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Department of Food Science
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-Ph.D. Thesis-

Setting up qualitative strategies for screening the composition of marker fractions from various wild growing plants in Friuli

by

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~To my parents~

Abbreviations and symbols

ANOVA	Analysis Of Variance
$AlCl_3$	aluminium chloride
a.s.l.	above sea level
$CHCl_3$	Chloroform
CH_2Cl_2	dichloromethane
CH_3COONa	sodium acetate
CH_3COCl meth.	acetyl chloride in methanol
CID	collision-induced dissociation
CV	coefficient of variation
CNG	cyanogenic glycoside
Da	Dalton
DOE	design of experiments
EI	electron impact
EIC	extracted ion chromatogram
ELSD	evaporative light scattering detector
ESI	electrospray ionization (MS)
EtOH	ethanol
GC-MS	gas chromatography
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography coupled with mass spectrometry
HPLC/UV-DAD	high performance liquid chromatography coupled with ultraviolet photodiode array detector
HMDS	hexamethyldisilazane
HS-SPME	head space solid phase microextraction
Hz	Hertz
i.d.	internal diameter
IT	ion trap
KOH meth.	Potassium hydroxide in methanol

LC	liquid chromatography
m/z	mass to electronic charge
MAE	microwave-assisted extraction
MeCN	acetonitrile
MeOH	methanol
MHz	megahertz
MS	mass spectrometry
MS^n	multiple stage MS
MW	molecular weight
NaOH	sodium hydroxide
ppm	parts per million
r. t.	room temperature
RP_{18}	Reversed Phase Silica with C-18 functional groups
RSD	relative standard deviation
sp	species (one)
spp	species (several)
SD	standard deviation
SL	sesquiterpene lactone
SPE	solid phase extraction
TFA	trifluoroacetic acid
TIC	total ion chromatogram
TMS	tetramethylsilane
TOF	time of flight
UAE	ultrasound-assisted extraction
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet
W	watt
λmax	wavelength of absorption maxima (nm)

Abstract

In view of developing qualitative strategies for screening the composition of marker fractions from various wild growing plants (*Cicerbita alpina, Asparagus acutifolius, Chenopodium bonus henricus, Levisticum officinale, Silene vulgaris and Spirea aruncus*), a working plan was previously set up. Since little amount of plant material and phytochemical data were available, the main challenge of the present investigation was to create working strategies designed to generate a valuable composition overview of the investigated plants. The selection of the wild species that are protected by two regional laws was done within a regional research project on the basis of their traditional culinary use in Friuli Venezia Giulia, a region in the North-eastern Italy. For this purpose, the present preliminary study concerned two main aspects: a rapid microwave-assisted total phenolics extraction by means of a design of experiments approach meant to evaluate the proper experimental conditions that allow toobtain the richest phenolic fraction; a reasonable use of various analytical tools able to address valuable information about the qualitative composition of the crude extracts. This study was conducted as a basic imperative part towards a more in-depth study and used an extended work strategy applied on one of the most commercially important plant species within the project. The proposed framework stood as reference for the final selective strategies applied on the other extracts. Moreover, in order to get more valuable data, several extracts from the same species, obtained by different

extraction techniques were submitted to several qualitative composition comparisons. The choice of the parameters and their ranges were established in accordance with literature data and were successfully applied to all the other plant material. Furthermore, due to their complementary specificity, the two different analytical tools, that were LC and GC-MS, were used to get a wide coverage of metabolites. With respect to the present literature data, several compounds belonging to various structural classes were detected and/or unambiguously identified by selective algorithms for the first time in the six plants.

Riassunto

E' stato messo a punto un piano di lavoro per la valutazione qualitativa e lo screening della composizione di frazioni marker di una serie di piante spontanee: *Cicerbita alpina, Asparagus acutifolius, Chenopodium bonus henricus, Levisticum officinale, Silene vulgaris e Spirea aruncus*. Poichè erano disponibili piccole quantità di materiale e i relativi dati fitochimici erano scarsi, l'obiettivo principale di questo studio è stato quello di mettere a punto strategie di lavoro tali da ottenere una stima significativa della composizione delle piante oggetto di studio. La selezione delle specie spontanee che sono protette da due leggi regionali è stata compiuta all'interno di un progetto regionale sulla base del loro uso nella cucina tradizionale del Friuli Venezia Giulia, regione situata nel nord est dell'Italia. Per questa ragione, il presente studio preliminare ha riguardato due principali aspetti: una rapida ed efficace estrazione dei composti fenolici attraverso le microonde sulla base di un disegno sperimentale per la valutazione delle condizioni sperimentali migliori che hanno consentito di ottenere frazioni fenoliche arricchite; un ragionevole uso dei metodi analitici in grado di fornire importanti informazioni riguardo la composizione qualitativa degli estratti grezzi. Questo studio è stato condotto come una parte preliminare che presuppone studi più approfonditi. La struttura proposta diventa un modello di riferimento da applicare per altri estratti. Inoltre, al fine di ottenere dati piu' significativi, molti estratti della stessa specie, ottenuti da differenti tecniche di estrazione, sono stati confrontati dal punto di vista qualitativo. La scelta dei parametri e dei loro range è stata fatta sulla base di dati di letteratura ed è stato applicata con successo a tutte le altre piante. Inoltre, a causa della loro

specifica complementarietà, le due tecniche analitiche (GC e LC-MS) sono state usate per individuare un largo numero di metaboliti. Sulla base dei dati di letteratura, molti composti appartenenti a diverse classi strutturali sono stati individuati e identificati per la prima volta in sei piante attraverso algoritmi discriminatori.

CONTENTS

I. Aim

The main goal of the present study was setting up a qualitative workflow analytical strategy applied to little investigate crude extracts obtained from aerial parts of different wild plant species grown mostly in alpine habitats (*Cicerbita alpina, Asparagus acutifolius, Chenopodium bonus henricus, Levisticum officinale, Silene vulgaris and Spirea aruncus*). These plants are used in the traditional gastronomy of Friuli Venezia Giulia, a region of the North-eastern part of Italy. This research was supported by "Bioinnoverbe" Regional (Friuli Venezia Giulia) research Project, regional Law on innovation n. 26/05, art. 17. In order to preserve the environmental flora of this region, these plants have undergone to special biological agronomical techniques. The selection criteria of the species has been made accordingly to their traditional culinary use and to their commercial value due to the limited diffusion safeguarded by two Friuli Venezia Giulia regional laws: of June the 3rd 1981, N.34, integrated by the law 19th of August 1996, N. 32 with following modifications. The traditional use together with the need to build up a phytochemical profile, have arisen the interest to initializing a metabolite profiling study, since little literature data has been reported so far on them. It should be stressed out that while previous studies reported information mostly regarding the sub-aerial parts of these species, the present investigation focuses exclusively on the aerial edible parts. Thus, common characteristics of the aerial parts could be underlined.

The most common class of secondary metabolites with a large diffusion within the aerial parts of plants are phenolic compounds. Firstly, the study was therefore conducted in order to evaluate their presence in the investigated plant extracts and secondly to detect the presence of other classes of metabolites. The preliminary challenge of this study was to obtain the richest phenolic profile possible from our samples despite the modest amount of plant material available. Even though the investigations were addressed to secondary metabolites, the analytical methods employed covered the detection of some primary metabolites as well. Every rigorous study on plant extracts starts with two crucial points: an up to date literature survey related to the research topic and a representative amount of plant material that has to be investigated.

Such studies are often being limited by the lack of the aforementioned basic requisites, as the present study is. What can be done if there is a lack of the promoting prerequisites? Further on, it is presented a multitask screening strategy meant to overcome these drawbacks by means of appropriate sample work-up techniques, such as non-conventional extraction techniques, various sample preparation approaches and analytical techniques, such as HPLC-UV, LC-MS, UHPLC-TOF-MS and GC-MS. The proposed analytical approaches reveal a broad scale capacity for detecting different categories of secondary metabolites of interest in crude extracts even though the goal was not directed towards an exhaustive study. Every step of the proposed strategy was dictated by the imperative necessity to use limited amounts of plant material and the goal to qualitatively screen their crude extracts composition, highlighting the

characteristic marker fraction profile revealed by various analytical approaches. Such preliminary steps are crucial for any subsequent in-depth study of plant matrices lacking of phytochemical data.

Introduction

II. Introduction

II.1 Primary and secondary metabolites in plants. Fundamentals of plant metabolomics.

Plants produce a diverse and complex assortment of organic compounds, the great majority of which do not participate directly in growth and development. Plants synthesize a complex array of compounds with a variety of physiological roles that are collectively referred to as secondary metabolites or natural products. Knowledge about plant's chemical composition provides useful information highlighting the importance of developing comprehensive analytical methods capable of screening a wide range of metabolites. The compounds present in plants are conveniently divided into two major groups: primary and secondary metabolites. Metabolites are the end products of cellular processes, and their levels represent the ultimate response of biological systems to genetic or environmental changes[1]. Primary metabolites are those produced by and involved in primary metabolic pathways such as respiration and photosynthesis. These components include processes such as glycolysis, the Calvin-Benson cycle, and the Krebs cycle and are virtually identical throughout the plant kingdom: they are mainly sugars, amino acids, organic acids, proteins, nucleic acids, and polysaccharides of plant cells[2]. Bioactive compounds extracted from plants are used as pharmaceuticals, flavor and fragrance ingredients, food additives, and pesticides. About 200,000 metabolites have been elucidated in plants[1], most of them having unknown function[3]. The secondary metabolites are known to play a

major role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals[4].
Secondary metabolites have no known function in the primary metabolism of plants. Formerly, they were considered to be a means of depositing excess carbon fixed by photosynthesis and have long been considered as defenses against pathogens or herbivores[5].
To distinguish these compounds from primary metabolites, in 1891, Kossel introduced the concept of "secondary metabolites", which are not necessary for the growth, survival or reproduction of their producers. In general, the terms plant secondary compounds, phytochemicals, antinutritional factors, and plant xenobiotics have been used in the literature to define this group of compounds [6]. Plant secondary metabolite is a generic term used for different substances which are exclusively produced by plant and stored in specific vesicles, such as pigments, alkaloids, phenolics, steroids, terpenoids, toxins, polymeric substances and so on. In a simplistic way, they can be classified in three main groups: the terpenes (originating from mevalonic acid, usually consisting of carbon, hydrogen and oxygen), phenolics (derivatives containing a phenolic unit), and nitrogen-containing compounds. These components are usually generated during a part of the vegetative cycle of the plant. Many secondary compounds possess signaling and hormone functions. They influence the activities of other cells, control their metabolic activities and co-ordinate the development of the whole plant. Other substances like flower colors serve to communicate with pollinators or protect the plants from feeding by animals or infections. Some plants produce specific phytoalexines after fungi infection that inhibits the spreading of the fungi mycelia within the plant. A number of substances is secreted and

influences the existence of other species. In contrast, primary metabolites (carbohydrates, fats, proteins, vitamins and mineral nutrients) are found in all living organisms because they perform essential functions in growth and development. Taiz defines the primary metabolism as a system that encompasses reactions involving those compounds which are formed as a part of the normal anabolic and catabolic processes[7]. These processes take place in most, if not all, cells of the organism. Secondary metabolites are grouped into chemical classes based on similar structures and common biosynthetic pathways. Figure 1[8] presents the main primary and secondary metabolic pathways occurring in plants.

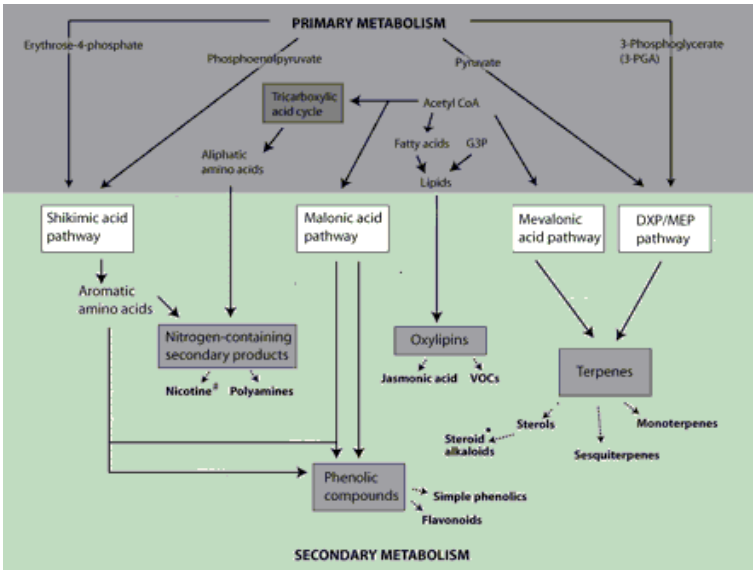


Figure 1 *Primary and secondary metabolism pathways in plants*
Source: Adapted from Schmidt D. et. al Plantphysiol. 2005, 138, 1763-1773.

While primary metabolites have identifiable functions and play known roles in physiological processes, the role of the

secondary metabolites remains partially unknown in the host organism. Besides the importance for the plant itself, metabolites influence the nutritional quality of food, color, taste and smell. Beside these, they possess also antioxidative, anticarcinogenic, antihypertension, anti-inflammatory, antimicrobial, immunostimulating, and cholesterol-lowering properties. Plants, being nature's most prolific biochemists [9], are a perpetual source of inspiration for industrial chemists in their research for novel bioactive molecules that can inspire the development of new synthetic drugs for the pharmaceutical industry, or biocides such as fungicides and insecticides for use in agriculture[10]. Recent advances in high-throughput techniques and technology has changed how metabolic processes are studied. Previously, most analytical methods were targeted to a limited group of metabolites usually on the basis of separation technology for a specific chemical class of compounds. However, the emergence of non-targeted analytical methods breaks this limitation, and now many different metabolites of different metabolic origins and chemical properties can be evaluated simultaneously from a single sample extract.

The measurement of all the metabolites in a given system is known as metabolomics and it provides primary information about biological responses to physiological or environmental changes. Metabolomics is a term coined at the end of the 1990s by Oliver et al.[11] and it deals with the separation, detection and quantification of 'all' metabolites in a sample using a platform of complementary technologies such as gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography (LC) coupled to mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) which have been applied to many areas of plant sciences so

far[12]. The MS based metabolite techniques covers a wide range of detectable metabolites, as it is shown in Figure 2[13].

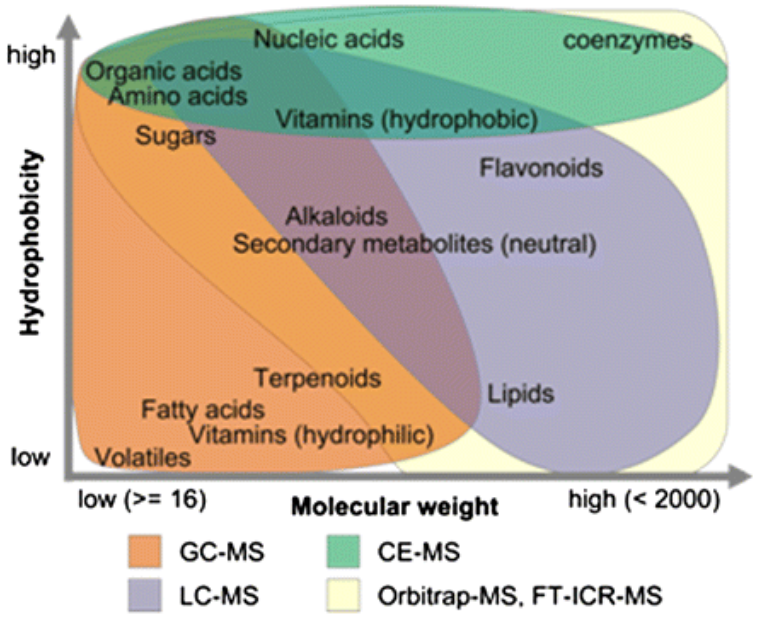


Figure 2 *Conceptual coverage of metabolite detection using MS-based metabolite techniques*
Source: Kusano M. et al, J.Exp. Bot 2011

In addition, metabolomics constitutes an integral part of system biology and extensive studies have been performed in various species such as microorganisms[14, 15] plants[9, 16, 17] and human[18]. Fiehn et al. proposed three different approaches within metabolomics: metabolite target analysis, metabolite profiling and metabolic fingerprinting[1]. Metabolic profiling is the most conventional approach that focuses on the analysis of a specific group of metabolites related to a metabolic pathway or class of compounds. The main feature of the metabolic profiling approach

implies a hypothesis-driven strategy rather than a hypothesis-generating one[19].

Metabolic fingerprinting is a non-targeted screening method which aims the high throughput analysis of a large number of samples. The initial goal is generally sample comparison and discrimination analysis.

Targeted analysis regards both qualitative and quantitative detection of chosen metabolites that have similar properties (e.g. amino acids) or share a common biosynthetic pathway (e.g. flavonoids). Such study requires a dedicated sample preparation and specific analytical methods applied on a certain part of the sample, in order to acquire a sensitive detection.

Nevertheless, the metabolic experiment implies not only the choice of a suitable combination of analytical techniques, but also a rigorous sample treatment. For this purpose, experimental designs are being used. In 2004, Bino et al proposed the Minimum Information about a Metabolomics Experiment (MIAMET), which should be reported in every metabolomics study, with the aim to facilitate the exchange of information among researchers[20]. The main steps that should be followed are:

- Sampling
- Sample preparation
- Sample analysis
- Data export
- Data analysis

Due to its complexity, metabolomics find applications in several directions, from medical science to agriculture. Discovery of biomarkers related to a physiological reaction, the early detection of diseases[21], medical diagnosis[22] and testing drugs[23] by evaluating the effects of metabolic

modifications or toxicity[24, 25] are just a few of the numerous applications. Indeed, metabolomics has an increasingly interest in the nutrition research field[26], such as food composition analysis.

II.2 Investigated plants

The framework of this subchapter consists in the pharmacognostic profile of the studied herbs, their traditional use and a brief phytochemical characterization based on the existing literature. Since they are used in the traditional gastronomy, the interest is focused on the aerial edible parts of these herbs.

II.2.1 *Cicerbita alpina* (L.) Wallr (Asteraceae)

Family

The Asteraceae or Compositae family (the sunflower family) belongs to the Asterales order and represents the largest family of flowering plants, comprising about 1,100 genera and 20,000 species which comprise herbaceous members, few shrubs and trees that are characterized by having the flowers reduced and organized into an involucrate[27]. The leaves are alternate, opposite or whorled; stipules are absent. The flowers which give this plant family its original name of Compositae are or made up of many individual flowers[28]. These flowers may be regular (disc florets), with all the petals the same size, or irregular (ray florets), with some petals larger than others. This family is ecologically and economically of great importance. Members of the family occur from the Polar Regions to the tropics, and may range over all habitats from dry desert to swamp, and from rainforest to mountain peaks. Together with other 34 species, *Cicerbita alpina* (L.) Wallr is a species that belongs to the genus Cicerbita.

Synonims: *Mulgedium alpina* (L.) Less., *Lactuca alpina* (L.) A. Gray, *Lactuca spicata* (Lam.) Hitchc, *Sonchus alpinus* L., *Sonchus spicatus* Lam.
Common name: Alpine Blue Sow Thistle

II.2.1.1 Botanical description

Figura 3 *Cicerbita alpina* L. *(*Asteraceae*)*

The Alpine Sow Thistle (Figure 3) is a species of rich soils that grows on hills and mountains. It prefers damp microclimatic conditions. It is a perennial herb, 50-250 cm high with attractive blue-flowers. In late summer its pale blue-violet, dandelion-like flower heads open in tight clusters at the top of the stem. *C. alpina* has an erect stem 50-250 cm, simple or branched. Alternate leaves, glabrous and glaucous, are 8–25 x 2-12 cm. Flower head is an elongated panicle and the peduncles with dense reddish glandular hairs. Involucre is 10-15 x 7-10 mm and petals are pale blue[29].

II.2.1.2 Chemical composition: literature review

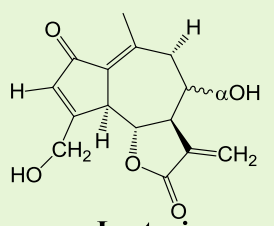
Table 1 sums up the main classes of secondary metabolites isolated and identified in *C. alpina* which have been reported in the literature so far: the sesquiterpene lactones, furanocoumarins and phenolic compounds. Table 2 reports the chemical structures of the compounds belonging to the three classes of secondary metabolites.

Table 1 Secondary metabolites occurring in *C. alpina*

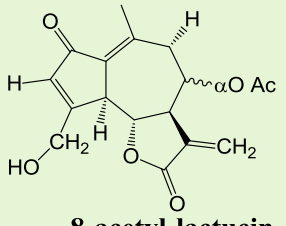
Plant organ	Extraction	Plant collection: Time, place, altitude	Isolated compounds	Chemical class of metabolites	References
Air dried Subaerial parts	MeOH	2002, Tyrol/Austria, 1900 m	8-acetyl-15β-D-glucopyranosyllactucin Sonchuside A	Sesquiterpene lactone (guaiane type) Sesquiterpene lactone (non guaiane type)	30, 31
Air dried root	EtOH	-	8-acetyl-lactucin 8-acetyl-11β,13-dihydrolactucin Lactucin 11β, 13-dihydrolactucin	Sesquiterpene lactones	32
Freeze-dried shoots	MeOH, $(CH_3)_2CO$ H_2O	Frisanchi (Centa, TN, Italy), 1078 m Monte Peller (Cles, Trento, Italy) 1950 m	8-O-Acetyl-15-β-D-glucopyranosyllactucin chlorogenic acid 3,5-dicaffeoylquinic acid Caffeoyltartric acid cichoric acid	Sesquiterpene lactone Phenolic acids	33
Dried roots Dried leaves	EtOH	1989, Pian della Mussa (Turin, Italy)	Imperatorin Isoimperatorin Oxypeucedanin Ostruthol 11β, 13-dihydrolactucin 8-Acetyl-15-β-D glucopyranosyllactun	Furanocoumarins Sesquiterpene lactones	34

Table 2 Short overview of the main classes of secondary metabolites within *C. alpina*

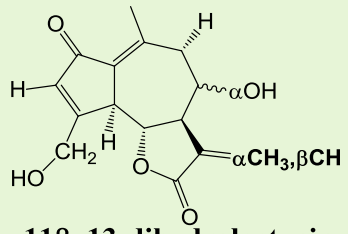
Brief description of the main classes secondary metabolites identified in *C. alpina*
Sesquiterpene lactones (SLs) • **SLs** constitute a large class of plant terpenoids which are biologically active plant chemicals. The biosynthetic basis for the terpene nomenclature is determined by the number of five-carbon isoprene units incorporated into the carbon skeleton. The condensation of three isoprene units and subsequent cyclization and oxidative transformation produce a *cis* or *trans*-fused lactone. These secondary compounds are primarily classified on the basis of their carbocyclic skeletons. The biosynthetically simplest sesquiterpene is farnesyl pyrophosphate, an unsaturated linear molecule which feeds into several alternative pathways, generating the major subclasses of sesquiterpene: guaianalides, germanocranolides, eudesmanolides etc[35]. SLs exhibit variety of skeletal arrangements. An individual plant species generally produces one skeletal type of SLs concentrated predominantly leaves and flower heads. • They are known to possess a series of biological and pharmacological activities such as antiviral, antimicrobial, cytotoxic, anti-inflammatory, antibacterial, antifungal activities, effects on the central nervous and cardiovascular systems as well as allergenic potency. Experimental studies suggest a role for SLs in reducing herbivore pressure as well[36,37,38]. These compounds are toxic to a variety of insects and can be shown experimentally to deter insect predation at concentrations equivalent to those commonly found in plant tissues[39,36]. The SLs serve as deterrents to grazing sheep and cattle, and in some cases are responsible for severe livestock losses. They have also demonstrated microbial growth inhibition, contact dermatitis initiation, and allelopathy[36, 40].



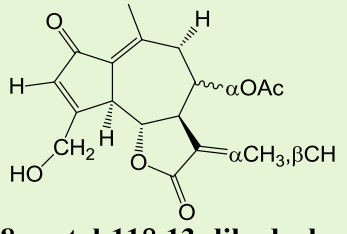
Lactucin



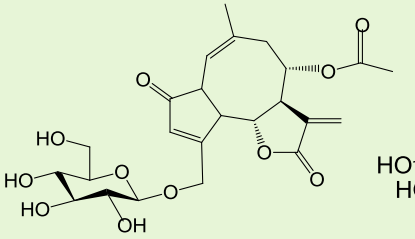
8-acetyl-lactucin



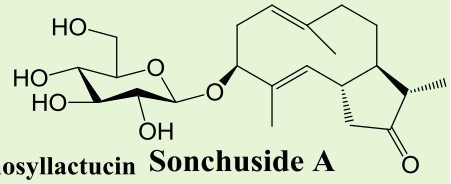
11β, 13-dihydrolactucin



8-acetyl-11β,13-dihydrolactucin



8-acetyl-15β–D-glucopyranosyllactucin



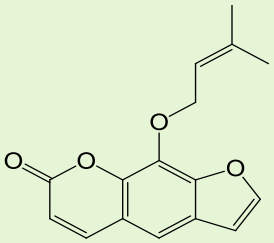
Sonchuside A

Furanocoumarins

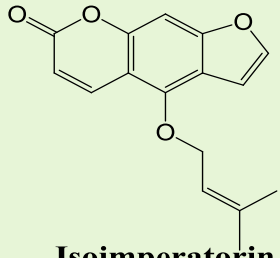
• **Furanocoumarins** are natural occurring compounds that consist of a five-membered furan ring attached to the coumarin nucleus, divided to linear and angular types with substituents at one or both of the remaining benzenoid positions. The furan may be fused to the coumarin in different ways, producing several isomers. The biosynthesis of furanocoumarins involves contributions from two pathways, the phenylpropanoid pathway and the mevalonic acid pathway. The immediate precursors for furanocoumarin synthesis are umbelliferone (7-hydroxycoumarin) and isoprene. Two categories of furanocoumarins are produced: the linear

furanocoumarins have the furan ring in line with the benz-2-pyrone nucleus, while the angular furanocoumarins have the furan ring oriented at an angle to the nucleus.

