol TE/ g of extract	µmol Fe(II)/ g of extract	µmol TE/ g of extract	µmol TE/ g of extract	µmol TE/ g of extract	mg GAE/ g of extract
1	*Tussilago farfara* L. (CN)	Flower buds residue	236.79 ± 8.93	332.27 ± 34.67	213.52 ± 20.80	392.74 ± 14.26	1046.86±158.16	34.06 ± 2.09
2	*Tussilago farfara* L. (CN)	Stalks residue	77.88 ± 0.15	219.81 ± 12.34	138,96 ± 7.40	194.25 ± 7.44	518.94 ± 44.60	17.65 ± 0.88
3	*Tussilago farfara* L. (FR)	Flower buds residue	275.81 ± 30.20	455.16 ± 16.00	301.44 ± 9.60	575.53 ± 10.64	1411.76 ± 42.36	45.98 ± 3.92
4	*Tussilago farfara* L. (FR)	Roots residue	70.23 ± 5.60	213.14 ± 4.33	134,96 ± 2.60	204.27 ± 9.67	436.40 ± 6.10	19.13 ± 1.47
5	*Calendula arvensis* L.	Roots residue	58.05 ± 3.86	119.12 ± 3.00	78.56 ± 1.80	207.35 ± 3.85	632.46 ± 79.66	24.35 ± 1.73
6	*Robinia pseudoacacia* L.	Flower residue	46.11 ± 2.92	189.47 ± 2.67	120.76 ± 1.60	425.41 ± 51.48	286.29 ± 14.32	24.15 ± 0.21
7	*Robinia pseudoacacia* L.	Leaves residue	354.57 ± 10.93	527.17 ± 42.68	344.64 ± 25.60	1036.72 ± 40.23	913.87 ± 124.69	62.62 ± 1.63
8	*Spartium junceum* L.	Flowers residue	135.90 ± 1.08	380.94 ± 10.67	242.72 ± 6.40	760.96 ± 25.59	1821.25 ± 150.37	57.66 ± 2.32
9	*Spartium junceum* L.	Flower buds residue	213.24 ± 2.06	459.63 ± 24.67	289.92 ± 14.80	867.01 ± 33.91	2244.94 ± 188.56	69.81 ± 2.31
10	*Spartium junceum* L.	Stem	96.07 ± 10.58	169.13 ± 11.34	108.56 ± 6.80	405.91 ± 18.34	945.67 ± 34.76	40.47 ± 1.12
11	*Cytisus scoparius* L.	Flowers residue	213.89 ± 16.18	442.29 ± 24.67	279.52 ± 14.80	979.54 ± 17.88	3166.98 ± 94.70	89.31 ± 1.77
12	*Cytisus scoparius* L.	Stem	372.68 ± 38.65	845.90 ± 40.01	535.84 ± 24.00	1783.97 ± 58.88	4480.04 ± 253.49	160.50 ± 10.10
13	*Saussurea costus*	Roots residue	29.74 ± 2.63	103.79 ± 7.00	69.36 ± 4.20	155.71 ± 12.72	700.20 ± 161.46	17.33 ± 0.47
14	*Citrus aurantium* L.	Fruits residue	51.39 ± 4.90	561.85 ± 24.00	365.44 ± 14.40	1801.23 ± 78.92	7515.54 ± 344.25	140.42 ± 10.54

TE: Trolox equivalent; GAE: Gallic acid equivalent; Results are expressed as a mean ± standard deviation (n=3 or 4).

3.3.4.1 DPPH radical scavenging assay

Methanolic extract directly from stems of *Cytisus scoparius* L. presented the highest AOC value (372.68 μmol TE / g extract) while roots residue extract of *Saussurea costus* had the lowest value (29.74 μmol TE / g extract). For all 14 samples, a low correlation (r = 0.2331) was found between TPC and AOC values. But for samples from one plant (*Tussilago farfara* L.), there was a high correlation (r = 0.9465) between TPC and AOC values.

3.3.4.2 ABTS radical cation decolourisation assays and FRAC assay

The reducing/antioxidant power in the FRAP assay were expressed both in Fe^{2+} equivalents (FE) and Trolox equivalents (TE). These two expressions gave different FRAP values but they gave almost same tendency for AOC of samples. There is a high correlation (r=0.9989) between FRAP-FE values and FRAP-TE values. The sample with the highest and the lowest FRAP values were extracted from stems of *Cytisus scoparius* L. and from the roots residue of *Saussurea costus*, respectively, which was in accordance with DPPH assay results. However, one difference was the antioxidant capacity of the residue extract from dry fruits of *Citrus aurantium* L. In the DPPH assay, its AOC value (51.39 μmol TE / g extract) was about 2 times that (29.74 μmol TE / g extract) of *Saussurea costus* residue extract. However, in FRAP assay, its FRAP value (561.85 μmol TE / g extract) was more than 5 times that (103.79 μmol TE / g extract) of *Saussurea costus* residue extract. As indicated in previous AOC evaluation of aqueous extracts, DPPH assay may not reflect the real antioxidant capacity of dry fruits of *Citrus aurantium* L. Consequently, in FRAP assay its antioxidant capacity greatly increased. Further confirmation was from ABTS radical cation decolourisation assay and ORAC assay in which the residue extract from dry fruits of *Citrus aurantium* L. showed the highest antioxidant capacity (1801.23 and 7515.54 μmol TE / g extract, respectively).

ABTS radical cation decolourisation assays results were also expressed in μmol of Trolox equivalents (TEs) / g of plant extract. The results showed that the extract obtained directly from stems of *Cytisus scoparius* L. possessed similar antioxidant capacity (1783.97 μmol TE / g extract) as that of extract from dry fruits of *Citrus aurantium* L., and roots residue extract from *Saussurea costus* was the less efficient (155.71 μmol TE / g extract) in AOC.

For FRAP and ABTS assay, strong correlations were found between TPC and AOC values(r=0.9583 for ABTS assay; r = 0.8036 and 0.7974 for FRAP-FE and FRAP-TE, respectively).

3.3.4.3 Oxygen radical absorbance capacity (ORAC) assay

ORAC assay results showed a different AOC tendency from the above-mentioned methods. Residue extract from dry fruits of *Citrus aurantium* L. (7515.54 μmol TE / g extract) gave the highest ORAC values followed by stem extract of *Cytisus scoparius* L. (4480.04 μmol TE / g extract). While flower residue extract from *Robinia pseudoacacia* L. (286.29 μmol TE / g extract) showed the lowest ORAC value. The correlation coefficient between TPC and ORAC value was 0.8195.

3.3.4.4 Total phenolic contents (TPC)

The TPC in extract was expressed as mg of gallic acid equivalents (GAE) / g of extract. TPC results of these 14 samples showed that stem extract of *Cytisus scoparius* L. and fruit residue extract of *Citrus aurantium* L. were two samples with highest total phenolic contents (160.50 and 140.42 mg GAE / g extract, respectively). Roots residue extract from *Saussurea costus* possessed the lowest TPC (17.33 mg GAE / g extract).

Based on comprehensive evaluation results by DPPH, ABTS, FRAP, ORAC and Folin-Ciocalteu assay, it could be concluded that methanolic extracts from stems of *Cytisus scoparius* L. and fruit residue of *Citrus aurantium* L. were two strongest antioxidant extracts among 14 samples. While methanolic extract of root residue from *Saussurea costus* showed the lowest antioxidant capacity. Other relatively strong antioxidant samples also included leaves residue of *Robinia pseudoacacia* L.

If putting together antioxidant capacity results of aqueous extracts and methanolic extracts of selected plant samples, it could be found that *Cytisus scoparius* L. including flowers and stems, dry fruits of *Citrus aurantium* L., flower buds of *Tussilago farfara* L. and leaves of *Robinia pseudoacacia* L. could be potential sources of natural antioxidants. In the previous literature, there have been some studies on the antioxidant capacity evaluation of these four plants but mainly with DPPH radical scavenging assay. The extracts tested in literature were directly extracted from plant samples using methanol, ethanol or mixed alcohol-water as solvent while in our study aqueous extracts were firstly obtained from by-product of

hydrodistillation and then methanolic extracts were obtained from residues after hydrodistillation or simultaneous distillation extraction.

For *Cytisus scoparius* L., several flavonoids could be responsible for antioxidant capacity of its extracts, including rutin, quercetin, quercitrin, isorhamnetin and kaempferol (Brum-Bousquet and Paris, 1974), 6"-*O*-acetyl scoparin (Brum-Bousquet *et al.*, 1977) and an isoflavone glycoside named sarothamnoside (Brum-Bousquet *et al.*, 1981).

For dry fruits of *Citrus aurantium* L., two flavonoid glycosides (naringin and neohesperidin) are supposed to play a big role in the strong antioxidant capacity of this plant sample. Actually, these two compounds have been selected as the standards to evaluate the quality of *Fructus aurantii*. According to Pharmacopoeia of People's Republic of China, the contents of naringin and neohesperidin in dry fruits of *Citrus aurantium* L. which are used medicinally shouldn't be less than 4% and 3%, respectively. Other responsible molecules in dry fruits of *Citrus aurantium* L. could be polymethoxylated flavones, e.g. nobiletin, tangeretin, natsudaidai and 5-hydroxy-6,7,8,4'-tetramethoxyflavone (Chen *et al.*, 2012).

In the flower buds of *Tussilago farfara* L., total flavonoids, polysaccharides and other phenolic compounds should be responsible for its antioxidant activity. Dicaffeoylquinic acids and quercetin pentoside have been identified as the major antioxidant compounds in extracts from aerial parts of *Tussilago farfara* L. (Dobravalskyte *et al.*, 2013).

Flavonoids in *Robinia pseudoacacia* L. are supposed to be responsible for its antioxidant capacity, e.g. five flavonoids including acacetin, secundiflorol I, mucronulatol, isomucronulatol and isovestitol (Tian and McLaughlin, 2000), four flavone glycosides such as luteolin and diosmetin (Veitch *et al.*, 2010), geranyl flavone called kuwanon S and a new geranyl flavonol named robipseudin A (Zhang *et al.*, 2013).

In the evaluation of AOC in our study, antioxidant activities of some samples including roots of *Tussilago farfara* L. and *Calendula arvensis* L. were not high but their AOC evaluations were made for the first time.

For the global valorisation of molecules in MAP-refinery, it is also necessary to have the knowledge about the molecules in each part, including aqueous extract, methanolic extract and final residue. Knowledge of antioxidant molecules in aqueous extracts and methanolic extracts will provide some guidance for their future application.

3.3.5 Correlation between AOC evaluation methods

Correlations between different AOC evaluation methods were shown in Table 11. FRAP-FE and FRAP-TE used the same method and were two expressions of FRAP values. The highest correlation between methods were FRAP and ABTS (r = 0.8236 and 0.8239). This could be explained by the fact that the basic principle of these two assays is very similar and in the FRAP assay a ferric ion acted as the oxidant, whose redox potential (0.70 V) is comparable to that of ABTS radical cation (0.68 V) (Huang *et al.*, 2005). In addition, these two methods are both electron transfer (ET) based evaluation methods. In fact, for samples from the same plant (Table 12), the correlations between FRAP and ABTS were even higher (r = 0.9969 and 0.9956).

Table 11 Correlation coefficients between AOC evaluation methods (14 samples)

r	DPPH	FRAP-FE	FRAP-TE	ABTS	ORAC
DPPH	-				
FRAP-FE	0.6005	-			
FRAP-TE	0.6039	**0.9989**	-		
ABTS	0.248	**0.8236**	**0.8239**	-	
ORAC	0.0253	0.4934	0.4949	**0.7966**	-

The lowest correlation was found between DPPH assay and ORAC assay, with correlation coefficient as only 0.0253, which means that these two methods have almost no correlation. However, it should be pointed out, all correlations between DPPH assay and other evaluation methods were not high. There is also relatively high correlation between ABTS assay and ORAC assay.

Table 12 Correlation coefficients between AOC evaluation methods (*Tussilago farfara* L.)

r	DPPH	FRAP-FE	FRAP-TE	ABTS	ORAC
DPPH	-				
FRAP-FE	0.9095	-			
FRAP-TE	0.8974	**0.9996**	-		
ABTS	0.9234	**0.9969**	**0.9956**	-	
ORAC	0.966	0.982	0.9763	0.9818	-

However, if we look at the relationships between different methods using the AOC data from samples of the same plant (4 samples from *Tussilago farfara* L.), high correlations could be observed among these methods. All correlation coefficients were equal to or greater than 0.9, which means that all these evaluation methods gave the same AOC tendency for these samples. Different samples from the same plant may contain antioxidant molecules of similar

chemical structures even if they can't contain exactly the same molecules. These molecules of similar chemical structure will interact with oxidants in AOC evaluation assays in a similar way so that these different methods could give similar tendency. But for samples from different plants, these methods could give quite different tendency for antioxidant capacity. With one method, one plant sample is the strongest in AOC but with another method, another plant sample may be the strongest. In conclusion, it is difficult to say which method is the best for the AOC evaluation and a comprehensive evaluation using different methods is quite necessary. To have a clear picture of antioxidant capacity of one sample, comparisons with some antioxidant standards at the same conditions are also necessary.

3.3.6 AOC comparison of aqueous extracts and methanolic extracts

The antioxidant capacities of aqueous extracts and methanolic extracts from 12 plant samples were presented in Table 13.

Table 13 AOC of aqueous and methanolic extracts by DPPH radical scavenging assay

N°	Plant name	Plant parts	Aqueous extracts µmol TE/ g	Methanolic extracts µmol TE/ g
1	*Tussilago farfara* L. (CN)	Flower buds	201.30 ± 1.66	236.79 ± 8.93
2	*Tussilago farfara* L. (CN)	Stalks	119.25 ± 1.33	77.88 ± 0.15
3	*Tussilago farfara* L. (FR)	Flower buds	488.48 ± 6.78	275.81 ± 30.20
4	*Tussilago farfara* L. (FR)	Roots	142.59 ± 1.61	70.23 ± 5.60
5	*Calendula arvensis* L.	Roots	50.59 ± 0.95	58.05 ± 3.86
6	*Robinia pseudoacacia* L.	Flower	114.90 ± 0.96	46.11 ± 2.92
7	*Robinia pseudoacacia* L.	Leaves	403.64 ± 5.70	354.57 ± 10.93
8	*Spartium junceum* L.	Flower	142.41 ± 0.80	135.90 ± 1.08
9	*Spartium junceum* L.	Flower buds	219.03 ± 1.91	213.24 ± 2.06
10	*Cytisus scoparius* L.	Flower	369.67 ± 3.77	213.89 ± 16.18
11	*Saussurea costus*	Roots	47.85 ± 0.49	29.74 ± 2.63
12	*Citrus aurantium* L.	Fruits	83.07 ± 1.64	51.39 ± 4.90

The AOCs of aqueous extracts and methanolic extracts were both evaluated by DPPH radical scavenging assay, however, the former were obtained by routine method using cuvettes and UV-vis spectrophotometer while the latter were obtained by high-throughput screening on the microplate. It was true that there are some deviations between AOC values obtained from these two systems, but it is still possible for a simple comparison between these two groups of data. For most samples, aqueous extracts possessed higher antioxidant capacity than methanolic extracts. Only for one sample (flower buds of *Tussilago farfara* L.),

methanolic extracts had higher antioxidant capacity than aqueous extracts. Considering the deviations introduced by two different methods, at least, it is safe to conclude that antioxidant capacities of aqueous extracts and methanolic extracts are in the same range. However, in the context of MAP-refinery, there will be a big difference. As indicated in Table 8, the contents of aqueous extracts in the selected plants ranged from 34.97% to 77.25% while the contents of methanolic extracts ranged only from 2.1% to 19.27%. In comparison, if these extracts will be potential sources of natural antioxidants, more attention should be paid to aqueous extracts because they contain more quantity of antioxidant molecules.

3.4 Conclusion

In this study, MAP-refinery was applied to several selected medicinal and aromatic plants from the Midi-Pyrénées and Chongqing for a valorisation of different molecules. Except two stem samples, all plant samples were processed into four parts, i.e. essential oil or volatile extract, aqueous extract, methanolic extract and final residue. Essential oils or volatile extracts accounted for up to 0.23% in the selected plants and were characterised by GC-FID and GC-MS. Volatile extract composition in the roots of *Tussilago farfara* L. and *Calendula arvensis* L., and flower buds of *Spartium junceum* L. were firstly identified.

Aqueous extracts were collected as by-products after hydrodistillation or simultaneous distillation extraction and accounted for 34.97% to 77.25% in the selected plants. Because of their high content, more attention should be paid to aqueous extracts. Methanolic extracts were obtained from residues and accounted for 2.1% to 19.27%. The antioxidant capacities of aqueous extracts and methanolic extracts are in the same range. Antioxidant capacity evaluation results showed that several plant samples like *Cytisus scoparius* L., *Tussilago farfara* L., *Citrus aurantium* L. and *Robinia pseudoacacia* L. were potential sources of natural antioxidants. A comprehensive evaluation of AOC using different methods was also done to get more complete information about the antioxidant capacity of methanolic extracts. The final residues obtained accounted for 20.65% to 83.50% in case of stems and could be proposed for agromaterials or biomaterials.

It should be pointed out that application of MAP-refinery to several plants presented in this study was the preliminary step. To realise real and complete valorisation of different molecules in selected medicinal and aromatic plants, more work should be done for each separated part. For aqueous extracts and methanolic extracts, further separation, purification and analysis would be necessary.

Chapter 4 Study of several water-based extraction technologies

4.1 Effects of different extraction techniques on the essential oil composition and antioxidants recovery from *Tussilago farfara* L.

4.1.1 Introduction

In the process of hydrodistillation (HD) or steam distillation (SD) of aromatic plants, Clevenger apparatus seems to be the most commonly-used essential oil separator, while in Poland, Deryng apparatus appears to be widely used (Polish Pharmacopoeia VI, 2002). Deryng apparatus has been reported to obtain more essential oil compounds than Clevenger apparatus (Sajewicz *et al.*, 2009; Rzepa *et al.*, 2012). On the other hand, apart from the odorous fraction, aromatic plants appeared as a valuable source for natural antioxidant molecules. If such components could be obtained after the collection of essential oils, the extraction residues which represent up to 99% of the raw distillated material could be considered as a potential complementary source for natural antioxidants. From perspective of a full utilization of aromatic plants, some by-products should be produced from the residues instead of leaving them as wastes. Some recent work has focused on the evaluation of the effects of essential oil extraction on the subsequent antioxidants recovery from rosemary residues of distillation (Navarrete *et al.*, 2011). Solvent free microwave extraction was found to be an efficient method for recovering antioxidants after essential oil extraction, compared with hydrodistillation and steam distillation. However, in the valorization of rosemary residues, only solid residues were considered. For the hydrodistillation process, a large quantity of antioxidant molecules could be lost in the liquid residues.

In this part, aiming at cross-evaluating the effects of two extraction processes [Hydrodistillation (HD) and Steam Distillation (SD)] combined with two essential oil separators [Clevenger Apparatus (CA) and Deryng Apparatus (DA)] on both essential oil composition and antioxidant molecules recovery from residues of distillation, *Tussilago*

farfara L. growing wildly in mountain Midi-Pyrénées was chosen as model plant. Its essential oils were extracted by these four combined techniques (HD-CA, HD-DA, SD-CA and SD-DA) and characterized by GC-MS. Extracts were obtained from the residues of distillation and tested for their antioxidant capacities and total phenolic contents. Results obtained by these four techniques were compared in terms of: (1) chromatographic profiles and compositions of essential oils; (2) antioxidant capacities and total phenolic contents of the corresponding extracts.

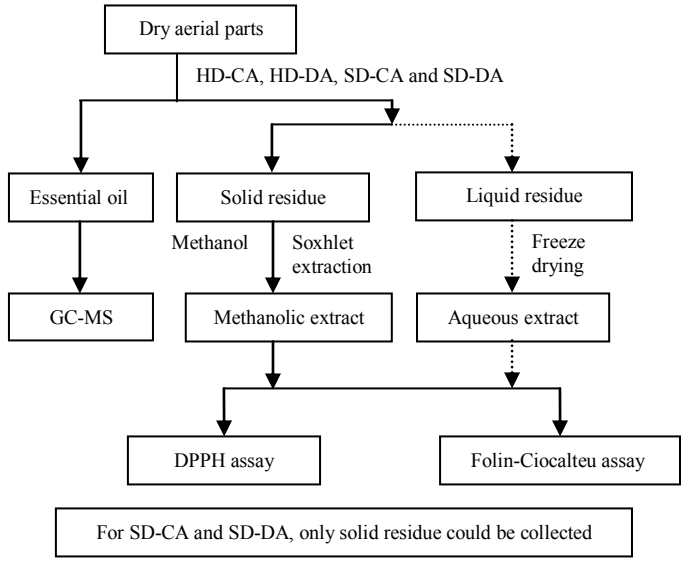


Figure 1 Scheme of preparation and analysis of the *Tussilago farfara* L. extracts

4.1.2 Materials and methods

4.1.2.1 Plant materials and chemicals

Aerial parts of *Tussilago farfara* L. were collected in the fields along the road near mountain Midi-Pyrénées on April, 2011 and identified by the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse. The aerial parts of *Tussilago farfara* L. were then placed in the shady room to be naturally dried. The fully-dried samples were cut into pieces before distillation.

n-pentane (HPLC grade, Scharlau, Spain), methanol (HPLC grade, Sigma-Aldrich), DPPH (95%, Sigma-Aldrich), anhydrous sodium carbonate (99.7%, VMR International, Belgium), Folin-Ciocalteu reagent (Sigma-Aldrich), deionized water, rosemary extract by SFE-CO_2 (Aroma Zone, France)

4.1.2.2 Distillation of Essential Oils

Hydrodistillation (HD) or steam distillation (SD) were carried out combined to an essential oil separator, either Clevenger apparatus (CA), or Deryng apparatus (DA), that is, HD-CA, HD-DA, SD-CA and SD-DA. For HD-CA and HD-DA processes, 15.0 g of dry aerial parts of *Tussilago farfara* L. were mixed with 1.1 L of water for hydrodistillation. For SD-CA and SD-DA processes, 1.2 L of water was put into the flask to generate the steam and 15.0 g of dry aerial parts of *Tussilago farfara* L. were carefully placed on the plate above the water for steam distillation. All the distillation processes lasted for 3 h. After the distillation, 1 mL of *n*-pentane was used to dissolve the small quantity of essential oil floating above the water in the essential oil separators because the essential oil yield of *Tussilago farfara* L. was very low.