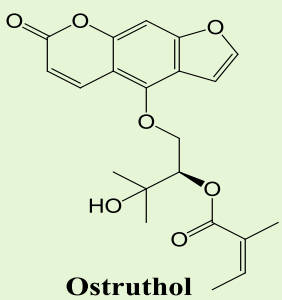
• Are considered to constitute a plant protection system against pests and adverse physicochemical factors such as desiccation, mechanical injuries, etc. In the presence of long-wave ultraviolet (UV) light, furanocoumarins yield transformation products that can interact with DNA to form mono- and di-adducts responsible for phytophotodermatitis in humans. In addition to their phototoxicity[41,42] furanocoumarins may demonstrate antifungal[43] and insecticidal[44] activities.



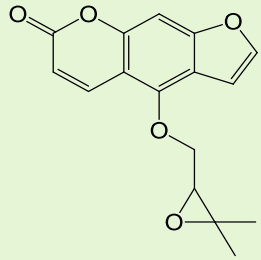
Imperatorin



Isoimperatorin



Ostruthol



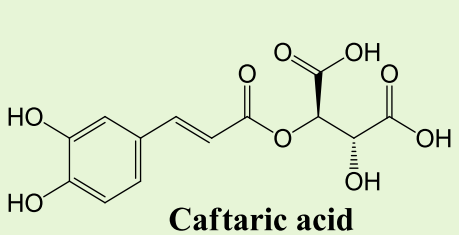
Oxypeucedanin

Phenolic compounds

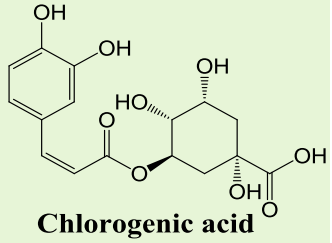
• **Phenolic compounds** are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways in plants[45]. These compounds are one of the most widely occurring groups of phytochemicals. Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents and range from simple

phenolic molecules to highly polymerized compounds[46].

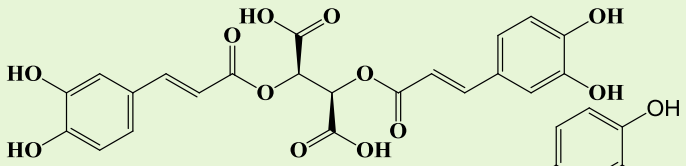
• Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, antiatherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects[47, 48, 49].



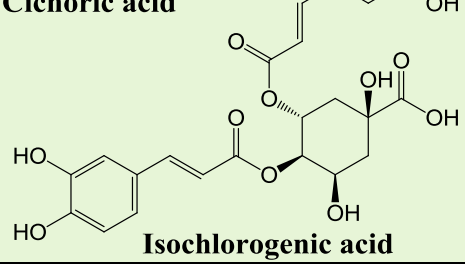
Caftaric acid



Chlorogenic acid



Cichoric acid



Isochlorogenic acid

II.2.1.3 Traditional use

In the traditional gastronomy of Friuli Venezia Giulia region, the fresh edible shoots of *C. alpina* are being used either as fresh vegetable for salads, cooked or preserved in oil. No traditional pharmacological properties have been reported so far.

II.2.2 *Asparagus acutifolius* L. (Liliaceae)

Family

The Liliaceae family (Liliales order) is extremely complex and botanists estimate 250 genera and 4,000 to 6,000 species worldwide. It consists mostly of perennial herbs, rarely of shrubs. Some species are edible while others are very poisonous. This family consists mainly of herbs with bulbs, rhizomes or tubers, long thin leaves that sprout from the ground. Flowers are hermaphrodite and the fruit is variable, fleshy or dry. Several species are cultivated as food: onions, shallots, garlic, green onions and leeks. Leaves are simple, alternate or whorled, often all basal, never very strong or very succulent, not persistent from year to year. Some species show spine-reduced leaves, like in asparagus sp. The genus *Asparagus* comprises up to 300 species. *Asparagus acutifolius* L. is a dioecious and native plant species, widely distributed in the Mediterranean Basin[50]. *Asparagus acutifolius* L., produces edible spears used in local diets of Mediterranean countries.

Synonim: Asparagus corruda Scop. , Asparagus ambiguus De Not. , Asparagus commutatus Ten.

Common name: Wild asparagus

II.2.2.1 Botanical description

Figure 4 *Asparagus acutifolius* L. (Liliaceae)

A. acutifolius is an armed climbing undershrub with woody stems and recurved or rarely straight spines. The tuberous succulent roots are 30 cm to 100 cm or more in length. Young stems are delicate, brittle and smooth. Leaves are reduced to minute chaffy scales and spines. Flowers are dioecious. In some Mediterranean regions flowering occurs in late summer from August through September. These plants grow near woods and in uncultivated places on dry and sunny soil. They can be found at an altitude of 0–1.300 meters above sea level.

II.2.2.2 Chemical composition: literature review

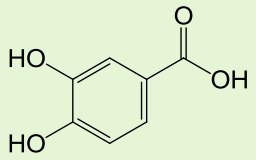
Asparagus roots contain protein 22%, fat 6.2%, Carbohydrate 3.2%, Vitamin B 0.36%, Vitamin C 0.04% and traces of Vitamin A. It contains several alkaloids and a number of antioxytocic saponins[51]. Leaves contain rutin, diosgenin and a flavonoid glycoside identified as quercetin-3-glucuronide[54]. Table 3 groups together the secondary metabolites identified in *A. acutifolius*.

Table 3 Chemical compounds occurring in *A. acutifolius*

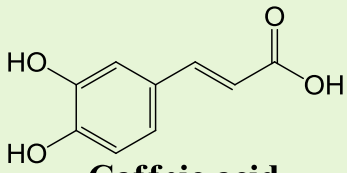
Chemical compounds occurring in *A. acutifolius*					
Plant organ	**Extraction**	**Plant collection: Time, place, altitude**	**Isolated compounds**	**Chemical class of metabolites**	**References**
Spears	MeOH	2009, Monti Tifatini, Caserta, Italy	Protocatehuic acid 3, 4 dimethoxyphenol Catechol	Phenolic acids	52
Aerial parts	80% MeOH	Ragusa, Italy	Caffeic acid Protocatechuic acid Isorhamnetin Kaempferol Myricetin Myricetin-3-glucoside Quercetin Quercetin-3-galattoside Quercetin-3-rhamnoside Quercetin-glucuronide	Phenolic acids Flavonoids	53
Roots	MeO:H_2O 7:3	-	Compounds A-G (see Table 4)	Steroidal saponins	54

Tabe 4 Short presentation of the main classes of secondary metabolites within *A. acutifolius*

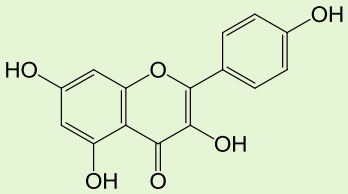
Brief description of the main classes secondary metabolites identified in *A. acutifolius*
Flavonoids • The term flavonoids is a collective noun for plant pigments, mostly derived from benzo-γ-pyrone, a C6-C3-C6 carbon framework, which is synonymous with chromone[55,56,57]. Depending on the position of the linkage of the aromatic ring to the benzopyran moiety, this group of natural products may be divided into three classes: the flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans) and the neoflavonoids (4-benzopyrans)[58]. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. The flavonoid pathway is part of the phenylpropanoid pathway, which produces a range of other secondary metabolites, such as phenolic acids, lignins, lignans, and stilbenes. The key flavonoid precursors are phenylalanine, obtained via the shikimate and arogenate pathways, and malonyl-CoA, derived from citrate produced by the TCA cycle. Flavonoids are universal within the plant kingdom; they are the most common pigments next to chlorophyll and carotenoids and they generally occur in plants as glycosylated derivatives and their physiological roles in the ecology of plants are diverse. Flavonoid glycosides are poorly absorbed until they have undergone hydrolysis by bacterial enzymes in the intestine, where their aglycones can be absorbed[59]. • Flavonoids possess antioxidant and free radical scavenging activities[60, 61, 62]. Epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer[63, 64, 65] and against many chronic diseases[66, 67, 68].



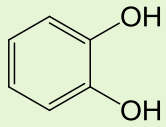
Protecatechuic acid



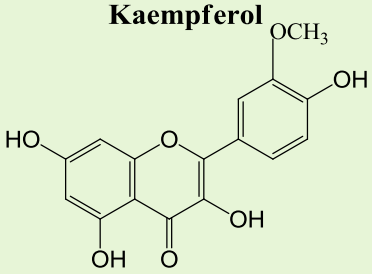
Caffeic acid



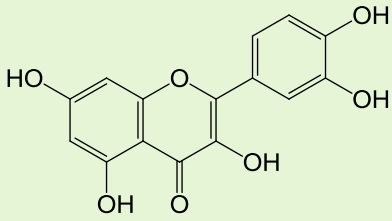
Kaempferol



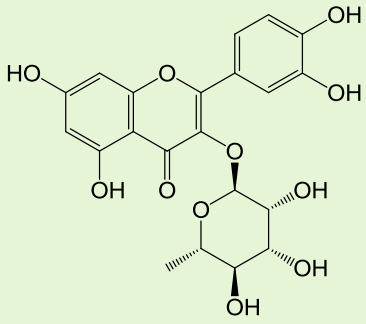
Catechol



Isorhamentin



Quercetin



Quercitrin

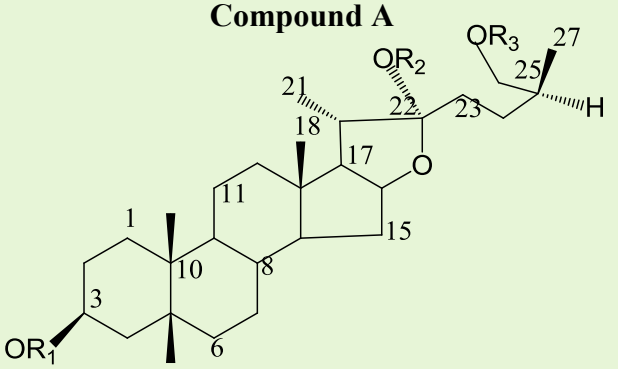
Steriodal saponins

• **Steroidal saponins** are tetracyclic molecules that are ultimately synthesized from acetyl coenzyme A (CoA). Based on the structure of the aglycone, saponins are classified into two types: the steroidal and the triterpenoid saponin, which are inherently

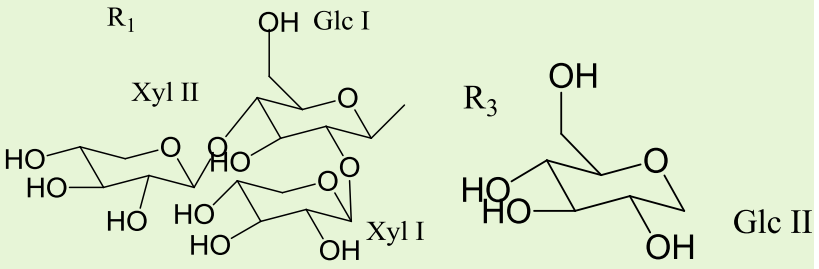
lipophilic. Due to the presence of the aglycone (apolar molecules) and a water soluble sugar chain in their structure (amphiphilic nature), saponins are surface active compounds with detergent and foaming properties. This property seems to improve the absorption of certain botanical constituents (including other saponins) from the gut, when they are ingested simultaneously with saponins[69].

• Saponins have various biological properties, namely being antioxidants[70], antihepatotoxic, antidiarrheal, anticarcinogenic[71], immunostimulants, antimicrobial[72], antiulcerogenic[73], antioxytocic and useful in diabetic retinopathy. They are included in a large group of protective molecules found in plants named phytoanticipins[74].

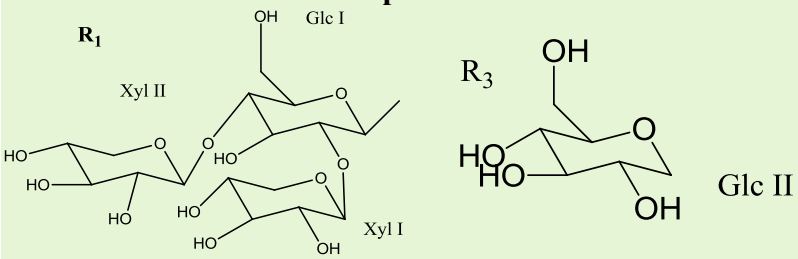
Compound A



R_2= Me

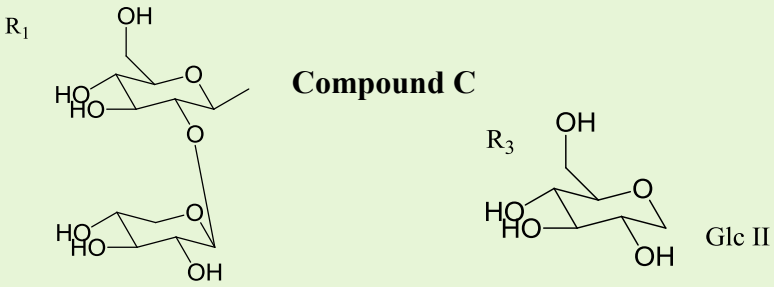


Compound B



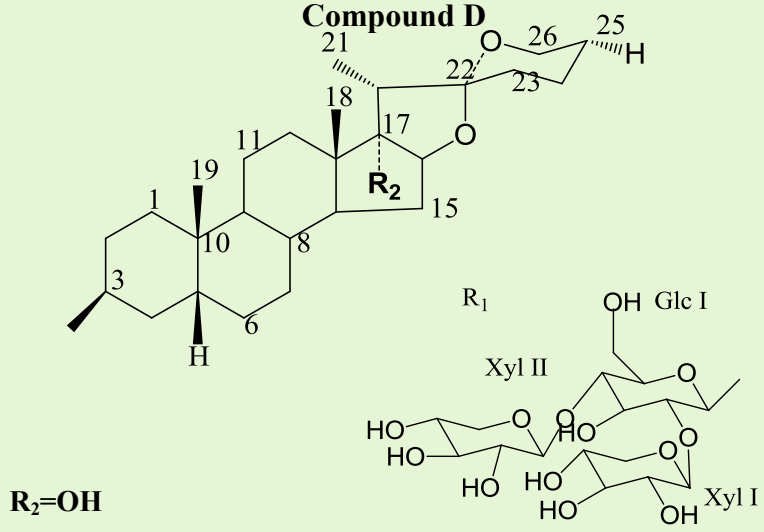
R_2= H

Compound C



R_2= Me

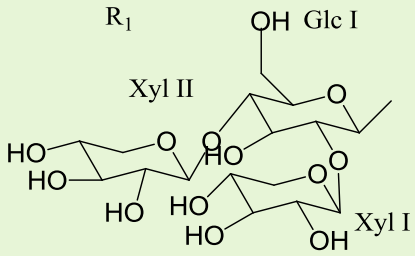
Compound D



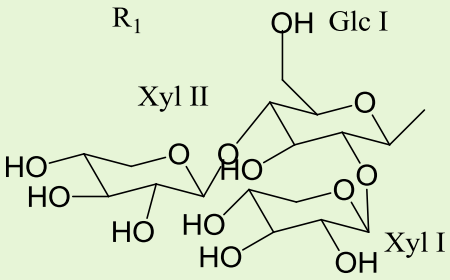
R_2=OH

Compound E

R_2= H

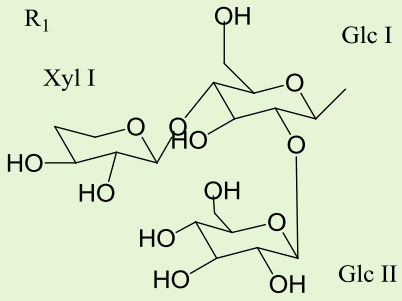


Compound F



R_2= OH

Compound G



R_2= H

II.2.2.3 Traditional use

Recently, interest has risen for its cultivation as frugal crop for niche markets, but only limited information exists on the nutritional values of this vegetable. From a biological point of view, some species were documented to exhibit antifungal[75], antiprotozoal[76] and cytotoxic[77, 78] activities. *Asparagus sp.* has widespread applications as diuretic, cooling agent and an excellent safe herbal medicine for pre-natal care. It is useful in nervous disorders, dyspepsia, diarrhoea, tumors, inflammations, burning sensation, hyperdipsia, ophthalmopathy, nephropathy, hepatopathy, throat infections, tuberculosis, cough, bronchitis, fatigue, hyperacidity, hemorrhoids, hypertension, abortion, cardiac and general debility[79]. Root is demulcent, diuretic, aphrodisiac, tonic, alterative, antiseptic, antidiarrheal, galactogogue and antispasmodic. Aerial part is spasmolytic, antiarrhythmic and anticancer[80].

II.2.3 *Chenopodium bonus henricus* L. (Amaranthaceae/Chenopodiaceae)

Family

The Amaranthaceae is a cosmopolite family belonging to the Caryophyllales order and occurs at disturbed, arid or saline areas. One of the characteristics that ensure their survival in unfriendly environments is the operation of C4 pathway of photosynthesis[81,82]. A broad definition of the Amaranthaceae family includes Chenopodiaceae which is supported in a total of 2000 species and 170 genera. The species are represented by

herbs, rarely shrubs or woody climbers with opposite or alternate leaves. Inflorescences are racemose or more or less paniculate, of single flowers or cymose flower clusters. Flowers are bisexual or unisexual, usually with two bracteoles and the fruit usually a nut, sometimes a capsule or berry[83]. Leaves and roots of some species of the family are edible (*Chenopodium* sp., *Spinacia oleracea*, *Beta vulgaris* and *Amaranthus* sp.[81]
Common name: Good King Henry, Perennial goosefoot

II.2.3.1 Botanical description

Figure 5 *Chenopodium bonus henricus* (Chenopodiaceae)

A perennial herbaceous plant with a fleshy, multi-head root mostly branched in 3-5 parts, reaching length of over 40 cm. Its above-ground parts are farinose of vesicular hairs. Stems 24-66 cm high, erect, multiridged, yellowish-green or green-reddish[84]. Leaves are cordate or hastate, not aromatic. Inflorescence mainly terminal, narrowly pyramidal and tapering, leafless except at base. Flowers mostly hermaphrodite (or female). The perianth is green to yellowish[85].

II.2.3.2 Chemical composition: literature review

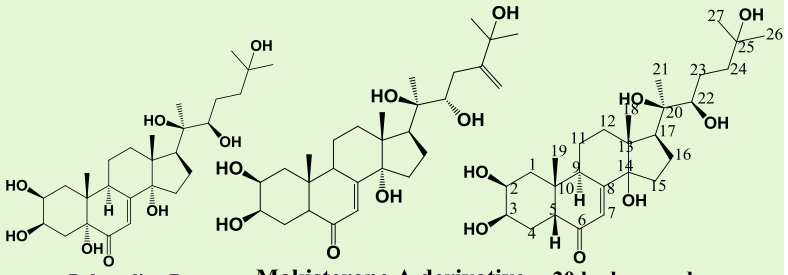
The medicinal properties of *C. bonus-henricus* are due to the presence of the terpene saponins localized in the roots which are known to have diuretic, laxative, enhancing secretory and expectorating effect. Its leaves contain ecdysteroids, saponins, fats, starch, vitamin C, and flavonids[86]. The phytochemical investigations of genus *Chenopodium* afforded the identification of compounds having a variety of structural patterns. The *Chenopodium* spp. were also reported to contain: minerals, primary metabolites such as carbohydrates, amino acids, nonpolar constituents, proteins, hormones and secondary metabolites like flavonoids, saponins, terpenes, sterols, alkaloids and vitamins[87]. Table 5 depicts some chemical constituents of *C. bonus-henricus* reported by a previous study[88].

Table 5 Chemical constituents of *C. bonus henricus*

Ecdysteroids
Ecdysteroids are the active principles in arthropod molting or ecdysis[89-91]. These molecules are involved in ecdysis of insects, crustaceans, spiders and ticks, which appear likely to be the molting hormone of all arthropods[92]. Phytoecdysteroids have a varied chemical structure derived from C27, C28 or C29 sterols. The basic chemical structure of ecdysteroids is a cyclopentano-perhydrophenanthrene skeleton, where the C/D ring junction is generally *trans*, while the A/B ring junction is normally *cis* (5β-H) and only rarely trans (5α-H). Most ecdysteroids contain a 14α-hydroxy group and a 7-en-6-one chromophore in ring B[93]. These phytoecdysteroids may exist in either free form or as polar conjugates of glucosides, sulfates, and phosphates or

nonpolar conjugates as acetates or benzoates[94,95]. In addition to these ecdysteroids, plants often make ecdysteroid-related compounds which are loosely defined based upon their structure and biological activity[94]. The distribution of phytoecdysteroids within an individual plant is related to the organ type and its state of development. A series of studies prior to 1985 on plant biosynthetic capacities supported the conversion of mevalonic acid and cholesterol to ecdysteroids by the A/B ring inversion, which is different from that observed in insects[96]. Studies performed on spinach revealed that phytoecdysteroid biosynthesis is a highly controlled pathway as demonstrated by the incorporation of [2-^{14}C] mevalonic acid, MVA, and other putative ecdysteroid biosynthetic intermediates[97,98].

A great number of studies tried to stress out the pharmacological effects of ecdysteroids in mammals and their influence upon physiological functions[93].



II.2.3.3 Traditional use

C. bonus-henricus, known also as "wild spinach" is traditionally used for its emollient, vermifuge and laxative properties. A poultice of the leaves is used to cleanse and heal chronic sores, boils and abscesses[99]. In the traditional gastronomy, it is cooked as spinach.

II.2.4 *Levisticum officinale* Koch. (Umbelliferae *sin*. Apiaceae)

Family

Herbaceous plants belonging to the order Apiales comprising up to 400 genera of plants distributed throughout a wide variety of habitats. Most members are aromatic herbs with alternate, feather-divided leaves that are sheathed at the base. The flowers are often arranged in a conspicuous umbel. Each small individual flower is usually bisexual, with five sepals and petals, and an enlarged disk at the base of the style. The fruits are ridged and are composed of two parts that split open at maturity[100].
Common names: Lovage

II.2.4.1 Botanical description

Figure 6 *Levisticum officinale* (Umbelliferae)

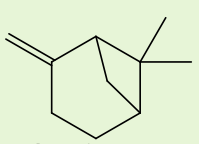
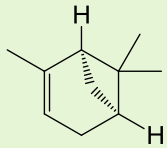
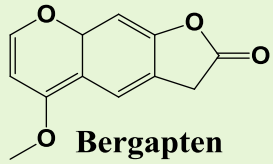
L. officinale is a lofty perennial aromatic plant. The herb bears dark green leaves and greenish yellow flowers. The plants grow up to 2 meters high. Its leaves are divided by sharply toothed leaflets. Its characteristic, strongly

aromatic odor resembles celery. Lovage is native to Europe, but it is found throughout the northeastern United States and Canada[101,102]. This plant should not be confused with *Oenanthe crocata* L. known commonly as water lovage and *O. aquatica* (L.) Lam. (water fennel), toxic members of the family Umbelliferae.

II.2.4.2 Chemical composition: literature review

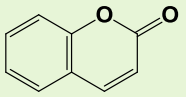
Lovage contains approximately 2% of a volatile oil responsible for its characteristic flavor and odor. This oil consists primarily of phthalide lactones (70%), (e.g. 3-butylphthalide 32%, *cis*- and *trans*-butyldenephthalide, *cis*- and *trans*-ligustilide 24%, senkyunolide and angeolide)[101,103,104], characteristic components of the adult root oil. In addition, lower amounts of compounds such as terpenoids (characteristic of the aerial part oil), volatile acids and furanocoumarins contribute to the flavor of the extract. Other compounds are bergapten, psoralen, caffeic and benzoic acids[105]. Two major components common to all aboveground plant parts are *β*-phellandrene (flowers and fruit containing the largest amount) and *α*-terpinyl acetate (leaves and stems containing the largest amount). Linalol was mainly found in stems along with considerable amounts of camphene, *α* and *β*-pinene[106]. Polyacetylenes such as falcarinol and falcarindiol are compounds present in *L. officinale* roots[107]. Quercetin and luteolin rhamnoglucoside were reported by Justensen as flavonoids found in lovage leaves[108]. Table 6 reports compounds found in *L. officinale* essential oil [106,107,114,115].

Table 6 Chemical constituents of *L. officinale*

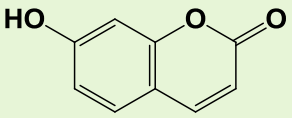
Chemical constituents identified in *L. officinale*
Terpenoids (Isoprenoids) **Terpenoids** are a large class of natural products consisting of isoprene (C5) unit. According to Croteau et al.[109], they are the most numerous and structurally diverse plant natural products. The term terpenoid refers to a terpene that has been modified, e.g by the addition of oxygen. Special isoprene rule states that the terpenoid molecule is constructed of two or more isoprene units joined in a 'head to tail' fashion. Most natural terpenoid hydrocarbon have the general formula (C5H8)n. There are two biosynthetic pathways, the mevalonate pathway and the mevalonic acid independent pathway[110]. In addition to their roles as end products in many organisms, terpenes are major biosynthetic building blocks within nearly every organism[111]. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, antiinflammatory, and immunomodulatory properties[112-117].  **β pinene**  **α pinene**  **Bergapten** **Coumarins** **Coumarins** belong to a group compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. Like other phenylpropanoids, coumarins arise from the metabolism of phenylalanine via cinnamic acid, p-coumaric acid[118,119]. The specificity of the process resides in the 2'-hydroxylation, followed by the photocatalysed isomerisation of

the double bond and lactonisation. In some rare cases, glucosylation of cinnamic acid occurs, precluding lactonisation. In such cases, coumarin only arises after tissue injury and enzymatic hydrolysis. The primary site of synthesis of coumarins is suggested to be the young, actively growing leaves, with stems and roots playing a comparatively minor role[120]. Although mainly synthesized in the leaves, coumarins occur at the highest levels in the fruits, followed by the roots and stems.