4.1.2.3 Gas Chromatography-Mass Spectrometry Analysis

These four essential oils were analyzed on Thermo Finnigan Trace DSQ GC-MS, equipped with Triplus autosampler and fused-silica capillary columns HP-5 MS (0.25 μm × 0.25 mm × 30 m). GC-MS parameters: helium gas as carrier gas (flow rate: 1.0 mL/min); oven temperature programming: at 60℃ for 2 min, rising to 220℃ at 6℃ /min and then held at 220°C for 6 min; injector temperature: 250℃ , ion source temperature: 200℃ ; electron ionization: 70 eV; mass spectra range: 50-350 amu and 1.6 scans/s; split ratio: 1/100; injection volume: 1 μL; starting time of MS detection: 3 min.

Identification of individual component in the essential oils was mainly based on the comparison of their mass spectra with those in the mass spectra library of data process software (NBS75K database and Wiley 7th NIST 98 EPA/NIH Mass Spectral Library), and also those found in published data. The identification was also aided by the comparison of calculated retention index of each component, with those found in the literature (Adams, 2007).

4.1.2.4 Preparation of extracts from residues of distillation

As shown in Figure 2, after the extraction of essential oils, the residue mixtures in the hydrodistillation (HD-CA and HD-DA) were filtered. The solid residues were collected and dried by using air-drying machine at around 60° C while liquid residues were collected and freeze-dried to obtain the aqueous extracts. For steam distillation (SD-CA and SD-DA), after

the extraction of essential oils, only solid residues could be collected and directly dried using air-drying machine at around 60° C. The Soxhlet extraction using methanol as solvent was carried out for all the solid residues to obtain the methanolic extracts. The extractions lasted for 5 h.

4.1.2.5 DPPH radical scavenging assay and Folin-Ciocalteu assay

Methanolic extract solution of 10 mg/mL was prepared by dissolving 10 mg of each extract in 1.00 mL of methanol while aqueous extract solution of 10 mg/mL was prepared by dissolving 10 mg of each extract in 1.00 mL of methanol/water solution (50%, v/v). DPPH radical scavenging assay and Folin-Ciocalteu assay of these extracts were performed according to procedures described in Chapter 3. The antioxidant capacity (AOC) of the extract was expressed in μ mol of Trolox equivalents (TE) / g of plant extract. The total phenolic content (TPC) of the extract was expressed as mg of gallic acid equivalents (GAE) / g of extract.

4.1.3 Results and discussion

4.1.3.1 Chemical composition of essential oils

The relative percentages of each component in the essential oils were given according to the normalisation results of peaks in GC-MS chromatograms. Generally, GC-MS are not used for the quantification because there is possibly great variability in response factor of each component, based on fragmentation by mass spectrometry. However, it is still possible for comparison of the same components in these four essential oils. Total Ion Chromatograms (TICs) of these four essential oils are shown in Figure 3-6 and chemical compositions of essential oils obtained by the four different techniques are given in Table 1.

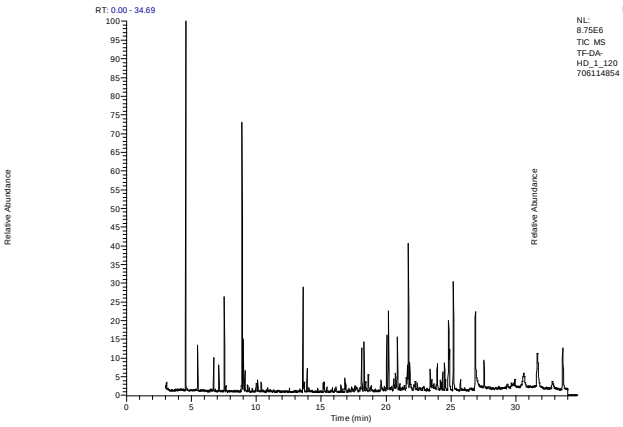


Figure 3 TIC of the essential oil obtained by HD-DA

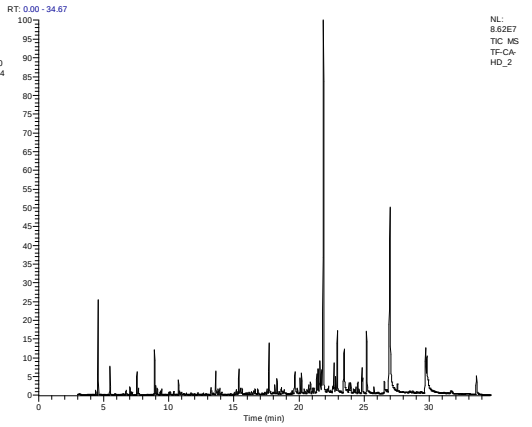


Figure 4 TIC of the essential oil obtained by HD-CA

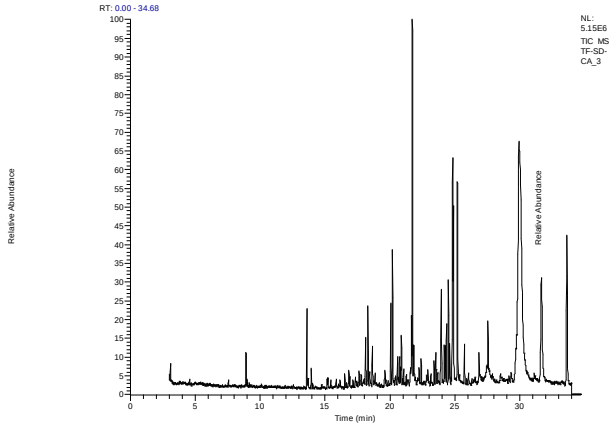


Figure 5 TIC of the essential oil obtained by SD-CA

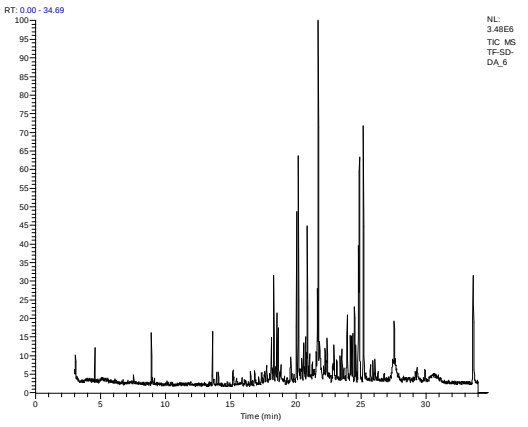


Figure 6 TIC of the essential oil obtained by SD-DA

Table 1 Main chemical compounds of essential oils from aerial parts of *Tussilago farfara* L. obtained by four different techniques of extraction

N°	Components	RI[a]	RIL[a]	Relative Percentage %				Identification
				HD-DA	HD-CA	SD-CA	SD-DA	
1	1-nonene	882	886	12.14	3.96	nd	0.94	MS, RI
2	α-pinene	936	932	1.68	1.59	nd	nd	MS, RI
3	1-decene	982	986	1.48	0.24	nd	nd	MS, RI
4	octanal	1003	988	nd	0.41	nd	nd	MS, RI
5	α-phellandrene	1001	1002	0.99	0.1	nd	nd	MS, RI
6	*p*-cymene	1019	1020	4	1.28	0.14	0.24	MS, RI
7	limonene	1031	1024	0.24	0.4	nd	nd	MS, RI
8	1,10-undecadiene	1074	1080	10.6	2.34	0.88	1.52	MS, RI
9	2-butylidene-bicyclo [2.2.1] heptane	1079	1084	2.14	0.54	0.19	0.25	MS, RI
10	1-undecene	1083	1086	0.94	0.61	0.1	0.17	MS, RI

N°	Components	RI[a]	RIL[a]	Relative Percentage %				Identification
				HD-DA	HD-CA	SD-CA	SD-DA	
11	linalool	1095	1095	0.37	0.24	nd	nd	MS, RI
12	pentylbenzene	-	1158	nd	0.81	nd	nd	MS
13	*(E)*-cycloundecene	1271	1279	4.28	1.42	1.96	1.67	MS, RI
14	carvacrol	1302	1298	0.19	0.1	0.08	0.45	MS, RI
15	valerophenone	1364	1364	nd	1.53	nd	nd	MS, RI
16	decanoic acid	1360	1363	nd	0.5	nd	nd	MS, RI
17	β-caryophyllene	1422	1417	0.74	0.33	0.51	0.36	MS, RI
18	α-humulene	1454	1454	nd	nd	0.51	nd	MS, RI
19	germacrene D	1482	1485	2.37	0.6	1.74	1.82	MS, RI
20	β-selinene	1486	1489	2.19	0.88	2.31	3.67	MS, RI
21	β-bisabolene	1505	1505	0.7	0.53	1	2.18	MS, RI
22	dodecanoic acid	1562	1565	nd	2.34	nd	nd	MS, RI
23	spathulenol	1579	1577	2.69	0.92	2.29	5.4	MS, RI
24	caryophyllene oxide	1586	1582	4.2	1.41	4.29	8.56	MS, RI
25	2,3,5,9-tetramethyl-tricyclo [6.3.0.0(1,5)] undec-2-en-4-one	-	-	2.74	0.68	1.5	5.3	MS
26	α-cadinol	1659	1652	nd	1.28	nd	nd	MS, RI
27	β-eudesmol	-	1654	nd	2.65	nd	1.3	MS
28	aromadendrene oxide-1	-	1702	nd	0.75	2.21	3.67	MS
29	myristic acid	1759	1762	1.19	5.31	0.54	0.74	MS, RI
30	hexahydrofarnesyl acetone	-	1835	2.53	0.79	5.08	8.96	MS
31	palmitic acid	1951	1959	6.57	28.5	0.9	nd	MS, RI
32	manool oxide	-	1991	1.56	nd	2.53	3.29	MS
33	linoleic acid	-	2130	nd	5.39	nd	nd	MS
34	oleic acid	-	2141	nd	5.14	nd	nd	MS
35	docosane	2200	2200	2.5	nd	10.12	nd	MS
36	tricosane	2300	2300	5.01	2.41	9.41	9.32	MS, RI
	Identified compounds			**25**	**33**	**21**	**20**	
	Monoterpene hydrocarbons			6.91	3.37	0.14	0.24	
	Oxygenated monoterpenes			0.56	0.34	0.08	0.45	
	Sesquiterpene hydrocarbons			6	2.34	6.07	8.03	
	Oxygenated sesquiterpenes			6.89	7.01	8.79	18.93	
	Oxygenated diterpene			1.56	0	2.53	3.29	

Aromatic compounds	0	2.34	0	0
Aliphatic compounds	52.12	60.58	30.68	28.87
Total (%)	**74.04**	**75,98**	**48.29**	**59.81**

RI[a]: Retention index calculated with reference to a series of *n*-alkanes using an apolar column; **RIL**[a]: Retention index on apolar column reported in literature; **RP**: relative percentage; **nd**: not detected; Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

In these four essential oils, 36 chemical compounds in total were identified. The chemical compounds identified in the essential oils obtained by HD-DA, HD-CA, SD-CA and SD-DA techniques were 25, 33, 21 and 20, accounting for 74.04%, 75.98%, 48.29% and 59.81% of the samples, respectively. More identified chemical compounds and higher percentages were observed in the case of the essential oil obtained by HD-CA.

Globally, all essential oils of *Tussilago farfara* L. were characterized by high percentages of aliphatic compounds. The highest relative percentage (60.58%) was registered in the essential oil obtained by HD-CA, which contained a high level of palmitic acid. In the chemical class of terpenoids, the relative percentages of monoterpenes and sesquiterpenes depend on the distillation process, with a predominance of monoterpenes in the oils obtained by HD while sesquiterpenes (mainly oxygenated) were most abundant when steam distillation was used.

The chemical compositions of the essential oils in the four cases were qualitatively very similar. 16 common chemical compounds could be found in all four essential oils, e.g. p-cymene, 1,10-undecadiene, 1-undecene, spathulenol, caryophyllene oxide and tricosane. But quantitatively, the relative percentage of these components varied greatly. In the essential oil obtained by HD-DA, the most abundant component was 1-nonene (12.14%) while in the essential oils obtained by SD-DA this aliphatic compound only accounted for 0.92%. In the essential oil obtained by SD-CA, 1-nonene was not even detected.

Finally, the chemical compositions of essential oils from aerial parts of *Tussilago farfara* L. were significantly different from those previously published. Some aliphatic compounds like tricosane and pentacosane were found to be the major compounds in essential oil from its flowers and stems in Lithuania (Asta and Jurga, 2011). The main volatile components obtained from flower buds of the same species grown in China were β-bisabolene (13.93%), (*E*)-cycloundecene (8.49%), 1-pentadecene (4.57%) and 1-undecene (4.83%) (Liu *et al.*, 2006). This variability could be explained by many factors, such as origin of plants, climate, parts of plants, collecting periods, extraction methods, etc.

4.1.3.2 Antioxidants recovery from residues of distillation

The extraction yields, antioxidant capacity and total phenolic contents of the extracts obtained by these four different techniques are shown in Table 2.

Hydrodistillation and steam distillation had significant impacts on the recovery of antioxidants. In the hydrodistillation process, water-soluble compounds including antioxidant ones were firstly recovered by freeze drying; the remaining antioxidant compounds were sequentially extracted from the solid residues by methanol, which increased the total recovery of antioxidant compounds (total yields reaching up to 53.77% for HD-CA and 55.93% for HD-DA). In the process including steam distillation then a Soxhlet extraction of the solid residue by methanol, the total yields were less important (35.32% for SD-DA and 32.88% for SD-CA). The higher extraction yield of aqueous extracts in HD process showed that water is a better solvent to extract the molecules from aerial parts of *Tussilago farfara* L. than methanol. The antioxidant capacity of aqueous extracts obtained by HD process was also comparable to that of methanolic extracts after steam distillation. In comparison, hydro distillation seemed to be a better method for recovery of water-soluble antioxidant compounds and also valorization of molecules in the plants: essential oil and antioxidant extracts could be extracted at the same time in the hydrodistillation process, which was an advantage that steam distillation doesn't have. However, steam distillation appeared to be better than hydrodistillation in recovering antioxidants from solid residue.

Table 2 Extraction yields, antioxidant capacity and total phenolic contents of different extracts

Samples	Extracting yield % DP	AOC µmol TE / g of extract	TPC mg GAE / g of extract
HD-CA aqueous extract	48.10	427.27 ± 7.77	85.43 ± 1.09
HD-DA aqueous extract	49.40	430.58 ± 7.99	86.26 ± 0.58
SD-DA methanolic extract	35.32	394.13 ± 9.01	82.60 ± 0.73
SD-CA methanolic extract	32.88	411.81 ± 2.72	84.51 ± 0.91
HD-CA methanolic extract	5.67	153.44 ± 3.90	39.57 ± 0.32
HD-DA methanolic extract	6.53	136.48 ± 6.34	38.05 ± 0.36
Rosemary extract	-	404.59 ± 1.53	63.12 ± 0.86

DP: dry plant

Regarding Deryng and Clevenger apparatus, their impacts on the recovery of antioxidants were unsurprisingly limited. Similar yields of extracts were obtained and the extracts also

showed similar antioxidant capacity and total phenolic contents.

Methanolic extracts after SD, and aqueous extracts obtained by HD seemed to be in the same range of antioxidant capacity than SFE-CO_2 rosemary extract, indicating that this species could be one promising source of natural antioxidant. However, the total phenolic content of SFE-CO_2 rosemary extract was significantly lower than those of methanolic extracts after SD, and aqueous extracts obtained by HD. It could be speculated that, in the rosemary extract, there are other molecules which contribute significantly to the antioxidant capacity apart from phenolic compounds, while for *Tussilago farfara* L., phenolic compounds are the main molecules which are responsible for the antioxidant capacity. This speculation was further supported by the high correlation coefficient (r = 0.9979) between antioxidant capacity and total phenolic contents of different extracts from *Tussilago farfara* L.

If comparing the antioxidant capacities of methanolic extracts after HD and those after SD, methanolic extracts after SD showed a higher antioxidant capacity. However, solid residues of hydrodistillation were quite different from those of SD. In the hydrodistillation process water has extracted the most polar compounds before the methanol extraction while in steam distillation process solid residues are only deodorized samples from which methanol could extract more polar compounds including antioxidant ones.

4.1.4 Conclusion

In this part, it has been shown that the chemical compositions of the essential oils obtained by HD-DA, HD-CA, SD-DA and SD-CA techniques were similar but the relative percentages of their components varied greatly. All essential oils presented a high percentage of aliphatic compounds, mainly in the hydrodistillated samples. In comparison, hydrodistillation extracted more monoterpenoid compounds while steam distillation extracted more sesquiterpenes.

Deryng apparatus and Clevenger apparatus had very limited effects on the recovery of antioxidants from extraction residues, in contrast with the processes of hydrodistillation and steam distillation. In comparison, hydrodistillation seemed to be a better method for recovery of water-soluble antioxidant compounds but steam distillation appeared to be better than hydrodistillation in recovering antioxidants from solid residue. The results also indicated that *Tussilago farfara* L. could be a promising source of natural antioxidants.

4.2 Effects of mineral contents in water on antioxidants recovery from residues of hydrodistillation

4.2.1 Introduction

In the process of hydrodistillation for obtaining essential oil and aqueous extract, tap water is often used. However, tap water is not pure water and contains some ion of low concentrations. Tap waters in different places are different in ion composition and concentration. At very low concentrations, ions in water (mineral content) seem to have very limited effects on essential oil extraction and antioxidant compounds in aqueous extract. What could be interesting is to figure out the concentrations at which these ions in water will have some significant effects on essential oil composition and antioxidants recovery from residues of hydrodistillation.

There have been some previous studies on salt effects in extraction of essential oil by hydrodistillation. In fact, some salts were added in the process of hydrodistillation to increase the oil yield. Shamspur and co-workers studied the effects of salt treatments on essential oil content and composition of *Rosa damascena* Mill (Shamspur *et al.*, 2012). It was found that, at the optimal amount (22 g of sodium chloride per 220 g of rose flower), the salt had no considerable effects on the essential oil composition but the oil yield increased by 21%. In another study, four salts including sodium chloride, calcium chloride, sodium carbonate and calcium carbonate (all at 3%) were used as additives in hydrodistillation to evaluate the quality and recovery of *Mentha pulegium* L. essential oil (Hassanpouraghdam *et al.*, 2012). Results showed that the percentages of several major components like pulegone and menthone were salt-dependant. More recently, ultrasonic enhanced salt-containing hydrodistillation of lavender essential oil was investigated. Addition of magnesium sulphate (11.5 g/L) greatly improved the essential oil yield (Yu *et al.*, 2013). However, in these studies, ions existed at a very high concentration because salts were added in large quantity, mainly to improve the recovery of essential oil and reduce its loss in the water. The role of salts in water is mainly to decrease the solubility of chemical compounds of essential oil though reactions between some oil compounds and some ions could occur.

The effects of ions in water on antioxidants recovery from residues of hydrodistillation will be a little different. More often antioxidants are polyphenols. On one hand, just like salt

effects in hydrodistillation of essential oil, ions like potassium, sodium and chlorides, will have some effects on the solubility of phenolic compounds. With the increase of salt concentration, the solubility of gallic acid was found to decrease (Noubigh *et al.*, 2007). That will lead to the speculation that high concentration of salts will have an influence on the distribution of polyphenols between aqueous extract and solid residue in hydrodistillation. But this kind of effects of ions on polyphenols is often observed using salts with high concentrations. On the other hand, some ion like calcium and magnesium can have complexion reactions with polyphenols even if their concentrations are not high. When complexion reactions occur, antioxidant capacity and total phenolic content of aqueous extract may decrease. In the tea infusion studies performed by Moisson *et al.* (2008 and 2010), it was found that mineral contents in water, especially calcium content, could decrease the extraction yield of total polyphenols.

In conclusion, based on previous literature, the effects of ions on essential oil are very limited when choosing commonly used water for hydrodistillation. However, for antioxidants, the effects of ions may be great even if ion concentrations (or mineral contents) are not high, due to possible complexion reactions. In our study, antioxidants recovery from residues is an important step in global valorisation of different molecules in the plants. Therefore, it seems necessary to examine the effects of mineral contents in water on antioxidant compounds recovery from aqueous and solid residues of hydrodistillation. *Geranium robertianum* L. was chosen as model plant because it showed high antioxidant capacity, with high content of polyphenols. The effects of mineral contents on antioxidants recovery will be easily observed by using this plant. Five mineralized waters with different mineral contents were selected for hydrodistillation. After hydrodistillation, aqueous residues and solid residues were collected respectively. For aqueous residues, extracts were obtained by removing water. Extracts from solid residue were obtained using Soxhlet extraction with methanol as solvent. All extracts were then tested for their antioxidant capacity using DPPH radical scavenging assay and also for their total phenolic contents using Folin-Ciocalteu assay.

4.2.2 Materials and methods

4.2.2.1 Plant materials and chemicals

Aerial parts of *Geranium robertianum* L. were collected in the fields along the road near mountain Midi-Pyrénées on June, 2012 and identified by the botanist Isabelle FOURASTE

from University of Paul Sabatier in Toulouse. The aerial parts of *Geranium robertianum* L. were then placed in the shady room to be naturally dried. The fully-dried samples were cut into pieces before distillation.

Methanol (HPLC grade, Sigma-Aldrich), DPPH (95%, Sigma-Aldrich), anhydrous sodium carbonate (99.7%, VMR International, Belgium), Folin-Ciocalteu reagent (Sigma-Aldrich), deionized water, Trolox (Sigma-Aldrich)

4.2.2.2 Hydrodistillation

Hydrodistillation (HD) was carried out using Clevenger apparatus. 30.0 g of aerial parts of *Geranium robertianum* L. was put into the flask and 1000 mL of mineralized water was added. Five mineralized waters with different mineral contents (25, 300, 844, 1280 and 2225 mg/L) were used. The selected mineralized waters have a large range in mineral contents: the mineral content in the highest one is almost 90 times that in the lowest one. The hydrodistillation process lasted for 3 h.

Table 1 Five mineralized waters with different mineral contents

Mineral contents	Mont Roucous	OGEU	VITTEL	VAUBAN	Saint Antonin
Total content mg/L	**25**	**300**	**844**	**1280**	**2225**
pH	5.85	7.8	7.5	7.2	7.0
Calcium mg/L	2.4	49	203.8	230	528
Magnesium mg/L	0.5	19	43.1	66	78
Sodium mg/L	3.1	27	5	40	9
Potassium mg/L	0.4	1	-	8	3
Sulphates mg/L	2.0	84	328.9	620	1342
Bicarbonates mg/L	6.3	170	399	280	329
Nitrates mg/L	3.0	1.5	4.3	< 1	-
Chlorides mg/L	3.0	23	-	58	9
Fluorides mg/L	< 0.1	-	-	1.3	1.3

4.2.2.3 Preparation of extracts from residues

After hydrodistillation, the mixture of aqueous and solid residues was filtered. Solid residues were collected and directly dried by the air-drying machine at around 60℃ . The

Soxhlet extraction using methanol as solvent was carried out for the solid residues to obtain the methanolic extracts. The extractions lasted for 5 h. The aqueous residues were freeze-dried to obtain the aqueous extracts.

4.2.2.4 DPPH radical scavenging assay and Folin-Ciocalteu assay

Methanolic extract solution of 10 mg/mL was prepared by dissolving 10 mg of each extract in 1.00 mL of methanol while aqueous extract solution of 10 mg/mL was prepared by dissolving 10 mg of each extract in 1.00 mL of methanol/water solution (50%, v/v). DPPH radical scavenging assay and Folin-Ciocalteu assay of these extracts were performed according to procedures described in Chapter 3. The antioxidant capacity (AOC) of the extract was expressed in μmol of Trolox equivalents (TE) / g of plant extract. The total phenolic contents (TPC) of the extract were expressed as mg of gallic acid equivalents (GAE) / g of extract.

4.2.3 Results and discussion

4.2.3.1 Effects of mineral contents in water on extraction yields

The yields of aqueous and methanolic extracts are presented in Table 1.

Table 1 Aqueous and methanolic extracts yields by different waters

Mineralized waters	Mineral content mg/L	Aqueous extracts yield % / DP	Methanolic extracts yield % / DP
Mont Roucous	25	33.67%	2.20%
OGEU	300	32.83%	2.09%
VITTEL	844	33.40%	1.95%
VAUBAN	1280	33.27%	2.61%
Saint Antonin	2225	31.00%	2.13%

DP: dry plants

For aqueous extracts, with the increase of mineral contents, there was a slight decrease of extracts yields. For methanolic extracts, the highest yield was observed in the case of VAUBAN while the lowest yield was in the case of VITTEL. However, there was no significant difference between extraction yields obtained by the five mineralized water, which

led to the conclusion that mineral contents in water had very limited effects on the yields of aqueous and methanolic extracts.

4.2.3.2 Effects of mineral contents in water on AOC and TPC

Antioxidant capacity and total phenolic content of aqueous and methanolic extracts are shown in Table 2 and Table 3. Significant effects of mineral contents on antioxidant capacity and total phenolic content were observed for both aqueous extracts and methanolic extracts. For aqueous extracts, when mineral content increased, both antioxidant capacity and total phenolic compounds decreased greatly. The lowest values of AOC and TPC were obtained in mineralized water with highest mineral content. For methanolic extracts, the tendency of AOC and TPC was a little different. With the increase of mineral content, AOC and TPC of methanolic extracts also decreased but the lowest AOC and TPC values were observed in the case of mineralized water VITTEL whose mineral content was in the middle place in these five waters.

Table 2 AOC and TPC of aqueous extracts

Mineralized waters	**Calcium mg/L**	**Magnesium mg/L**	**Bicarbonate mg/L**	**pH mg/L**	AOC µmol TE/ g of extract	TPC mg GAE/ g of extract
Mont Roucous	2.4	0.5	6.3	5.85	1040.57 ± 13.25	154.01 ± 2.68
OGEU	49	19	170	7.8	954.30 ± 9.75	135.15 ± 2.17
VITTEL	203.8	43.1	399	7.5	746.03 ± 8.31	97.59 ± 0.98
VAUBAN	230	66	280	7.2	784.13 ± 5.66	110.64 ± 3.35
Saint Antonin	528	78	329	7	654.57 ± 4.24	82.38 ± 2.10

It could be concluded that ions in water did have effects on AOC and TPC of extracts but different ions contribute to the effects differently. Of all ions listed in Table 1, three ions including calcium ion, magnesium ion and bicarbonate ion were supposed to be ions which were responsible for the effects. As is known, antioxidant compounds are often polyphenols. For aqueous extracts and methanolic extracts, there were high correlation ($r = 0.9917$ and 0.9526 for two extracts, respectively) between AOC and TPC, which means that the decreasing effects of AOC were due to interactions between polyphenols and these three ions.

Calcium and magnesium ions can complex with polyphenols while bicarbonate ion can have acid-base reaction with polyphenols whose hydroxyl groups are acidic. Another ion in water should also be considered, hydrogen ion (or hydroxide ion) whose concentration could be indicated by pH. In the five selected mineralized water, Saint Antonin is neutral, Mont Roucous is a little acidic and the other three mineralized waters are a little alkaline. The concentration of hydrogen ion (or hydroxide ion) is so low that its effects on polyphenols could be neglected.

Table 3 AOC and TPC of methanolic extracts

Mineralized waters	**Calcium mg/L**	**Magnesium mg/L**	**Bicarbonate mg/L**	**pH mg/L**	AOC µmol TE/ g of extract	TPC mg GAE/ g of extract
Mont Roucous	2.4	0.5	6.3	5.85	637.23 ± 5.77	93.12 ± 1.48
OGEU	49	19	170	7.8	604.59 ± 3.96	86.48 ± 0.33
VITTEL	203.8	43.1	399	7.5	380.71 ± 1.98	53.76 ± 0.29
VAUBAN	230	66	280	7.2	521.38 ± 5.24	78.01 ± 0.58
Saint Antonin	528	78	329	7	481.76 ± 7.92	76.37 ± 1.30

For calcium and magnesium ions, as the concentrations of calcium are 3-7 times that of magnesium in the five mineralized waters, calcium seems to play a more important role than magnesium in decreasing AOC and TPC through complexion. If comparing the cases of VITTEL and VAUBAN, the results of AOC and TPC seemed to be quite strange. The mineral content and calcium concentration of VAUBAN were higher than those of VITTEL, but the decreasing effects of AOC and TPC caused by VITTEL were stronger than those caused by VAUBAN, which means that there was another ion which had much stronger decreasing effect on AOC and TPC than calcium. This ion was bicarbonate ion which can react with polyphenols in the way of acid-base reaction. In the mineralized water VITTEL, the concentration of bicarbonate ion is the highest, followed by Saint Antonin. In fact, polyphenols in the methanolic extracts were more sensitive to the bicarbonate ion. If AOC and TPC of methanolic extracts by different waters were arranged in the decreasing order, it would be Mont Roucous, OGEU, VAUBAN, Saint Antonin and VITTEL. The same order of

mineralized waters could be obtained if arranging the concentrations of bicarbonate in the increasing order.

In conclusion, mineral content in waters, especially calcium ion and bicarbonate ion, had significant decreasing effects on antioxidants recovery from residues of hydrodistillation. Though magnesium ion may also have strong decreasing effects, our results could not give a clear conclusion because the concentration range of magnesium in selected mineralized waters is not large. For the aqueous extracts, the stronger decreasing effects (37.1% for AOC and 46.5% for TPC) were observed in the case of Saint Antonin (528 mg/L of calcium ion and 329 mg/L of bicarbonate ion) while for the methanolic extracts, the stronger decreasing effects (40.3% for AOC and 42.3% for TPC) were found in the case of VITTEL (203.8 mg/L of calcium ion and 399 mg/L of bicarbonate ion). According to our results, for a better recovery of antioxidants from residues of hydrodistillation, the concentrations of calcium, magnesium or bicarbonate should be kept low in the water. In addition, for the same mineralized water, AOC and TPC of the aqueous extracts were higher than those of the methanolic extracts. If also considering that aqueous extracts (around 33%) accounted for a much higher percentage than methanolic extracts (around 2%) in *Geranium robertianum* L., aqueous extracts will be an important factor for recovery of antioxidants from residues.

4.2.4 Conclusion

In this part, five mineralized waters with different mineral content were used in the hydrodistillation to examine the effects of mineral contents on antioxidants recovery from residues of *Geranium robertianum* L. It was found that mineral contents in water had very limited effects on yields of aqueous extracts and methanolic extracts. However, mineral contents, especially concentrations of calcium and bicarbonate, had significant decreasing effects on antioxidant capacity and total phenolic content of both aqueous and methanolic extracts. Therefore, for a better recovery of antioxidants from residues of hydrodistillation, some attention should be paid to water used so that the interferences from calcium, magnesium or bicarbonate could be as low as possible. In comparison, aqueous extracts will be an important factor for recovery of antioxidants from residues because of their higher percentages in *Geranium robertianum* L., high antioxidant capacity and total phenolic content.

4.3a Effects of waters of different physical states on essential oil composition and antioxidants recovery from *Calendula arvensis* L.

4.3a.1 Introduction

In the previous part, we examined the effects of hydrodistillation (HD) and steam distillation (SD) on the essential oil composition and recovery of antioxidants from the residues of *Tussilago farfara* L. In this part, besides liquid water and water vapor in the cases of HD and SD respectively, water of another physical state (subcritical water) was also employed to examine its effects on essential oil composition and antioxidants recovery. Based on the results in literature and our preliminary experimental results, no real essential oil could be observed or directly collected in subcritical water extraction. The effects of waters of different physical states on essential oil composition will be investigated mainly by comparison of HD and SD results. Subcritical water extraction results will be combined with HD and SD processes for examining the effects on antioxidants recovery. For an interesting comparison, the temperature of subcritical water extraction (at 110°C) was chosen to be closer to that in hydrodistillation and steam distillation (both at around 100°C). As shown in the water phase diagram (Figure 1), subcritical water zone is surrounded by liquid zone, vapor zone and supercritical fluid zone. Hydrodistillation is mainly carried out in liquid and vapor states of water, steam distillation is done in vapor state of water while subcritical water extraction is operated in subcritical state of water. Choosing *Calendula arvensis* L. as the model plant, HD, SD and subcritical water extraction (SWE) were applied to its aerial parts to obtain different extracts. Essential oils obtained by HD and SD would then be characterized by GC-FID and GC-MS. Aqueous solutions obtained by HD and SWE were freeze-dried to obtain aqueous extract (HD) and aqueous extract (SWE). All solid residues of HD, SD and SWE were extracted with methanol to get methanolic extracts. Antioxidant capacity (AOC) of different extracts from *Calendula arvensis* L. was evaluated by DPPH radical scavenging assay and total phenolic contents (TPC) of these extracts were calculated by Folin-Ciocalteu assay.

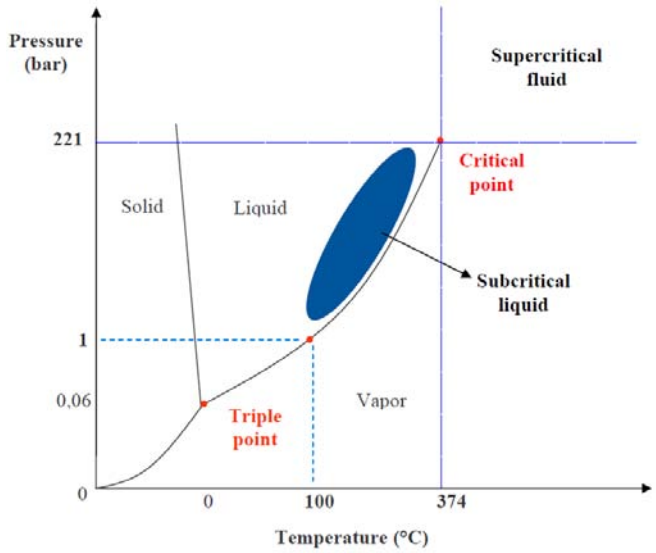


Figure 1 Water phase diagram

4.3a.2 Materials and method

4.3a.2.1 Plant material and chemicals

Aerial parts of *Calendula arvensis* L. were collected in the fields along the road about 30 km from Toulouse on April, 2012. This plant was identified by the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse. Its aerial parts were then placed in the shady room to be naturally dried. The fully-dried plants were cut into pieces before distillation or extraction.

n-pentane (HPLC grade, Scharlau, Spain), methanol (HPLC grade, Sigma-Aldrich), DPPH (2,2-Diphenyl-1-picrylhydrazyl, 95%, Sigma-Aldrich), anhydrous sodium carbonate (99.7%, VMR International, Belgium), Folin-Ciocalteu reagent (Sigma-Aldrich), deionized water, Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%, Sigma Aldrich)

4.3a.2.2 Essential oil extraction by Hydrodistillation and Steam distillation

Hydrodistillation 15 g of dry plants were put into the flask followed by the addition of 1 L of water. The hydrodistillation was carried out by use of Clevenger apparatus. The extraction lasted for 3 h and three repetitions were made. After the distillation, 1 mL of *n*-pentane was used to dissolve the small quantity of essential oil floating above the water in the essential oil

separator. The liquid residue was collected and freeze-dried to obtain the aqueous extracts (HD). And the solid residue was collected and dried using the drying machine at around 60°C.

Steam Distillation 15 g of dry plants were placed on the plate in the steam-generating flask into which 1 L of water was added. The extraction lasted for 3 h and three repetitions were made. After the distillation, 1 mL of *n*-pentane was used to dissolve the small quantity of essential oil of *Calendula arvensis* L. The solid residue was collected and dried using the drying machine at around 60°C.

4.3a.2.3 Subcritical water extraction

Subcritical water extraction was carried out on the accelerated solvent extractor (Dionex ASE350, Thermo Scientific). 1 g of sample powder was mixed with sand and the mixture was then loaded into a 10-mL SST-type cell. Milli-Q water was used as the solvent. The experiments were conducted under the conditions: temperature: 110°C; static time: 5 min; heating time: 6 min; purge time: 100 s; Cycles: 1. The pressure in Dionex ASE 350 cannot be changed and is fixed at 110 bar. Aqueous solutions were finally collected in the bottle and freeze-dried to obtain the aqueous extract (SWE). The solid residue of the plant was dried using drying machine at around 60°C. Three repetitions were made.

4.3a.2.4 GC-FID and GC-MS analysis

Essential oils from aerial parts of *Calendula arvensis* L. were analyzed by GC-FID and GC-MS. The analyses and identification of chemical compounds were performed according to the procedures described in Chapter 3.

4.3a.2.5 Preparation of extracts from solid residues

The three solid residues of HD, SD and SWE were subjected to Soxhlet extraction using methanol as solvent to obtain methanolic extracts. The extraction lasted for 5 h.

4.3a.2.6 DPPH radical scavenging assay and Folin-Ciocalteu assay

For aqueous extracts obtained by HD and SWE, solutions of 10 mg/mL were prepared by dissolving 10 mg of each extract in 1.00 mL of methanol/water solution (50%, v/v) while for methanolic extracts, solutions of 10 mg/mL were prepared by dissolving 10 mg of each

extract in 1.00 mL of methanol. DPPH radical scavenging assay and Folin-Ciocalteu assay of these extracts were performed according to procedures described in Chapter 3. The antioxidant capacity (AOC) of the extract was expressed in μmol of Trolox equivalents (TE) / g of plant extract. The total phenolic content (TPC) of the extract was expressed as mg of gallic acid equivalents (GAE) / g of extract. Extracted quantity of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) / g of dry plant.

4.3a.3 Results and discussion

4.3a.3.1 Essential oils composition

Chemical compounds of the essential oils obtained by hydrodistillation and steam distillation are shown in Table 1. In total, 47 chemical compounds were identified in two essential oils. 28 and 35 compounds were identified in HD oil and SD oil, respectively, accounting for 80.1% and 89% of total essential oils. 16 common chemical compounds were identified in both essential oils. The major compounds of HD oil were α-cadinol (27.8%), *epi*-α-muurolol (14.6%), *epi*-cubebol (8.8%), cubebol (5.5%) and α-bisabolol (4.1%) while major compounds in SD oil were α-cadinol (17.9%), δ-cadinene (17.9%), *epi*-cubebol (11.9%), *epi*-α-muurolol (9.2%), cubebol (7.6%), palmitic acid (4.7%) and α-bisabolol (3.8%). The biggest difference in relative percentages of these compounds existed in δ-cadinene whose percentage was 17.9% in SD oil but only 1% in HD oil. The percentage of α-cadinol (27.8%) in HD oil was much greater than that in SD oil (17.9%).

Table 1 Main chemical compounds of essential oils from aerial parts of *Calendula arvensis* L. obtained by HD and SD

N°	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %		Identification
						HD	SD	
1	1-nonene	890	886	922	931	t	0.5	RI, MS
2	α-pinene	935	932	1028	1032	t	t	RI, MS
3	sabinene	977	969	1127	1132	t	nd	RI, MS
4	β-pinene	982	974	1117	1113	t	nd	RI, MS
5	α-phellandrene	1001	1002	1162	1166	nd	t	RI, MS
6	*para*-cymene	1029	1020	1275	1280	t	t	RI, MS
7	limonene	1030	1024	1218	1203	t	t	RI, MS
8	1,8-cineole	1026	1031	1214	1214	t	nd	RI, MS

N°	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %		Identification
						HD	SD	
9	γ-terpinene	1060	1054	1249	1244	nd	t	RI, MS
10	2,5-dimethyl styrene	-	1099	-	-	nd	t	MS
11	nonanal	1105	1100	1395	1400	nd	t	RI, MS
12	carvotanacetone	1244	1244	1665	1686	2.7	nd	RI, MS
13	undecan-2-one	1283	1293	1598	1606	2.2	nd	RI, MS
14	(*2E,4Z*)-decadienal	-	1293	-	-	nd	t	MS
15	α-cubebene	-	1345	-	1480	nd	t	MS
16	α-copaene	1377	1374	1489	1488	0.6	0.4	RI, MS
17	β-cubebene	-	1387	-	1558	nd	t	MS
18	β-caryophyllene	1421	1417	1601	1594	nd	t	RI, MS
19	undecan-2-yl acetate	1419	-	-	-	0.6	nd	MS
20	α-humulene	1457	1452	-	-	nd	0.4	RI, MS
21	γ-gurjunene	1475	1475	-	-	nd	0.7	RI, MS
22	γ-muurolene	1478	1478	-	1681	nd	0.5	RI, MS
23	germacrene D	1486	1484	1739	1705	0.9	0.7	RI, MS
24	*epi*-cubebol	1497	1493	1884	-	8.8	11.5	RI, MS
25	α-muurolene	1501	1500	1708	1727	1.9	1.8	RI, MS
26	γ-cadinene	1513	1512	1757	1752	2.2	nd	RI, MS
27	cubebol	1518	1514	1937	-	5.5	7.6	RI, MS
28	*trans*-calamenene	1521	1521	1816	-	0.8	nd	RI, MS
29	δ-cadinene	1526	1522	1757	1749	1	17.9	RI, MS
30	cadina-1,4-diene	1534	1533	-	1786	nd	0.9	RI, MS
31	α-cadinene	1540	1537	1757	-	0.5	0.5	RI, MS
32	germacrene D-4-ol	1579	1574	-	-	nd	0.6	RI, MS
33	spathulenol	1582	1577	-	2129	nd	0.4	RI, MS
34	gleenol	1585	1586	-	2025	0.6	nd	RI, MS
35	cubeban-11-ol	1598	1595	-	-	nd	2.2	RI, MS
36	ledol	1609	1602	-	2028	nd	0.6	RI, MS
37	1-*epi*-cubenol	1632	1627	-	2090	2.3	2.7	RI, MS
38	*epi*-α-muurolol	1646	1640	2183	-	14.6	9.2	RI, MS
39	α-cadinol	1661	1652	2242	2167	27.8	17.9	RI, MS
40	*trans*-calamenen-10-ol	1668	1668	-	-	1.6	nd	RI, MS
41	α-bisabolol	1684	1685	2226	2153	4.1	3.8	RI, MS
42	10-nor-calamenen-10-one	1702	1702	-	-	0.8	nd	RI, MS
43	myristic acid	1753	1759	-	2690	nd	0.4	RI, MS
44	aristolone	1773	1762	-	2284	0.6	nd	RI, MS
45	palmitic acid	1957	1959	-	2899	nd	4.7	RI, MS

N°	Components	RI^a	RIL^a	RI^p	RIL^p	RP %		Identification
						HD	SD	
46	eicosane	2000	2000	-	-	nd	0.4	RI, MS
47	phytol	-	2118	-	2603	t	2.7	MS
	Number of chemical compounds					**28**	**35**	
	Monoterpene hydrocarbons					**t**	**t**	
	Oxygenated monoterpenes					**2.7**	**0**	
	Sesquiterpene hydrocarbons					**7.9**	**23.8**	
	Oxygenated sesquiterpenes					**65.9**	**56.5**	
	Aromatic compounds					**0.8**	**t**	
	Aliphatic compounds					**2.8**	**8.7**	
	Total					**80.1**	**89**	

RI^a and **RI^p**: Retention index calculated with reference to a series of n-alkanes using apolar and polar columns; **RIL^a** and **RIL^p**: Retention index on apolar and polar columns reported in literature; **RP**: relative percentage; **t**: trace (<0.1); **nd**: not detected; Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

From perspective of chemical class of these compounds, both HD and SD extracted very small quantity of monoterpene hydrocarbons. Oxygenated monoterpenes were only found in HD oil. The main class of identified compounds in both oils was oxygenated sesquiterpenes, which accounted for 65.9% and 56.5% in HD oil and SD oil, respectively. SD oil contained higher percentages of sesquiterpene hydrocarbons and aliphatic compounds than HD oil.