Their physiological, bacteriostatic and anti-tumour activity makes these compounds attractive for further backbone derivatisation and screening as novel therapeutic agents. Weber et.al showed that coumarin and its metabolite 7-hydroxycoumarin have antitumour activity against several human tumour cell lines[121-123].



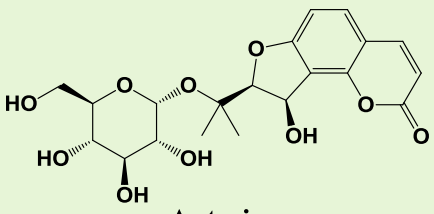
Coumarin



Umbelliferone
(7 hydroxycoumarin)

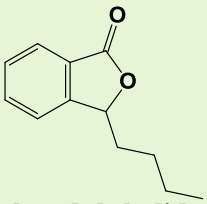
Phthalides

67 phthalides, including dihydro, tetrahydro, and hexahydro derivatives, as well as associated dimers, have been isolated from plants belonging to Umbelliferae family[124]. These taxa have been reported as exhibiting a wide range of bioactivities against experimental models of several illnesses and physiological conditions, including microbial[125] and viral infections, stroke[126], tuberculosis, and vasoconstriction.

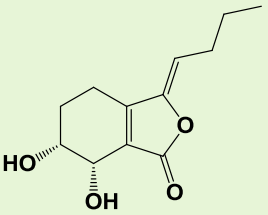


Apterin

Ligustilide



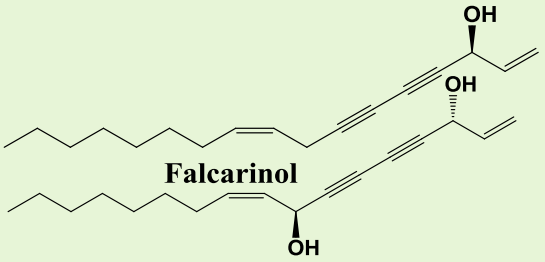
3-butylphthalide



senkyunolide

Polyacetylenes

Polyacetylenes belong to a class of molecules containing two or more triple bonds and constitute a group of relatively unstable, reactive and bioactive natural products[127]. Aliphatic C17-polyacetylenes of the falcarinol type such as falcarinol and falcarindiol are widely distributed in the Umbelliferae family. The structures of most polyacetylenes indicate biosynthesis from unsaturated fatty acids. Polyacetylenes of the falcarinol-type are formed from oleic acid by dehydrogenation leading to the C18-acetylenes crepenynic acid and dehydrocrepenynic acid, which is then transformed to C17-acetylenes by oxidation. Further oxidation and dehydrogenation leads to falcarinol and related C17-acetylenes of the falcarinol-type[128,129]. These molecules possess antitumor, anti-inflammatory, antibacterial and antifungal activity[130].



Falcarinol

Falcarindiol

II.2.4.3 Traditional use

Lovage has several uses, including medicinal and culinary. Several researches have shown that lovage has beneficial properties for the digestive and respiratory systems. The underground parts of the herb - rhizome and roots – are used for their therapeutic properties. The roots of lovage have been used as a folk medicine, especially for their diuretic and carminative properties since the 14th century. It is used for diaphoretic, expectorant, stomachic, anti-dyspeptic and stimulant activities[106].

II.2.5 *Silene vulgaris* Moench. (Caryophyllaceae) Family

Caryophyllaceae family belongs to the order Caryophyllales and comprises 86 genera and 2,200 herbaceous species mainly of north temperate distribution. The members are diverse in appearance and habitat, most of them having swollen leaf and stem joints[131]. The leaves are simple, entire, usually without stipules, and almost always opposite. The flowers are radially symmetric and usually bisexual. The inflorescence is cymose, sometimes simple but often highly compound[132,133]. Few genera have a single terminal flower. The fruit is a capsule that opens by teeth at the top of the ovary. Common name: Silene cucubalus, Silene inflata, Bladder campion

II.2.5.1 Botanical description

Figure 7 *Silene vulgaris* Moench. (Caryophyllaceae)

S. vulgaris is a perennial herb which stems up to 60 cm and can be found in weedy places, semi-dry turf and open dry places. The stem is glabrous and makes few branches while leaves are hairless, opposite and usually decussate. The white flowers are dioecious. The bladder-like calyx is a large inflated structure pale green or green yellow[134,135,136].

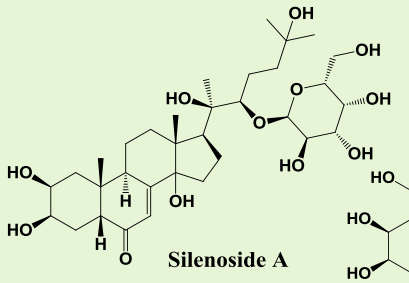
II.2.5.2 Chemical composition: literature review

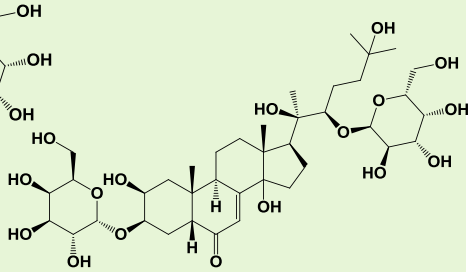
Few studies have been conducted on the chemical composition of *S. vulgaris* seeds and roots. The roots contain triterpenoid saponins while leaves and seeds contain fatty acids. *S. vulgaris* intact aerial plant contains a pectic polysaccharide called silenan. Table 7 displays the chemical structures of the main compounds identified in *S. vulgaris*[139,146-148].

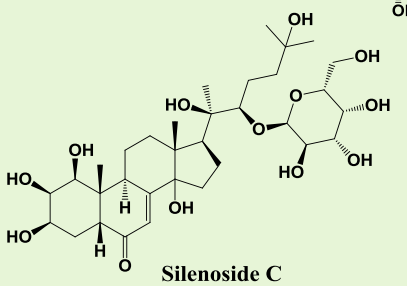
Table 7 Chemical constituents of *S. vulgaris*

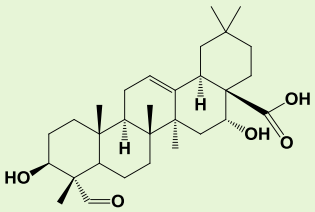
Chemical constituents identified in *S. vulgaris*

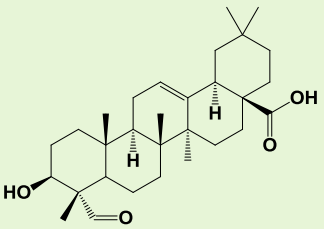
Triterpenoid saponins[141,142]











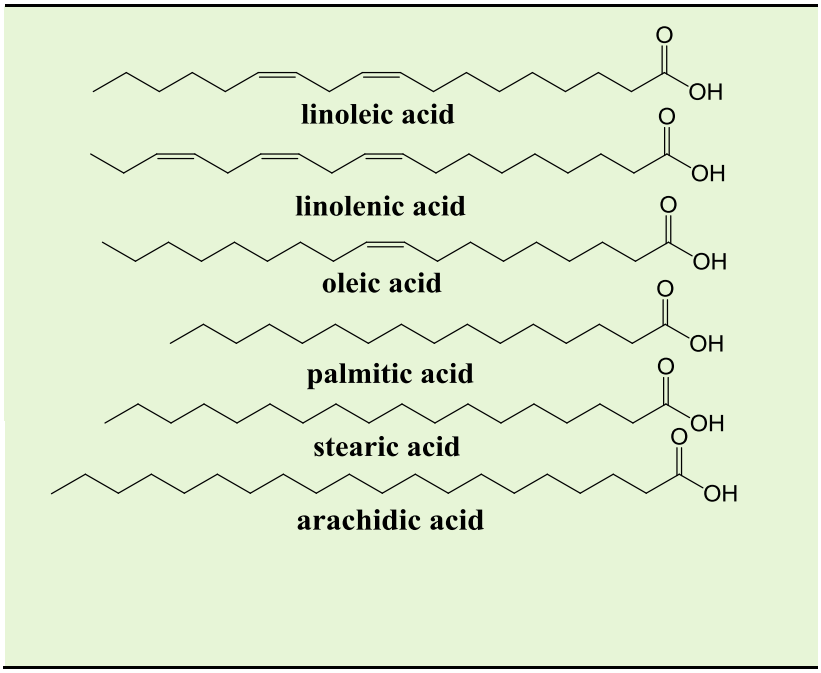
Polysaccharides

The pectic polysaccharide silenan contains a linear α-(1→4)-D-galacturonan backbone with 2-substituted α-rhamnopyranose residues and ramified regions. The silenan side chains are composed of blocks containing terminal α-(1→5)-linked arabinofuranose and β-(1→4)-linked galactopyranose residues[137]. Silenan was shown to contain homogalacturonan segments as the

backbone or "smooth regions" and rhamnogalacturonan fragments as ramified or "hairy regions". The sugar chain of silenan was found earlier to contain residues of D galacturonic acid, arabinose, galactose, and rhamnose as the main constituents[138]. The silenan possesses immune-modulating activity, in particular, by the stimulation of phagocytic activities of neutrophils and macrophages[139]. Silenan enhances oxygen metabolism of peritoneal macrophages influencing functional activity of the cell receptors. The stimulatory action of silenan on blood neutrophils and peritoneal macrophages in vitro is manifested in increasing secretory function without changes in cell adhesive properties[140].

Fatty acids[143-148]

Fatty acids are almost entirely straight chain aliphatic carboxylic acids. The broadest definition includes all chain lengths, but most natural fatty acids are C4 to C22 with C18 most common. Naturally occurring fatty acids share a common biosynthesis. The chain is built from two carbon units, and *cis* double bonds are inserted by desaturase enzymes at specific positions relative to the carboxyl group[143]. This results in even-chain-length fatty acids with a characteristic pattern of methylene interrupted *cis* double bonds. Two enzyme systems are utilized in the synthesis of long chain fatty acids: acetyl-CoA carboxylase and fatty acid synthase[144]. The end products of this synthesis are usually the saturated fatty acids palmitate and stearate with the latter predominating. Once the long chain acids have been produced they can be subject to elongation, desaturation and further modifications[145].



II.2.5.3 Traditional use

Young shoots and leaves are used raw or cooked. The young leaves are sweet and usually are used in salads. The cooked young shoots, harvested when about 5 cm long have a flavour similar to green peas but with a slight bitterness[134]. This bitterness can be reduced by blanching the shoots as they appear from the ground. The leaves should be used before the plant starts to flower. The plant is emollient and is used in baths or as a fumigant[135]. The juice of the plant is used in the treatment of ophthalmia. The root is used as a soap substitute. The soap is obtained by simmering the root in hot water[136].

II.2.6 *Spirea aruncus* L. (Rosaceae)

Family

Rosaceae family, in the order Rosales, is a large plant family containing more than 100 genera and 2,000 species of trees, shrubs, and herbs. Most of these species have leaves with serrated margins and a pair of stipules where the leaf joins the stem. Flowers in this family are typically radially symmetrical flat discs (actinomorphic) and contain both male and female floral structures in a single flower[149].

Common name: Aruncus dioicus, Aruncus sylvestris, Goat's beard

II.2.6.1 Botanical description

Figure 8 *Spirea aruncus* (Rosaceae)

S. aruncus is a perennial plant which occurs in moist woodlands, especially in mountainous regions. The plant can grow up to 1.8 m tall and has alternate, pinnately compound leaves with two stipules at the base, on thin, stiff stems.

Flowers are dioecious. This plant can be found throughout Europe, Asia, and eastern and western North America[150,151].

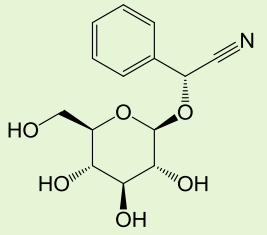
II.2.6.2 Chemical composition: literature review

Bohm reported the presence of flavonoid compounds based upon kaempferol, quercetin and eriodictyol in two species of *Spirea*[152]. The aerial parts of *S. aruncus* afforded five new monoterpenoids: aruncin A, aruncin B, aruncide A, aruncide B, aruncide C[153]. Prunasin is a cyanogenic glycoside which was isolated from the leaves and roots of *S. aruncus*[154]. Compounds identified in *S. aruncus* are described in Table 8.

Table 8 Chemical constituents from *S. aruncus*

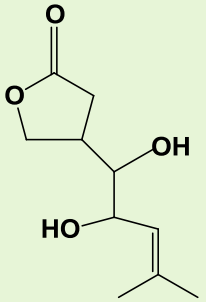
Chemical constituents identified in *S. aruncus*
Cyanogenic glycosides
Cyanogenic glucosides (CNGs) are phytoanticipins known to be present in more than 2500 plant species. They are considered to have an important role in plant defense against herbivores due to their bitter taste and release of toxic hydrogen cyanide upon tissue disruption[157]. CNGs are β-glucosides of α-hydroxynitriles derived from the aliphatic protein amino acids l-valine, l-isoleucine and l-leucine, from the aromatic amino acids l-phenylalanine and l-tyrosine and from the aliphatic non-protein amino acid cyclopentenyl-glycine. When plant tissue is disrupted by the herbivore attack for example, CNGs are brought into contact with β-glucosidases and α-hydroxynitrile lyases that hydrolyze the CNGs with the release of toxic hydrogen cyanide (HCN) known also as prussic acid. Cyanide is a toxic substance, mainly due to its affinity for the terminal cytochrome oxidase in the mitochondrial respiratory pathway[158]. Cyanide is detoxified in the body, by the enzyme rhodanese in the presence of sulphur-containing amino acids, to produce thiocyanate In plants,

cyanogenic glucosides serve as good chemotaxonomic markers for plant relatedness: the more closely related two plant species are, the more similar their cyanogenic glucosides are[159]

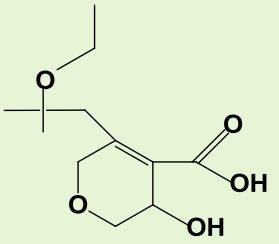


prunasin

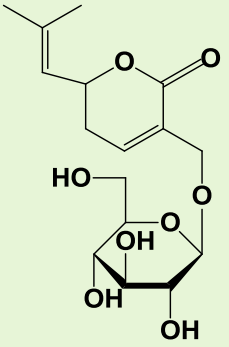
Monoterpenoids



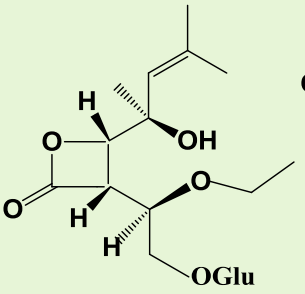
aruncin A



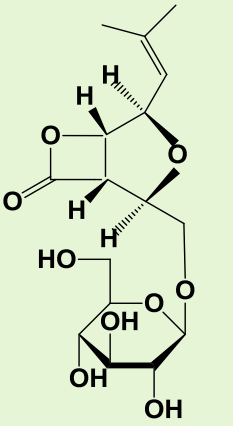
aruncin B



aruncide A



aruncide B



aruncide C

II.2.6.3 Traditional use

The poulticed root is applied to bee stings[155,156]. The infusion of the roots is used to allay bleeding after child birth, to reduce urination in excess and to treat stomach ache, diarrhoea, gonorrhoea, fevers and internal bleeding[155,156]. The tea is used externally to bathe swollen feet and rheumatic joints. A salve made from the root ashes is rubbed onto sores.

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II.3 Comprehensive non-conventional extraction methods of secondary metabolites. Design of experiments: a valuable tool for the extraction of targeted molecules

Plants are complex matrices which contain a wide range of secondary metabolites with different functional groups and polarities. Extraction represents the first basic step toward a reliable qualitative and quantitative analysis of the analytes present in plant matrix. Phytocompounds may occur in a higher or a lower abundance, thus arising the need to develop high performance extraction and analysis techniques[1]. The methods used to extract metabolites for a metabolomic study are those commonly used for phytochemical studies[2]. Solvent characteristics, ratio solvent and sample, duration of extraction and temperature are important aspects in an extraction procedure. The problem in metabolomics is that the metabolome consists of a wide variety of compounds at very different levels and polarities. At present, there is not a single solvent with the property to dissolve the whole range of compounds. Ideally, an extraction procedure should be exhaustive with respect to the constituents to be analyzed, rapid, simple, and for routine analysis amenable to automation. Nowadays, besides the solvent-free extraction methods, the tendency is to perform extractions using non-conventional techniques such as accelerated solvent extraction, ultrasound solvent assisted extraction, microwave solvent assisted extraction, supercritical fluid extraction, aimed to improving all the

parameters involved in an extraction process. In many instances, classic solvent extraction procedures such as maceration, percolation, Soxhlet extraction, extraction under reflux and steam distillation are used for comparison purposes with the aforementioned techniques. Although they are relatively simple methods, they suffer from such disadvantages as long extraction times, relatively high solvent consumption, and often unsatisfactory reproducibility[3]. Even though the non-conventional techniques eliminate most of the above mentioned drawbacks of the traditional extraction methods, they may differ in their extraction effectiveness. A crucial point is when facing a low level of an analyte in the raw material. The large chemical variation in plants exists between both different plant species and different tissues of a single plant. According to Krishnan et al.[4], a typical cell may contain 5000 metabolites, so the target of a sample-preparation method is to trap as many metabolites as possible. A number of methods using organic and/or aqueous solvents are employed in the extraction of natural products. Although water is used as solvent in many traditional protocols, organic solvents of varying polarities are preferred in order to exploit the different solubility of plant constituents. The dynamics of the extraction process can be simplified by dividing it into different steps. In the first instance, the solvent has to diffuse into cells, in the following step it has to solubilize the metabolites, and finally it has to diffuse out of the cells enriched in the extracted metabolites. Further down, fundamentals of two non-conventional extraction methods used in this study are shortly presented.

Microwaves are non-ionizing electromagnetic waves with frequencies ranging from 300 MHz to 300 GH that heat

up the molecules by dual mechanism of ionic conduction and dipole rotation[5]. When they interact with polar solvents, these two effects, hence the heating process may occur individually or simultaneously. Microwave-assisted extraction (MAE) offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and the solid matrix, efficiently and homogenously[6]. Microwave extraction efficiency increases with the increase of analytes' polarity and with solvents' dielectric constant. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the recovery of the secondary metabolites[7]. The solvent of choice in MAE is dictated by the solubility of the compounds of interest and by the microwave absorbing properties of the solvent. Literature data report that MAE gives yields comparable to Soxhlet extraction methods, but in much less time. In general, microwave-assisted extractions are carried out on 1 to 10 g samples, using an appropriate solvent and irradiation powers. A broad spectrum of phytochemicals has been extracted by MAE[1]. Compared to classic extraction methods, higher extraction yields were achieved in a shorter time for artemisinin from *Artemisia annua* L[8], for glycyrrhizic acid from licorice root[9], for tea polyphenols[10]. Azadirachtin-related limonoids were extracted from 2 g of neem seed kernel[11] and ginsenosides were extracted from 2 g of ginseng root in 5 minutes[12]. On the other hand, the ultrasound-assisted extraction (UAE) uses sound waves with frequencies superior to 20 KHz. Unlike electromagnetic waves, sound waves must travel in a matter, involving expansion and compression cycles. Vinatoru states that the mechanism of the ultrasonic assisted

extraction consists in the swelling and hydration of plant materials that can subsequently cause enlargement of the pores of the cell wall[13]. Better swelling will improve the rate of mass transfer breaking the cell walls sometimes, thus resulting in increased extraction efficiency. It is commonly applied to facilitate the extraction of intracellular metabolites from plant cell cultures[14]. The external glands of plant secretory structures can be easily destroyed by sonication, thus facilitating release of metabolites into the extraction solvent[13]. UAE was recently used for the polysaccharides[15], and bioactive phytochemicals[16], including menthol[17], cardiac glycosides[18], pyrethrins[19], and camptothecin[20].

A selective extraction can also be performed sequentially with solvents of increasing polarity. This has the advantage of allowing a preliminary separation of the metabolites present in the material within distinct extracts[21]. In an extraction referred to as ''total,'' a polar organic solvent or an aqueous alcoholic mixture is employed in an attempt to extract as many compounds as possible. This is based on the ability of alcoholic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium polarity to apolar constituents. Due to its broad applicability in sample preparation and to its versatility, MAE was used as the method of choice for the present study. The challenge was to design a microwave-assisted extraction method from aerial plant material belonging to species little known for their phytochemical profile. The focus of the design of experiments used is the evaluation of the total chemical compounds that absorb at 280 nm, roughly presuming that all of them have a phenolic structure. The proposed method can be applied for the detection of classes of compounds commonly encountered during plant extraction, including selected groups of

secondary metabolites. Certain analytical techniques require special sample preparation in order to avoid thermal degradation of some compounds developed during the extraction process. For this reason, several selective extractions have been sequentially carried out using the ultrasound- assisted extraction by means of a probe system consisting of an immersion titanium horn and an ice bath.

One of the main tasks required of any biological or analytical test is a systematic approach to the experiments. In order to create a protocol for the analysis of metabolites or to design an experiment for metabolic profiling of a plant, several factors that affect the response can be identified. Often, the goal for the analytical systems is to find the settings that maximize response and reproducibility. The classical way to investigate and find optimal conditions in an experimental approach (the experimental 'area' that is defined by the variation of the experimental variables) is to Change One Separate factor at a Time, also known as the COST approach[22]. Finding true optima is not aboveboard within this approach, because of its inefficiency in requiring unnecessarily large numbers of runs, ignoring interactions, generating knowledge relatively slowly and not outlining the experimental space. Design of experiments[23-25] (DOE) is a procedure where the effects of the factors to be analyzed are evaluated using regression models. In contrast to the COST approach, DOE allows the causal effect of each factor in the experimental domain to be elucidated in a reduced number of experiments. Design of experiments (DOE) is a statistical methodology that handles how to plan and conduct experiments in order to extract the maximum amount of information in the fewest number of experimental runs. The basic idea is to vary all relevant factors simultaneously, over a set of planned experiments, and then connect the results by

means of a mathematical model. This model is then used for interpretation, predictions and optimization. DOE selects a diverse and representative set of experiments in which all factors are independent of each other despite being varied simultaneously. The result is a causal predictive model showing the importance of all factors and their interactions. These models can be summarized as informative contour plots highlighting the optimum combination of factor settings[23]. DOE is used for three primary objectives: screening (sets the factors which are most influential and the corresponding range), optimization and robustness testing. The experimental cycle consists of three phases:

• The design phase where factors and their ranges to be varied, their responses, objective, design and model are to be defined.

• The analysis phase for exploring the data, review the raw data and the fit, review diagnostics in plots and lists, refine and interpret the model.

• The prediction phase where the model is used to predict the optimum area for operability.

Screening is the first stage of an investigation where the goal is just to identify the important factors. A factor is considered important when its variation causes substantial effects in the response. In the screening stage one uses simple models (linear or linear with interactions), and experimental designs that allows the identification of the factors with the largest effects in the fewest possible number of experimental runs. If the combinations of k factors are investigated at two levels, a factorial design will consist of 2^k experiments[23]. A factorial design explores the influence of all experimental variables, factors, and interaction effects on the response or responses. The responses obtained from the experiments are

commonly elaborated using Partial Least Squares Regression[26,27] (PLS). PLS can be used when the number of experimental factors is equal to or fewer than the number of experiments and the design factors are not correlated[28]. Depending on the design setup, different equations can be applied to give an approximation of the response surface. The model can also be used to make predictions to validate the model.

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II.4 Analytical platforms

Due to the high complexity and diversity of the secondary metabolites, for their investigation, it is highly recommended the engagement of a platform of analytical technologies. No single technique is comprehensive, selective and sensitive enough to measure all the metabolites[1]. The monitoring of such techniques becomes meaningful when the processing of the generated raw data is supported by different algorithms, specific databases and chemometric approaches. Handling large amounts of data can be accomplished in three steps: data processing, data pre-treatment and data analysis. Profiling refers to a detailed analysis using both simple (e.g. direct infusion mass spectrometry[2], liquid chromatography[3,4]) and hyphenated techniques-mass spectrometry platforms (e.g. gas chromatography[5-7]), resulting in an extended coverage of the metabolic diversity, as they provide complementary information[8,9]. Such techniques provide detailed chromatographic profile of the sample, which differences stay in their resolution power and the specificity of the detection technique[10]. Broad metabolite profiling provides data for a wide range of chemical classes, but the methods represent a compromise in terms of chemical sensitivity, selectivity and speed of the different techniques[11] and do not provide the same data quality for all of the metabolites covered. In order to build up a reliable information framework on the samples' composition, the preliminary study employed the features of the most relevant chromatographic techniques, e.g. GC/MS and LC/MS

technologies. Figure 9 depicts the trade-off between metabolic coverage and the quality of metabolic analysis.

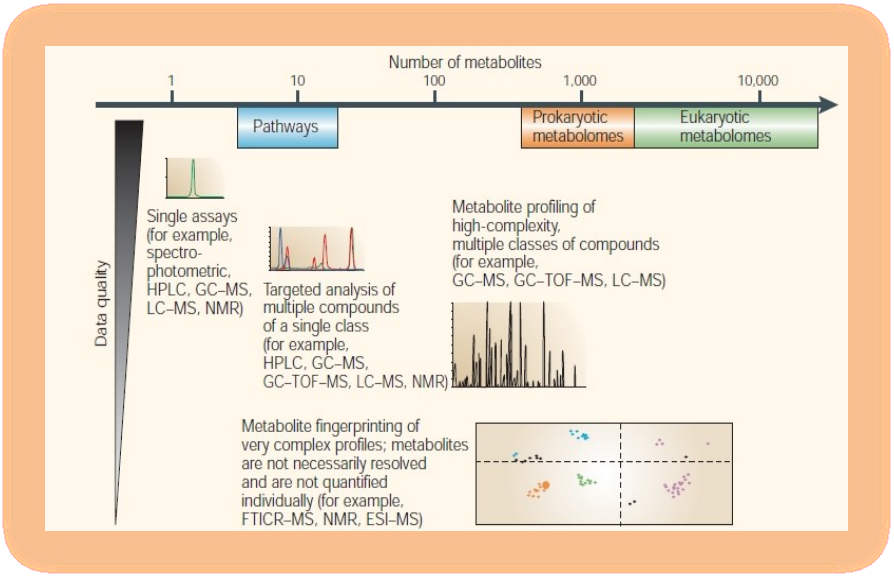

Figure 9 *The trade-off between metabolic coverage and the quality of metabolic analysis*

Source: Fernie, A. et al. Nature reviews 2004, 5, 4.

II.4.1 HPLC approaches for metabolite profiling

The choice of the appropriate detection method in HPLC is crucial because of the diversity of natural products and the fact that there is no single technique for their efficient detection[12]. Traditionally, HPLC has been coupled to ultraviolet and visible light (UV/VIS) or diode-array detectors[13] (DAD). HPLC with UV detection is a common

method used for targeted analysis of plant materials and for metabolic profiling of individual classes. DAD provides UV spectra directly online and is particularly useful for the detection of natural products with characteristic chromophores[14,15]. For example, polyphenols can be efficiently detected by this method because they possess characteristic chromophores[16]. Selection of compounds arises initially from the type of solvent used for extraction and then from the type of column and detector. The large polarity differences of the constituents in a plant extract usually require gradient elution[17] which cause a shift of the baseline at low wavelengths. HPLC profiling methods rely to a great extent on comparisons with reference compounds. Comparison of retention data and spiking with known standards is required in order to provide evidence of composition, but this may lead to erroneous results as absolute identification is not possible[18]. The full UV spectrum gives some useful information on the nature of compounds in complex profiles, but often indicates the class of the compound rather than its exact identity. HPLC-UV methods can be used to compare the fingerprint profiles of closely related species or the same species from different locations. In contrast to UV, ELSD (evaporative light-scattering detection) is a mass-dependent detector, which is a concentration-dependent detector, and the generated response does not depend on the spectral or physicochemical properties of the analyte. In theory, this means that ELSD generates a similar response for equal amounts of mass present and thus a universal response factor. ESLD has been used mainly for the detection of compounds with weak chromophores such as terpenes, in both aglycone[19] and glycosidic forms[20], saponins[21], and some alkaloids[22]. UV performs better than ELSD for the detection of natural

products with strong cromophores, the latter being more dedicated to the analysis of non-UV-absorbing compounds. The advantage of HPLC resides in the large diversity of separation mechanisms including normal phase (silica), reverse phase (C_{18}, C_8, C_4 and phenyl), hydrophilic interaction and ion exchange chromatography[23].

II.4.2 HPLC-MS, MS^n and UHPLC-TOF/MS approaches for metabolite profiling

Hyphenated MS-based techniques have been proven to be extremely powerful tools in natural product analysis, as they permit the fast screening of crude natural product extracts or fractions for detailed information about metabolic profiles, with a minimum amount of material. For this preliminary study of plant extracts, three techniques have been employed as tools in the dereplication of compounds by the on-line spectroscopic information. By providing an unique combination of resolving power, sensitivity and specificity, LC/MS and particularly LC/MS^n offers the possibility to identify target metabolites in complex mixtures. The two most widely used interfaces, especially in relation to natural product analysis, are electrospray (ESI) and atmospheric pressure chemical ionization (APCI). In conjunction with these interfaces, different types of low resolution e.g. quadrupole, ion trap (IT), and high resolution e.g. time of flight (TOF) analyzers can be used, and they offer various degrees of mass accuracy and MS/MS possibilities. Besides detection, a mass spectrometer gives the possibility of generating either nominal mass molecular ions or accurate mass measurements for the determination of empirical formulas[24]. The choice of mass analyzer is dictated

by many factors whether the focus is on targeted vs. untargeted analysis, high vs. low sample throughput, high or low mass resolution and cost of the analysis. Comprehensive metabolomics analysis requires the use of multiple mass analyzers. IT mass analyzers in various selective ion scanning modes (precursor ion scanning, neutral loss and multiple reaction monitoring) can be useful for targeted metabolomics analysis. IT analyzers employ a combination of direct-current and radio frequency potentials to keep ions within a certain, narrow *m/z* range in a stable trajectory. Structural information by controlled ion fragmentation (collision induced dissociation (CID)) is the application of an ion trap which enables the possibility to perform MS^n experiments as mass analyser. Ions are "trapped" with these devices by electrodynamic focusing and can be forced to leave the trap by putting them in unstable orbits by increasing the voltage. High resolution MS detectors provide highly specific chemical information that is directly related to the chemical structure, such as accurate mass, isotope distribution pattern for elemental formula determination, and characteristic fragment-ions for structural elucidation or identification via spectral matching to authentic compound data. TOF analyzers register the time difference between the signal start (e.g. acceleration of the ion) and the pulse generated when the ion hits the detector. Prior separation in most complex matrices can be greatly improved if ultra-high performance LC (UHPLC) is used. The main advantage compared to conventional HPLC is the higher separation efficiency achieved by the use of sub-2-μm particle sorbents that UHPLC uses[25], which represent a step forward in crude extract profiling. The UHPLC methods typically offer higher separation efficiency, together with

substantial reductions in run time and solvent consumption compared to HPLC. It is worth mentioning that the interpretation of the MS/MS spectra often requires the analysis of many related products[26] in order to extract structurally relevant information and establish rules that can be used for structure prediction. Examples of hyphenated MS-based techniques applied for metabolite profile purpose are: the model plant *Arabidopsis thaliana* L. has been investigated by LC/MS[27-29]; Dan et al. investigated the metabolite profile in different plant organs of *Panax notoginseng* Wall. by UHPLC-qTOF/MS identifying numerous saponins[30]; Jensen et al.[31] and Xie et al.[32] used LC-APCI/MS methods to evaluate the contents of bilobalides and ginkgolides of commercial Ginkgo preparations while a capillary LC/IT was applied by Ding et al. for a fingerprinting of Ginkgo biloba preparations[33]. An HPLC-MS/MS method has been developed for a fingerprint profiling for seven different black cohosh (*Cimicifuga*) species and six different commercial products by Wang et. al[34].

II.4.3 GC-MS approaches for metabolite profiling

Gas chromatography coupled to mass spectrometry (GC-MS) is considered a standard tool for metabolite profiling in plants[35]. It can be used to analyze a wide range of volatile compounds and through chemical derivatisation it is possible to analyze also semi-volatile metabolites. Routinely, GC-MS analysis is performed on single-quadrupole mass spectrometers which provide nominal mass information. The capillary columns used in GC enable the separation of more

than 100 compounds in a single analysis, e.g. alcohols, sugars, lipids, amino and non-amino organic acids, and others[36, 37]. The typical ionization techniques are chemical ionization which minimizes fragmentation, and fragmentation through electron impact[38]. The application of GC-MS in metabolite profiling regards the naturally occurring volatile metabolites and metabolites rendered volatile through chemical derivatisation, a chemical reaction carried out to reduce the polarities of the functional groups. Headspace sampling (HS) is a solvent-free technique used in combination with GC and GC-MS to characterize the volatile fraction of different matrices including food matrices and aromatic and medicinal plants[39]. Solid-phase microextraction (HS-SPME) developed and applied to HS sampling by Zhang and Pawliszyn in 1993[40] maximizes sampling opportunities. GC-MS metabolite profiling has been applied in various studies: tomato[41], pumpkin[42],alfalfa[43,44],Arabidopsis.

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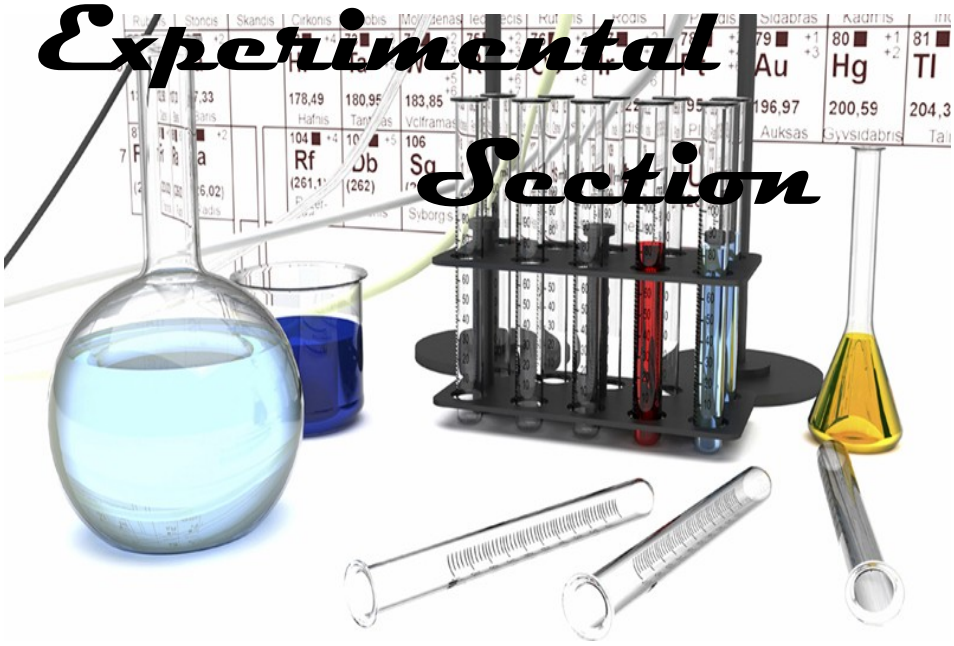
Experimental Section



III. Experimental

III.1 Material and equipment

III.1.1 Plant material and extraction. Sample preparation

Leaves and edible shoots of *Cicerbita alpina* L. Wallr., *Asparagus acutifolius* L., *Chenopodium bonus henricus* L., *Levisticum officinalis* Koch., *Silene vulgaris* Moench., *Spirea aruncus* L. were collected in the Udine province (northeastern Italy) and were provided by ERSA (Udine, Italy). Plants were collected in the wild at different sites during the summer of 2009. In addition, a comparative investigation has been conducted on samples belonging to cultivated shoots of *C. alpina* collected from the experimental orchard and samples collected in the wild. Exact dates and locations of collection sites are given in Table 9. The shoots were collected at the developmental stage suitable for human consumption that is when they reached 8-10 cm height. A batch of cleaned fresh samples were immersed in liquid nitrogen, stored at -80°C and successively freeze-dried, while the second batch consisted in air-dried samples. Prior to extraction, the samples were reduced to a fine powder using a grinding mixer (Analytical grinder A10 3250000, Ika, Germany). The methods of extraction employed were:

- Microwave-assisted extraction (MAE): 30 mL of solvent MeOH/H_2O (50:50 and 80:20 v/v) were added to 3.0 g of plant material in the inner vessel, for 5 minutes, at 90°C and 300 W irradiation power;

- Ultrasound-assisted extraction (UAE): 50 mL of solvent MeOH/H_2O (50:50, v/v) were added to 5.0 g of plant material, for 1 h, at 70 W and 21.4 Hz; maceration using 50 mL EtOH added to 5.0 g of plant material. All the extracts were filtered and the filtrates were evaporated under vacuum.

- Microdistillation of the *C. alpina* and *L. officinale* aerial parts according to the method described in the 5th edition of the European Pharmacopoeia[1]. A sample (10 g) of dried plant material was suspended in 200 mL water in a 500 mL reactor for 1 h and then submitted to hydrodistillation in a Clevenger micro-apparatus for 2 h[2]. The resulting essential oil was left to stabilize for 1 h and then analyzed.

Table 9 Samples under investigation

Sample	Collection site	Date of collection	Altitude (a.s.l)
C. alpina w.	Collina	27th May, 2009	1800 m
C.alpina c.	Piani di Vas (Rigolato)	19th May, 2009	1400 m
A. acutifolius w.	Sistiana	22nd April, 2009	100 m
C. bonus Henricus c	Curiedi	28th April, 2009	700 m
L. officinale w.	Tarcento	26th April, 2009	750 m
S. vulgaris w.	Sauris	16th May, 2009	230 m
S. aruncus w.	Pedrosa	7th April, 2009	1200 m
			176 m

[w] wild [c] cultivated

- Sample preparation for the UHPLC-TOF-MS analyses consisted in the SPE elution protocol, selected following consideration of both the nature of the analytes and MS compatibility. The SPE cartridge (Sep-Pak C_{18} 100 mg, Waters) was flushed with CH_3OH 85% (1 mL), then the sample was loaded, and finally the cartridge was washed with CH_3OH 85%. The second fraction was collected by elution with CH_3OH 100%.
- Samples (200 mg) of air-dried *C. alpina* and *L. officinale* were hermetically sealed in a 7.5 mL vial, introduced into a thermostatic bath and submitted to HS-SPME sampling.

III.1.2 Chemicals and solvents

The standards used in this study were: flavonoids and phenolic acids e.g. acacetin, apigenin, diosmin, eriodictyol, hesperetin, kaempferol, luteolin, caffeic acid, ferulic acid, gallic acid, para hydroxybenzoic acid, syringic acid and two flavanone glicosides, apigetrin (apigenin 7-*O*-glucoside) and hesperidin (hesperetin 7-rhamnoglucoside); glucose, fructose; aminoacids e.g. hydroxy L-proline, L-aspartic acid, L-tryptophan, L-leucine, L-lysine, L-arginine, L-isoleucine, L-serine, alfa phenyalanine, L-alanine, methionine, L-tyrosine, L-threonine, L-proline, L-valine, glycine, L-asparagine, L-glutamic acid, D(-) isoleucine, D(-) cysteine, D serine, D-leucine; fatty acids e.g. oleic acid, stearic acid, palmitic acid. HPLC-grade methanol, HPLC-grade formic acid and Milli-Q grade water (Milli-Q Plus system, Milipore, Bedford, USA) were used as solvents for HPLC and HPLC-MS analyses, whereas acetonitrile and water for the UPLC-ESI-TOF/MS analyses were ULC/MS grade purchased from Biosolve (Valkenswaard, The Netherlands). All the chemicals were purchased from Sigma Aldrich (Milan, Italy).

III.1.3 Instrumentation

MAE experiments were carried-out in a professional microwave oven (Mars 1200 W, 2450 MHz, CEM Corporation, Matthews, North Carolina, USA). The

instrument has an internal temperature control system with an optical fiber thermometer and a pressure control system. UAE experiments were performed in a sonochemical apparatus consisting in a probe system with an immersion titanium horn (21.4 kHz).

III.2 Methods

III.2.1 Colorimetric assay for total phenolic content

Phenolic content was determined, according to the method developed by Cicco et. al[4]. The proposed method is a variation on the conventional Folin-Ciocalteu method which uses a new combination among time, temperature, alkali and alcohol for the spectrophotometric evaluation of low-concentration phenolics in methanol extracts. 100 μL of properly diluted samples were pipetted into separate tubes and 100 μL of Folin-Ciocalteu reagent was added. The mixture was mixed well and allowed to equilibrate for 2 minutes. Subsequently, 800 μL of a 5% (w/v) sodium carbonate solution were added and finally, the tubes were placed in a temperature bath, at 40°C for 20 minutes. The absorption of the final mixtures was measured at 740 nm, in a 1 cm cuvette, on a UV/VIS Varian Cary 1E spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Quantification was carried out on the basis of a standard curve which was prepared using different dilutions of a 1 mg mL^{-1} solution of gallic acid in methanol as the reference phenolic compound. Total phenol values are

expressed as gallic acid equivalents (mg g^{-1} GAE of dry weight DW).

III.2.2 Colorimetric assay for total flavonoids

The aluminum chloride colorimetric method was used for the determination of flavonoid content in the crude extracts[5]. The diluted samples (0.5 mL) were mixed with MeOH (1.5 mL), 10% $AlCl_3$ (0.1 mL), 1 M CH_3COONa (0.1 mL) and distilled water (2.8 mL). Absorbance at 415 nm was recorded after 30 min of incubation at room temperature. A standard calibration curve was generated at 415 nm using known concentrations of rutin. Total flavonoid values in the test samples were calculated from the calibration curve and expressed as rutin equivalents per g of sample.

III.2.3 High performance liquid chromatography coupled with ultraviolet detection/diode array detection

High performance liquid chromatography coupled with a diode array UV detector (HPLC/UV-DAD) was used to analyze qualitatively the investigated extracts by providing preliminary information about the UV profile.

A binary pump Shidmazu LC-6A, connected to a photodiode array UV-VIS detector Shimadzu SPD-M6A (Kyoto, Japan) and a Rheodyne 7125 injection valve with a 20 μL loop were used. The extracts were analyzed by HPLC using a Nova-Pak C_{18} column (150 x 3.9 mm i.d., 4 μm, Waters, Milford USA) eluted with a gradient of methanol (A)–water (B)

containing 0.1% formic acid from 5-95 % B in 70.0 min. The flow rate was 1.0 mL min^{-1}; UV detection was set at 280 and 323 nm. The injection volume was 10 µL. All samples were filtered through a 0.45 mm membrane filter (Econofilter, Agilent Technologies, Cernusco sul Naviglio, Italy). HPLC system was controlled by the LabSolution software.

III.2.4 Liquid chromatography coupled with mass spectrometry

LC/ESI-MS analyses and MS^n experiments were performed on a Finnigan LXQ linear ion trap operating in negative ion mode coupled with a Finnigan Surveyor LC Pump Plus equipped with a Finnigan Surveyor Autosampler Plus (Thermo Scientific, San Jose, CA, USA). The LC separations were performed on a C_{18} Nova-Pack column (150 x 3.9 mm, 4 µm) operating at 30°C, 1 mL min^{-1} flow. The injection volume was 10 µL. Collision-induced dissociation (CID) multiple MS spectra (MS^n experiments) were acquired using helium as the collision damping gas in the ion trap at a pressure of 1 mTorr. Elution from the LC column was carried out using the same mobile phase and gradient, as described previously for the HPLC-UV method. Optimized instrument tune parameters were: transfer line capillary at 275°C, ion spray voltage at 4.70 kV, sheath, auxiliary and sweep gas flow rates at 22, 4 and 0 arbitrary units, respectively. CID was carried out by isolating the precursor ions in the ion trap (isolation width 1.2 *m/z*), and subjecting them to the following typical conditions:

normalized collision energy between 15 and 25%, selected to preserve a signal of the precursor ion in the order of 5%; activation Q 0.25 and activation time 30 ms. Mass selection of the analyte by *m/z* was followed by fragmentation and fragment analysis. The full mass spectra were recorded in the 100-1000 *m/z* range. Data were processed using Xcalibur 2.0 software (Thermo Electron). The components were identified by comparison of their mass spectra to those of authentic samples and with data in the literature.

III.2.5 Ultra performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC/HR-TOF-MS)

UHPLC/HR-TOF-MS analyses were performed on a Micromass-LCT Premier Time of Flight mass spectrometer from Waters (Milford, MA, USA) with an electrospray (ESI) interface coupled with an Acquity UPLC system from Waters. Detection was performed separately in positive and negative in the range *m/z* 100-1000 in centroid mode with a scan time of 0.3 s. ESI conditions in positive mode were capillary voltage 2800 V and negative mode were capillary voltage 2400 V, cone voltage 40 V, source temperature 120°C, desolvation temperature 330°C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. For internal calibration, a solution of leucine-enkephalin from Sigma-Aldrich (Steinheim, Germany) at 1 μg/mL was infused through the lock-mass probe at a flow rate of 10 μL/min,

using a second Shimadzu LC-10ADvp LC pump (Duisburg, Germany). The separation was performed on a 150 mm × 2.1 mm i.d., 1.7 μm, Acquity BEH C_{18} UPLC column (Waters) in the gradient mode at a flow rate of 460 μL min^{-1} with the following solvent system: A) 0.1 % formic acid-water, B) 0.1 % formic acid acetonitrile; 2-95% B in 40.0 min and holding at 95% for 5 min. The temperature was set at 40 °C. The injected volume was 20 μl. Data were processed by MassLynx Software 4.1 (Waters, MA, USA). The identification of the compounds resulting in the greatest peak abundance was conducted by means of an algorithm of formula filtering of the elemental compositions computed iteratively for each compound and database searches (e.g Dictionary of Natural Products). The algorithm is based on the "seven heuristic rules" ((1) restriction for the number of elements, (2) Lewis and Senior chemical rules, (3) isotopic pattern, (4) hydrogen/carbon ratios, (5) element ratio of nitrogen, oxygen, phosphor and sulphur versus carbon, (6) element ratio probabilities, (7) presence of trimethylsilylated compounds), accurately described by Kind et al[6].

III.2.6 Gas chromatography coupled with mass spectrometry

GC-MS analyses were carried out on three instruments:

- an Agilent Technologies 6850 Network GC System with 5973 Network Mass Selective Detector operating in electron impact mode, using a capillary column (HP-5MS 5% phenyl-95% methylsiloxane, length 30 m; i.d. 0.25 mm; film thickness 0.25 μm).

- GC conditions were: injection split 1:20, injector temperature 250°C, detector temperature 280°C; He as carrier gas at 1.0 ml/min; Temperature programmes: 1) 150°C for 4 min, raised 10°C/min to 315°C and held for 10 min (method adapted with slight modifications from Füzfai and Molnár)[7]; 2)70°C for 3 min, 10°C/min to 150°C, raised 8°C/min up to 320°C and held for 5 min.
- a Trace GC Ultra combined with a Trace DSQ (Dual Stage Quadrupole) mass spectrometer operating in electron impact mode at 70 eV. An AI 3000 auto injector was used for sample injections. All instruments were supplied by Thermo Electron, Milano, Italy. MXLATOR. GC data were acquired with Xcalibur Data System Software (ver. 1.4 Thermo Electron Corporation) and handled with Hyper Chrom Card ver. 2.3.3. (Thermo Electron Corporation). Ion source temperature: 250°C, transfer line: 250°C. Automatic tuning was used. GC analyses were carried out on a HP-5MS capillary column. For the volatile fraction, the GC oven temperature programme was set at 50°C for 1 min, raised 3°C/min to 250°C and held for 5 min.
- an Agilent 7890A equipped with a Mass Selective Detector 5975C (Agilent Technologies, Little Falls, DE, U.S.A.) and an autosampler Gerstel MPS (Gerstel, Mülheim a/d Ruhr, Germany). MS operated in electron impact ionization mode at 70 eV, ion source temperature 250°C, transfer line 280°C. The column employed was a HP-5MS. The GC oven temperature programme was set at 50°C for 1 min, raised 3°C/min to 250°C and held for 5 min. Three

incubation temperatures were used for the HS-SPME analyses: 35°C, 50°C and 80°C for 15 min. Helium was used as carrier gas at a flow rate of 1 mL/min. Split ratio 1:10 was used for the analyses performed with HS-SPME and 1:20 for the all the rest. Injection volume was 1 μL for all the runs. Data acquisition and data handling were performed by ChemStation G1701 DA D 03.00 software. Mass spectra of all detected compounds were compared both to spectra of pure standards and spectra available in commercial MS spectra libraries through the match quality index calculated by the NIST Similarity and Identity Spectrum Search algorithm (NIST 08 and Wiley MS 275).

III.2.7 Acidic hydrolysis

Aliquots of crude extracts were submitted to hydrolysis with TFA 2M, at room temperature for 15 min, 30 min, 1h, 2h, 3h, 4h[7]. Hydrolysis was also carried out using 20 mL of HCl 0.05 N at 65°C for 1h.

III.2.8 Enzymatic hydrolysis

Extracts were treated with β-D-glucosidase for three days at room temperature. The final reaction mix was divided in two fractions extracted with dichloromethane.

III.2.9 Derivatisation reactions for GC analyses

Derivatisation was performed on dehydrated crude extracts obtained from samples belonging to freeze-dried and air dried plant material (15 mg of dehydrated crude extract were treated with 500 μL pyridine (containing 2.5 g hydroxylamine hydrochloride/100 mL) and were heated for 30 min, at 70 °C. The cooled samples were then trimethylsilylated with 900 μL HMDS and 100 μL TFA in the same vials for 60 min, at 100 °C[7]):

- derivatisation of crude extracts not subjected to a preliminary treatment;
- derivatisation of hydrolyzed extracts for various periods of time;
- derivatisation of extracts obtained selectively with solvents having increasing polarity (hexane, dichloromethane, ethyl acetate, MeOH 80%) using both maceration and UAE techniques.
- derivatisation method (for fatty acids) adapted from Lepage and Toy with modifications made by Xu et al.[8,9]: the dried extract (15 mg) was suspended in 1 ml methanolic NaOH solution 0.5N and heated at 75° for 10 min; after cooling the sample, 1 ml methanolic solution CH_3COCl_3 3M was added to the mixture, then again heated at 75°C for 10 min; after cooling the sample, 1 ml of water and 1 ml of hexane were added to the mixture. The heterogeneous sample was vortexed. After phase separation, the hexane phase was transferred into vials prior to GC analysis.

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Results

and

Discussions

IV. Results and discussions

IV.1 Design of experiment of microwave-assisted extraction. 2^3 Full factorial design

A MAE set was designed for the extraction of phenolics from the cultivated *C. alpina* edible shoots. The variables of the study include methanol/water ratio, sample quantity, extraction temperature and time, whereas the response is expressed in the total chromatographic area of the peaks monitored at 280 nm. The coded values which correspond to the upper (+1), intermediate (0) and the lower level (-1) ranges for each variable, are listed in Table 10.