In literature, there have been studies on essential oil from aerial parts of *Calendula arvensis* L. One publication concerned the chemical composition of the essential oil of *Calendula arvensis* L. growing in Corsica (Paolini *et al*., 2010). 85 components were identified, with α-cadinol (15.1%) and δ-cadinene (12.4%) as the major compounds, which was similar to the results in our SD oil with α-cadinol (17.9%) and δ-cadinene (17.9%) as the two major compounds. In our previous study on volatile extract from roots of *Calendula arvensis* L., the major compounds were spathulenol (27.4%), α-cadinol (6.9%) and manool oxide (5.7%). In comparison, the chemical compounds of essential oil from aerial parts of *Calendula arvensis* L. were different from those of volatile extract from roots of this plant.

4.3a.3.2 Antioxidant capacity (AOC) and total phenolic content (TPC) of extracts

Extraction yields, antioxidant capacity (AOC) and total phenolic contents (TPC) of extracts, extracted quantity of phenolic compounds are shown in Table 2. HD, SD and SWE appeared

to have significantly different impacts on antioxidants recovery. In terms of extraction yield, aqueous extract obtained by HD presented a higher percentage (51.75%) than that obtained by SWE (18.35%). But the results were reasonable, boiling water extraction in the hydrodistillation process lasted for 3 h while subcritical water extraction lasted only around 15 min. Methanolic extract after SD had higher yield (24.42%) than those after HD and SWE (5.04% and 6.00%, respectively).

Table 2 Extraction yields, AOC and TPC of extracts, and extracted quantity of phenolic compounds from *Calendula arvensis* L.

Extracts	Extraction yield % / DP	AOC TE µmol / g extract	TPC mg GAE / g extract	Extracted quantity of PC mg GAE / g DP
Aqueous extract (HD)	51.75	70.80 ± 0.66	16.21 ± 0.17	8.39 ± 0.09
Methanolic extract after HD	5.04	66.85 ± 0.53	27.78 ± 0.08	1.40 ± 0.004
Methanolic extract after SD	24.42	148.97 ± 1.87	36.01 ± 0.42	8.79 ± 0.10
Aqueous extract (SWE)	18.35	124.39 ± 3.22	39.06 ± 1.07	7.17 ± 0.20
Methanolic extract after SWE	6.00	18.56 ± 0.71	7.30 ± 0.10	0.44 ± 0.10

DP: dry plant; **TE**: Trolox equivalent; **GAE**: gallic acid equivalent; **PC**: phenolic compounds

Methanolic extract after SD presented the highest antioxidant capacity followed by aqueous extract obtained by SWE. The methanolic extract after SWE gave the lowest antioxidant capacity. As for total phenolic contents of extracts, surprisingly, methanolic extract after SD was not the highest one. The highest and lowest antioxidant capacities were found in aqueous extract obtained by SWE and methanolic extract after SWE, respectively. High total phenolic content in aqueous extract obtained by SWE showed that subcritical water had higher selectivity for phenolic compounds than ordinary water in HD. Both aqueous extract obtained by HD and methanolic extract after HD showed moderate antioxidant capacity and total phenolic contents. If we looked at the extracted quantity of phenolic compounds obtained by different methods (expressed as mg GAE / g of dry plant), methanolic extract after SD presented the highest values, followed by aqueous extract obtained by HD. However, extracted quantity of phenolic compounds in aqueous extract obtained by SWE was still comparable to those of aqueous extract obtained by HD and methanolic extract after SD. If taking into account the time for each process, hydrodistillation for 3 h, steam distillation for 3 h and following Soxhlet extraction for 5 h but subcritical water extraction only for 15 min, subcritical water extraction seemed to be a very promising method for quick extraction of phenolic compounds from aerial parts of *Calendula arvensis* L. In this study, subcritical water

extraction conditions were not optimized because we would like to examine the effects of these three water-based techniques at closer conditions. Temperature of SWE was chosen to be at 110°C which was close to around 100°C in HD and SD but more importantly gave a confirmed subcritical state of water for extraction. For a better extraction efficiency of phenolic compounds from *Calendula arvensis* L., some follow-up work could be done on optimization of subcritical water extraction conditions.

4.3a.4 Conclusion

In this part, three water-based techniques, hydrodistillation, steam distillation and subcritical water extraction, were applied to aerial parts of *Calendula arvensis* L. for examining their effects on essential oil composition and antioxidants recovery. Results showed that hydrodistillation and steam distillation exerted limited influence on essential oil composition. The chemical compounds in two essential oils were qualitatively similar. SD oil contained more sesquiterpene hydrocarbons and aliphatic compounds than HD oil. But HD oil contained more oxygenated sesquiterpenes than SD oil. In addition, oxygenated monoterpenes were only found in HD oil.

HD, SD and SWE were found to have significantly different impacts on the recovery of antioxidants. HD showed higher extraction yields than SD and SWE. At the conditions chosen for subcritical water extraction, total phenolic content in aqueous extract obtained by SWE was slightly lower than aqueous extract obtained by HD and methanolic extract after SD. However, the time for SWE was much shorter than HD and SD processes. After optimization of extraction conditions, subcritical water extraction could be a very promising method for extraction of phenolic compounds from aerial parts of *Calendula arvensis* L., with high selectivity as well as short extraction time.

4.3b Subcritical water extraction of phenolic compounds from *Calendula arvensis* L. and *Geranium robertianum* L.

4.3b.1 Introduction

In our previous study on the comparison of hydrodistillation, steam distillation and subcritical water extraction (SWE), SWE showed some advantages compared to hydrodistillation but the optimization of experimental conditions for extraction of phenolic compounds from *Calendula arvensis* L. was not done. From the bibliographic studies and our results, extracts from *Geranium robertianum* L. showed high antioxidant capacity. As far as we could ascertain, at present, no studies have been conducted on subcritical water extraction of phenolic compounds from *Calendula arvensis* L. and *Geranium robertianum* L. Therefore, SWE of phenolic compounds from these two plants is really worth investigating.

In this study, the phenolic compounds from aerial parts of *Calendula arvensis* L. and *Geranium robertianum L.* were extracted using subcritical water. The extracts were also tested for their antioxidant capacity using DPPH radical scavenging assay, and total phenolic contents of extracts were calculated by Folin-Ciocalteu assay. The effects of extraction temperature and extraction time on extracts yield, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds were examined. The optimized results were also compared with the extracts obtained by boiling water extraction in hydrodistillation. Thin layer chromatography analysis combined with DPPH test was used to separate and qualify the antioxidant compounds in different extracts.

4.3b.2 Materials and method

4.3b.2.1 Plant materials and chemicals

Aerial parts of *Calendula arvensis* L. were collected in the fields about 30 km from Toulouse on April, 2012 while aerial parts of *Geranium robertianum* L. were collected in the fields near mountain Midi-Pyrénées on June, 2012. These two plants were identified by the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse. Their aerial parts

were then placed in the shady room to be naturally dried. The fully-dried plants were well ground before subcritical water extraction.

Methanol (HPLC grade, Sigma-Aldrich), DPPH (2,2-Diphenyl-1-picrylhydrazyl, 95%, Sigma-Aldrich), anhydrous sodium carbonate (99.7%, VMR International, Belgium), Folin-Ciocalteu reagent (Sigma-Aldrich), Milli-Q water, gallic acid (Sigma-Aldrich), Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%, Sigma Aldrich)

4.3b.2.2 Subcritical water extraction

Subcritical water extraction was carried out on the accelerated solvent extractor (Dionex ASE350, Thermo Scientific). 1 g of sample powder was mixed with fine sand and the mixture was then loaded into a 10-mL SST-type cell. Milli-Q water was used as the solvent. The experiments were conducted under the conditions indicated in Table 1. The pressure in Dionex ASE 350 is fixed at 110 bar. Aqueous solutions were finally collected in the bottles and freeze-dried to obtain the subcritical water extracts. Three repetitions were made.

Table 1 Experimental conditions of subcritical water extraction

No.	Temperature / °C	Heating time / min	Cycle	Static time / min	Total static time / min
1	110	6	1	5	5
2	130	7	1	5	5
3	150	7	1	5	5
4	170	8	1	5	5
5	170	8	2	5	10
6	170	8	3	5	15
7	190	9	1	5	5

4.3b.2.3 DPPH radical scavenging assay and Folin-Ciocalteu assay

The solutions of subcritical water extracts (10 mg/mL) were prepared by dissolving 10 mg of each extract in 1.00 mL of methanol/water solution (50%, v/v). Ultrasonic bath was used to accelerate the dissolution. DPPH radical scavenging assay and Folin-Ciocalteu assay of these extracts were performed according to procedures described in Chapter 3. The antioxidant capacity (AOC) of extracts was expressed in μmol of Trolox equivalents (TE) / g of plant extract. The total phenolic contents (TPC) of extracts were expressed as mg of gallic acid

equivalents (GAE) / g of extract. Extracted quantity of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) / g of dry plant.

4.3b.2.4 Thin Layer Chromatography analysis

TLC analysis of subcritical water extracts from *Calendula arvensis* L. was performed on silica gel $60F_{254}$ TLC plates (Merck, Darmstadt, Germany). A methanol/water (75/25, v/v) solution of each extract (10 mg/mL, 5μL) was spotted onto the plate. After drying for 5 min, TLC plate was placed in the developing chamber saturated with mixed solvent (chloroform/methanol/water, 20:70:10, v/v/v). When the solvent front reached around 1 cm from the top of the plate, TLC plate was taken out and air-dried for 5 min, followed by spraying of 0.05% DPPH solution (methanol). Bands or spots showing antioxidant capacity could be observed to be yellow on a purple background. If the bands or spots were not clear, the quantity of extract solution spotted on the plate could be increased to 10 μL, 15 μL and 20 μL. The moving distance of each separated compound and that of developing solvent were measured to calculate the retention factor R_f. Before DPPH colourisation, each TLC plate was also monitored under UV light at 254 nm. All experiments were carried out at room temperature and repeated three times.

4.3b.3 Results and discussion

Aerial parts of *Calendula arvensis* L. and *Geranium robertianum* L. were extracted at five temperatures, 110, 130, 150, 170 and 190°C. Fixing temperature at 170°C, the aerial parts were also extracted at three extraction times (total static time, 5, 10 and 15 min). Extracts yields, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds from these two plants are shown in Table 2 and 3. To have a common basis for comparison, extracted quantity of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) / g of dry plant.

4.3b.3.1 Effects of extraction temperature and extraction time in SWE of *Calendula arvensis* L.

Extraction temperature had a significant effect on extracts yield, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds from

Calendula arvensis L. When the temperature was raised from 110℃ to 190℃, extracts yields had almost tripled. Especially from 170°C to 190°C, extracts yields greatly increased. The use of higher temperature greatly increased the capacity of water to solubilise chemical compounds in the plant materials. In fact, subcritical water at higher temperature is even able to solubilise and degrade hemicellulose (Ingram *et al.*, 2009) and lignin (Liu and Wyman, 2003). The increase of temperature also resulted in the increase of antioxidant capacity and total phenolic content of extracts, which means that SWE had higher selectivity for phenolic compounds in *Calendula arvensis* L. at high temperature. Extracted quantity of phenolic compounds at 190°C was more than five times that at 110°C. However, some of increased phenolic compounds may come from the degradation of lignin.

Table 2 Extracts yield, AOC and TPC of subcritical water extracts, and extracted quantity of phenolic compounds from *Calendula arvensis* L. at different experimental conditions

Extraction temperature / °C	Extracts yield % / DP	AOC of extracts µmol TE / g extract	TPC of extracts mg GAE / g extract	Extracted quantity of PC mg GAE / g DP
110	18.35 ± 0.35	124.39 ± 3.22	39.06 ± 1.07	7.17 ± 0.24
130	26.37 ± 0.91	125.09 ± 1.43	39.01 ± 1.11	10.28 ± 0.46
150	24.77 ± 0.83	142.8 ± 1.91	46.24 ± 0.96	11.45 ± 0.45
170	28.84 ± 1.37	185.43 ± 3.37	68.14 ± 1.45	19.65 ± 1.02
170 (2)	47.98 ± 0.55	207.02 ± 5.49	60.57 ± 0.36	29.06 ± 0.38
170 (3)	49.31 ± 0.64	223.08 ± 5.51	67.48 ± 0.22	33.27 ± 0.45
190	50.76 ± 0.75	247.67 ± 2.10	77.41 ± 1.57	39.29 ± 0.99
100 (HD)*	51.75	70.80 ± 0.66	16.21 ± 0.17	8.39 ± 0.09

TE: Trolox equivalent; GAE: Gallic acid equivalent; DP: dry plant; PC: phenolic compounds.
170: one cycle; 170 (2): two cycles; 170 (3): three cycles; HD: hydrodistillation;
100 (HD)*: The data was from the results in **Chapter 4.2**.

Extraction time was also found to have a significant effect on extracts yields, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds, which was not in accordance with a previous study in which extraction time seemed to have no significant effect on extracts yield and antioxidant capacity of extracts from thyme (Vergara-Salinas *et al.*, 2012). At 170°C, when total static time changed from 5 min (1 cycle) to 15 min (3 cycles), extracts yields increased by more than 20%. Antioxidant

capacity of extracts also increased. Extracted quantity of phenolic compounds almost doubled at 3 cycles (15 min) compared with that at 1 cycle (5 min).

The optimal experimental conditions were found to be at 190°C and 1 cycle (5 min). On one hand, higher temperature may increase the extraction yield of phenolic compounds; On the other hand, high temperature and longer exposure times will also reduce some phenolic compounds because some polyphenols are thermally sensitive and will be destroyed at high temperature. Therefore a higher temperature (190°C) and a shorter extraction time (1 cycle: 5 min) were finally selected.

Compared with results obtained by boiling water extraction in hydrodistillation, subcritical water extraction showed many advantages. Though extracts yields of both methods were very similar (51.75% for HD and 50.76% for SWE), extracted quantity of phenolic compounds by SWE was around five times that obtained by boiling water extraction. Antioxidant capacity of subcritical water extracts was also much greater than that of extracts by boiling water extraction. SWE presented a really high recovery of phenolic compounds from *Calendula arvensis* L. Also important is that, hydrodistillation lasted for 3h while subcritical water extraction took only 15 min including heating time, thus saving a lot of energy. In accordance with previous studies, subcritical water extraction is really an efficient method for the extraction of phenolic compounds from plants.

4.3b.3.2 Effects of extraction temperature and extraction time in SWE of *Geranium robertianum* L.

Extraction temperature showed great effects on extracts yield, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds from *Geranium robertianum* L. With the increase of temperature from 110°C to 190°C, the extracts yields increased by almost two times. However, antioxidant capacity and total phenolic content of extracts gradually decreased, which was different from the results in SWE of *Calendula arvensis* L. The decrease of AOC and TPC of extracts means that subcritical water at higher temperature extracted more other chemical compounds than phenolic compounds. But the extracted quantity of phenolic compounds still increased when the temperature was raised.

Longer extraction time increased the extracts yields from 33.42% at 5 min to 48.04% at 15 min. The antioxidant capacity and total phenolic content of extracts increased from 5 min to

10 min but decreased from 10 min to 15 min. However, the extracted quantity of phenolic compounds increased when extraction time was prolonged.

At 170°C and 3 cycles (15 min), optimal results were obtained, with the highest extracted quantity of phenolic compounds. While at 190°C and 1 cycle (5 min), the highest extracts yields were obtained. If comparing the optimal results with those obtained by boiling water extraction in hydrodistillation, subcritical water extraction presented shorter extraction time, higher extracts yields, and higher extracted quantity of phenolic compounds. These results showed that subcritical water extraction was also a promising method for extraction of phenolic compounds from *Geranium robertianum* L.

Table 3 Extracts yield, AOC and TPC of subcritical water extracts, and extracted quantity of phenolic compounds from *Geranium robertianum* L. at different experimental conditions

Extraction temperature / °C	Extracts yield % / DP	AOC of extracts μ mol TE / g extract	TPC of extracts mg GAE / g extract	Extracted quantity of PC mg GAE / g DP
110	19.17 ± 0.62	1068.94 ±12.40	188.24 ± 3.47	36.08 ± 1.34
130	23.29 ± 0.86	956.55 ± 18.93	172.36 ± 1.49	40.14 ± 1.53
150	29.09 ± 0.62	863.52 ± 12.24	158.40 ± 2.05	46.08 ± 1.15
170	33.42 ± 0.74	796.98 ± 4.97	157.51 ± 0.26	52.63 ± 1.17
170 (2)	39.92 ± 0.21	913.70 ± 6.55	161.10 ± 0.79	64.30 ± 0.46
170 (3)	48.04 ± 0.70	787.67 ± 10.87	144.17 ± 1.64	69.25 ± 1.28
190	52.68 ± 0.60	719.68 ± 9.86	121.04 ± 0.61	63.76 ± 0.80
100 (HD)*	34.95	932.92 ± 7.14	151.36 ± 2.40	52.90 ± 0.84

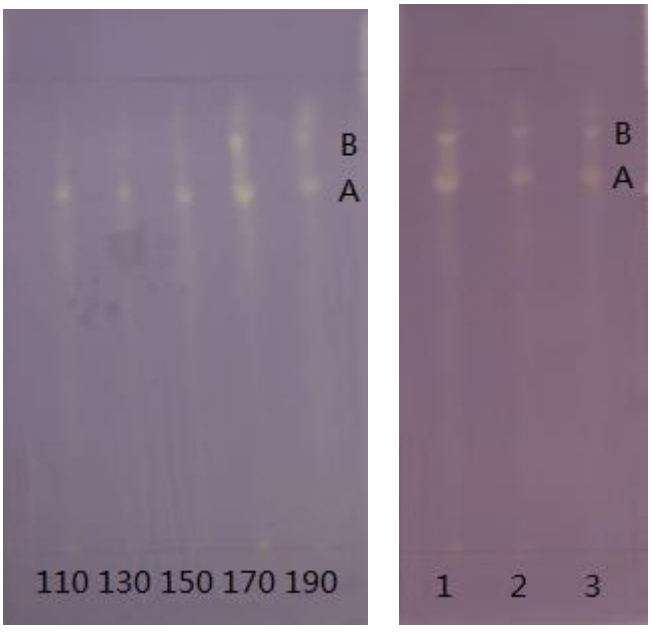
TE: Trolox equivalent; GAE: Gallic acid equivalent; DP: dry plant; PC: phenolic compounds.
170: one cycle; 170 (2): two cycles; 170 (3): three cycles; HD: hydrodistillation
100 (HD)*: The obtaining process of the data was seen in **Chapter 5**

4.3b.3.3 TLC analysis

TLC-DPPH method is an easy-to-use screening test for antioxidant capacity of the compounds present in different extracts. Thin layer chromatograms of subcritical water extracts from *Calendula arvensis* L. at different extraction temperatures and extraction time are shown in Figure 1. Figure 1(a) showed the separation of subcritical water extracts at 100, 130, 150, 170 and 190°C. The yellow spots on the purple background were well-separated

compounds with antioxidant capacity. The yellow bands are poorly-separated chemical compounds with antioxidant capacity. It can be seen that there were two main chemical compounds which showed obvious antioxidant capacity: compound A (R_f= 0.75 ± 0.03) and compound B (R_f = 0.87 ± 0.02). At temperature 110, 130 and 150°C, compound A was mainly responsible for antioxidant capacity of extracts. When temperature was raised to 170°C and even 190°C, compound B showed up and was responsible for antioxidant capacity of extracts, together with compound A. However, the origin of compound B was unknown: it may exist in the plant and high-temperature water has extracted it; it may be newly-formed compounds from degradation of lignin or from degradation of other compounds. On the whole, the high temperature led to the extraction of more species of phenolic compounds. But further increase of temperature will certainly destroy these antioxidant compounds, thus reducing the species of antioxidant compounds. Therefore there was an optimal temperature for better recovery of phenolic compounds from plant materials.

Figure 1(b) showed the separation of extracts at three extraction times (170°C). Two compound A and B were responsible for antioxidant capacity of extracts. No significant different could be observed between the thin layer chromatograms of these three extracts.

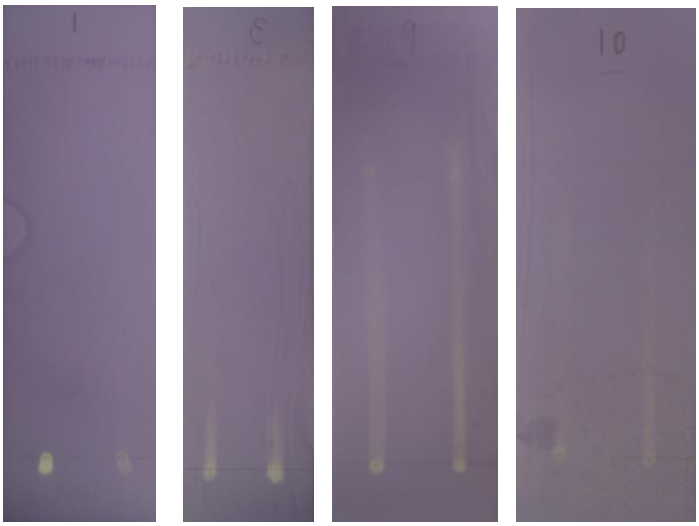


a b

Figure 1 (a) Thin layer chromatogram of subcritical water extracts from *Calendula arvensis* L. at different temperatures (b) Thin layer chromatogram of subcritical water extracts from *Calendula arvensis* L. under different extraction time at 170℃

For the thin layer chromatography of subcritical water extracts from *Geranium robertinaum* L., unfortunately, no satisfactory separation could be achieved. Trial and error method was

used to find out the best developing solvent. More than 10 mixed solvents of different polarity were tried without finding one for better separation. The figure 2 presents four thin layer chromatograms of *Geranium robertianum* L. extracts developed by three solvent systems. In the TLC of extracts from *Geranium robertianum* L., when increasing the polarity of solvents, some antioxidant compounds reached the solvent front while some antioxidant compounds still stayed on the original spot. In different solvent systems, no yellow spots could be observed. Only long yellow band could be observed. Reduced sample loading and length-increased TLC plate could not solve the problem, only making yellow bands not clear enough. Even very polar solvents like methanol/water (75/25, v/v) could not fully move the samples from the original spots. However, it is very difficult to further increase the polarity because too much water will destroy the TLC plate. Actually water is generally not recommended in TLC developing solvents. The TLC results led to the conclusion that extracts from *Geranium robertianum* L. contained very complex antioxidant compounds with different polarity. This was confirmed by the previous results in published literature. In aerial parts of *Geranium robertianum* L., there exist more than 10 antioxidant compounds. Ordinary TLC could not fully separate these compounds. The better separation of these compounds could be done by use of HPTLC or HPLC.



a b c d

Figure 2 (a) TLC of *Geranium robertianum* L. extracts developed by solvent chloroform/methanol (95/5, v/v); (b) TLC of *Geranium robertianum* L. extracts developed by chloroform/methanol/water (65/35/5, v/v/v); (c) TLC of *Geranium robertianum* L. extracts developed by methanol/water (90/10, v/v); (c) TLC of *Geranium robertianum* L. extracts developed by methanol/water (75/25, v/v)

4.3b.4 Conclusion

In this study, phenolic compounds in aerial parts of *Calendula arvensis* L. and *Geranium robertianum* L. were extracted using subcritical water extraction. Extraction temperature and extraction time were found to have significant effects on extracts yields, antioxidant capacity of extracts, total phenolic content of extracts and extracted quantity of phenolic compounds. At optimized extraction temperature and time, SWE showed higher recovery of phenolic compounds from these two plants than boiling water extraction, especially for *Calendula arvensis* L. SWE also required much less extraction time. In conclusion, SWE is a very efficient method for extraction of phenolic compounds from *Calendula arvensis* L. and *Geranium robertinum* L. Though TLC analysis located two antioxidant compounds in subcritical water extracts from *Calendula arvensis* L., some additional work should still be done to identify and quantify the chemical compounds which are responsible for antioxidant capacity in extracts from these two plants, especially *Geranium robertianum* L. which contained more antioxidant compounds.

4.4 Improved MAP-refinery

4.4.1 Introduction

In our previous studies, MAP-refinery was applied to several plants originated in Midi-Pyrénées and Chongqing. The final residues could be proposed for agromaterials or biomaterials or directly for fuels. However, in the subcritical water extraction of *Calendula arvensis* L. and *Geranium robertianum* L., subcritical water at high temperature was found to induce very high recovery for antioxidant compounds or phenolic compounds from these two plants. This led to the thought that subcritical water extraction may be able to extract some antioxidant compounds from the final residues in MAP-refinery. Therefore the improved MAP-refinery was developed, as indicated in figure 1. Based on improved MAP-refinery, raw plant materials will be processed into five parts, essential oil (or volatile extract), aqueous extract, methanolic extract, subcritical water extract and final residue. In this way, more antioxidant extracts may be obtained than original MAP-refinery, which will give more added values to these medicinal and aromatic plants because natural antioxidants are increasingly sought after in food or cosmetics industry.

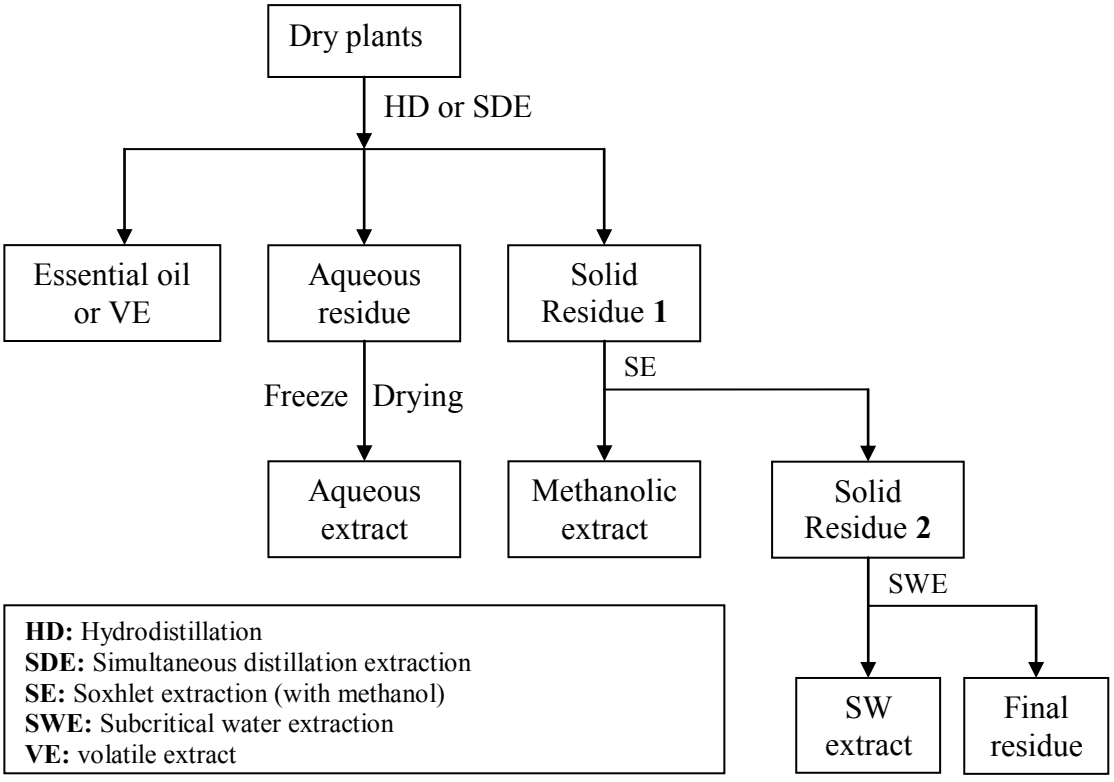


Figure 1 Improved MAP-refinery

In this study, *Geranium robertianum* L. was selected to examine the feasibility of improved MAP-refinery. Firstly aerial parts of *Geranium robertianum* L. were subjected to hydrodistillation to obtain the volatile extracts. The aqueous residue was collected and freeze-dried to obtain aqueous extract. The solid residue **1** was then extracted by methanol to obtain methanolic extract. The subcritical water extraction of solid residue **2** was carried out at 170℃ and 3 cycles (15 min), which are the optimal experimental conditions for subcritical water extraction of phenolic compounds directly from aerial parts of *Geranium robertianum* L. The antioxidant capacity of different extracts was tested using DPPH radical scavenging assay and total phenolic content of extracts were calculated by Folin-Ciocalteu assay.

4.4.2 Materials and methods

4.4.2.1 Plant material and chemicals

Aerial parts of *Geranium robertianum* L. were collected in the fields along the road near mountain Midi-Pyrénées on June, 2012 and identified by the botanist Isabelle FOURASTE from University of Paul Sabatier in Toulouse. The aerial parts of *Geranium robertianum* L. were then placed in the shady room to be naturally dried. The fully-dried samples were cut into pieces before distillation.

n-pentane (HPLC grade, Scharlau, Spain), methanol (HPLC grade, Sigma-Aldrich), DPPH (2,2-Diphenyl-1-picrylhydrazyl, 95%, Sigma-Aldrich), anhydrous sodium carbonate (99.7%, VMR International, Belgium), Folin-Ciocalteu reagent (Sigma-Aldrich), deionized water, Milli-Q water, Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane -2-carboxylic acid, 97%, Sigma Aldrich) , gallic acid (Sigma-Aldrich)

4.4.2.2 Volatile extracts extraction

Aerial parts of *Geranium robertianum* L. were subjected to hydrodistillation using Clevenger apparatus. 20 g of dry plants were put into the flask followed by the addition of 1 L of water. The extraction lasted for 3 h and three repetitions were made. After the distillation, 1 mL of *n*-pentane was used to dissolve small quantity of yellow substance above the water in the Clevenger apparatus. The pentane solution of volatile extracts was stored in fridge for the following analysis.

4.4.2.3 GC-FID and GC-MS analysis

Volatile extract from aerial parts of *Geranium robertianum* L. was analyzed by GC-FID and GC-MS. The analyses and identification of chemical compounds were performed according to the procedures described in Chapter 3.

4.4.2.4 Preparation of different extracts

Aqueous and methanolic extracts: After hydrodistillation, the aqueous residue was collected and freeze-dried to obtain the aqueous extract while the solid residue was collected and dried using the drying machine at around 60℃ . Then dried solid residue **1** was subjected to Soxhlet extraction using methanol as solvent to obtain methanolic extract. The extraction lasted for 5 h. The solid residue after Soxhlet extraction was collected and dried for follow-up subcritical water extraction.

Subcritical water extract: Subcritical water extraction was carried out on the accelerated solvent extractor (Dionex ASE350, Thermo Scientific). 1 g of dried solid residue **2** after Soxhlet extraction was mixed with sand and the mixture was then loaded into a 10-mL SST-type cell. Milli-Q water was used as the solvent. The experiments were conducted under the conditions: temperature: 170℃ ; static time: 5 min; heating time: 8 min; purge time: 100 s; Cycles: 3; Total static time: 15 min; Rinse volume: 100%. The pressure was fixed at 110 bar. Aqueous solution was finally collected in the bottle and freeze-dried to obtain the subcritical water (SW) extract. Three repetitions were made.

4.4.2.5 DPPH radical scavenging assay and Folin-Ciocalteu assay

For aqueous extract (HD) and SW extract, solution of 5 mg/mL was prepared by dissolving 5 mg of each extract in 1.00 mL of methanol/water solution (50% v/v) while for methanolic extract, solution of 5 mg/mL was prepared by dissolving 5 mg of extract in 1.00 mL of methanol. DPPH radical scavenging assay and Folin-Ciocalteu assay of these extracts were performed according to procedures described in Chapter 3. The antioxidant capacity (AOC) of the extract was expressed in μmol of Trolox equivalents (TE) / g of plant extract. The total phenolic contents (TPC) of the extract were expressed as mg of gallic acid equivalents (GAE)

/ g of extract. Extracted quantity of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) / g of dry plant.

4.4.3 Results and discussion

4.4.3.1 Volatile extract analysis

Table 1 showed the chemical compounds in the volatile extract of *Geranium robertianum* L. 32 compounds were identified, reaching up to 80.6% of total volatile extracts. The major compounds were fatty acids, palmitic acid (33.4%), lauric acid (10.3%), myristic acid (7.0%) and linoleic acid (3.7%). Besides, phytol accounted for 4.8%. From the perspective of chemical class, no monoterpene hydrocarbons were detected. The relative percentages of oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes were relatively low, accounting for 2.5%, 6.7% and 10.1%, respectively. The major identified compounds belong to aliphatic compounds.

Table 1 Main chemical compounds of volatile extract from aerial parts of *Geranium robertianum* L.

NO.	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %	Identification
1	linalool	1100	1095	1545	1553	1.1	RI, MS
2	1-nonanol	1170	1165	1656	-	0.1	RI, MS
3	terpinen-4-ol	1180	1174	1602	1611	0.1	RI, MS
4	α-terpineol	1193	1186	1694	1706	0.1	RI, MS
5	decanal	1205	1201	1499	1510	0.1	RI, MS
6	geraniol	1253	1249	-	1843	0.1	RI, MS
7	nonanoic acid	1272	1267	-	2144	0.1	RI, MS
8	(*2E*,*4Z*)-decadienal	1296	1292	-	1749	0.1	RI, MS
9	decanoic acid	1369	1364	-	2272	0.1	RI, MS
10	α-copaene	1385	1374	-	1489	0.1	RI, MS
11	β-elemene	1395	1389	1590	1595	0.1	RI, MS
12	dodecanal	1413	1408	-	1704	0.1	RI, MS
13	longifolene	1415	1407	1569	1574	0.1	RI, MS
14	β-caryophyllene	1423	1417	1598	1594	0.4	RI, MS
15	geranyl acetone	1453	1453	-	1853	0.2	RI, MS
16	1-dodecanol	1473	1469	1962	-	1.5	RI, MS
17	germacrene D	1485	1484	1709	1705	0.9	RI, MS
18	(*E*)-β-ionone	1488	1487	1939	-	0.9	RI, MS
19	cubebol	1518	1514	1937	-	1.5	RI, MS

NO.	Components	RI[a]	RIL[a]	RI[p]	RIL[p]	RP %	Identification
20	γ-cadinene	1520	1516	-	1752	1.1	RI, MS
21	δ-cadinene	1527	1522	1757	1749	4.0	RI, MS
22	lauric acid	1565	1565	-	2488	10.3	RI, MS
23	caryophyllene oxide	1592	1588	1991	1962	0.7	RI, MS
24	cubeban-11-ol	1599	1595	-	-	0.4	RI, MS
25	*epi*-α-muurolol	1646	1642	2183	-	1.8	RI, MS
26	α-cadinol	1658	1652	2242	2167	3.5	RI, MS
27	α-bisabolol	1695	1685	2226	2153	0.9	RI, MS
28	myristic acid	1761	1762	-	2690	7.0	RI, MS
29	(*5E, 9E*)-farnesyl acetone	1921	1913	-	2384	1.3	RI, MS
30	palmitic acid	1967	1959	-	2899	33.4	RI, MS
31	phytol	2120	2118	-	2603	4.8	RI, MS
32	linoleic acid	2136	2132	-	2845	3.7	RI, MS
	Oxygenated monoterpenes					**2.5**	
	Sesquiterpene hydrocarbons					**6.7**	
	Oxygenated sesquiterpenes					**10.1**	
	Aliphatic compounds					**61.3**	
	Total					**80.6**	

RI[a] and **RI[p]**: Retention index calculated with reference to a series of n-alkanes using apolar and polar columns; **RIL[a]** and **RIL[p]**: Retention index on apolar and polar columns reported in literature; **RP**: relative percentage; Identification: **RI:** identification based on comparison of retention index with those of published data; **MS**: identification based on comparison of mass spectra with those found in database or literature.

Essential oil composition of *Geranium robertianum* L. has been previously studied. However, essential oil content of this plant is very low. In fact, in our study, no real essential oil could be observed. The major compounds in essential oil of *Geranium robertianum* L. from Netherlands were reported to be linalool (22.9%), γ-terpinene (13.9%), germacrene-D (7.8%), limonene (5.3%), geraniol (4.4%), α-terpineol (3.8%) and phytol (3.8%) (Pedro *et al.*, 1992). However, essential oil of *Geranium robertianum* L. from Serbia showed noticeably different compounds, with palmitic acid, pentacosane, hexahydrofarnesyl acetone and caryophyllene oxide as the most abundant ones (Radulovic *et al*., 2012). The volatile extract from aerial parts of *Geranium robertianum* L. in our work had some common compounds with these previous results, but the relative percentages of these compounds varied greatly. Despite of these differences, it is understandable that essential oil composition from the same species could be quite different because the secondary metabolites in the plants, e.g., essential

oil, are influenced by many factors, such as climate, soil, collection season and even extraction methods.

4.4.3.2 AOC and TPC of different extracts

Percentages, antioxidant capacity (AOC) and total phenolic content (TPC) of the different extracts obtained from *Geranium robertianum* L. are shown in Table 2, along with extracted quantity of phenolic compounds. It can be seen that subcritical water extracted quite a lot of chemical compounds from solid residue after methanol extraction, accounting for 12.58% of raw plant materials. Total phenolic content of the SW extract was slightly lower than that of methanolic extracts, but antioxidant capacity of the SW extract was only half of that of methanolic extract. This could be tentatively explained by that subcritical water may extract different phenolic compounds from those in methanolic extract, and phenolic compounds in the SW extract were less strong antioxidants than those in methanolic extract. The extracted quantity of phenolic compounds in the SW extract reached up to 10.55 mg GAE/ g DP, which was around three times that in methanolic extract. By applying subcritical water extraction to the final residue in MAP-refinery, more antioxidant compounds or phenolic compounds were obtained. Therefore the improved MAP-refinery is an efficient method for better valorisation of chemical compounds in the aerial parts of *Geranium robertianum* L.

Table 2 AOC and TPC of extracts, and extracted quantity of phenolic compounds from *Geranium robertianum* L.

Different parts	Content % / DP	AOC µmol TE / g extract	TPC mg GAE / g extract	Extracted quantity of PC mg GAE/ g DP
Volatile extract	**t**	-	-	-
Aqueous extract	**34.95**	932.92 ± 7.14	151.36 ± 2.40	52.90 ± 0.84
Methanolic extract	**3.70**	566.69 ± 6.71	99.70 ± 1.03	3.69 ± 0.04
SW-extract	**12.58**	282.56 ± 10.68	83.85 ± 1.75	10.55 ± 0.22
Final residue	**48.77**	-	-	-
		Total		**67.14**
SW-extract	48.04	787.67 ± 10,87	144.17 ± 1,64	**69.25 ± 1,28**

t: trace; DP: dry plant; **PC:** phenolic compounds
SW-extract: extract obtained by direct SWE of *Geranium robertianum* L. (Data was from Chapter 4)

Because of low percentage of essential oil in aerial parts of *Geranium robertianum* L., the more valuable compounds in extracts obtained by the improved MAP-refinery were

antioxidant compounds. If only taking into account phenolic compounds, direct subcritical water extraction of *Geranium robertianum* L. seemed to have similar recovery (69.25 mg GAE / g DP) compared to the accumulated results in the improved MAP-refinery (67.14 mg GAE / g DP). However, the improved MAP-refinery also played the role of fractionation of antioxidant compounds: the raw plant materials were sequentially extracted by boiling water, methanol and subcritical water to obtain different extracts containing different antioxidant compounds. Also importantly, the relatively low extraction temperature of boiling water and methanol could prevent the degradation of some antioxidant compounds. If the improved MAP-refinery is applied to aromatic plants with high percentage of essential oil, this process will allow the extraction of valuable essential oil and more antioxidant compounds.

4.4.4 Conclusion

In this study, an improved process for the valorisation of chemical compounds in the plants was developed by using subcritical water to extract antioxidant compounds from the final residue in MAP-refinery. Aerial parts of *Geranium robertianum* L. were subjected to the process of the improved MAP-refinery to obtain volatile extracts, aqueous extract, methanolic extract, subcritical water extract and final residue. The results showed that the improved MAP-refinery significantly increase the recovery of antioxidant compounds compared with original MAP-refinery. This promising process will allow a better valorisation of essential oil and antioxidant compounds in the aromatic plants at the same time.

Conclusion and prospects

Main conclusions

During this work, the concept of MAP-refinery was proposed and applied to several underutilized medicinal and aromatic plants in Midi-Pyrénées and Chongqing regions. Several water-based green extraction technologies of natural products (e.g. hydrodistillation, steam distillation and subcritical water extraction) were also investigated to look at their effects on essential oil composition and antioxidants recovery from selected plants.

Firstly, lists of forgotten or underutilized medicinal and aromatic plants in both regions were established according to the rules of selection. From the lists, six plants in the Midi-Pyrénées region (*Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Geranium robertianum* L., *Cytisus scoparius* L. and *Spartium junceum* L.) and three plants in the Chongqing region (*Tussilago farfara* L., *Citrus aurantium* L. and *Saussurea costus*) were finally selected for investigations.

Then the MAP-refinery was applied to the selected plants in two regions in order to realise their global valorisation. Volatile extracts composition in the roots of *Tussilago farfara* L. and *Calendula arvensis* L., as well as flower buds of *Spartium junceum* L. were firstly investigated. The main chemical compounds in volatile extract from *Tussilago farfara* L. roots were sesquiterpene hydrocarbons and aliphatic compounds while main chemical compounds in volatile extract from *Calendula arvensis* L. roots were oxygenated sesquiterpenes, oxygenated monoterpenes and oxygenated diterpenes. The volatile extract from flower buds of *Spartium junceum* L. was mainly composed of aliphatic compounds. Antioxidant capacity evaluation results showed that several plant samples like *Cytisus scoparius* L., *Tussilago farfara* L., *Citrus aurantium* L. and *Robinia pseudoacacia* L. could be potential sources of natural antioxidants.

Comparisons of hydrodistillation (HD), steam distillation (SD) and subcritical water extraction (SWE) showed that HD and SD had limited effects on essential oil composition but HD, SD and SWE had significant impacts on the recovery of antioxidants. Hydrodistillation seemed to be a better method for recovery of water-soluble antioxidant compounds than steam distillation but steam distillation appeared to be better than hydrodistillation in recovering antioxidants from solid residue. However, for direct extraction of antioxidant molecules (or

phenolic compounds) from plants, SWE appeared to be a very efficient method. In the hydrodistillation process, mineral contents in water were found to have very limited effects on yields of extracts but calcium and bicarbonate ions had significant decreasing effects on antioxidant capacity and total phenolic content of both aqueous and methanolic extracts. Therefore, for a better recovery of antioxidants from residues of hydrodistillation, the concentrations of calcium, magnesium or bicarbonate should be kept low in the water.

Finally, an improved MAP-refinery was developed. Subcritical water was used for further extraction of antioxidant compounds from residues in original MAP-refinery. In this way, five parts could be obtained from plant materials: volatile extract, aqueous extract, methanolic extract, subcritical water extract and the final solid residue. The results showed that the improved MAP-refinery significantly increased the recovery of antioxidants compared with original MAP-refinery. This promising process will also allow a better valorisation of the final solid residue due to the lower content of residual water.

Prospects

Due to the limited duration, my thesis work mainly focused on preliminary application of MAP-refinery and several green extraction techniques of natural products. A lot of work remains to be done. According to the concept of the improved MAP-refinery, plant materials could be separated into five parts: essential oil (volatile extract), aqueous extract, methanolic extract, subcritical water extract and the final solid residue. However, these five parts are still mixtures of different molecules. Further separation and purification are needed for each part for a real valorisation, i.e. the concept of MAP-refinery should be further developed.

An important part of the thesis is the extraction and analysis of essential oils (or volatile extracts) of the selected plants, with low yields for their recovery. The identification of odour type is very important for the application of these volatile extracts. GC-olfactometry could be used to complete the information and to check their interest in this area.

Concerning the evaluation of antioxidant capacity of different extracts from selected plants, the molecules which are responsible for antioxidant capacity were not clearly identified. Especially for the extracts showing significant antioxidant capacity, it would be interesting to isolate and identify the active components.

In our study, only one biological activity assay (antioxidant activity) was done to screen out the valuable plants. For a comprehensive evaluation of these forgotten plants, more biological

activities assays should be tested, e.g. antimicrobial activity assay. Antimicrobial activity assay could be performed by using thin layer chromatography (TLC) bioautography.