Table 10 Factors and coded values applied in MAE of total phenolics

Independent variabile	Lower level (-1)	Intermediate level (0)	Upper level (+)
X1: Temperature	40°	65°	90°
X2: Extraction time (min)	5	15	25
X3: Extraction solvent % (binary mixtures MeOH/H_2O)	50	75	100
X4: Sample quantity (g)	1	2	3

Modde procedures for design of experiments (Modde ver. 8.02, MKS Umetrics AB, Sweden) were employed to design and analyze the experimental data. 19 experiments were used to optimize the four aforementioned factors in the 2^3

full factorial design applied to the MAE of phenols from *C. alpina* edible shoots.The response values together with the experimental combinations are listed in Table 11.

Table 11 Screening factorial design in terms of coded values and response values for total chromatographic area at 280 nm

Experiment no.	X1 (°C)	X2 (min)	X3 (%)	X4 (g)	Response (A.U)
1	-1	-1	-1	-1	55594
2	1	-1	-1	-1	59216
3	-1	1	-1	-1	53012
4	1	1	-1	-1	49516
5	-1	-1	1	-1	41344
6	1	-1	1	-1	48943
7	-1	1	1	-1	46335
8	1	1	1	-1	45469
9	-1	-1	-1	1	66283
10	1	-1	-1	1	74554
11	-1	1	-1	1	56078
12	1	1	-1	1	57059
13	-1	-1	1	1	51481
14	1	-1	1	1	55857
15	-1	1	1	1	41218
16	1	1	1	1	44596
17	0	0	0	0	49821
18	0	0	0	0	47781
19	0	0	0	0	46312

The most important factors and their interactions are depicted as contour plots of the MAE process. All runs were randomly performed in triplicate and the chromatographic area averages at 280 nm were taken as the response.

The model fit was evaluated by examining the summary of the fit, R^2 (measures fit), Q^2 (measures predictive power), model validity (indicates if the model is appropriate) and reproducibility (assesses replicate variation), coefficients, ANOVA and the effect plots for screening designs. Model adequacy was further assessed using a plot of residuals against predicted values. The goal of the screening factorial design was to reduce the number of factors down to only those with the largest effect on the response. The MAE total chromatographic area at 280 nm ranged from 41218 to 74554 A.U.

The response contour plots that show the different interactions between the variables are shown in Fig. 10.

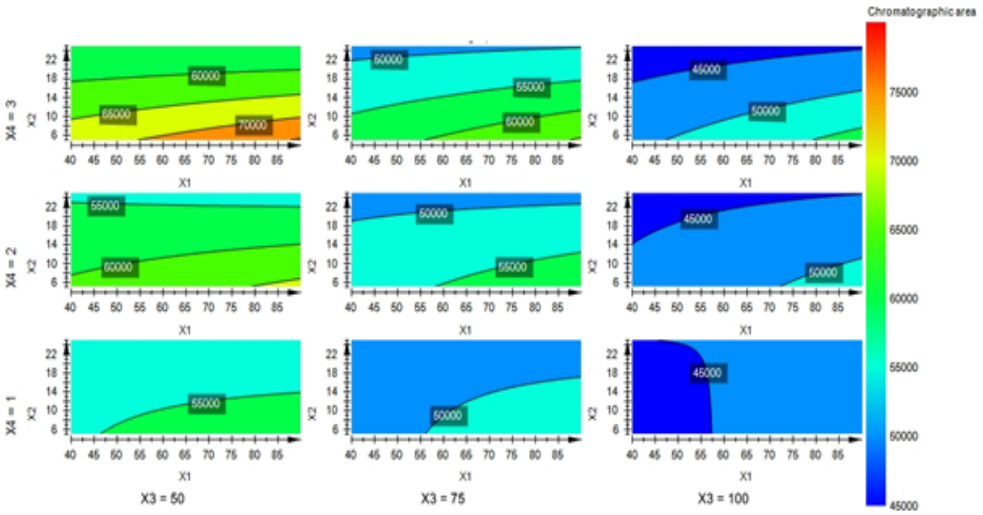


Figure 10 Contour plots for the effects of the variables X1, X2, X3, X4 on the chromatographic area

The ANOVA of the linear model showed that the values of the determination coefficient, R^2, and the adjusted determination coefficient, R^2 adj., were 0.9242 and 0.8295 respectively, which demonstrates a high degree of correlation between the observed and the predicted values. ANOVA also showed that the *p*-value of the model was 0.002 indicating that the model was statistically significant at the 95% confidence level ($p<0.05$).

In view of these results, the optimum MAE conditions were: extraction temperature (90°C), extraction time (5 min), solvent ratio (50%) and sample quantity (3 g). At higher temperature, solvent viscosity decreased enhancing the diffusivity and, thus, extraction efficiency. The reason for the higher extractability of phenols is the introduction of water to methanol. The increase in plant tissue permeability can be explained by the presence of water which enables better mass transfer via diffusion. The volume of solvent used was indeed important because a higher volume of solvent generally increases recovery in conventional extraction techniques, but studies concerning recoveries in MAE show that in this case, lower recoveries were achieved. The highest amount of phenolic compound was achieved after 5 minutes of MAE irradiation, thus, it can be supposed that prolonged exposure to the irradiation leads to the degradation of the phenols as the solute/solvent system overheats.

IV.1.1 Validation of the model

Fig. 11 presents the assessment of the model adequacy in the form of a plot of residuals against predicted values. Under the optimal conditions, the model predicted a response of 76335 A.U. and a mean value of 74820 A.U., RSD= 0.02% (*n*=3). The model adequacy was confirmed by the good correlation between the results.

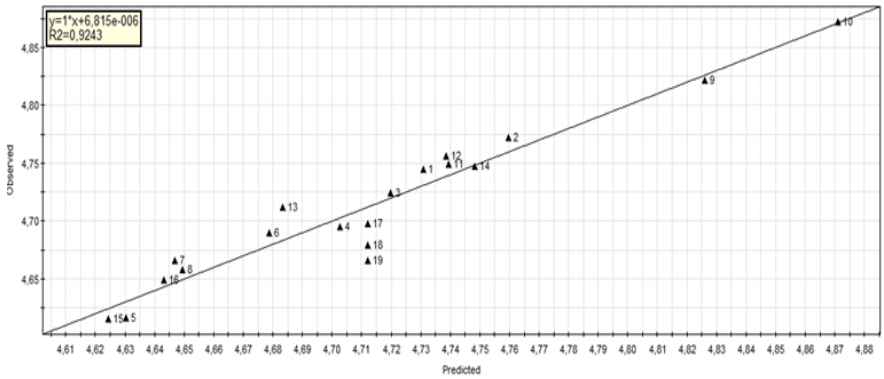


Figure 11 Plot of residuals against predicted values

IV.2 Preliminary qualitative phytochemical survey of the edible shoots of *C. alpina*

IV.2.1 Influence of various extraction techniques and solvent mixtures on the yield of *C.alpina* crude extracts

The freeze-dried fresh shoots of the cultivated *C alpina* yielded the highest amount of crude extract under the microwave-assisted extraction, using a mixture MeOH/H_2O 50:50 v/v (Figure 12). For comparative purposes with the non-conventional methods, maceration (3 days, r.t.) was used as a classical extraction technique. The efficiency of the UAE was evaluated in the presence of the solvent mixture aforementioned. The choice resorted to maceration using ethanol as "green" solvent and a prolonged contact sample-solvent time (24h) at constant temperature (r.t). The influence of the extraction methods on the investigated samples is outlined by three observations: following the same extraction technique and conditions, the yield of crude extract belonging to the cultivated herb is higher (25.42%) compared to the yield of crude extract belonging to the wild herb (21.30%); the crude extracts deriving from the air-dried samples yielded lower values; maceration gave the lowest yield of crude extract. The extractions performed with the mixture MeOH/H_2O 1:1 resulted in the highest amount of crude extract. MAE proved to be more efficient as extraction technique than UAE. In order to explore the differences between the seven extracts of the same species, they were subjected to further analytical investigation.

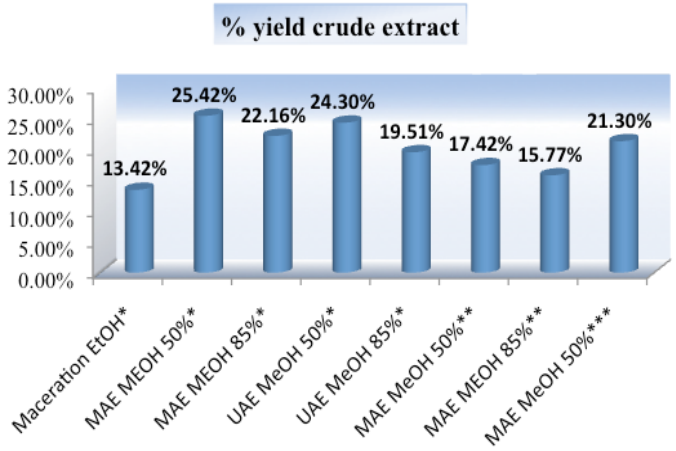


Figure 12 Yields of crude extracts of *C.alpina* obtained with different extraction techniques
*freeze-dried edible shoots of cultivated *C.alpina*
**air-dried edible shoots of cultivated *C. alpina*
*** freeze-dried edible shoots of wild *C.alpina*

IV.2.2 Colorimetric investigations and UV profiles of the crude extracts

In order to roughly estimate the overall amount of phenolics and flavonoids in the crude extracts, two basic spectrophotometric assays were employed. The total phenolic content for the extracts under investigation was calculated from the regression equation Y= 0.1045x + 0.0219, R^2=0.9987 and results are reported in Table 12. The total flavonoid content was calculated from the equation Y=0.0031x + 0.0878, R^2= 0.9857 and results are shown in Table 13. All measurements were repeated five times and expressed as averages ± SD. The highest amount of both total phenolics and flavonoids was obtained from the freeze-dried shoots of the cultivated herb extracted under

microwave irradiation with MeOH 50%. The lowest amounts were obtained from the freeze-dried shoots of the sample collected in the wild. The great difference within the same species growing in two different habitats is also illustrated by four UV profiles of the crude extracts monitored at 280 nm and 323 nm (Figure 13). According to Schieber et *al.*[1], Folin-Ciocalteu method leads to an overestimation of the total phenolic content in plant extracts, due to interference from reducing substances, so a chromatographic approach is essential for a better estimation of the phenolic content.

Table 12 Total phenolic content of the crude extracts

Extraction technique	**Phenolic content (mg GAE/g DW ± SD)[a]**
Maceration EtOH*	55.71±0.83
MAE MeOH 50%*	93.58±0.47
MAE MeOH 85%*	91.30±0.74
UAE MeOH 50%*	84.50±0.91
UAE MeOH 85%*	70.30±0.45
MAE MeOH 50%**	69.83±0.87
MAE MeOH 85%**	62.07±0.51
MAE MeOH 50%***	33.92±0.94

[a]Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; Mean and standard deviation, *n*= 5

Table 13 Total flavonoid content of the crude extracts

Extraction technique	Flavonoid content (mg rutin/g DW ± SD)[a]
Maceration EtOH*	71.51±1.18
MAE MeOH 50%*	145.06±0.87
MAE MeOH 85%*	144..30±1.04
UAE MeOH 50%*	85.50±0.97
UAE MeOH 85%*	79.30±0.44
MAE MeOH 50%**	139.22±0.81
MAE MeOH 85%**	110.07±1.98
MAE MeOH 50%***	46.73±1.55

[a]Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample.; Mean and standard deviation, *n*= 5

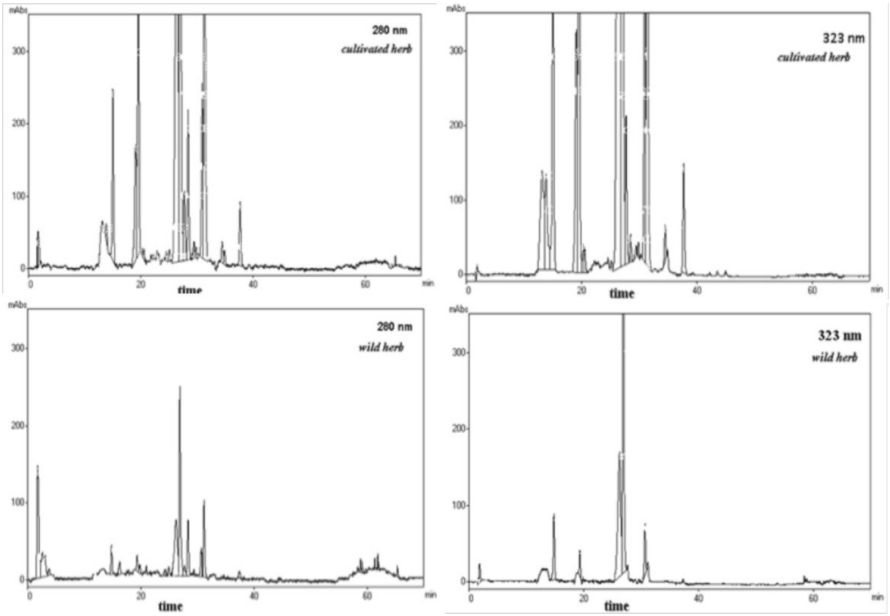


Figure 13 UV profiles corresponding to the crude extracts of the cultivated and wild *C. alpina* edible shoots

The first step of the present investigation offered preliminary information about the extracts that were subsequently subjected to a deeper analysis. The results obtained by the colorimetric assays are in good agreement with the UV profile monitored at specific wavelengths.

IV.2.3 LC-MS^n analysis of the cultivated *C. alpina* crude extract

The LC analysis of a mixture of common phenolic standards available in our laboratory was carried out before, in order to evaluate the fragmentation behavior in the product-ion spectra. According to Justensen[2], the product-ion spectra of the standard aglycones provided information about their specific A-ring and B-ring fragmentation. The A-ring fragments *m/z* 151 and *m/z* 107 were present as common fragments in the product-ion spectra of the main flavanone (eriodyctiol, hesperitin) and flavone (apigenin, luteolin, acacetin) standard compounds. The specific B-ring fragments observed for flavones and flavanones were: *m/z* 117 for apigenin, *m/z* 133 for luteolin and *m/z* 135 for eriodyctiol. Mass spectra of the methoxylated flavonoids, acacetin and hesperitin, revealed base peaks whose *m/z* values correspond to the loss of $\bullet CH_3$ from the deprotonated ion $[M-H-15]^{\bullet -}$ and did not provide B-ring fragments (Fig. 14) .

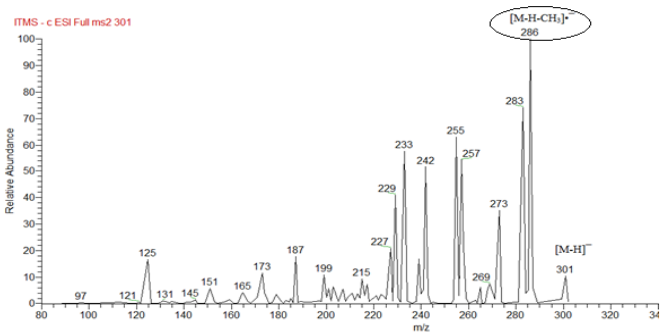


Figure 14 Product-ion spectra of hesperitin (precursor ion *m/z* 301)

In order to obtain structure information, multiple stage LC-MS analyses were performed on the "richest" crude extract revealed by the previous chemical surveys, whose components possess strong chromophore systems. Fifteen peaks, numbered as 1-15, were detected and tentatively assigned as belonging to both flavonoid and phenolic acid classes. Characterization of the phenolic compounds was based on the mass spectra obtained under ESI and multistage MS, with reference compounds and with literature data[7-14]. The direct injection mass spectra and the HPLC-MS profile of the *C. alpina* extract under investigation are presented in Fig. 15. Even though plant matrix hampers the analysis of many metabolites, a previous screening analysis by direct mass injection was carried out before the chromatographic separation. MS^n analyses in negative mode were performed on each molecule and the data obtained are summarized in Table 14.

In negative MS analysis, the pseudomolecular ion occurs due to the glycosidic *O* linkage with a concomitant H arrangement in the 7-position. In such way, dehydrated

monosaccharide residues (162 amu) or disaccharide residues (324 amu) [3] are eliminated. The MS spectra of compounds 1-4 revealed specific fragments that characterize the presence of three free phenolic acids (2-4) and a diglucoside of quinic acid (1) which is highlighted by the fragment MS^2 191, the loss of two hexose units (324 amu) and a water molecule.

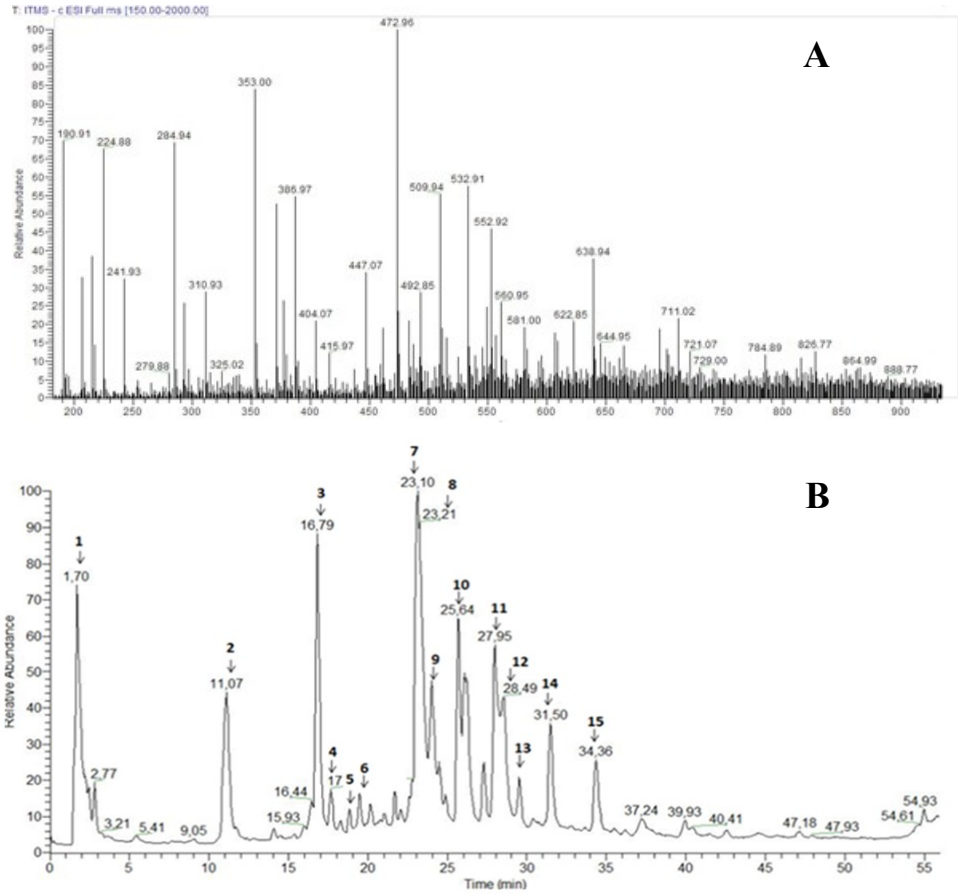


Figure 15 Profile of in-source fragment ions (**A**) and total ion chromatogram of the cultivated *C. alpina* crude extract (**B**

Table 14 Tentative peak assignments of *C. alpina* crude extract

			m/z of the main fragments (% relative abundance)		
Peak	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS^2 ions**	**MS^3 ions**	**Tentative assignment**
1	1.7	533	191 (29)	-	Quinic acid derivative
2	11.07	311	179 (65)	149 (30)	Caftaric acid
3	16.79	353	179 (9), 191 (100)	-	Caffeoylquinic acid
4	17.01	179	161 (54), 135 (100), 143 (69	-	Caffeic acid
5	18.81	447	401 (2)	285 (100)	Luteolin derivative
6	19.38	431	385 (100)	-	1-O-β-D-glucopyranosyl sinapate
7	23.1	463	287 (100)	-	Eriodyctiol glucuronide
8	23.21	473	311 (100), 293 (92), 179 (2)	-	Caffeic acid apiosyl glucoside
9	24.07	473	311 (100)	179 (58)	Cichoric acid
10	25.64	609	447 (7), 285 (100)	-	Kaempferol- 3', 7' di-O-glucoside
11	27.95	515	353 (100)	179 (53), 191 (100)	3', 5' O- dicaffeoylquinic acid
12	28.49	461	285 (100)	-	Luteolin glucuronide
13	29.33	593	447 (100), 285 (7)	-	Luteolin-rhamnoglucoside
14	31.5	431	269 (57)	-	Apigenin-7-O-β-glucoside
15	34.36	533	489 (100)	285 (100)	Luteolin acetyl glucoside

Based on literature data[4], compounds 2 and 3 were tentatively assigned as caftaric acid ((*m/z*) 311→179→149) and caffeoylquinic acid ((*m/z*) 353→179→191). Caffeic acid ((*m/z*) 179→161→135→143) was identified by comparing its mass spectra with its authentic sample. Another mass fragmentation pattern, similar to the one found for compound 3, can be seen in the case of compound 11 ((*m/z*) 515→353→179→191). In this case, the fragmentation pattern of 3', 5' dicaffeoylquinic acid is identical to the same compound reported in other studies[5].

The caffeic acid derivative found in the mass spectra of compound 8 is revealed by the presence of the fragment MS^2 179 that corresponds to caffeic acid and by the losses of 162 amu (hexose unit) and 132 amu (pentose moiety). Data previously reported in literature[6] and the mass fragmentation of compound 9 revealed a derivative of caffeic acid (Mr 180) and tartaric acid (Mr 150), namely cichoric acid. The

product ion spectra for peak 7 and 12 exhibited the loss of a 176 amu which can be attributed to a glucuronide loss. Negative ESI-MS^n spectra of components 5, 9, 11 and 2 are shown in Fig. 16. On the basis of literature data[8-14] and of the mass spectra analyses, four compounds (5, 12, 13 and 15) were tentatively assigned as luteolin derivatives. In all cases, the fragmentation of the deprotonated ion resulted in fragments having in common the aglycone *m/z* 285. The mass spectrum of luteolin was compared to the mass spectra of the relative reference standard, thus confirming its identity. It is well known that flavonoids are generally stable compounds, and their fragmentation pathways depend on the substitution pattern of the aromatic rings, producing product ions at low level of intensity, and maintaining high level of unfragmented precursor ions. The heterocyclic linkage between rings A and B of flavonoids (Fig. 17) is more prone to fragmentation, this site being less easily fragmented when a C-2–C-3 double bond is present, as in flavones, or when a further hydroxyl is present at C-3 (flavonols). Such a fragmentation, known also as the retro Diels Alder reaction (RDA) which may occur in the six-membered C-ring cyclic structures containing a double bond can be noticed for the aglycones of compounds 10 and 14. This reaction leads to 151 *m/z*, 225 *m/z* and 117*m/z* specific negative ions for apigenin (Fig.17).

The assignment of compound 14 as apigenin-7-*O*-β-glucoside was done by comparison with the authentic sample.Kaempferol 3'-7'-di-*O*-glucoside was unambiguously assigned to compound 10, on the basis of literature data and comparison to the aglycone (kaempferol) authentic sample. The publicly available Massbank database[14] was used as additional support for the putative identification process of the reported compounds.