As there are limited scientific studies on some selected medicinal and aromatic plants, a systematic chemical component investigation on one or two plants could be worth doing. Some unknown compounds may be isolated from these plants.

The major problem encountered in this work was the availability of the selected plants. Even if some selected plants could be collected in the fields, their availability was not sufficient for scaling up all the experiments. As a result, some parts in the MAP-refinery process could not be performed, especially the materials fabrication from the final residues. Therefore, if some forgotten medicinal and aromatic plants are found to be interesting in terms of chemical compounds, cultivation of these plants should be considered in order to obtain them in larger quantity. However, MAP-refinery should firstly focus on underutilized medicinal and aromatic plants which have already been cultivated, like *Tussilago farfara* L.

In the process of improved MAP-refinery, subcritical water extraction was only performed on the accelerated solvent extractor to examine its feasibility. For the real application, a pilot device for subcritical water extraction should be set up.

Finally, depending on their characteristics, the final solid residues of different plants could also be employed to produce agromaterials, biomaterials or pellet fuels.

Compared with some well-developed biorefinery concepts, the MAP-refinery has a long way to go. Compared with other biomasses, medicinal and aromatic plants have their unique characteristic: they contain highly valuable chemical compounds with relatively low content. These valuable compounds could find wide applications in food, cosmetic and pharmaceutical industries. Some of these compounds are very sensitive and there are also strict regulations for these compounds. Therefore, extraction techniques are very important for these compounds. Green and high-efficiency extraction and purification technologies of these compounds should be applied and even developed within the context of MAP-refinery.

For the medicinal and aromatic plants with low contents of essential oils, a new MAP-refinery concept could be proposed and applied in the future research work (as indicated in Fig.1). After hydrodistillation process, essential oil will not be separated. Instead, hydrosol containing aroma compounds and water-soluble antioxidant compounds, will be directly collected and tested for its application. For the solid residue, subcritical water extraction will be directly used to obtain the subcritical water extracts. Compared with original and improved MAP-refinery, the new MAP-refinery concept removes the freeze-drying process which requires high energy consumption to eliminate water and avoids the use of toxic solvent like

methanol. To put it in another way, the new MAP-refinery is ‘greener’ and more sustainable according to the princples of green chemistry.

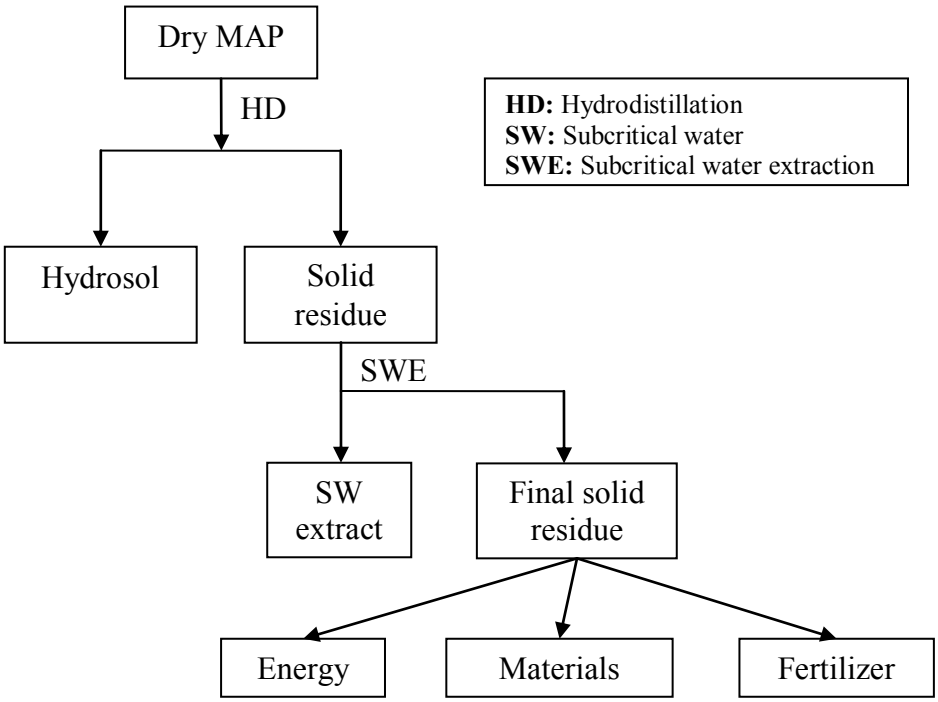


Figure 1 The concept of new MAP-refinery

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1. Study on subcritical water extraction of phenolic compounds and development of Medicinal and Aromatic Plants-Refinery concept (***submitted to International Journal of Food Science and Technology***)
2. Effects of different extraction techniques on essential oil composition and antioxidants recovery from *Tussilago farfara* L. (***submitted to Journal of Essential Oil Research***)

Oral communication

Study of antioxidant activity of various coltsfoot extracts originated from France, China and Lithuania. Zhao, T.M., Dobravalskyte, D., Menut, C., Venskutonis, R. and Talou, T. ***7th Baltic Conference on Food Science and Technology***, Kaunas (LT), May 17-18, 2012

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Résumé étendu de la thèse

1. Introduction

1.1 Contexte scientifique et technique

Depuis les temps anciens les plantes aromatiques et médicinales (MAP) sont traditionnellement utilisées comme une source de médicaments dans plusieurs systèmes de médecine traditionnelle et plus récemment comme sources de compléments alimentaires, d'arômes et parfums voire d'ingrédients cosmétiques. Cependant, certaines de ces PAM, en particulier celles qui ne sont pas cultivées, ont de moins en moins eu d' utilisations au fil du temps, au point d'être considérées comme oubliées. Comme on le sait, les plantes contiennent un grande variété de molécules diverses, en particulier les métabolites primaires (cellulose, hémicellulose, lignine, lipides, protéines,...) et les métabolites secondaires (terpénoïdes, alcaloïdes, composés phénoliques,...). Outre ces derniers, qui sont généralement des molécules présentant des activités biologiques et à ce titre très recherchées par les industries alimentaire, cosmétique et pharmaceutique, ces PAM oubliées, peuvent contenir des molécules odorantes non classiques pouvant être à la base de formulations aromatisantes ou parfumantes originales.

Historiquement, la région de Midi-Pyrénées (France) est riche en plantes aromatiques et médicinales. Cependant, la plupart de ces plantes ne sont pas actuellement pleinement valorisées et même pour les plantes cultivées, où seulement les huiles essentielles ou certains principaux composés actifs étant extraits (qq%), une grande quantité de résidus reste à éliminer. De même dans la région de Chongqing (Chine), plus de 6000 genres différents de plantes peuvent être trouvées, mais moins de 15% de ces plantes ont été étudiées et utilisées. Avec ses plantes médicinales abondantes, Chongqing est un important producteur de plantes m dicinales traditionnelles chinoises en Chine. Cependant, l'utilisation de ces plantes médicinales reste, comme en Europe, loin d'être optimale. En effet, de nombreuses plantes médicinales cultivées sont souvent utilisées pour produire seulement une ou deux molécules cibles, par exemple l'art misinine dans le cas d'*Artemisia annua* L.) tandis que les résidus d'extraction ne sont pas valorisés. De plus dans la médecine traditionnelle chinoise (TCM) à l'inverse de l'européenne, les huiles essentielles ne sont pas considérées comme des molécules

d'intérêt et sont souvent éliminées via un processus de pré-traitement, l'activité des plantes étant considérée venir des autres constituants de la plante. Par conséquent, une valorisation globale des différentes molécules de ces plantes ne pourrait que renforcer leur intérêt économique et favoriser leur remise en culture, les faisant passer du statut d'oubliées à cultivées.

Dans l'extraction des produits naturels, l'emploi de solvants organiques tels que l'hexane, l' ther de p trole et le m thanol, est fr quent lors de la mise en oeuvre des proc d s conventionnels. Ce qui génère des préoccupations croissantes vis a vis de la sécurité des extraits obtenus en raison de possibles résidus mais aussi du fait des caractères énergivores et polluants des procédés mis en oeuvre. De ce fait, on voit apparaitre une demande croissante d' innovations technologiques dans les procédés d'extraction et de séparation des produits naturels à partir de plantes, afin de les rendre plus respectueux de l'environnement.

Par ailleurs, les frontières entre les plantes médicinales et les plantes aromatiques ne sont pas clairement définies: certaines plantes médicinales peuvent contenir des huiles essentielles en forte teneur tandis certaines plantes aromatiques contiennent des molécules à plus haut poids moléculaire qui pourraient être utilisées à des fins médicinales. Si les huiles essentielles sont des extraits naturels qui sont largement utilisés dans les produits cosmétiques, sanitaires, agricoles et alimentaires. Si leur hydrophobicité permet une séparation aisée lors de leur extraction, leur faible teneur dans les plantes (de l'ordre de 1%) a comme corrolaire de générer 99 % de résidus après extraction. Aussi afin d'augmenter l'aspect durabilité de tels procédés, il convient d'envisager une valorisation de ces déchets, les faisant passer du statut de résidus a celui de co-produits. Cette approche est réaliste car ces résidus d'extraction peuvent contenir en quantités significatives des métabolites secondaires, ce qui en fait des sources potentielles de molécules bioactives, notamment des molécules anti-oxydantes ou antimicrobiennes. Et in fine, le résidu solide final pourra trouver une valorisation pour la fabrication d'agromatériaux ou de biofertilisants ou directement en tant que biocombustible.

Parmi les voies de valorisation des différentes molécules es résidus d'extraction, celle des anti-oxydants semble être prometteuse car les industries alimentaire et cosmétiques manquent cruellement de sources d'anti-oxydants naturels tandis que l'utilisation des anti-oxydants synthétiques, bien que strictement réglementée, crée une certaine défiance des consommateurs vis a vis des produits qui en contiennent. Certaines huiles essentielles contiennent des molécules anti-oxydantes (par exemple, des extraits de romarin et de sauge), permettant leur emploi directement ou après fractionnement en industries alimentaire et cosmétique. Néanmoins, le potentiel en de telles molécules dans les résidus d'extraction pouvant s'avérer

important, il convient d'envisager leur valorisation de façon plus optimale. Ainsi, il doit être envisager, pour une meilleure utilisation des différentes molécules actives des plantes aromatiques et médicinales oubliées ou sous-utilisées, de développer des recherches selon deux directions: (a) développer une approche de valorisation globale pour extraire séquentiellement huiles essentielles, anti-oxydants et autres molécules à partir des plantes; (b) appliquer des techniques d'extraction dites vertes, afin de faciliter la valorisation ultérieure des sous-produits. Ces deux voies correspondent aux concepts de chimie verte et de bioraffinerie et notre objectif serait de les coupler.

1.2 Développement du concept de MAP-raffinerie

La raffinerie du pétrole est un procédé industriel bien connu visant à transformer le pétrole brut en carburants, chaleur, électricité, produits chimiques et matériaux. En substituant le pétrole par la biomasse, on obtient la bioraffinerie . Selon la définition de l' IEA Bioenergy Task 42, la bioraffinerie (ou bioraffinage) est le traitement durable de la biomasse en un spectre de bio-produits (alimentation humaine et animale, produits chimiques et matériaux) et de de bioénergie (biocarburants, électricité et chaleur). Comme la biomasse est une ressource renouvelable, le développement de la bioraffinerie industrielle a été identifiée comme une solution potentielle qui permettrait de r pondre à la demande croissante d' nergie, carburants, produits chimiques et matériaux (King *et al.*, 2010).

Les plantes aromatiques et médicinales pouvant être considérées comme une biomasse renouvelable, la bioraffinerie pourrait donc être une approche appropriée pour une valorisation globale des différentes molécules extractibles de ces plantes. Par conséquent, en s'appuyant sur les principes de la bioraffinerie, le concept d'agroraffinage des plantes aromatiques et médicinales ou MAP-raffinerie a été développé. Il s'agit d'une valorisation de la plante entière par des extractions séquentielles des molécules d'intérêt comme le montre la figure 1. Dans un premier temps, une huile essentielle (ou un extrait volatil) est obtenue par hydrodistillation (HD) ou distillation-extraction simultanée (SDE), utilisée lorsque le rendement en huile essentielle est trop faible. Ensuite, la concentration des molécules solubles de l'hydrolat (extrait aqueux) permet l'obtention d'un extrait dit aqueux tandis que le traitement au méthanol du résidu solide génère l'extrait méthanolique et un résidu solide final, future base pour la fabrication d'agromat riaux, de fertilisants ou de biocombustibles. Il convient de souligner, que la MAP-raffinerie propos e ici n'est qu'un processus de valorisation préliminaire dans lesquelles les plantes sont traitées en plusieurs étapes. En effet,

pour une application finale, la séparation voire la purification des composants des différents extraits pourront s'avérer nécessaire. Cette approche d'une valorisation globale des PAM permettrait une réelle économie des ressources naturelles et la mise en place d'un procédé répondants aux exigences du développement durable.

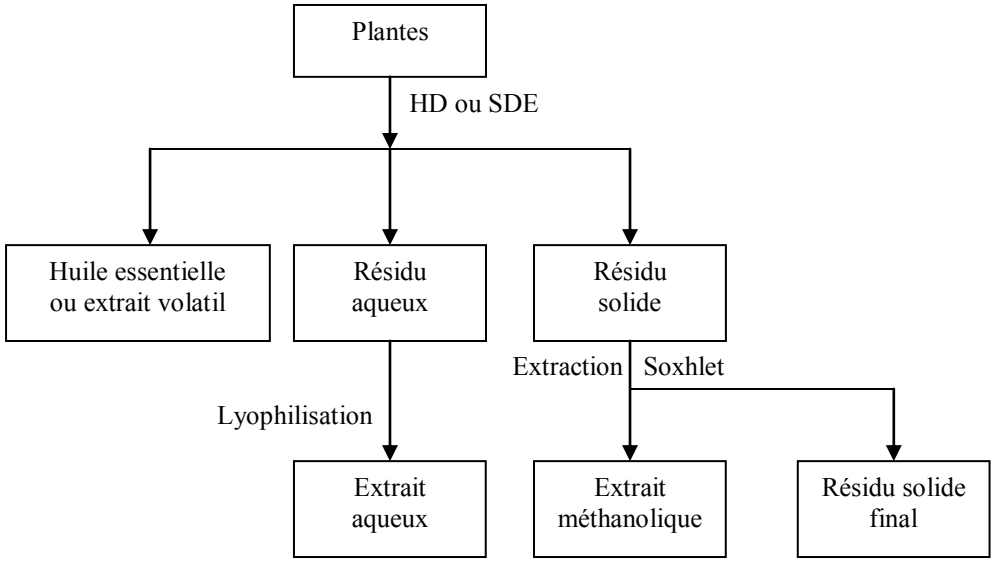

Figure 1 Schéma du concept de MAP-raffinerie

Lorsque la MAP-raffinerie est appliquée aux plantes aromatiques et médicinales pour une valorisation globale, l'extraction et la s paration constituent les tapes cl s du proc d . En d'autres termes, il peut s'av rer n cessaire de mettre en oeuvre des m thodes d'extraction dites vertes pour pallier les lacunes des m thodes d'extraction conventionnelles. Dans cette optique, Chemat et coll. ont proposés un concept d'extraction verte des produits naturels. Basé sur le conceptd de chimie verte, l'extraction verte des produits naturels est d finie: ''*Green extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product*'' (Chemat *et al.*, 2012). Dans le développement de technologies d'extraction vertes de produits naturels, l'utilisation de solvants alternatifs (principalement l'eau et les agro-solvants) est au coeur de la réflexion.

De ce fait, en plus de développer une agroraffinerie appliquée aux plantes aromatiques et m dicinales, nos travaux mettront l'accent sur plusieurs technologies d'extraction utilisant l'eau comme solvant vert: hydrodistillation, distillation à la vapeur et extraction par eau sub-critique, dont l'impact tant sur la composition des huiles essentielles que sur la récupération des molécules anti-oxydantes des résidus d'extraction sera évalué.

1.3 Objectifs de la thèse

La thèse a plusieurs objectifs: (1) sur la base des études bibliographiques, d'établir une liste de plantes aromatiques et médicinales oubliées ou sous-utilisées des régions de Midi-Pyrénées et de Chongqing, selon des règles de sélection pré-définies; (2) de proposer des approches pour une valorisation globale des différents molécules et de les appliquer aux plantes sélectionnées; (3) d' tudier plusieurs technologies d'extraction utilisant l'eau comme solvant et en particulier d'examiner leur impact sur la composition des huiles essentielles et la récupération des molécules anti-oxydantes à partir des résidus d'extraction.

2. Sélection des plantes candidates

En premier lieu, des règles ont été mises en place pour sélectionner les plantes aromatiques et médicinales oubliées ou sous-utilisées dans les régions de Midi-Pyrénées et de Chongqing: (1) peu d'études scientifiques réalisées sur la plante, en particulier sur les huiles essentielles et la capacité anti-oxydante des extraits de cette plante ; (2) la plante doit contenir des huiles essentielles au sens botanique du terme; (3) la plante n'est pas cultivée (en général, les plantes aromatiques et médicinales cultivées ont été largement étudiées); (4) la plante ne contient pas de molécules reconnues comme toxiques (5) la plante peut être cueillie plus ou moins facilement (pas de plantes classées comme protégées). Cependant, dans le processus de sélection des plantes candidates, le plus gros problème rencontré est celui de la disponibilité des plantes sélectionnées. Plusieurs plantes sous-utilisées et potentiellement intéressantes ont dû être exclues de notre liste parce qu'elles ne pouvaient pas être identifiées et/ou collectées dans les régions de Midi-Pyrénées et de Chongqing. Par conséquent, les plantes qui ont finalement été sélectionnées dans les deux régions sont issues d'un compromis alliant théorie et contraintes de terrain.

2.1 Sélection des plantes candidates dans la région de Midi-Pyrénées

La sélection des plantes candidates de la région Midi-Pyr n es a commenc à partir d'une liste comprenant 20 plantes aromatiques et médicinales oubliées (El Kalamouni, 2010). De cette liste, une seule plante a été sélectionnée: *Tussilago farfara* L car elle est d'une cueillette aisée, que la plante a peu été étudiée d'un point de vue composition chimique et qu'elle se

trouve également dans la région de Chongqing. Puis une étude bibliographique, complétée par des entretiens avec la botaniste Isabelle Fourasté de l'Université Paul Sabatier de Toulouse, a permis de sélectionner cinq autres plantes aromatiques et médicinales: *Calendula arvensis* L., *Geranium robertianum* L., *Robinia pseudoacacia* L., *Cytisus scoparius* L. et *Spartium junceum* L. Les six plantes sélectionnées ont été recueillies en plante entière dans les champs près de Toulouse ou dans les Pyrénées pendant les périodes de Février à Juin 2011 et 2012.

2.2 Sélection des plantes candidates dans la region de Chongqing

Comme il est difficile de trouver des botanistes pour identifier et collecter les plantes sauvages dans la région de Chongqing, les plantes candidates n'ont pû être choisies que parmi celles cultivées mais de façon artisanale (plantes sous-utilisées). Des études bibliographiques sur une liste de 48 plantes médicinales produites dans la région de Chongqing ont été réalisées, prenant en compte les rendements en huiles essentielles, les techniques d'extraction et de s paration, les techniques d'analyse et les compos s principaux des huiles essentielle identifiés (cf Annexe A: études bibliographiques de 48 plantes médicinales dans la région de Chongqing). Selon ces résultats bibliographiques, 48 plantes ont été classées en trois groupes: (1) 17 plantes médicinales avec de nombreuses études scientifiques sur les huiles essentielles; (2) 14 plantes médicinales avec un nombre limité d'études scientifiques sur les huiles essentielles; (3) 17 plantes médicinales avec quasiment pas d' études scientifiques sur les huiles essentielles. Les plantes du groupe **1** et **3** ont été éliminées respectivement en raison des trop nombreuses études sur les huiles essentielles ou l'absence de publications, généralement dues au fait que ces plantes ne comportent pas d'organes secréteurs des huiles. En prenant en compte la disponibilité des plantes, trois plantes candidates ont été choisies dans le groupe **2**: *Tussilago farfara* L. (une plante commune avec la région Midi-Pyrénées), *Fructus aurantii* et *Saussurea lappa*. Ces trois plantes ont été cueillies, avec l'aide de chercheurs de l'Université de Chongqing, dans des jardins de plantes médecinales traditionnelles chinoises de la région Chongqing.

3. Le concept de MAP-raffinerie appliquée aux plantes aromatiques sélectionnées

Les régions de Midi-Pyrénées (France) et de Chongqing (Chine) sont riches en plantes aromatiques et médicinales dites oubliées (ou médiévales). Afin de valoriser pleinement les différentes bio-molécules extractibles de ces plantes, la MAP-raffinerie a été développée et

appliquée à plusieurs plantes sélectionnées: 5 plantes de la région Midi-Pyrénées, à savoir *Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Spartium junceum* L. et *Cytisus scoparius* L., 3 plantes de la région de Chongqing, à savoir *Tussilago farfara* L., *Fructus aurantii* et *Saussurea lappa*. Selon le concept de MAP-raffinerie, les plantes seront traitées afin d'en extraire quatre parties: une huile essentielle (ou un extrait volatil), un extrait aqueux, un extrait méthanolique et un résidu solide final. Les huiles essentielles seront analysées par GC-MS et GC-FID. Les extraits aqueux et méthanoliques seront testés pour leur capacité anti-oxydante en utilisant diff rentes m thodes d' valuation. Les applications possibles de résidus solides finaux seront également proposées, basées sur l'expertise du laboratoire dans le domaine ainsi que d'après des études bibliographiques. Comme les tiges de *Spartium junceum* L. et *Cytisus scoparius* L ne contiennent pas d'huiles essentielles, l'extraction au solvant de type Soxhlet est mise en oeuvre afin d'obtenir des extraits méthanoliques. Le tableau 1 résume les échantillons sélectionnées et les méthodes d'extraction d'huile essentielle (ou extrait volatil).