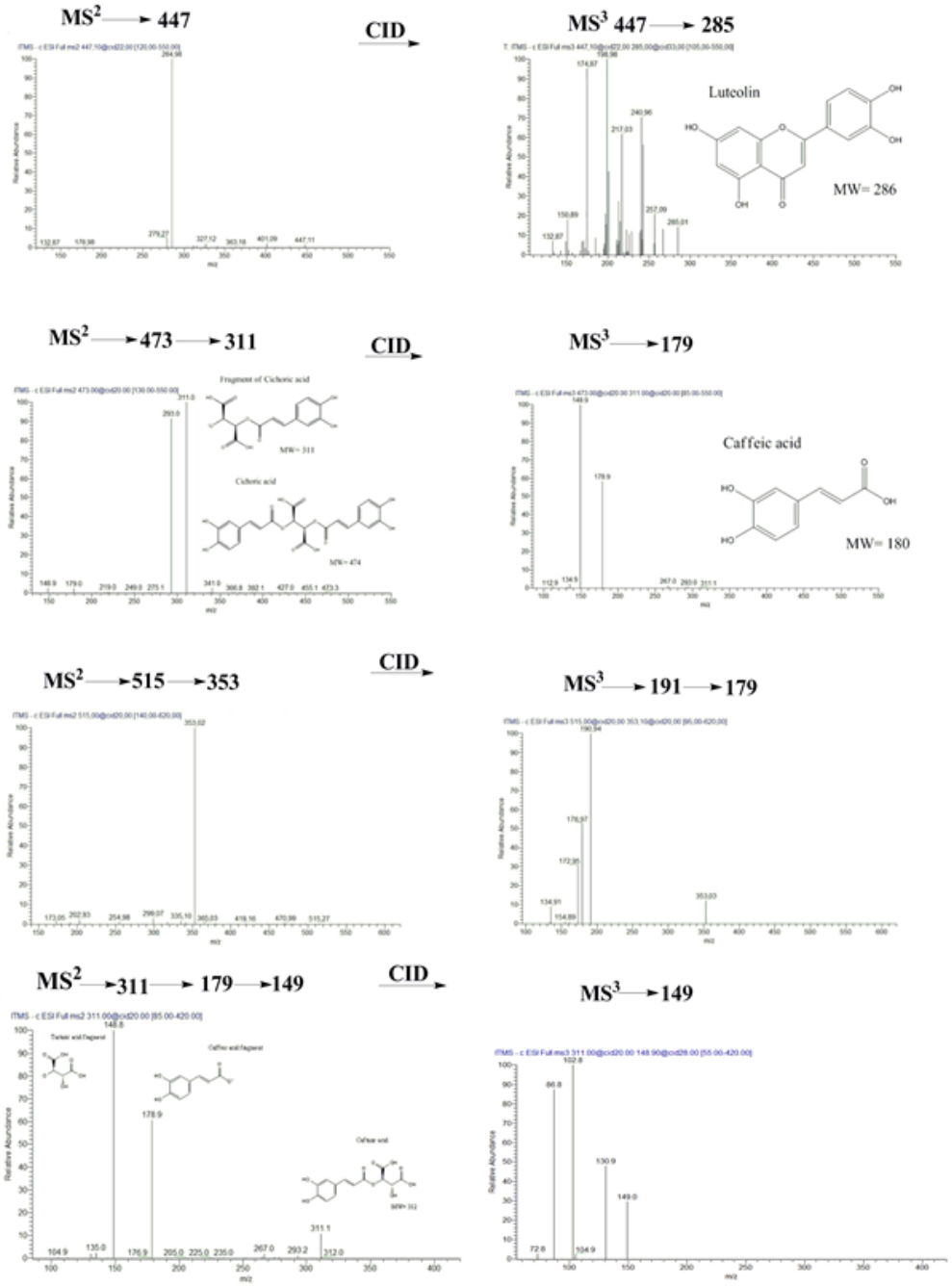
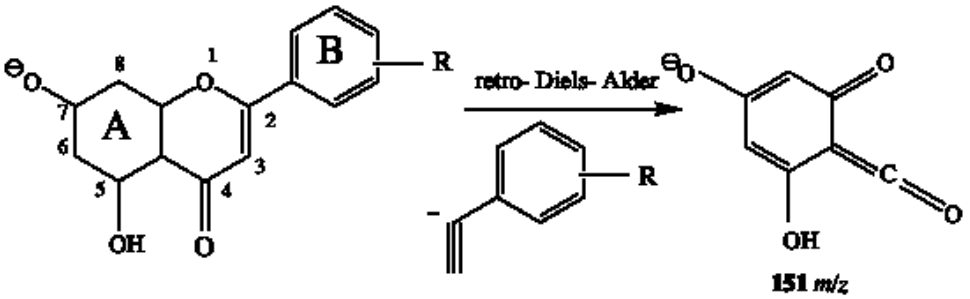


Figure 16 Negative ESI-MS^n spectra of components 5, 9, 11 and 2



Common RDA fragmentation patterns of flavonoids

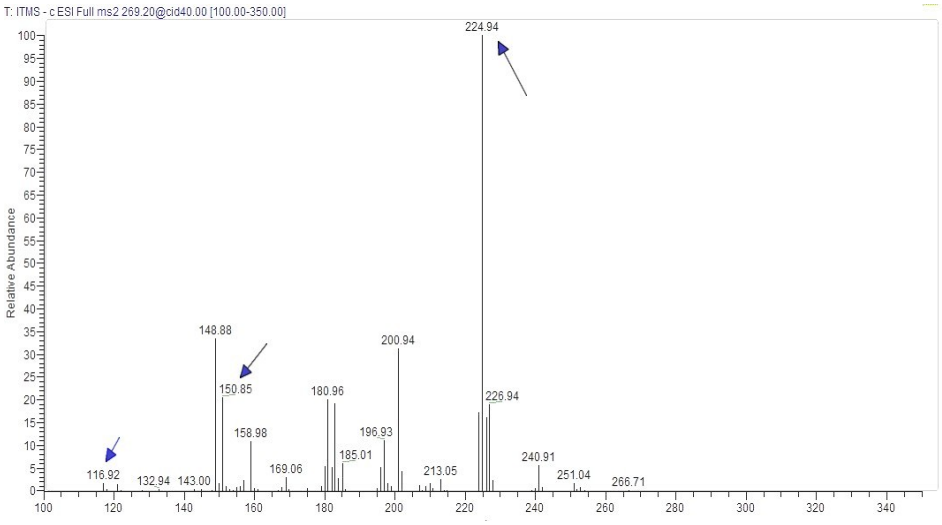


Figure 17 Negative ESI-MS^2 spectra of apigenin

IV.2.4 Comparative putative metabolite identification in crude extracts by UHPLC- TOF-MS

Because of the convoluted nature of the crude plant extracts and in order to prevent the unambiguous assignment of peaks caused by co-elution, thus increasing the certainty of the peak identification, the use of HR-MS was considered mandatory. High resolution chromatographic techniques and database searching represent two compulsory tools acting at the earliest possible stage in the dereplication strategies. Calculation of the chemical combinations that fit a certain accurate mass is generally one of the first steps to obtain a set of alternatives that can lead to the identity of the metabolite detected. By combining both mass and isotopic ratio determination, unknown peaks are conveniently characterized. The main challenge when dealing with mass spectra interpretation is the detection of the molecular ion. The peak at the highest *m/z* ratio is not always the molecular ion species, because adducts with solvent and acid molecules or molecular complexes can be generated. For instance, the solution to reduce the incidence of both adducts and complex formation could be increasing the cone voltage [15]. In pursuance of the aforementioned considerations, a step-by-step formula filtering using the seven golden rules is presented in Fig. 18. For the sake of simplicity, the whole algorithm presented in Fig. 18 was illustrated for one deprotonated ion chosen from the TIC of the crude extract and represents an example. This procedure was extended for all the other pseudomolecular ions in both negative and

positive mode in order to get the correct elemental formulas. Good formula fit was indicated by the low values of the i-FIT scores which showed the good pattern match between every individual peak and the relative predicted isotope peaks. Five crude extracts of *C. alpina* were analyzed by UHPLC-TOF-MS and their profiles were compared to the profile of the crude extract previously analyzed by LC-MS^n, taking into consideration the most abundant peaks revealed by this technique (Fig. 19). A comparative UHPLC-TOF-MS investigation of three crude extracts resulting in significant qualitative differences is reported in Table 15. Eighteen compounds characterized by their elemental composition obtained using the algorithm for formula filtering and their putative assignment show the great difference between the two samples. The presence of the pseudomolecular ions was checked in both negative and positive mode, in order to get more reliable information. Prior to analysis, all the crude extracts were purified by the SPE protocol described in the experimental section.

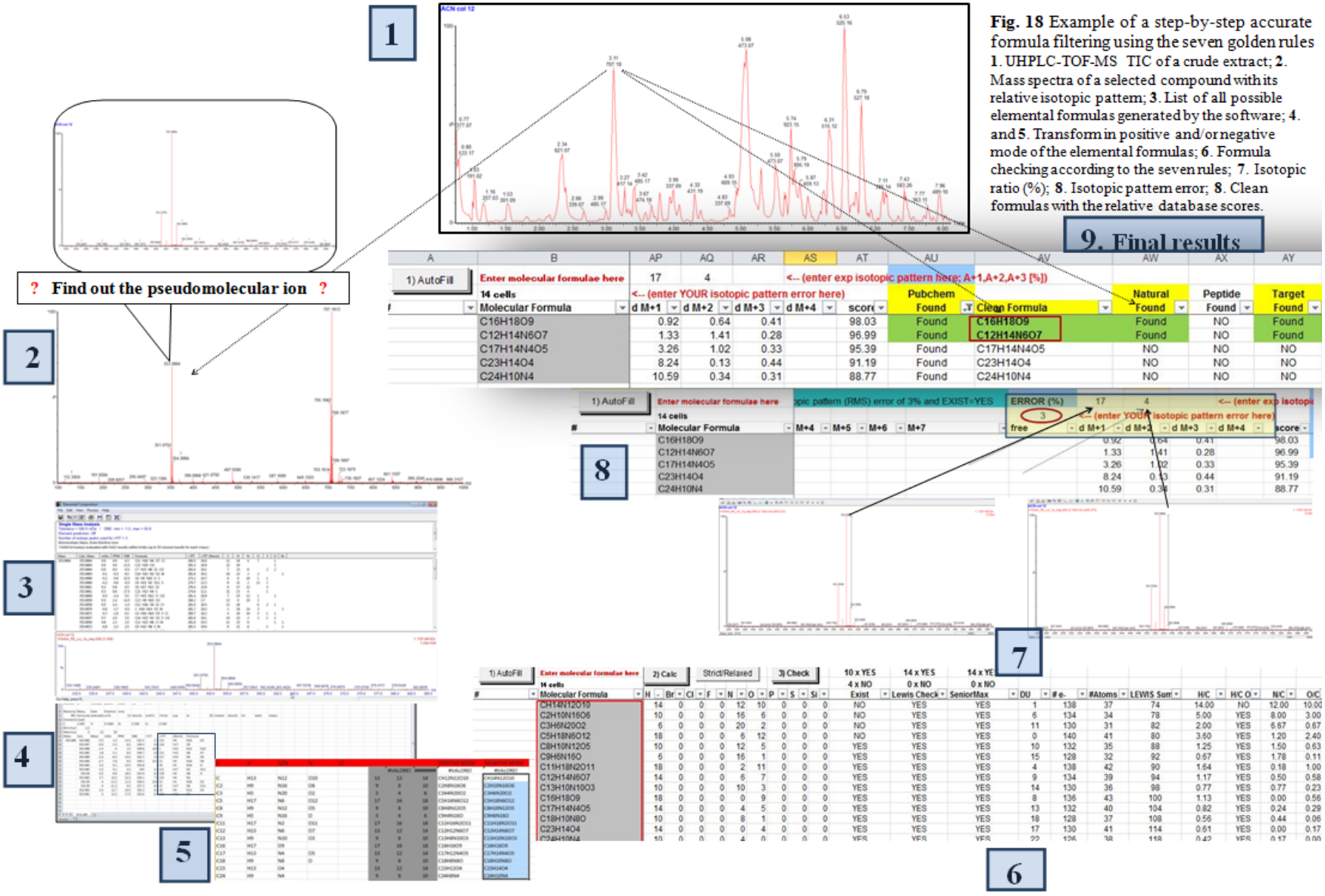


Fig. 18 Example of a step-by-step accurate formula filtering using the seven golden rules **1**. UHPLC-TOF-MS TIC of a crude extract; **2**. Mass spectra of a selected compound with its relative isotopic pattern; **3**. List of all possible elemental formulas generated by the software; **4**. and **5**. Transform in positive and/or negative mode of the elemental formulas; **6**. Formula checking according to the seven rules; **7**. Isotopic ratio (%); **8**. Isotopic pattern error; **8**. Clean formulas with the relative database scores.

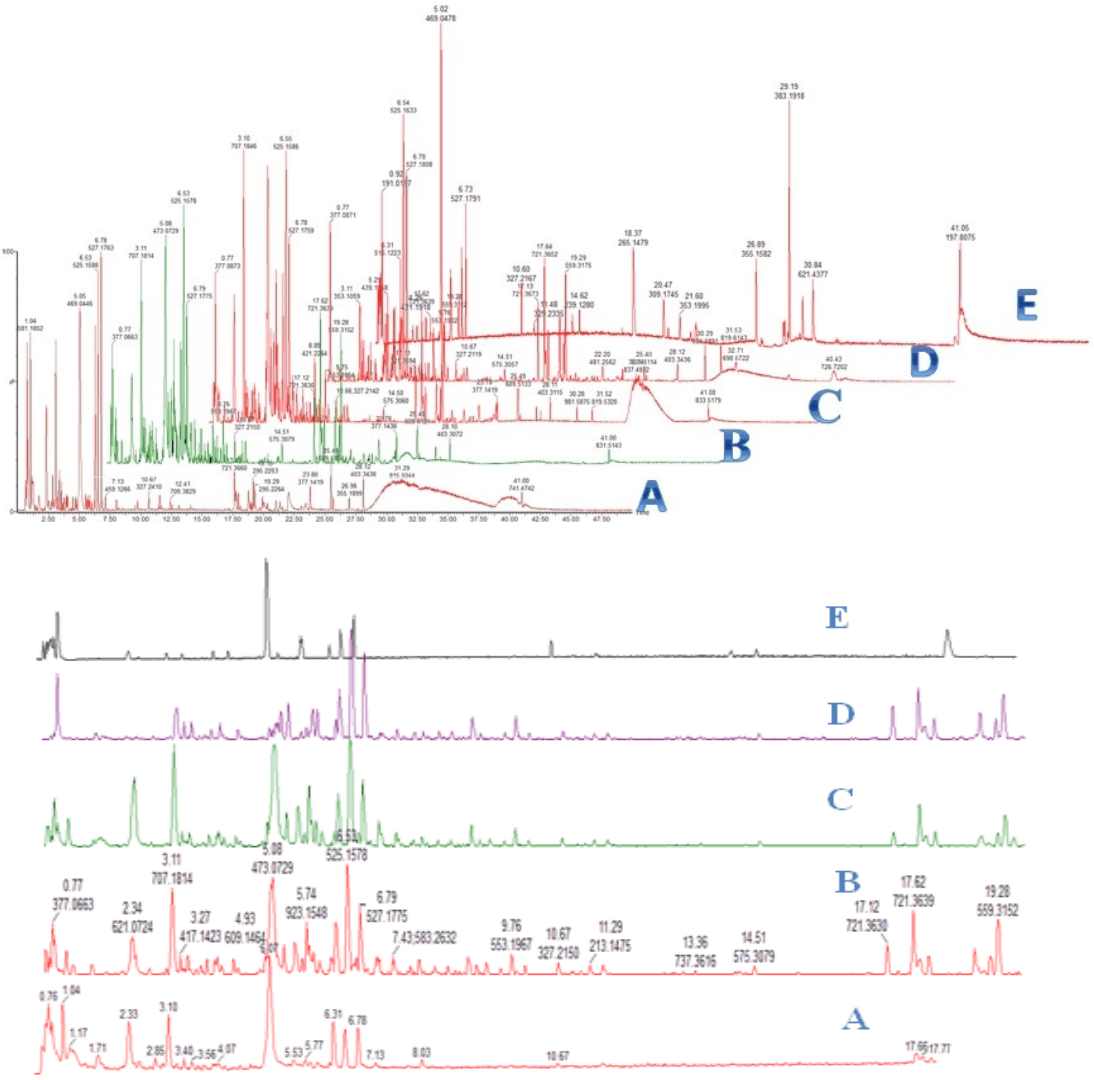


Figure 19 ESI (-) - TOF MS total ion chromatogram profiles of different crude extracts of *C. alpina*. **A.**Wild freeze-dried herb MAE MeOH 50%; **B**. cultivated freeze-dried herb MAE 50%; **C.** cultivated freeze-dried herb UAE MeOH 50%; **D**. cultivated freeze-dried herb MACE EtOH; **E**. air dried herb MAE 50%

The qualitative profiles of the crude extracts of *C. alpina* edible shoots highlighted three important aspects:

- sample storage (freeze-dried *vs*. air dried herb) had a great influence upon the chromatographic profiles (see Fig. 19, extracts B and E);
- the great qualitative difference between the wild and the cultivated species (extracts A and B);
- the insignificant qualitative differences between the profiles of the freeze-dried cultivated herb extracted with two different extraction techniques, using the same solvent mixture (MAE *vs* UAE, see extracts B and C) and the slight qualitative difference when compared to a classic extraction technique (extract D).

As a result of this qualitative profiling survey, once again extract B was remarkably distinguished by the higher number of compounds in contrast with the other extracts, thus, in this case it was considered the "reference" extract for further investigations. Therefore, a limited number of the most prevalent peaks of extract B were characterized (see Table 15) and their presence was checked in the other extracts. Data was not shown for the profiles where minor differences were noticed. Moreover, in order to get more information about the detected compounds, cone voltage fragmentation (50V) to induce fragmentation of parent ions was applied (e.g cichoric acid Fig. 20). UHPLC profiles of the extracted ions present in extract B with their high resolution mass spectra are shown in Fig. 21.

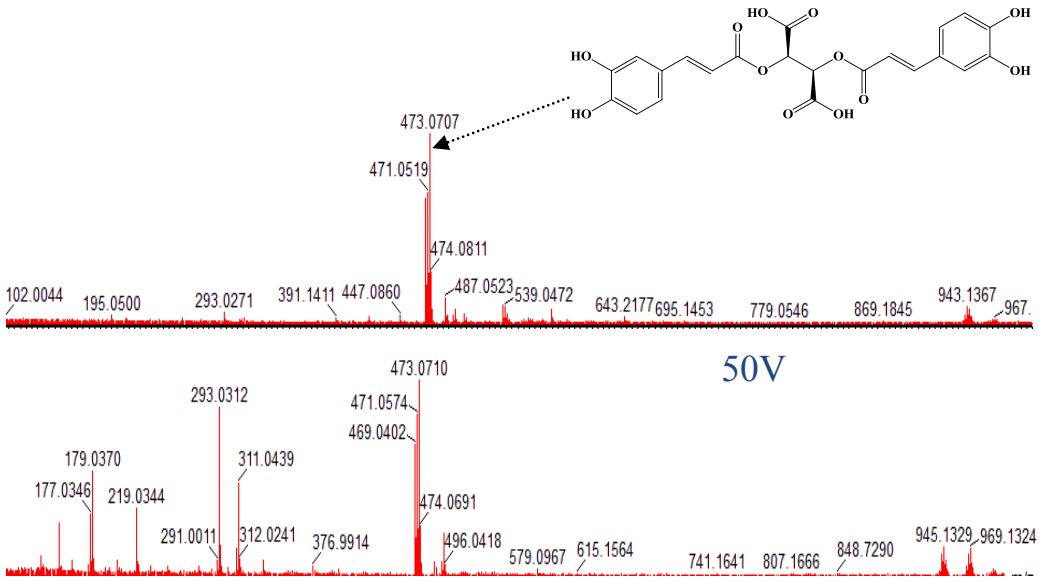


Figure 20 Influence of the cone voltage fragmentation on the fragmentation of parent ions in negative mode (e.g. cichoric acid)

Table 15 UHPLC-TOF-MS comparative investigation of three extracts of *C. alpina* edible shoots (MAE MeOH 50%)

Compound	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1*	0.79	211	191[c]	325 (193 $[M+132+H]^+$)	-	191.0572	$C_7H_{21}O_6$	Quinic acid
1**				-	-	191.0547		
1***				193		191.0197		
2*	2.31	253; 327	309 (177 $[M+132-H]^-$)[c]	-	309.0465; 177.03	177.0194	$C_9H_6O_4$	Aesculetin derivative
2**				-		177.0162		
2***				-		177.0188		
3*	2.66	216	339	-	703.189; 339.0976; 177.0304	339.0728	$C_{15}H_{16}O_9$	Aesculin[b]
3**			-	-		-		-
4*	3.11	218; 246; 325	707 (353)	355	707.1758; 487.0520; 353.0833;191.0534;179.0316	353.0859	$C_{16}H_{18}O_9$	Caffeoylquinic acid derivative[b]
4**			353			353.089		
4***			353			353.0859		
5*	3.58	235; 323	179	181	135.0441	179.0336	$C_9H_8O_4$	Caffeic acid*
5**			179	181		179.0317		
6*	4.39	216	655 (321)	-	609.146; 447.0329; 321.0950	321.0972	$C_{15}H_{16}O_5$	Lactucin
6**			-	-		-	-	-
7*	4.93	-	609	611	609.1421; 285.0409; 477.0933; 177.0185	609.1508	$C_{27}H_{30}O_{16}$	Kaempferol 3', 7' di- *O*-glucoside

7**			-	-		-		-
7***			609	611		609.1469		
8*	5.3	218; 324	439 (393 $[M+FA-H]^-$)	417 (395 $[M+22+H]^+$)	439.1782; 393.176; 377.0232; 293.0278; 179.034	393.170	$C_{17}H_{30}O_{10}$	Not identified
8**			-	-		-		
8***			439			393.1402		
9*	5.5	218; 327	473	-	945.1439;473.07013 11.0399; 293.0283; 219.0282; 179.0376	473.0759	$C_{22}H_{18}O_{12}$	Cichoric acid
9**			-	-		-	-	-
10*	5.74	211; 254;	923 (461 $[2M-H]^-$)	463	923.1478;461.0666 285.0396	461.0717	$C_{25}H_{18}O_8$	Luteolin 3 *O* glucuronide
10**		348	-	-		-	-	-
11*	5.79	211; 254; 348	895 (447 $[2M-H]^-$)	449	895.1910; 447.0863; 285.0374	447.0943	$C_{25}H_{22}O_8$	Luteoloside B[b]
11**			-	-		-		
12*	5.87	218	459	-	941.2441;481.1043 459.1235;447.0913 25.0915	459.1331	$C_{23}H_{24}O_{10}$	Not identified
12**			-	-		-	-	-
13*	6.31	218; 327	515[c]	517[c]	515.152; 353.0834; 271.0969	515.1234	$C_{25}H_{24}O_{12}$	Isochlorogenic acid
13**						515.1222		

14*	6.53	215; 256	525 (<u>479</u> [M+FA-H]$^-$)	<u>481</u>	959.3136; 525.1594;	479.1599	$C_{23}H_{28}O_{11}$	8 acetyl 15 β D glucopyranosyl lactucin
14**			<u>479</u>	-	479. 1290; 257.08; 239.0696;195.0788	479.1577		
14***			525 <u>(479)</u>			479.1625		
15*	6.79	213;	527 (<u>481</u>	<u>483</u>	963.3494;	481.1776	$C_{23}H_{30}O_{11}$	Not identified
15**		253;	[M+FA-H]$^-$)[c]		533.0864;489.0987	481.1752		
15***		342			481.1694; 421.147; 353.083; 285.0376; 215. 105; 197.0946	481.1790		
16*	9.76	220	553 (<u>507</u>)	<u>509</u>	-	507.1932	$C_{25}H_{32}O_{11}$	Not identified
16**			-	-		-		
17*	17.94	222	<u>293</u>	<u>277</u>	609.4135;	293.2176	$C_{18}H_{30}O_3$	Not identified
17**				<u>279</u>	361.1994; 293.2114	293.2112		
18*	19.28	222	<u>559</u>	353 (<u>515</u>)	559.3132; 513.3079; 476.2775; 295.2286; 277.2168	559.3097	$C_{27}H_{46}O_9$	Not identified
18**			295 (<u>559</u>)	279 (<u>515</u>)		559.3133		

*cultivated herb crude extract **wild herb crude extract *** air dried cultivated herb

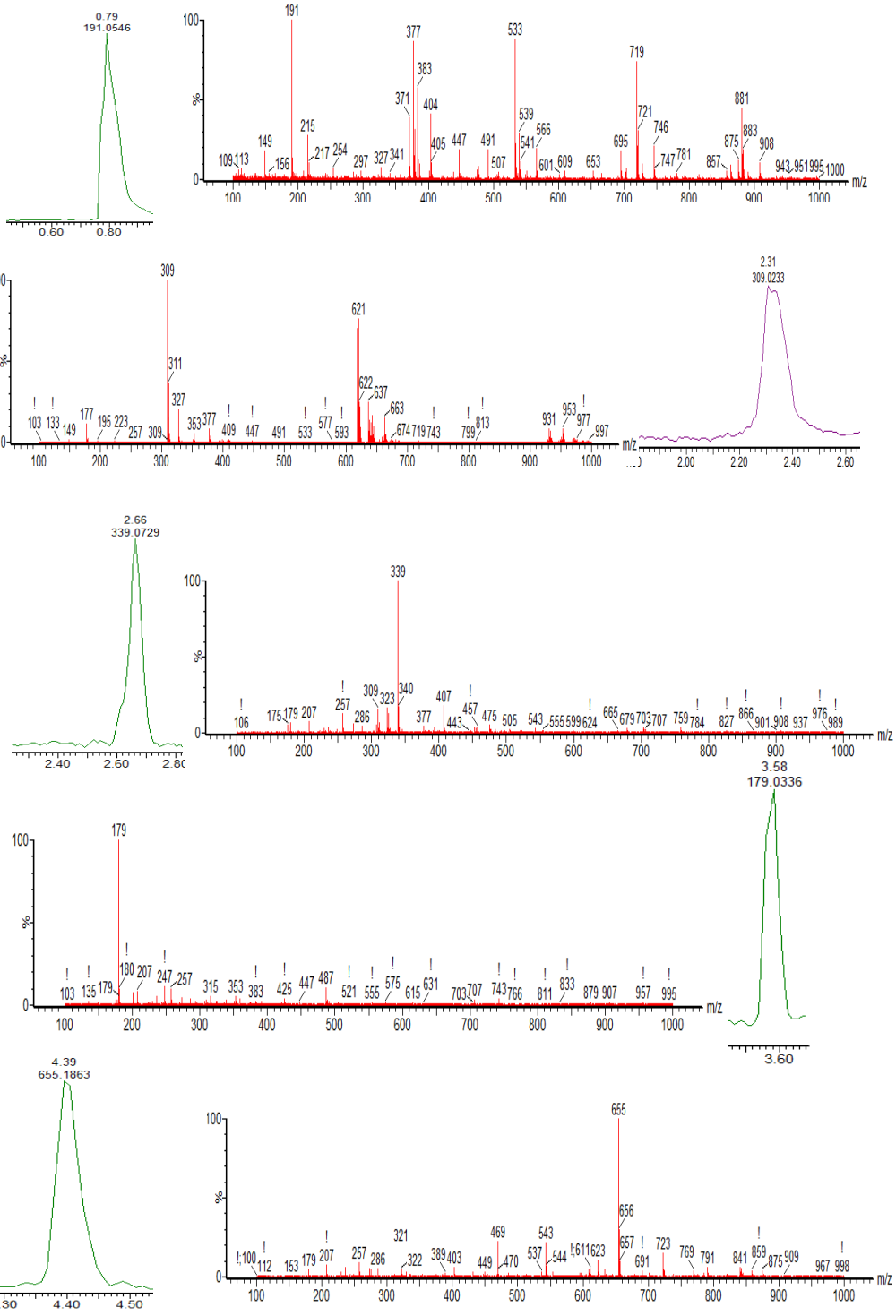
[a] Found accurate mass for the [M-H]$^-$ ion

[b] Compounds identified on the basis of comparison with authentic standards

[c] Common pattern for all the extracts

The base peak is followed by the molecular ion which is underlined.

All the elemental formulas are correspondent to one or more records found in the Dictionary of Natural Products.


0.79
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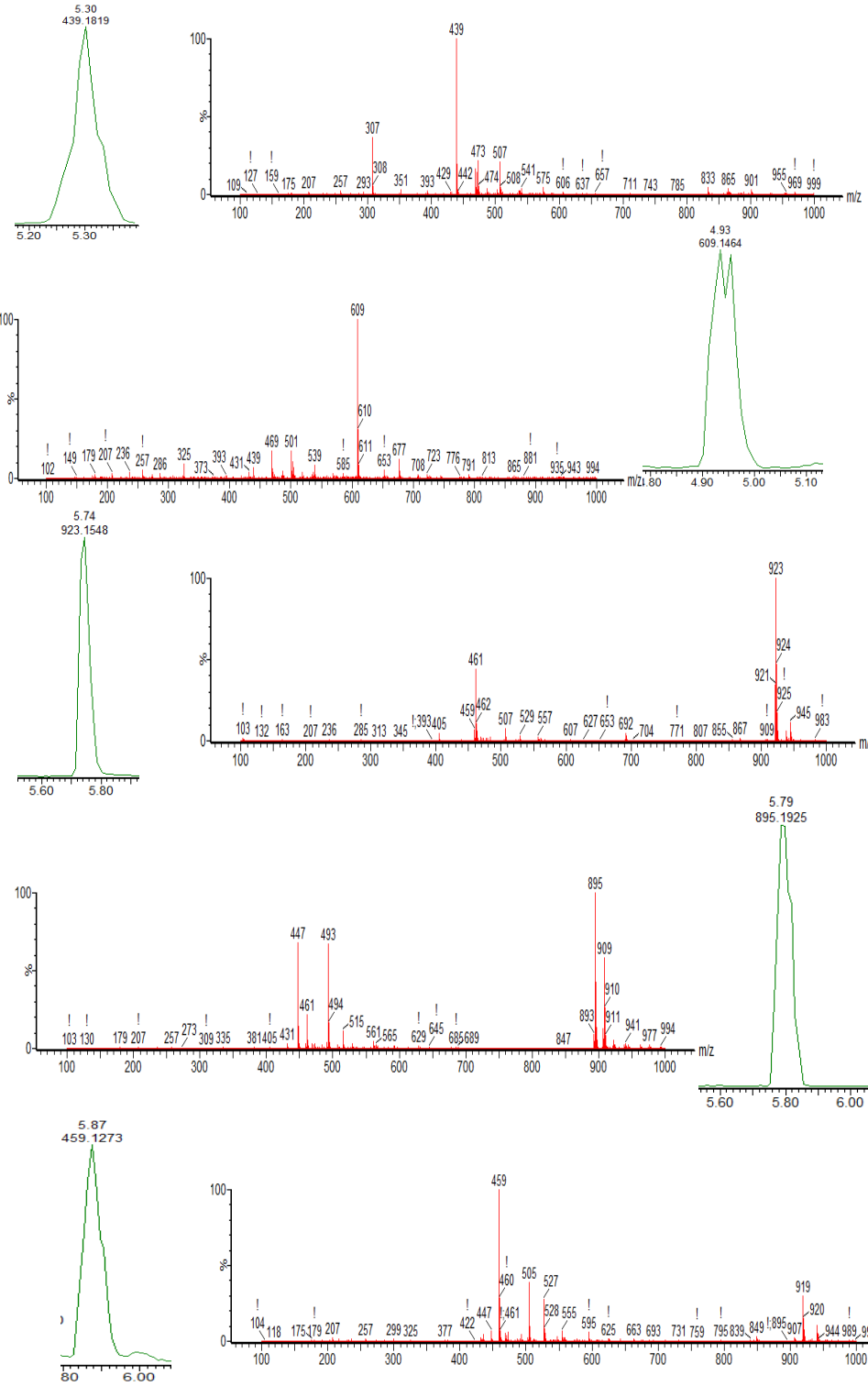
2.31
309.0233
2.66
339.0729
3.58
179.0336
4.39
655.1863
m/z


5.30
439.1819
5.20
5.30
439
307
473
507
308
442
474
508
541
575
606
637
657
711
743
785
833
865
901
955
969
999
m/z
4.93
609.1464
609
610
611
469
501
539
585
663
677
708
723
776
791
813
865
881
935
943
994
4.80
4.90
5.00
5.10
5.74
923.1548
5.60
5.80
923
924
921
925
461
459
462
507
529
557
607
627
653
692
704
771
807
855
867
909
945
983
5.79
895.1925
895
909
910
893
911
447
493
461
494
515
561
565
629
645
685
689
847
941
977
994
5.60
5.80
6.00
5.87
459.1273
80
6.00
459
460
505
527
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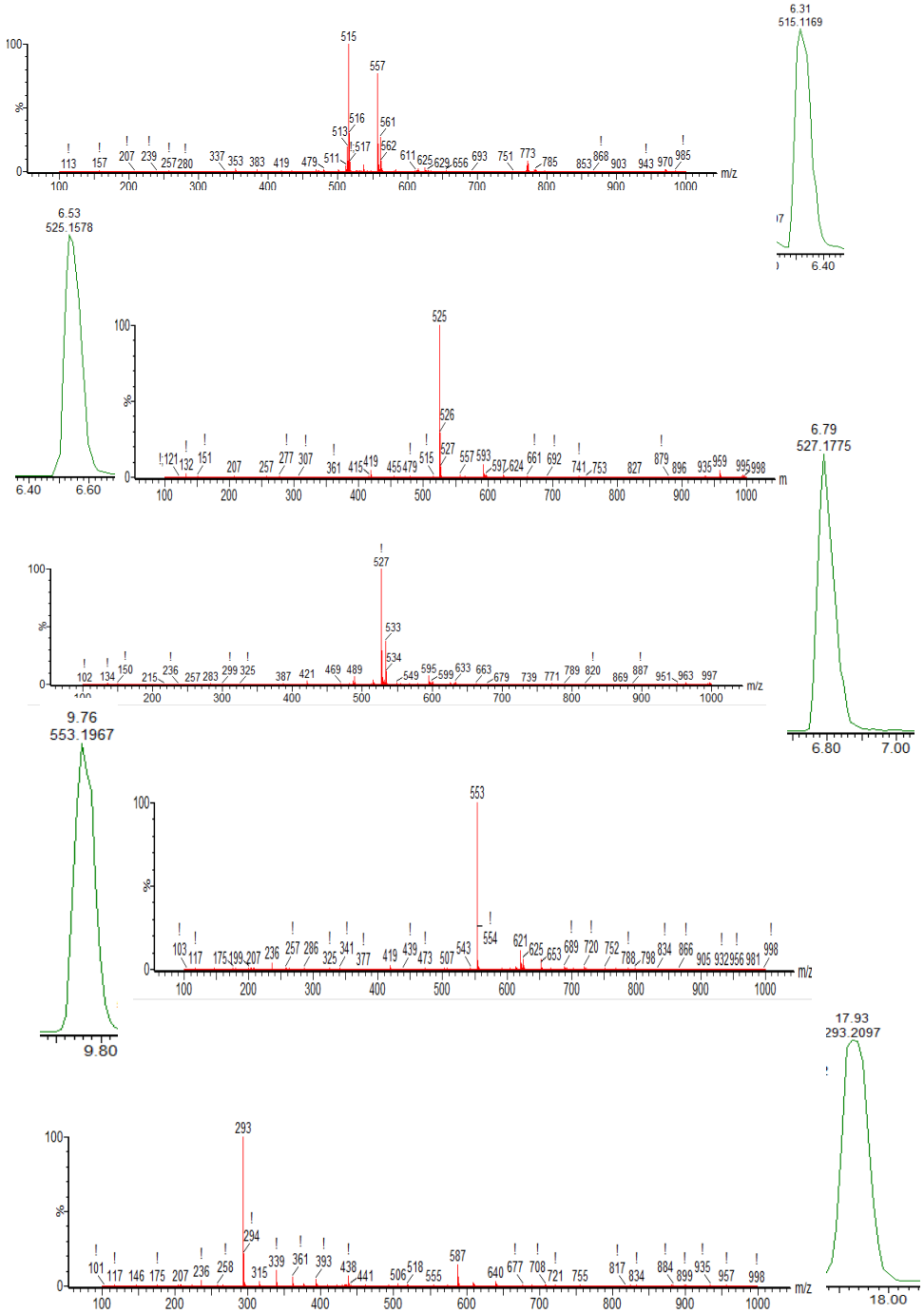
849
895
907
919
920
944
989
999


6.31
515.1169
6.40
6.53
525.1578
6.40
6.60
6.79
527.1775
6.80
7.00
9.76
553.1967
9.80
17.93
293.2097
18.00
m/z
%

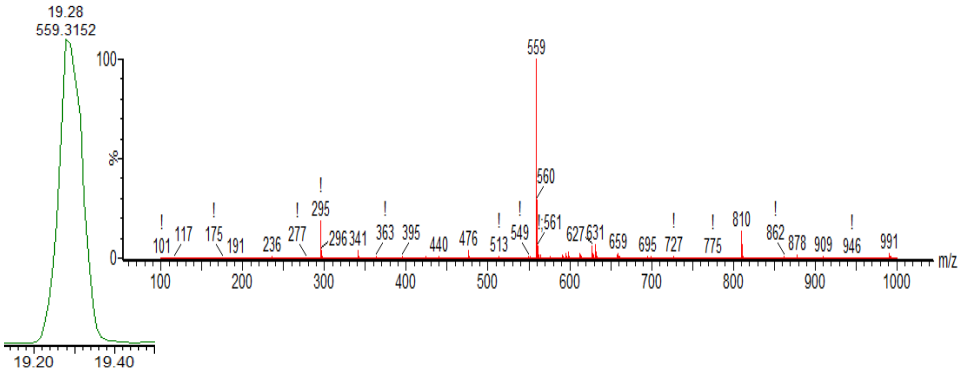


Figure 21 Extracted ions and ESI-TOF-MS spectra in negative mode (extract **B**)

The results summarized in Table 15 underline the valuable contribution of the high resolution mass spectrometry when facing the dereplication of natural compounds in crude extracts. Although only twelve compounds were tentatively identified, to each of the other six the elemental formulas were assigned. The detected compounds might be:

- phenolic acids (quinic acid, caffeoylquinic acid, caffeic acid, cichoric acid, isochlorogenic acid)
- flavonoids (Kaempferol 3', 7' di- *O*- glucoside, Luteolin 3- *O* glucuronide, Luteoloside B)
- coumarin derivatives (aesculetin and aesculin)
- Two sesquiterpene lactones (lactucin, 8 acetyl 15 *β* D- glucopyranosyllactucin).

Since validation is always performed by authentic standards, the algorithm for formula filtering did not generated definite identifications, but delivered very good annotation scores. Dictionary of Natural Products, Mass bank database and literature data were the main sources for compound assignments. The most obvious finding to emerge from these analyses is not only the great composition difference

between the wild and cultivated sample which may be due to climatic variations, but also the difference between the profiles within the same sample stored as freeze-dried and air-dried. The latter difference may be the reason of possible degradation reactions that can occur within the drying process.

IV.2.5 GC-MS approaches for metabolite profiling

Two sample preparation approaches were adopted for the GC-MS analysis of *C. alpina* edible shoots: the first consisted in the derivatisation of both total crude extracts and of different fractions obtained by solvent extractions with increased polarities; the second consisted in the analysis of the volatile fraction. Direct derivatization of crude hydroalcoholic/alcoholic extracts without preliminary fraction isolation by LLE helps to roughly investigate the extract composition and may represent the fastest screening choice when facing new plant matrix. Selective fraction extraction targets specific classes of compounds, thus covering a wide range of polarities. The crude extracts submitted to derivatisation were:

1. Crude extract of the freeze-dried cultivated edible shoots of *C. alpina* obtained under MAE (MeOH 50%) - **G1** (**G1a**: profile of the crude extract derivatized without oximation; **G1b**: profile of crude extract obtained in two steps derivatization).
2. Crude extract of the freeze-dried wild edible shoots of *C. alpina* obtained under MAE (MeOH 50%) - **G2**

3. Crude extract of the freeze-dried cultivated edible shoots of *C. alpina* obtained under maceration (EtOH) - **G3**
4. Crude extract of the air-dried cultivated edible shoots of *C. alpina* obtained under MAE (MeOH 50%) - **G4**
The GC-MS profiles of the four derivatized extracts without preliminary sample preparation are shown in Fig. 22.

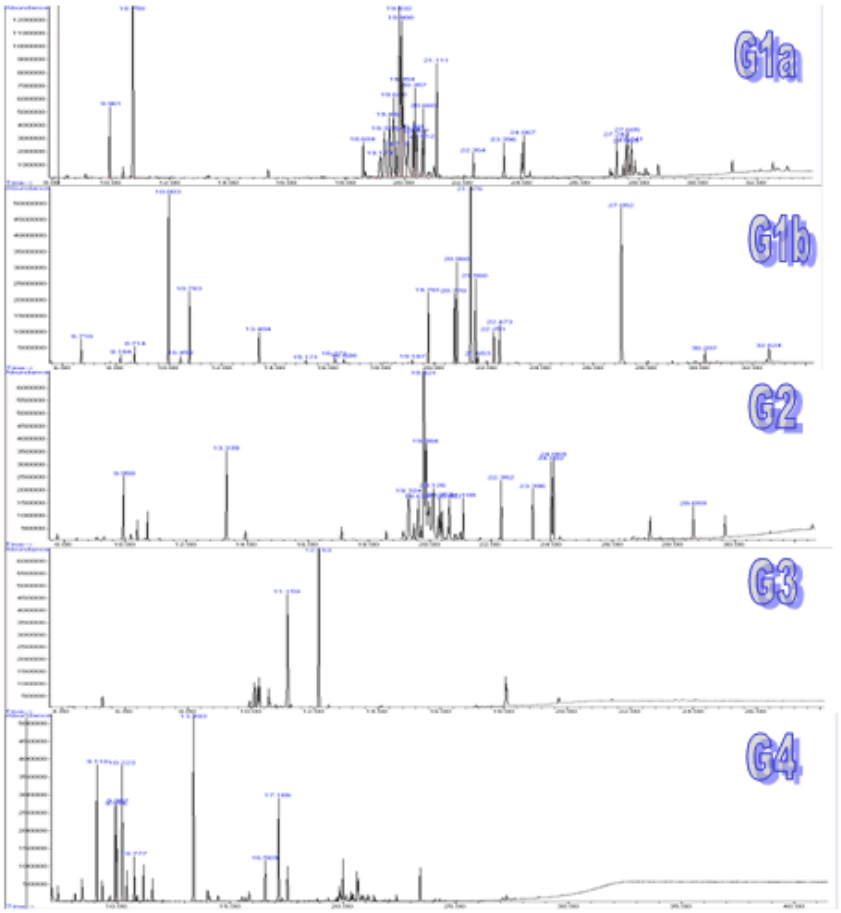


Figure 22 GC-MS profiles of the derivatized crude extracts

At the first glance, the GC profiles of the four extracts are totally different. Table 16 lists the compounds identified in the four extracts.

Table 16 Compounds tentatively identified in G1a, G2, G3 and G4 crude extracts by GC-MS

Extract label	R_t (min)	Tentative assignment*
G1a	10	glycerol
	10.45	Butanedioic acid
	10.77	Propanoic acid
	13.43	(DL) Malic acid
	16.27	2 ketoglutaric acid
	16.42	Benzophenone
	16.6	Succinic acid
	18.24	Isovanilic acid
	19.18	Citric acid
	20.68	Glucose[a]
	20.75	Fructose[a]
	21.13	Talose
	21.36	Galactose
	21.45	Hexadecanoic acid
	21.65	Inositol scyllo
	21.98	11 Octadecenoic acid
	22.24	Myo inositol
	22.48	Caffeic acid
	23.06	9, 12- Octadecadienoic
	23.08	acid (Z,Z)
	23.12	9, 12, 15- Octadecatrienoic acid (Z,Z)
		Octadecanoic acid
G2	10.18	L-isoleucine
	10.2	L-proline
	10.42	Butanedioic acid
	10.74	Propanoic acid
	11.12	L-serine
	13.33	Malic acid
	13.96	Pyroglutamic acid
	16.45	Glutamic acid
	17.11	Succinic acid

	17.5	L-Asparagine
	19.72	Glucofuranose
	20.12	Arabinofuranose
	20.35	Glucofuranose
	21.1	Glucopyranose
	22.35	Hexadecanoic acid
	23.39	Caffeic acid
	24.00	9, 12- Octadecadienoic
	24.06	acid (Z,Z)
	24.27	9, 12, 15- Octadecatrienoic acid (Z,Z)
		Octadecanoic acid
G3	10.6	β D galactofuranose
	11.15	Mannose
	11.88	Inositol muco
	12.13	D-glucose
	12.16	Glucose
	12.46	Hexadecanoic acid
	18.04	Sucrose[a]
G4	7.36	L-alanine
	7.61	glycine
	8.14	L-leucine
	8.45	L-isoleucine
	8.46	L-leucine
	9.07	L-valine
	9.66	Serine
	9.89	L-leucine
	10.19	isoleucine
	10.43	Butanedioic acid
	10.75	Propanoic acid
	10.88	2 butenedioic acid
	11.14	serine
	11.55	L threonine
	12.02	α aspartic acid
	13.28	2 pyrolidone 5 carboxylic acid
	13.4	(DL) malic acid
	14.02	α proline
	14.08	α aspartic acid
	14.44	Phenylalanine
	15.4	Threonic acid
	15.83	α asparagine
	16.86	Xylonic acid γ lactone
	17.16	L(+) Tartaric acid
	17.53	α asparagine

	18.65	D(+) arabitol
	19.51	α D galactofuranose
	19.9	D fructose
	21.37	tyrosine
	22.35	hexadecanoic acid
	23.41	caffeic acid
	24.02	9, 12 octadecadienoic
	24.08	(Z,Z)
	24.3	9, 12,15- Octadecatrienoic acid (Z,Z)
		octadecanoic acid
	27.1	Sucrose
	32.08	β sitosterol

* Compounds present as trimethylsilyl ether/esther derivatives and assigned by the best matching mass spectra from commercial NIST and Wiley libraries
[a] Compounds identified on the basis of comparison with authentic standards
The presence of all the amino acids was confirmed by their authentic standards

The preliminary GC-MS analyses highlighted the prevalence of five classes of compounds:

- saccharides
- organic acids
- fatty acids
- amino acids
- phytosterols

Extracts G2 and G4 were mainly characterized by the presence of amino acids, whilst extract G1a and G3 highlighted the presence of saccharides. Considering the previous results obtained by liquid chromatography, the present gas chromatographic separation of the crude extract without hydrolysis expected to be resulted in the identification of the free aglycones and sugar moieties simultaneously, but in our case only sugars could be

assigned. Therefore, the next step consisted in performing hydrolysis of the crude extracts with the aim to produce possible cleavages of glycosidic linkages in saccharides and decomposition of esters. In addition, a two-step derivatization procedure including methoximation followed by silylation was applied on the hydrolyzed extracts. Since natural matrices are very complex, strategies against chemical rearrangements of different functional groups must be considered. Therefore, the oximation step was introduced because it protects α keto acids against decarboxylation and locks sugars in open-chain conformation.[16] For instance, reducing sugars such as fructose and glucose lead to a number of different peaks related to cyclic and open-ring structures that can be controlled by the oximation step. Oxime derivatives can be formed as *syn* and *anti* isomers depending on the orientation at the carbon-nitrogen double bond. A disadvantage of this method is that complete derivatization seems to be limited to compounds bearing from one to four acidic protons. Hydrolyses of the crude extracts were performed as following:

- **G1** undergone both acidic and enzymatic hydrolysis
- **G2** and **G4** undergone acidic hydrolysis

Profiles of the hydrolyzed extracts are illustrated in Fig. 23 and detected compounds after hydrolysis are summarized in Table 17.

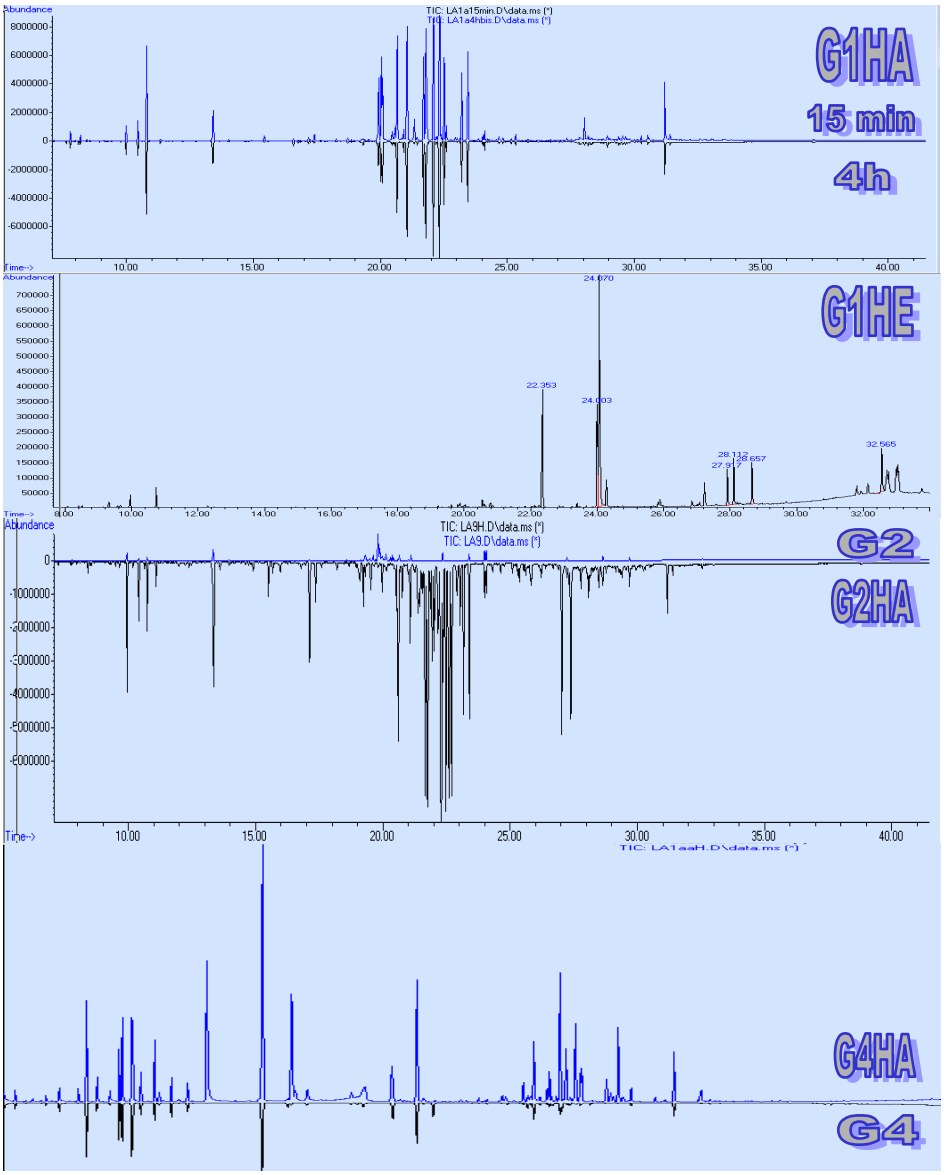


Figure 23 GC-MS profiles of the crude extracts before and after the hydrolysis. Extract G1 after 15 minutes and 4h acidic hydrolysis (**G1HA**); Extract G1 after enzymatic hydrolysis (**G1HE**); Extract G2 after acidic hydrolysis (**G2HA**); Extract G4 after acidic hydrolysis (**G4HA**)

Table 17 Compounds detected after the hydrolysis in G1HA, G2HA and G4HA

A						B		
Label	**R_t (min)**	**Tentative assignment***	**Label**	**R_t (min)**	**Tentative assignment***	**Label**	**R_t (min)**	**Tentative assignment***
G1HA	10,46	Butanedioic acid	G1HE	10.74	Propanoic acid	G2HA	13.33	Butanedioic acid
	10,8	Propanoic acid		22.35	Hexadecanoic acid		13.42	Malic acid
	11,35	β angelica lactone					17.11	Succinic acid
	13,4	DL malic acid		23.39	Caffeic acid		17.35	Gluconic acid
	15,43	2,3,4 trihydroxybutyric acid		24.00	9, 12-Octadecadienoic acid (Z,Z)		19.23	Xylitol
							20.71	Dglucosamine
	17,16	Tartaric acid		28.64	Octadecanoic acid		21.68	Fructose
	17,19	L (+) tartaric acid					23.17	Epi-inositol
	18,73	Xylonic acid γ lactone		32.55	β sitosterol		23.4	Caffeic acid
	19,88						24.00	9, 12-Octadecadienoic acid (Z,Z)
	20,46	1 ciclohexene 1 carboxyl acid					24.27	Octadecanoic acid
	20,52	β D galactofuranose						
	20,89	D (-) fructose						
	21,05	Gulonic acid						
	21,68	β D Glucopyranose						
	21,84	Fructose						
	22,08	Myoinositol						
	22,11	D glucose						
	22,39	Glucopyranose						
	23,45	Hexadecanoic acid						
	24,02	Caffeic acid						
	24,08	9,12 Octadecadienoic acid (Z,Z)						

* Compounds present as trimethylsilyl (oxime) ether/esther derivatives and assigned by the best matching mass spectra from commercial NIST and Wiley libraries

Despite the fact that an increased amount of constituents could be noticed after hydrolysis, data reported in Table 17 show that no remarkable qualitative information was attained. Hydrolysis of extract G4 did not produce profiling changes.

In an attempt to decrease the complexity of the samples, selective extraction fractionation using solvents of increased polarity was conducted.