Tableau 1 Echantillons sélectionnés et les méthodes d'extraction d'huile essentielle (ou extrait volatil)

N°	Nom latin de plante	Partie de plante	Méthode d'extraction
1	*Tussilago farfara* L. (CN)	Boutons de fleurs	SDE
2	*Tussilago farfara* L. (CN)	Tiges	SDE
3	*Tussilago farfara* L. (FR)	Boutons de fleurs	SDE
4	*Tussilago farfara* L. (FR)	Racines	SDE
5	*Calendula arvensis* L.	Racines	SDE
6	*Robinia pseudoacacia* L.	Fleurs	SDE
7	*Robinia pseudoacacia* L.	Feuilles	SDE
8	*Spartium junceum* L.	Fleurs	SDE
9	*Spartium junceum* L.	Boutons de fleurs	SDE
10	*Spartium junceum* L.	Tiges	-
11	*Cytisus scoparius* L.	Fleurs	SDE
12	*Cytisus scoparius* L.	Tiges	-
13	*Saussurea lappa*	Racines	HD
14	*Fructus aurantii*	Fruits	HD

CN: plante originiare de Chongqing (Chine); **FR**: plante originaire de Midi-Pyrénées (France); **HD**: hydrodistillation; **SDE**: distillation et extraction simultanée

3.1 Analyse des huiles essentielles (et extraits volatils)

Pour les plantes aromatiques et médicinales sélectionnées dans les deux régions, seules les huiles essentielles de *Fructus aurantii* et de *Saussurea lappa* ont pû être collectées

directement avec des rendements d'extraction respectivement de 0.21% et 0.23%. Pour les autres plantes sélectionnées, il est très difficile de recueillir directement les huiles essentielles en utilisant l'hydrodistillation, et donc la Distillation Extraction Simultan e a t utilis e pour obtenir les extraits volatils correspondants. L'étude des compositions chimiques des extraits volatils des racines de *Tussilago farfara* L. et de *Calendula arvensis* L., ainsi que des boutons de fleurs de *Spartium junceum* L. a été réalisée pour la première fois. Les principaux composés chimiques identifiés dans les extraits volatils de racines de *Tussilago farfara* L. sont des hydrocarbures sesquiterpéniques et des composés aliphatiques tandis que les principaux composés chimiques dans l'extrait volatil de racines de *Calendula arvensis* L. sont des sesquiterpènes oxygénés, des monoterpènes oxygénés et des diterpènes oxygénés. L'extrait volatil de boutons de fleurs de *Spartium junceum* L. est quant à lui principalement composé de molécules aliphatiques. Ces résultats sont résumés dans les tableaux 2,3 et 4.

Tableau 2 Principaux composés chimiques dans l'extrait volatil de racines de *Tussilago farfara* L. (FR)

N°	Composé	RI[a]	RIL[a]	PR%	Identification
1	1-nonène	882	886	5.7	RI, MS
2	α-pinène	930	932	0.7	RI, MS
3	benzaldéhyde	957	952	t	RI, MS
4	2-pentyl furane	985	984	0.7	RI, MS
5	α-phellandrène	1001	1002	0.9	RI, MS
6	*para*-cymène	1021	1020	0.6	RI, MS
7	1-undécène	1084	1086	1.5	RI, MS
8	(*E*)-cinnamaldéhyde	1268	1267	0.6	RI, MS
9	7-*épi*-silphiperfol-5-ène	1345	1345	0.7	RI, MS
10	eugénol	1355	1356	0.8	RI, MS
11	β-caryophyllène	1422	1417	1.1	RI, MS
12	*(E)*-β-farnésène	1452	1454	22.9	RI, MS
13	β-selinène	1486	1489	5.8	RI, MS
14	β-bisabolène	1505	1505	4.5	RI, MS
15	spathulenol	1579	1577	1.5	RI, MS
16	β-oplopenone	1612	1607	1.9	RI, MS
17	silphiperfol-6-en-5-one	1627	1624	0.8	RI, MS
18	ac tate d'khusinol	1825	1823	1.5	RI, MS
19	palmitate de méthyle	1916	1921	1.6	RI, MS
20	acide palmitique	1951	1959	17.8	RI, MS
21	linoléate de methyle	-	2095	4.5	MS
	Hydrocarbures monoterpéniques			**1.5**	
	Hydrocarbures sesquiterpéniques			**35.0**	
	Sesquiterpènes oxygénés			**5.7**	

Composés aromatiques	**1.4**
Composés aliphatiques	**32.5**
Total	**76.1**

RI[a]: indice de rétention calculé par référence à une série de n-alcanes en utilisant une colonne apolaire; **RIL**[a]: indice de rétention sur une colonne apolaire rapportés dans la littérature; **PR**: pourcentage relatif; **t**: trace (<0,1); **Identification**: **RI**: identification basée sur comparaison de l'indice de rétention avec ceux des données publiées; **MS**: identification basée sur la comparaison des spectres de masse avec ceux trouvés dans la base de données ou la littérature.

Tableau 3 Principaux composés chimiques dans l'extrait volatil de racines de *Calendula arvensis* L.

N°	Composé	RI[a]	RIL[a]	PR%	Identification
1	1-nonène	882	886	1.0	RI, MS
2	2-pentyl furane	982	984	0.4	RI, MS
3	δ-2-carène	999	1001	0.6	RI, MS
4	α-phellandrène	1001	1002	0.2	RI, MS
5	δ-3-carène	1007	1008	0.2	RI, MS
6	*para*-cymène	1019	1020	0.5	RI, MS
7	β-phellandrène	1027	1029	0.4	RI, MS
8	linalol	1093	1095	t	RI, MS
9	*cis*-*p*-menth-2-en-1-ol	1116	1118	1.9	RI, MS
10	*trans*-*p*-menth-2-en-1-ol	1134	1136	1.9	RI, MS
11	*p*-méthyle acétophénone	1179	1179	0.6	RI, MS
12	*p*-cymen-8-ol	1179	1179	0.8	RI, MS
13	α-terpinéol	1187	1186	0.8	RI, MS
14	*cis*-pipéritol	1191	1195	0.6	RI, MS
15	*trans*-pipéritol	1202	1207	1.2	RI, MS
16	pipéritone	1252	1249	0.1	RI, MS
17	α-terpinène-7-al	1271	1287	1.8	RI, MS
18	dictamnol	1425	1429	1.3	RI, MS
19	(*Z*)-β-farnésène	1438	1440	0.5	RI, MS
20	spirolepechinène	1450	1452	4.0	RI, MS
21	dodecanol	1483	1469	4.4	RI, MS
22	α-farnesene + *epi*-cubebol	1490	1494	t	RI, MS
23	δ-cadinène	1522	1522	t	RI, MS
24	spathulenol	1578	1577	27.4	RI, MS
25	*epi*-α-muurolol	1638	1640	0.1	RI, MS
26	**A** (MW =206) + α-cadinol	1652	1654	6.9	RI-MS
27	eupatriochromene	-	-	t	MS
28	hydroxy calamenène	-	-	2.3	MS
29	oxyde de manool	1996	1987	5.7	RI, MS
30	**B** (MW = 304)	-	-	2.6	MS (b)
31	nezukol	-	2132	2.5	MS (c)
32	**C** (MW = 306)	-	-	4.6	MS (d)
33	**D** (MW = 288)	-	-	13.6	MS
34	n-tétracosane	2400	2400	t	MS

Hydrocarbures monoterpéniques	**1.9**
Monoterpènes oxygénés	**9.1**
Hydrocarbures sesquiterpéniques	**4.5**
Sesquiterpènes oxygénés	**38.0**
Diterpènes oxygénés	**8.2**
Composés aromatiques	**1.0**
Composés aliphatiques	**5.4**
Total	**68.1**

RI[a]: indice de rétention calculé par référence à une série de n-alcanes en utilisant une colonne apolaire; **RIL**[a]: indice de rétention sur une colonne apolaire rapportés dans la littérature; **PR**: pourcentage relatif; **t**: trace (<0,1); **Identification**: **RI**: identification basée sur comparaison de l'indice de rétention avec ceux des données publiées; **MS**: identification basée sur la comparaison des spectres de masse avec ceux trouvés dans la base de données ou la littérature; **MW**: poids moléculaire

MS (b): m/z (%) = 43 (80), 55 (65), 69 (41), 81 (31), 95 (31), 109 (24), 123 (27), 135 (100), 151 (10), 165 (12), 177 (4), 201 (13), 271 (11), 289 (19)

MS (c): m/z (%) = 43 (100), 55 (70), 69 (68), 81 (52), 95 (41), 109 (28), 123 (25), 135 (13), 149 (15), 164 (23), 175 (7), 190 (12), 201 (8), 208 (6), 255 (10), 273 (7), 291 (33)

MS (d): m/z (%) = 43 (100), 55 (36), 69 (28), 81 (30), 95 (18), 109 (14), 119 (14), 135 (9), 147 (8), 153 (6), 163 (4), 175 (6), 190 (26), 201 (8), 218 (4), 243 (1), 255 (8), 273 (35)

Tableau 4 Principaux composés chimiques dans l'extrait volatil de boutons de fleurs de *Spartium junceum*

N°	Composé	RI[a]	RIL[a]	PR%	Identification
1	*(E)*-2-hexénal	843	846	0.5	RI, MS
2	1-nonène	882	886	3.8	RI, MS
3	α-pinène	936	932	t	RI, MS
4	benzaldéhyde	957	952	0.9	RI, MS
5	1-octène-3-ol	971	974	0.7	RI, MS
6	2-pentyl furane	986	984	0.8	RI, MS
7	α-phellandrène	1003	1002	0.6	RI, MS
8	*para*-cymène	1022	1020	0.6	RI, MS
9	phénylacétaldéhyde	1041	1042	1.5	RI, MS
10	linalol	1095	1095	1.2	RI, MS
11	2-phényl ethanol	1111	1116	1.4	RI, MS
12	α-terpinéol	1185	1186	t	RI, MS
13	salicylate de méthylé	1189	1190	t	RI, MS
14	safranal	1196	1196	t	RI, MS
15	2,3-dihydrobenzofurane	1212	1223	0.9	RI, MS
16	indole	1292	1290	0.5	RI, MS
17	*p*-vinyl-gaïacol	1312	1309	0.5	RI, MS
18	γ-nonalactone	1357	1358	t	RI, MS
19	(*Z*)-β-damascénone	1361	1361	t	RI, MS
20	laurate de méthyle	1524	1524	1.6	RI, MS
21	acide laurique	1658	1659	0.7	RI, MS
22	myristate de méthyle	1718	1722	2.6	RI, MS

23	acide myristique	1759	1762	21.8	RI, MS
24	palmitate de méthyle	1919	1921	2.9	RI, MS
25	acide palmitique	1957	1959	24.7	RI, MS
26	linoléate de méthyle	-	2095	2.1	MS
27	acide oléique	-	2141	5.3	MS
	Hydrocarbures monoterpéniques			**1.2**	
	Monoterpènes oxygénés			**1.2**	
	Composés aromatiques			**6.5**	
	Composés aliphatiques			**66.7**	
	Total			**75.6**	

RI[a]: indice de rétention calculé par référence à une série de n-alcanes en utilisant une colonne apolaire; **RIL**[a]: indice de rétention sur une colonne apolaire rapportés dans la littérature; **PR**: pourcentage relatif; **t**: trace (<0,1); **Identification**: **RI**: identification basée sur comparaison de l'indice de rétention avec ceux des données publiées; **MS**: identification basée sur la comparaison des spectres de masse avec ceux trouvés dans la base de données ou la littérature;

3.2 Evaluation de la capacité anti-oxydante des extraits aqueux et méthanoliques

La capacité anti-oxydante et la teneur totale en phénols des extraits aqueux ont été déterminées respectivement par les tests au radical libre DPPH et celui dit de "Folin-Ciocalteu". La capacité anti-oxydante de 14 extraits méthanoliques a quant a elle été évaluée tests au radical libre DPPH et par 3 autres tests complémentaires: test ORAC (capacité anti-oxydante au radical d'oxygène), test au radical cation ABTS et test FRAP (pouvoir anti-oxydant en réduisant ferrique) afin d'avoir une évaluation plus complète.

Les extraits aqueux, recueillis en tant que sous-produit liquide de l'hydrodistillation (ou de la distillation et extraction simultanée), représentent de 34.97 % à 77.25 % en masse des plantes sélectionnées tandis que les extraits méthanoliques , obtenus à partir des résidus solides, représentent de 2.1 % à 19.27 %. Il est à noter que les capacités anti-oxydantes des extraits aqueux et m thanoliques sont dans la même gamme. Des r sultats de l' valuation des capacités anti-oxydantes ont montrés que plusieurs plantes telles que *Cytisus scoparius* L., *Tussilago farfara* L., *Fructus aurantii* et *Robinia pseudoacacia* L. sont de réelles sources potentielles de molécules anti-oxydantes naturelles. In fine, les résidus solides finaux représentent 20.65% à 83.50 % et une voie de valorisation possible pourrait être la fabrication d'agromat riaux ou biomat riaux.

L'application de MAP-raffinerie aux différentes plantes présentées dans cette étude constitue seulement une étape préliminaire. En effet, pour réaliser une réelle et complète valorisation des différentes molécules, une optimisation devra être menée pour chacune des

étapes du process. Par exemple, en ce qui concerne les extraits aqueux et méthanoliques, une séparation et une purification des composés chimiques obtenus pourrait s'aveérer serait nécessaire.

4. Développement de technologies d'extraction en utilisant l'eau comme solvant vert

4.1 Impact de différentes techniques d'extraction tant sur la composition de l'huile essentielle que sur la récupération des molécules anti-oxydantes es *Tussilago farfara* L.

Dans les procédés d'hydrodistillation (HD) ou de distillation à la vapeur (SD) de plantes aromatiques, l'appareil Clevenger est le s parateur d'huiles essentielles le plus couramment utilisé du fait qu'il est recommandé par les Pharmacopées Française et Européenne, tandis qu'en Pologne, l'appareil Deryng semble être largement utilis (Polish Pharmacopoeia VI, 2002). La différence la plus notable entre ces deux appareils concerne la partie de condensation des vapeurs (Figure 2), celle de l'appareil Deryng tant consid r e comme tant la plus efficace. D'après la litt rature, l'appareil Deryng permettrait l'obtention de fractions en composés chimiques volatils plus riches qu'avec l'appareil Clevenger (Sajewicz *et al.*, 2009; Rzepa *et al.*, 2012). D'autre part, en plus de la fraction odorante, les plantes aromatiques peuvent être des sources en molécules anti-oxydantes naturelles, tant au niveau des huiles essentielles que des fractions extractibles à partir des r sidus d'extraction (qui repr sentent rappellons le jusqu'à 99% de la matière v g tale trait e). Des travaux r cents rapportent l'influence des techniques d'extraction de l'huile essentielle sur la r cup ration des molécules anti-oxydantes à partir de résidus de distillation de romarin (Navarrete *et al.*, 2011). L'extraction sous micro-ondes sans solvant s'est av r être une m thode efficace pour la récupération des molécules anti-oxydantes après l'extraction d'huile essentielle, par rapport à l'hydrodistillation et la distillation à la vapeur. Cependant, dans la valorisation des r sidus de romarin, seuls les r sidus solides ont t pris en compte. Or dans le cas de l'hydrodistillation, une quantité notable de molécules anti-oxydantes pourrait être présente dans les résidus liquides, qu'il convient donc de valoriser.

(a) (b) (c)

Figure 2 L'appareil Clevenger (a); L'appareil Deryng (b) et sa partie de refroidissement (c)

Afin d' valuer l'impact des deux proc d s d'extraction [hydrodistillation (HD) et distillation à la vapeur (SD)] combin es aux deux s parateurs d'huiles essentielles [appareil Clevenger (CA) et appareil Deryng (DA)] tant sur la composition de l'huile essentielle que sur la récupération des molécules anti-oxydantes à partie de résidus de distillation, *Tussilago farfara* L. qui pousse à l'état sauvage dans les Pyrénées a été choisie comme plante modèle. Ses huiles essentielles ont été extraites par ces quatre techniques combinées (HD-CA, HD-DA, SD-CA et SD-DA) et caractérisées par GC-MS. Les extraits ont été obtenus quand à eux à partir des résidus de distillation et testés pour leur capacité anti-oxydante et leur teneur totale en phénols. Les résultats obtenus par ces quatre techniques ont été comparés en termes de: (1) profils chromatographiques et compositions d'huiles essentielles; (2) capacit anti-oxydante et teneur totale en phénols des extraits correspondants.

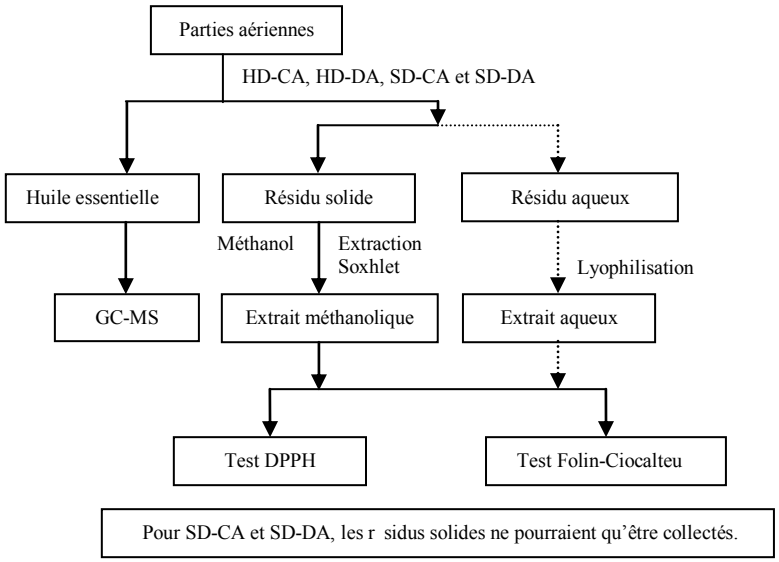

Figure 3 Schéma de préparation et d'analyse des extraits de *Tussilago farfara* L.

Les résultats ont montrés que les compositions chimiques des huiles essentielles obtenues par les techniques HD-DA, HD-CA, SD-CA et SD-DA sont similaires, même si les pourcentages relatifs de leurs constituants peuvent varier notablement. Toutes les huiles essentielles présentent un pourcentage élevé en composés aliphatiques, principalement dans les chantillons hydrodistill s, l'hydrodistillation extrayant n anmoins plus de monoterpénoïdes tandis que la distillation à la vapeur plus de sesquiterpènes.

Si les appareils Deryng et Clevenger ont des effets très limités sur la récupération des molécules anti-oxydantes à partir de r sidus d'extraction, il n'en est pas de même pour les procédés d'hydrodistillation et de distillation à la vapeur. En effet, l'hydrodistillation semble être une meilleure méthode pour la récupération des composés anti-oxydants et donc pour une valorisation de ces molécules. Les résultats ont également montrés que *Tussilago farfara* L. pourrait être une source prometteuse de molécules anti-oxydantes naturelles.

4.2 Impact de différents états physiques de l'eau tant sur la composition de l'huile essentielle que sur la récupération des molécules anti-oxydantes es *Calendula arvensis* L.

Dans la partie pr c dente, nous avons examin les effets de l'hydrodistillation (HD) et de la distillation à la vapeur (SD) tant sur la composition de l'huile essentielle que sur la récupération des molécules anti-oxydantes à partir des résidus de *Tussilago farfara* L. Dans cette partie, en sus de l'eau liquide et de la vapeur d'eau, respectivement dans les cas de HD et de SD, l'eau dans un autre tat physique (sub-critique) a également été utilisée afin d' tudier l'impact du couple Temp rature-Pression sur la composition de l'huile essentielle et sur la récupération des molécules anti-oxydantes. Sur la base des résultats de la littérature et de résultats exp rimentaux pr liminaires, aucune huile essentielle r elle n'ayant pu être directement recueillie dans l'extraction par eau sub-critique, les effets des trois différents tats physiques de l'eau sur la composition d'huile essentielle seront principalement étudiés par les comparaisons des résultats obtenus par HD et SD tandis que les résultats de l'extraction par eau sub-critique seront combinés avec ceux obtenus par HD et SD pour examiner leurs effets sur la récupération des molécules anti-oxydantes. Afin de permettre une meilleure comparaison, la temp rature de l'extraction par eau sub-critique (110 °C) a été choisie pour être plus proche de celles de l'hydrodistillation et de la distillation à la vapeur (voisine de 100°C). Comme le montre le diagramme de phase de l'eau (Figure 4), la zone

sub-critique de l'eau est une large zone entour e de la zone liquide, de la zone vapeur et de la zone supercritique. L'hydrodistillation est principalement r alis e dans les tats liquide et vapeur, la distillation à la vapeur se fait dans l' tat vapeur alors que l'extraction par eau sub-critique est effectu e dans l' tat sub-critique. Choisissant *Calendula arvensis* L. comme plante modèle, la HD, la SD et l'extraction par eau sub-critique (SWE) ont été appliquées à ses parties aériennes. Les huiles essentielles obtenues par HD et SD seront ensuite caractérisés par GC-FID et GC-MS. Les solutions aqueuses obtenues par SWE et HD seront lyophilisées pour obtenir les extraits dits aqueux. Tous les résidus solides de HD, SD et SWE sont ensuite extraits par du méthanol pour obtenir les extraits dits méthanoliques. La capacité anti-oxydante des différents extraits de *Calendula arvensis* L. a été évaluée par le test au DPPH et la teneur total en phénols de ces extraits a été calculée au moyen du test Folin-Ciocalteu.

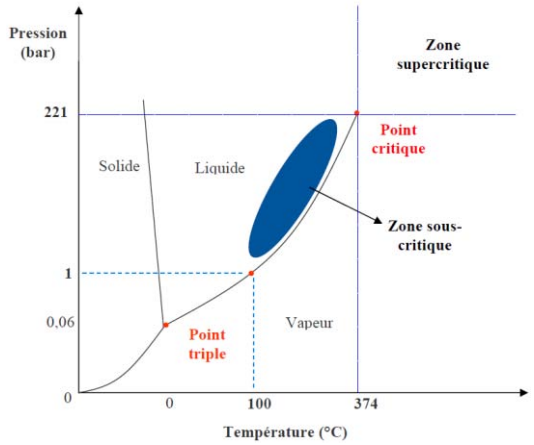

Figure 4 Le diagramme de phase de l'eau

Il ressort de nos travaux que la distillation à la vapeur d'eau et l'hydrodistillation ont une influence limitée sur la composition de l'huile essentielle, les composés chimiques de ces deux huiles essentielles étant qualitativement similaires. Néanmoins, celle obtenue par SD continet plus d'hydrocarbures sesquiterpéniques et de composés aliphatiques que celle obtenue par HD. A l'inverse, l'huile essentielle obtenue par HD contient plus de sesquiterpènes oxyg n s que l'huile obtenue par SD tandis que des monoterpènes oxyg n s ne sont trouv s que dans l'huile essentielle obtenue par HD.

La HD, la SD et la SWE ont par contre des effets très différents sur la récupération des molécules anti-oxydantes: la HD permettant des rendements d'extraction plus lev s que la SD et la SWE. Dans les conditions choisies pour l'extraction par eau sub-critique, la teneur totale en ph nols de l'extrait aqueux est l gèrement plus faible que celle de l'extrait aqueux obtenu par HD et de l'extrait méthanolique après SD. Cependant, le temps d'extraction pour

SWE était beaucoup plus court que dans le cas de HD et de SD. Après une optimisation des conditions d'extraction, l'extraction par eau sub-critique pourrait être une méthode très prometteuse pour l'extraction des compos s ph noliques de parties a riennes de *Calendula arvensis* L., avec une meilleure sélectivité et un gain de temps notable d'extraction.

4.3 Extraction par eau sub-critique de composés phénoliques es *Calendula arvensis* L. et *Geranium robertianum* L.

Dans l' tude pr c dente sur la comparaison de l'hydrodistillation, la distillation à la vapeur et l'extraction par eau sub-critique (SWE), la SWE a montré certains avantages par rapport à l'hydrodistillation. D'après à la fois des tudes bibliographiques et certains de nos r sultats préliminaires, des extraits de *Geranium robertianum* L. ont montrés une capacité anti-oxydante lev e. A notre connaissance, aucune tude n'a t men e sur l'extraction par eau sub-critique des composés phénoliques de *Calendula arvensis* L. et *Geranium robertianum* L. Par conséquent, la SWE des composés phénoliques de ces deux plantes ainsi que l'optimisation des conditions expérimentales sont des voies que nous avons explorées.

Les composés phénoliques des parties aériennes de *Calendula arvensis* L. et *Geranium robertianum* L. ont été extraits par eau sub-critique. Les extraits ont été également testés pour leur capacité anti-oxydante et leur teneur totale en phénols. Les effets de la température d'extraction et du temps d'extraction sur les rendements des extraits, la capacit anti-oxydante des extraits, la teneur totale en phénols des extraits et la quantité extraite de composés phénoliques ont été examinés. Les résultats optimisés ont également été comparés avec ceux obtenus par l'extraction à l'eau bouillante lors de l'hydrodistillation. L'analyse chromatographique en couche mince (CCM) combinée avec le test DPPH a été utilisée pour séparer et qualifier les composés anti-oxydants dans différents extraits.

Le temps d'extraction et la temp rature d'extraction ont eus des effets significatifs à la fois sur les rendements d'extraction, la capacité anti-oxydante de ces extraits, leur teneurs totales en ph nols et sur la quantit extraite de compos s ph noliques. Aux temp ratures d'extraction et au temps d'extraction optimis s, la SWE montre une meilleure r cup ration des compos s ph noliques pour ces deux plantes que l'extraction à l'eau bouillante en particulier pour *Calendula arvensis* L. tout en requiérant un temps d'extraction. En conclusion, la SWE s'avère être une technologie prometteuse pour l'extraction directe des mol cules anti-oxydantes à partir de *Calendula arvensis* L. et de *Géranium robertinum* L. L'analyse par CCM ayant permis de localiser deux composés anti-oxydants majeurs dans les extraits à l'eau sub-critique

de *Calendula arvensis* L., leurs identification et quantification reste à effectuer, en particulier dans le cas de *Geranium robertianum* L. qui contient le plus fort taux de composés anti-oxydants.

4.4 Impact de la composition minérale de l'eau sur la récupération des molécules anti-oxydantes de résidus d'hydrodistillation

Dans le proc d d'hydrodistillation, l'eau utilis e est classiquement de l'eau du robinet. Or cette eau n'est pas un eau pure et contient donc des ions à concentrations variables, fonction des lieux d'approvisionnement. Or si d'après la littérature et nos travaux, la composition minérale semble avoir des effets très limit s sur l'extraction des huiles essentielles, il n'en est pas de même pour celle des molécules anti-oxydantes, en raison de possibles réactions de complexation. Dans notre approche de raffinage, la récupération des molécules anti-oxydantes à partir des résidus est une étape importante dans la valorisation globale des différentes mol cules es planta. Par cons quent, il semble n cessaire d'examiner les effets de la composition min rale de l'eau sur l'efficacit de la r cup ration des compos s anti-oxydants des r sidus aqueux et solides après l'hydrodistillation. *Geranium robertianum* L. a été choisie comme plante modèle, du fait des capacités anti-oxydantes élevées de ses extraits. Cinq eaux naturellement minéralisées (de type source ou minérale) ont été sélectionnées pour l'étude centr e sur l'hydrodistillation.

Si la composition min rale de l'eau a eu des effets très limit s sur les rendements tant en extraits aqueux que extraits méthanoliques, les teneurs en ions calcium et bicarbonate ont néanmoins des effets significatifs décroissants sur la capacité anti-oxydante et sur la teneur totale en phénols de ces deux types d'extraits. De ce fait, afin d'envisager une meilleure récupération des molécules anti-oxydantes à partir des r sidus d'hydrodistillation, une attention particulière devra être accord e à la qualit de l'eau utilis e afin de minimiser l'impact des ions calcium et bicarbonate mais aussi à la récupération des extraits aqueux, du fait de leur richesse en molécules anti-oxydantes, notamment dans le cas de *Geranium robertianum* L.

5. Concept de MAP-raffinerie améliorée

Dans nos études précédentes, la MAP-raffinerie a été appliquée à plusieurs plantes issues de Midi-Pyrénées et Chongqing avec comme perspective une valorisation des résidus solides finaux pour la fabrication d'agromat riaux ou biomat riaux voire directement sous forme de combustibles (plaquettes). Or, dans l'extraction par eau sub-critique de *Calendula arvensis* L. et *Geranium robertianum* L., l'eau sub-critique à haute température s'est avérée induire une meilleure récupération des composés anti-oxydants (ou des composés phénoliques) de ces deux plantes. Cela nous conduit à proposer d'inclure une nouvelle étape à notre concept de MAP-raffinerie, à savoir l'extraction par eau sub-critique des composés anti-oxydants à partir des résidus solides finaux. Ainsi, un nouveau diagramme du concept de MAP-raffinerie améliorée est proposé comme rapporté dans la figure 5. Basé sur ce concept, les plantes seront donc traitées en cinq étapes: une huile essentielle (ou un extrait volatil), un extrait aqueux, un extrait m thanolique, un extrait à l'eau sub-critique et in fine un résidu solide. De cette façon, il devrait être possible de maximaliser la récupération des molécules anti-oxydantes par rapport à la MAP-raffinerie originale, ce qui donnera plus de valeur ajoutée à ces plantes aromatiques et médicinales du fait de la forte demande en molécules anti-oxydantes naturelles par les industries agro-alimentaire et cosmétique.

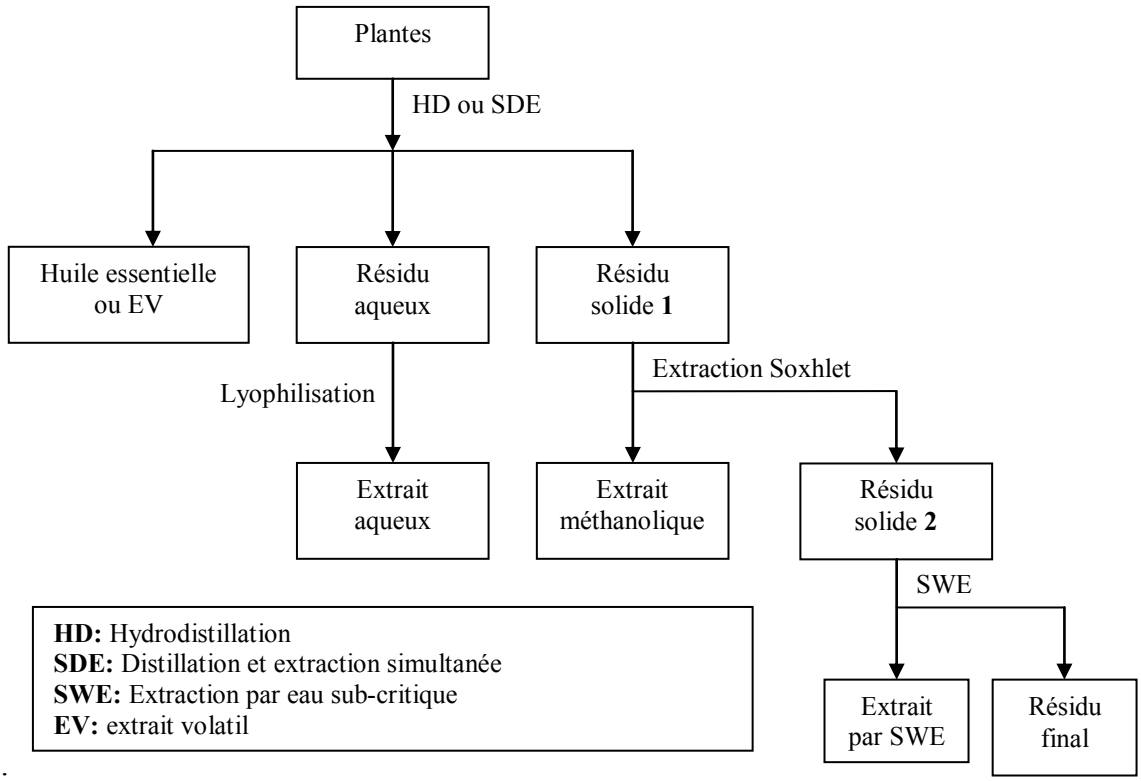

Figure 5 Concept de MAP-raffinerie améliorée

Geranium robertianum L. a été choisie comme plante modèle pour étudier la faisabilité de la MAP-raffinerie améliorée. Les parties aériennes de *Geranium robertianum* L. ont été soumises à l'hydrodistillation en vue d'obtenir un extrait volatil tandis que le r sidu aqueux a été recueilli et lyophilisé pour obtenir l' extrait aqueux. Le résidu solide **1** a ensuite été extrait par du méthanol pour obtenir l'extrait méthanolique. Puis l'extraction par eau sub-critique du résidu solide **2** a été réalisée à 170 °C et 3 cycles (15 min), conditions expérimentales optimales pour l'extraction de l'eau sub-critique des composés phénoliques directement à partir de parties aériennes de *Geranium robertianum* L. La capacité anti-oxydante des différents extraits a été testée par le test DPPH et la teneur totale en phénols des extraits a été calculée par le test Folin-Ciocalteu.

Les résultats montrent que la MAP-raffinerie améliorée a considérablement augmentée la récupération en composés anti-oxydants par rapport à MAP-raffinerie originale. Ce procédé prometteur devrait permettre à n'en pas douter une meilleure valorisation du potentiel en molécules actives (huile essentielle et molécules anti-oxydantes) des plantes aromatiques.

6. Conclusion

Au cours de ce travail de thèse, le concept de MAP-raffinerie a été créé et appliqué à une sélection de plantes issues de deux régions française et chinoise (Midi-Pyrénées et Chongqing). Plusieurs technologies d'extraction utilisant l'eau comme solvant vert (hydrodistillation, entrainement à la vapeur et extraction par eau sub-critique) ont ainsi été mises en oeuvre et leur impact tant sur la composition des huiles essentielles que sur la récupération des molécules anti-oxydantes a été évalué.

Dans un premier temps, une liste de plantes aromatiques et médicinales oubliées, voire sous-utilisées dans les deux régions a été établie selon des règles de sélection prédéfinies. Six plantes modèles de la région de Midi-Pyrénées (*Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Geranium robertianum* L., *Cytisus scoparius* L. et *Spartium junceum* L.) et trois plantes de la région de Chongqing (*Tussilago farfara* L., *Fructus aurantii* et *Saussurea lappa*) ont finalement été retenues.

Puis, le concept de MAP-raffinerie a été appliqué à ces plantes afin d'étudier leur possible valorisation globale. L'étude des compositions chimiques des extraits volatils des racines de *Tussilago farfara* L. et de *Calendula arvensis* L., ainsi que des boutons de fleurs de *Spartium junceum* L. a été réalisée par GC et GC-MS pour la première fois.

Par ailleurs, les r sultats de l' valuation des capacit s anti-oxydantes des extraits (par les tests DPPH, ABTS, FRAP, ORAC et Folin-Ciocalteu) ont montrés que plusieurs plantes comme *Cytisus scoparius L.*, *Tussilago farfara* L., *Fructus aurantii* ou *Robinia pseudoacacia* L. pourraient être des sources potentielles de molécules anti-oxydantes naturelles.

D'un point de vue technologique, les comparaisons de l'utilisation de l'hydrodistillation (HD), de la distillation à la vapeur (SD) et de l'extraction par eau sub-critique (SWE) ont montrées que si la HD et la SD ont des effets limités sur la composition des huiles essentielles, la HD semble être une méthode plus efficace pour la récupération des composés anti-oxydants à partir des résidus de distillation que la SD tandis que la SWE s'avère être une technologie prometteuse pour l'extraction directe de ces mol cules à partir des plantes.

Si la composition minérale de l'eau lors de l'hydrodistillation n'a que des effets très limités sur les rendements d'extraction, les teneurs en ions calcium et bicarbonate des eaux ont par contre des effets significatifs décroissants sur la capacité anti-oxydante et sur la teneur totale en phénols tant des extraits aqueux que méthanoliques.

De ce fait, au vue de ces résultats, un concept de MAP-raffinerie améliorée a été développé en intégrant une étape d'extraction à l'eau sub-critique des résidus d'extraction solides primaire en vue d'une meilleure extraction des composés anti-oxydants. Selon ce nouveau concept, cinq extraits peuvent être obtenus à partir de la matière végétale: un extrait volatil, un extrait aqueux, un extrait m thanolique, un extrait à l'eau sub-critique et in fine un résidu solide. Les premiers résultats ont montrés que la "MAP-raffinerie améliorée" augmente de manière significative la récupération des molécules anti-oxydantes par rapport à la MAP-raffinerie originale tout en permettant d'envisager une valorisation plus facile du résidu solide en agro-matériaux ou biomateriaux, du fait de sa faible teneur en eau résiduelle.

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Abstract

In both Midi-Pyrénées region (France) and Chongqing region (China), there are rich and underutilized medicinal and aromatic plants (MAP). Aiming at fully exploiting different molecules in these plants, the concept of MAP-refinery was developed and applied to several underutilized medicinal and aromatic plants in these two regions. Several water-based green extraction technologies of natural products (e.g. hydrodistillation, steam distillation and subcritical water extraction) were also investigated to look at their effects on essential oil composition and antioxidants recovery from selected plants.

Firstly, lists of forgotten or underutilized medicinal and aromatic plants in both regions were established according to the rules of selection. From the lists, six plants in the Midi-Pyrénées region (*Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Geranium robertianum* L., *Cytisus scoparius* L. and *Spartium junceum* L.) and three plants in the Chongqing region (*Tussilago farfara* L., *Citrum aurantium* L. and *Saussurea costus*) were finally selected for investigations.

Then the MAP-refinery was applied to the selected plants in two regions in order to realise their global valorisation. Volatile extracts composition in the roots of *Tussilago farfara* L. and *Calendula arvensis* L., as well as flower buds of *Spartium junceum* L. were firstly investigated. The main chemical compounds in volatile extract from *Tussilago farfara* L. roots were sesquiterpene hydrocarbons and aliphatic compounds while main chemical compounds in volatile extract from *Calendula arvensis* L. roots were oxygenated sesquiterpenes, oxygenated monoterpenes and oxygenated diterpenes. The volatile extract from flower buds of *Spartium junceum* L. was mainly composed of aliphatic compounds. Antioxidant capacity evaluation results (by DPPH, ABTS, FRAC, ORAC and Folin-Ciocalteu tests) showed that several plant samples like *Cytisus scoparius* L., *Tussilago farfara* L., *Citrum aurantium* L. and *Robinia pseudoacacia* L. could be potential sources of natural antioxidants.

Comparisons of hydrodistillation (HD), steam distillation (SD) and subcritical water extraction (SWE) showed that HD and SD had limited effects on essential oil composition but HD, SD and SWE had significant impacts on the recovery of antioxidants. Hydrodistillation seemed to be a better method for recovery of antioxidant compounds from residues of distillation than steam distillation. However, SWE appeared to be a more efficient method for direct extraction of antioxidant molecules (or phenolic compounds) from plants. In the hydrodistillation process, mineral contents in water were found to have very limited effects on yields of extracts but calcium and bicarbonate ions, had significant decreasing effects on antioxidant capacity and total phenolic content of both aqueous and methanolic extracts.

Finally, an improved MAP-refinery was developed. Subcritical water was used for further extraction of antioxidant compounds from residues in original MAP-refinery. In this way, five parts could be obtained from plant materials: volatile extract, aqueous extract, methanolic extract, subcritical water extract and the final residue. The results showed that the improved MAP-refinery significantly increased the recovery of antioxidants compared with original MAP-refinery. This promising process will also allow a better valorisation of the final solid residue due to the lower content of residual water.

Key words

Medicinal and aromatic plants, MAP-refinery, Hydrodistillation, Steam distillation, Simultaneous distillation extraction, Antioxidant capacity, Essential oil, Subcritical water extraction, Phenolic compounds, Deryng apparatus, Clevenger apparatus, Volatile extract, Aqueous extract, Methanolic extract, Improved MAP-refinery

Résumé

Les régions de Midi-Pyrénées (France) et de Chongqing (Chine) sont riches en plantes aromatiques et médicinales dites oubliées (ou médiévales). Afin de valoriser pleinement les différentes bio-molécules extractibles de ces plantes, le concept de MAP-raffinerie a été créé et appliqué à une sélection de plantes issues de ces deux régions. Plusieurs technologies d'extraction utilisant l'eau comme solvant vert (hydrodistillation, distillation à la vapeur et extraction par eau sub-critique) ont ainsi été employées et leur impact tant sur la composition des huiles essentielles que sur la récupération des molécules anti-oxydantes a été évalué.

Dans un premier temps, une liste de plantes aromatiques et médicinales oubliées, voire sous-utilisées dans les deux régions a été établie selon des règles de sélection prédéfinies. Six plantes modèles de la région de Midi-Pyrénées (*Tussilago farfara* L., *Calendula arvensis* L., *Robinia pseudoacacia* L., *Geranium robertianum* L., *Cytisus scoparius* L. et *Spartium junceum* L.) et trois plantes de la région de Chongqing (*Tussilago farfara* L., *Citrus aurantium* L. et *Saussurea costus*) ont finalement été retenues.

Puis, le concept de MAP-raffinerie a été appliqué à ces plantes afin d'étudier leur possible valorisation globale. L'étude des compositions chimiques des extraits volatils des racines de *Tussilago farfara* L. et de *Calendula arvensis* L., ainsi que des boutons de fleurs de *Spartium junceum* L. a été réalisée par GC et GC-MS pour la première fois. Les principaux composés chimiques dans l'extrait volatil de racines de *Tussilago farfara* L. étaient des hydrocarbures sesquiterpéniques et des composés aliphatiques tandis que les principaux composés chimiques dans l'extrait volatil de racines de *Calendula arvensis* L. étaient des sesquiterpènes oxygénés, des monoterpènes oxygénés et des diterpènes oxygénés. L'extrait volatil de boutons de fleurs de *Spartium junceum* L. était principalement composé de composés aliphatiques.

Par ailleurs, les résultats de l'évaluation des capacités anti-oxydantes des extraits (par les tests DPPH, ABTS, FRAP, ORAC et Folin-Ciocalteu) ont montrés que plusieurs plantes comme *Cytisus scoparius L.*, *Tussilago farfara* L., *Citrus aurantium* L. ou *Robinia pseudoacacia* L. pourraient être des sources potentielles d'anti-oxydants naturels.

D'un point de vue technologique, les comparaisons de l'utilisation de l'hydrodistillation (HD), de la distillation à la vapeur (SD) et de l'extraction par eau sub-critique (SWE) ont montrées que si la HD et la SD ont des effets limités sur la composition des huiles essentielles, la HD semble être une méthode plus efficace pour la récupération des composés anti-oxydants à partir des résidus de distillation que la SD tandis que la SWE s'avère être une technologie prometteuse pour l'extraction directe de ces molécules à partir des plantes.

Si la composition minérale de l'eau lors de l'hydrodistillation n'a que des effets très limités sur les rendements d'extraction, les teneurs en ions calcium et bicarbonate des eaux ont par contre des effets décroissants significatifs sur la capacité anti-oxydante et sur la teneur phénolique totale des extraits aqueux et méthanoliques.

Au vue de ces résultats, un concept amélioré de MAP-raffinerie a été développé en intégrant une extraction à l'eau sub-critique pour l'extraction des composés anti-oxydants des résidus d'extraction primaire. Selon ce nouveau concept, cinq extraits peuvent être obtenus à partir des matières végétales: un extrait volatil, un extrait aqueux, un extrait méthanolique, un extrait à l'eau sub-critique et in fine un résidu solide. Les premiers résultats ont montrés que la "MAP-raffinerie améliorée" augmente de manière significative la récupération des anti-oxydants par rapport à la MAP-raffinerie originale et permet d'envisager une valorisation plus facile du résidu solide en agro-matériaux du fait de sa faible teneur en eau résiduelle.

Mots clés

Plantes aromatiques et médicinales, MAP-raffinerie, Hydrodistillation, Distillation à la vapeur, Distillation et extraction simultanée, Capacité anti-oxydante, Huile essentielle, Extraction par eau sub-critique, Composés phénoliques, Appareil Deryng, Appareil Clevenger, Extrait volatil, Extrait aqueux, Extrait méthanolique, MAP-raffinerie améliorée