Selective extractions with dichloromethane, ethyl acetate and MeOH 85% resulted in different GC profiles, as it is shown in Fig. 24 for extracts from the freeze-dried herb (A) and air-dried herb (B).

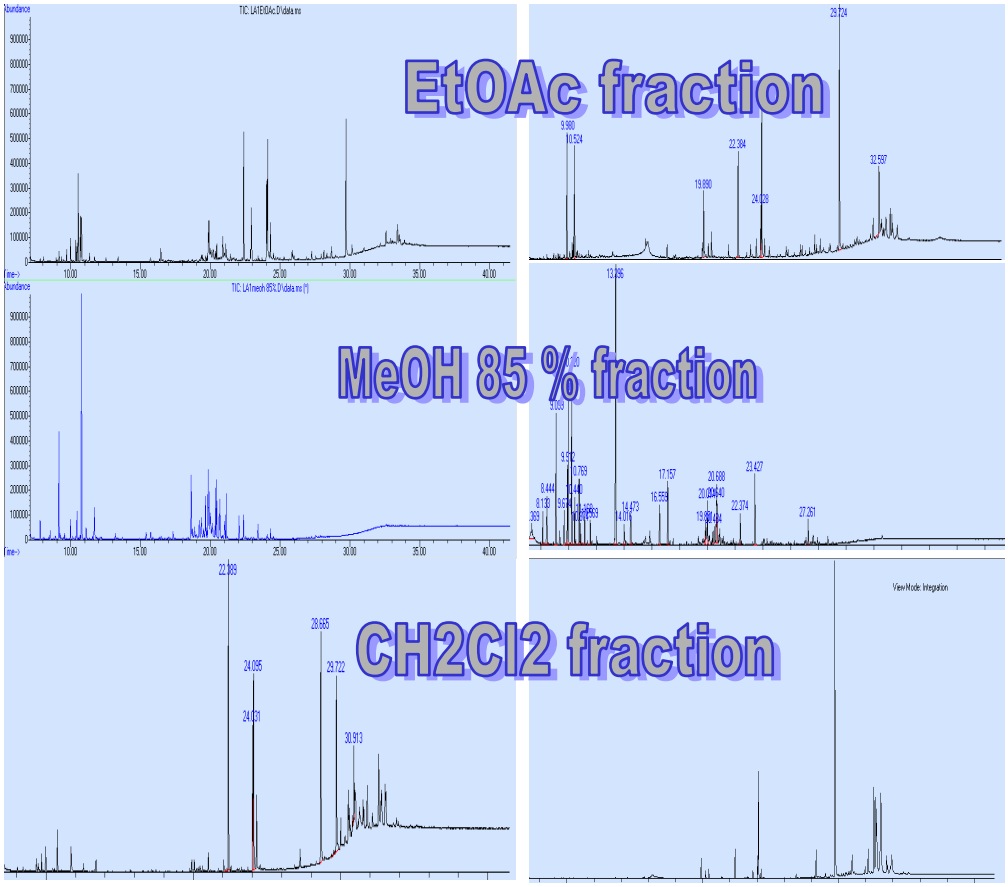


Figure 24 GC profiles of different derivatized fractions originating from the freeze-dried herb (**A**) and the air- dried herb (**B**)

Table 18 Compounds identified in the fractions obtained with different polarity solvents

R_t (min)	A_{CH2Cl2}	R_t (min)	A_{EtOAc}	R_t (min)	$A_{MeOH\ 85\%}$
22.36	Hexadecanoic acid	10.45	Succinic acid	19.52	Arabinofuranose
24.02	9,12Octadecadienoic acid (Z,Z)	10.77	Propanoic acid	20.31	Glucopyranose
24.1	9, 12, 15- Octadecatrienoic acid (Z,Z)	13.4	Malic acid	22.06	β D glucose
24.3	Octadecanoic acid	16.44	Benzophenone	23.42	Caffeic acid
32.74	β amyrin	22.86	9, 12, 15- Octadecatrienoic acid (Z,Z)		
		22.93	9-octadecenoic acid		
		23.42	Caffeic acid		
		24.3	Octadecanoic acid		
R_t (min)	B_{CH2Cl2}	R_t (min)	B_{EtOAc}	R_t (min)	$B_{MeOH\ 85\%}$
22.38	Hexadecanoic acid	9.32	Butanoic acid	8.14	L-leucine
24.02	9,12 Octadecadienoic acid (Z,Z)	9.98	glycerol	9.1	L-valine
		10.43	Malic acid	9.68	Serine
24.1	9, 12, 15- Octadecatrienoic acid (Z,Z)	20.06	Tetradecanoic acid	10.43	Malic acid
24.3	Octadecanoic acid	22.36	Hexadecanoic acid	11.57	α threonine
32.18	Stigmasterol	24.03	9,12 Octadecadienoic acid (Z,Z)	12.08	Aspartic acid
32.6	β sitosterol			14.10	L-proline
33.05	β amyrin			14.48	Phenylalanine
				17.14	Tartaric acid
				17.54	L-asparagine
				23.44	Caffeic acid

The last attempt to reasonably separate classes of compounds based on their different polarities did not reveal new compounds compared to those already detected by the other sample preparation strategies. Anyhow, the repeated detection and retention times of some of the reported compounds within every set of sample preparation may play an important role in the identification process and further designation of marker compounds. The nature of the detected compounds is in accordance with their origin that is the aerial part of the plant. Considering that the air-dried herb has a characteristic slight fragrance, both the essential oil and the volatile fraction captured by HS-SPME were investigated and their profile is shown in Fig. 25. Pyrazine and benzenacetaldehyde were common to both fractions.

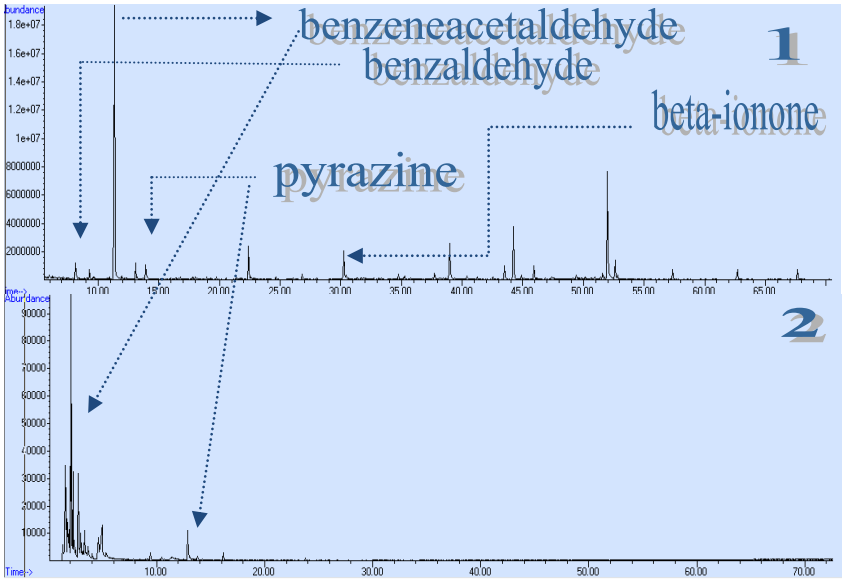


Figure 25 GC profile of the essential oil (**1**) and HS-SPME profile (**2**) of *C. alpina* edible shoots

Conclusions

The aim to set up qualitative strategies for screening the composition of marker compounds in *C. alpina* crude extracts lies in the need to limit the drawbacks originating in the low amount of plant material available and to propose alternatives when facing such problems. Considering both the gastronomical and commercial values in the local area of the edible shoots of *C. alpina*, their crude extracts obtained were used as testing model for the strategy.

IV.3 Preliminary analytical screening of various plant extracts by HPLC-UV, UHPLC-TOF-MS and GC-MS: *Asparagus acutifolius, Chenopodium bonus henricus, Levisticum officinale, Silene vulgaris and Spirea aruncus*

As stressed out before, facing various crude extracts from different plants requires a clear work strategy possibly based on a preliminary extended investigation of a crude extract taken as reference, in case of lacking material. Despite of the great differences between natural matrices, testing the qualitative response of a plant taken as reference within a multitask approach furnishes many data sets and may constitute a reliable starting point towards the study of other plant extracts.

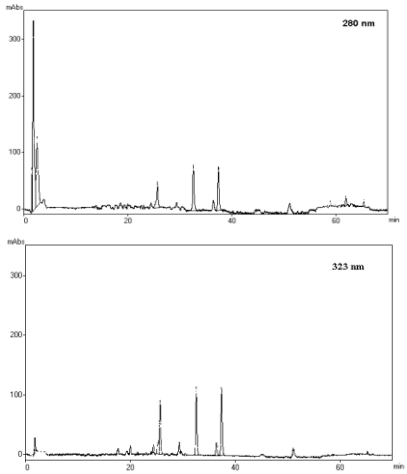
The second part of the present investigation dealt with the application of the most important analytical techniques to five different species of traditional culinary use in Friuli. All five crude extracts were obtained under the previously developed MAE method. The results reported for every extract point up the most representative following data: yield of extraction; colorimetric and HPLC-UV at 280nm and 323 nm screening; UHPLC-TOF-MS and GC-MS profiling.

Asparagus acutifolius

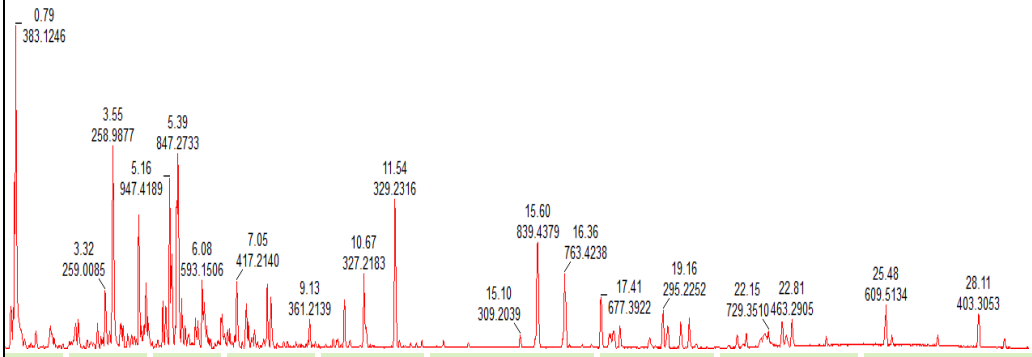
Yield of extraction, colorimetric results and characteristic HPLC-UV profiles

Yield of extraction (%)	Phenolic content (mg GAE/g DW ± SD)[a]	Flavonoid content (mg rutin/g DW ± SD)[b]
40.72%	40.8 ± 0.96	29 ± 1.01

Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; [b] Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample. Mean and standard deviation, *n*= 5



UHPLC-TOF-MS total ion chromatogram of *A. acutifolius*



No	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1	2.31	216; 327	353	-	353.0954 191.0553 179.0344	353.0863	$C_{16}H_{18}O_9$	Caffeoylquinic acid
2	3.28	218; 325	357 (287)	773 (289)	771.2043 593.1523 445.0847 357.0327 311. 0276	287.0584	$C_{15}H_{12}O_6$	Eriodictyol

					287.0585 135.2721			
3	3.43	218; 325	367	369	367.1025 193.0503	367.0999	$C_{17}H_{20}O_9$	3-O feruloylquinic acid
4	3.6	235; 323	179	181	-	179.0341	$C_9H_8O_4$	Caffeic acid[b]
5	4.65	211; 255; 353	755	757	755.2024 609.1465 519.1808 357.1006 301.0305	755.2051	$C_{33}H_{40}O_{20}$	Quercetin derivative
6	5.37	211; 256; 353	609	611	609.1431 301.0318 274.5596	609.1448	$C_{27}H_{30}O_{16}$	Rutin[b]
7	5.39	259	463	465	463.085 301.0331	463.0857	$C_{21}H_{20}O_8$	Myricitrin[b]
8	5.6	211; 256; 353	463	465	463.0851 301.0331	463.0857	$C_{21}H_{20}O_{12}$	Isoquercitrin[b]
9	6.27	218; 353	623	625	623.1606 315.0498	623.1616	$C_{28}H_{32}O_{16}$	Isorhamnetin rhamnoglucoside
10	10.76	221	413	415	413.1243 398.1 249.07 193.0498	413.1213	$C_{22}H_{22}O_8$	Ferulic acid derivative
11	21.91	223	433	435	433.2337 353. 1990 279.2320 171.004 152.9931	433.2337	$C_{24}H_{16}O_4$	Not identified

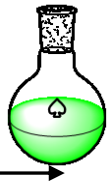
a. Found accurate mass for $[M-H]^-$ ion

b. Compounds identified on the basis of comparison with authentic standards

c. All the elemental formula are correspondent to one or more records found in the Dictionary of Natural Products

GC-MS tentative assignment of compounds present in the crude extract of *A. acutifolius* before (A) and after hydrolysis (B)

Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*
A. acutifolius	13.33	Malic acid	*A. acutifolius*	21.63	Fructose
	22.35	Hexadecanoic acid		22.25	Glucose
	24.00	9,12 Octadecadienoic acid (Z,Z)		22.44	Galactose
				24.00	9,12 Octadecadienoic acid (Z,Z)
	24.06	9,12, 15 Octadecatrienoic acid (Z,Z)		22.35	
				24.05	Trans 9-octadecenoic acid
				24.08	9,12, 15 Octadecatrienoic acid (Z,Z)
				24.14	Cis 13 octadecenoic acid
				24.27	Octadecanoic acid
				32.55	β sitosterol

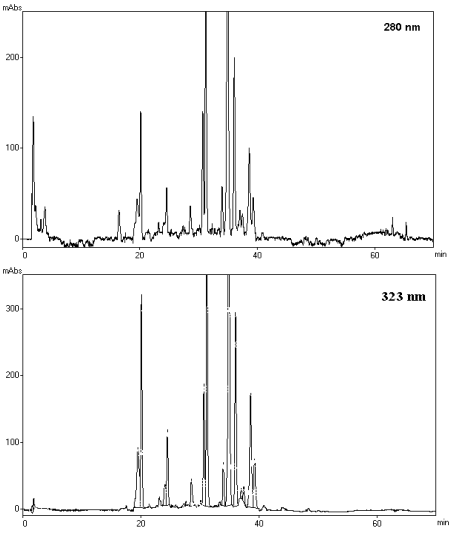


Chenopodium bonus henricus

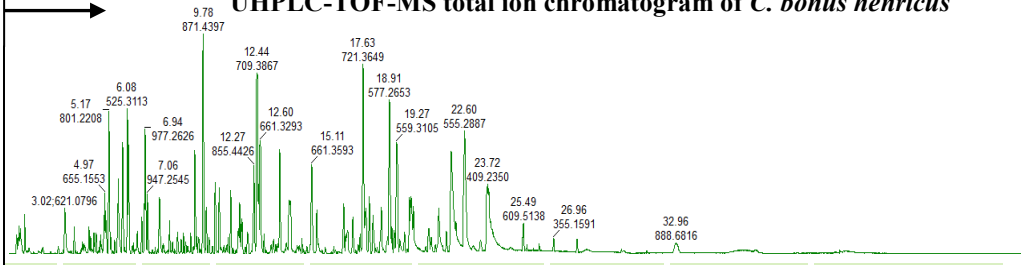
Yield of extraction, colorimetric results and characteristic HPLC-UV profiles

Yield of extraction (%)	Phenolic content (mg GAE/g DW ± SD)[a]	Flavonoid content (mg rutin/g DW ± SD)
49.35%	19.53 ± 0.88	49 ± 1.01

Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; [b] Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample. Mean and standard deviation, *n*= 5



UHPLC-TOF-MS total ion chromatogram of *C. bonus henricus*



No	Rt (min)	λ_{max} (nm)	[M-H]$^-$ (*m/z*)	[M+H]$^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1	3.02	216; 253 320	621	218 (623)	621.0525 311.0414 233 179.0324 149.0669	311.0405	$C_{13}H_{12}O_9$	Caftaric acid
2	5.17	215; 353	801	803	801.2098 279.0509	801.2208	$C_{38}H_{42}O_{19}$	Not identified
3	5.63	218; 325	639 (315)	317	639.1519 342.1335 315.0490	315.8413	$C_{16}H_{12}O_7$	Isorhamnetin
4	6.09	220	525	961	959.6108	479.2897	$C_{27}H_{44}O_7$	Ecdysterone

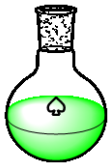
			(479)	(481)	593. 2927 525.3026 479.2958			
5	6.14	220	541 (495)	497	541.2993 495.2905 477.1027 342.1334	495.2988	$C_{27}H_{44}O_8$	Polypodine B
6	6.55	211; 256; 353	963 (477)	479	963.2448 477.1028 315.0489	477.1044	$C_{22}H_{22}O_{12}$	Isorhamnetin glucoside
7	6.78	218; 346	507	509	507.1143 345.0606	507.1160	$C_{23}H_{24}O_{13}$	Syringetin- 3-O-β-D- glucoside
8	9.78	211	871 (664)	666	871.4294 825.4363 664.3676	664.6404	$C_{36}H_{56}O_{11}$	Medicagenic acid-3-O-glucopyranoside

a. Found accurate mass for $[M-H]^-$ ion

All the elemental formula are correspondent to one or more records found in the Dictionary of Natural Products

GC-MS tentative assignment of compounds present in the crude extract of *C. bonus henricus*

Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*
C. Bonus henricus	10.41	Butanedioic acid	*C. Bonus henricus*	21.64	Fructose
	10.45	Propanoic acid		22.25	Glucose
	13.33	DL Malic acid		22.35	Hexadecanoic acid
	15.35	L threonic acid		22.44	Galactose
	20.3	L galactopyranose		23.15	Myo-inositol
	20.41	β galactofuranose		23.98	9,12 octadecadienoic acid (Z,Z)
	20.86	Gulonic acid lactone			
	22.28	D-gluconic acid		24.14	Trans 9 octadecenoic acid
	22.35	Hexadecanoic acid		24.27	Octadecanoic acid

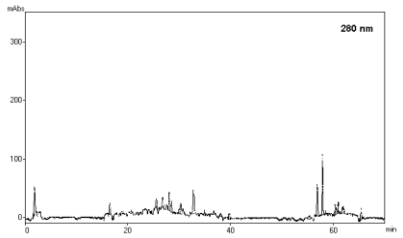


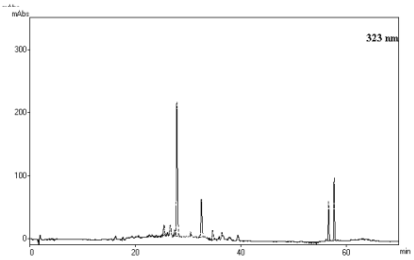
Levisticum officinale

Yield of extraction, colorimetric results and characteristic HPLC-UV profiles

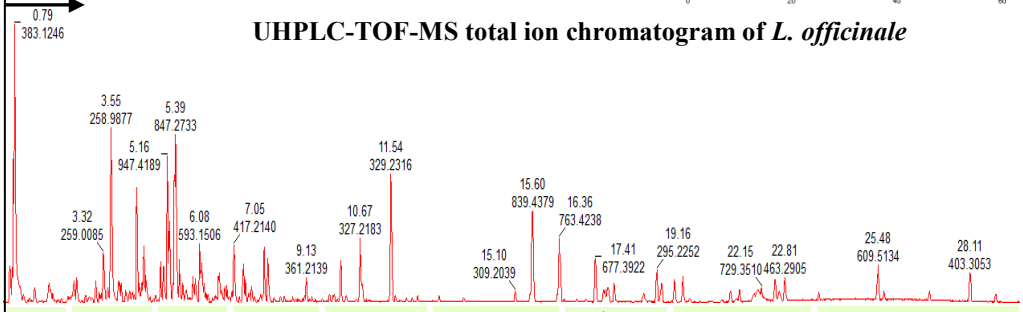
Yield of extraction (%)	Phenolic content (mg GAE/g DW ± SD)[a]	Flavonoid content (mg rutin/g DW ± SD)[b]
45.94%	10.9 ± 0.41	19.63 ± 0.12

Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; [b] Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample. Mean and standard deviation, *n*= 5





UHPLC-TOF-MS total ion chromatogram of *L. officinale*



No	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1	0.79	211	325 (191)	218 (193)	-	191.0573	$C_7H_{21}O_6$	Quinic acid
2	5.37	211; 348	609	303	609.1426 423.1281 301.0356	303.0491	$C_{15}H_{10}O_7$	Quercetin rhamnoglucoside
3	5.39	211; 337	847 (423)	425	847.2681 609.1454 423.1276	423.1292	$C_{20}H_{24}O_{10}$	Apterin

					243.0630			
4	6.08	220	593 (285)	595 (287)	593.1466 285.0391	285.0391	$C_{15}H_{10}O_7$	Luteolin rhamnoglucoside
5	7.05	220	417	-	417.2107 353.1013 289.0773	417.2140	$C_{20}H_{35}O_9$	Not identified
6	7.9	-	413	-	413.2171 371.2083	413.2184	$C_{21}H_{34}O_8$	Not identified
7	8.03	220; 319	569	571	569.1630 423.1273	569.1661	$C_{29}H_{30}O_{12}$	Not identified
8	17.41	-	677 (631)	439 (633)	677.3009 631.3869	631.3851	$C_{36}H_{56}O_9$	Momordin

a. Found accurate mass for $[M-H]^-$ ion
All the elemental formulas are correspondent to one or more records found in the Dictionary of Natural Products

GC-MS tentative assignment of compounds present in the crude extract of *L. officinale* before (A), after hydrolysis (B) and volatile fraction obtained by HS-SPME (C)

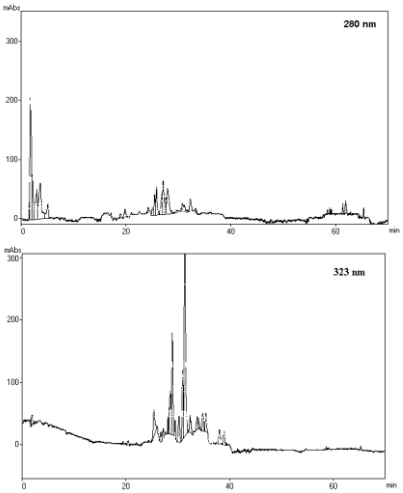
Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*
A *L officinale*	10.4	Butanedioic acid	B *L officinale*	21.64	Fructose	C *L. officinale HS-SPME*	7.44	Alpha pinene
	13.34	DL Malic acid		22.25	Glucose		7.95	camphene
	15.36	L threonic acid		22.33	Hexadecanoic acid		8.36	Benzaldehyde
	20.3	L galactopyranose		22.46	Galactose		9.53	β myrcene
	21.13	β galactofuranose		24.00	9,12 octadecadienoic acid (Z,Z)		10.03	Beta phellandrene
	22.35	Hexadecanoic acid		23.15			10.86	Para-cymene
	24.00	9,12 octadecadienoic acid (Z,Z)		23.98			11.15	Sabinene
	24.06	9,12,15 octadecatrienoic acid (Z,Z)		24.06	9,12,15 octadecatrienoic acid (Z,Z)		11.47	Cis ocimene
				24.27	octadecadienoic acid		11.89	Trans β ocimene
							12.3	γ terpinene
							13.56	α terpinolene
							15.38	allo ocimene
							22.37	(-)- bornyl acetate
							25.23	α terpinene
							25.39	α terpinenyl acetate
							25.33	terpinolene
							25.46	2 - β-pinene
							26.46	(+)-2- carene
							26.31	α copaene
							26.65	geranyl acetate
							26.98	β elemene
							28.65	γ elemene
							30.55	germacrene-D
							30.73	βselinene
							32.27	delta cadinene
							33.53	germacrene B

Silene vulgaris

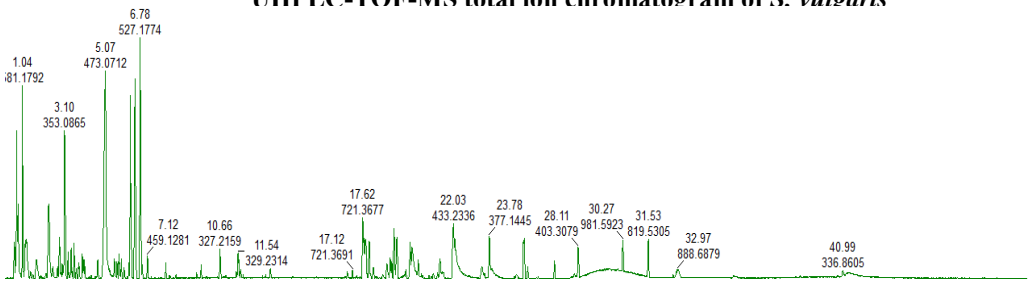
Yield of extraction, colorimetric results and characteristic HPLC-UV profiles

Yield of extraction (%)	Phenolic content (mg GAE/g DW ± SD)[a]	Flavonoid content (mg rutin/g DW ± SD)[b]
47.85%	3.9 ± 0.88	9.06 ± 0.8

Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; [b] Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample. Mean and standard deviation, *n*= 5



UHPLC-TOF-MS total ion chromatogram of *S. vulgaris*



No	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1	3.1	218; 325	707 (353)	355.1016	-	353.0865	$C_{16}H_{18}O_9$	Caffeoylquinic acid derivative
2	3.5	218 324	179	222 (181)	-	179.0324	$C_9H_8O_4$	Caffeic acid
3	5.07	220 251 327	473	-	-	473.0712	$C_{22}H_{18}O_{12}$	Caffeoyltartaric acid

4	5.7	220	461	463	-	461.0710	$C_{21}H_{18}O_{12}$	Not identified
5	5.74	220	447	449	-	447.0902	$C_{21}H_{20}O_{11}$	Not identified
6	6.3	220; 327	515 (353)	-	547.2357 372.1438 353.0903	353.0863	$C_{16}H_{18}O_9$	Isochlorogenic acid

a. Found accurate mass for $[M-H]^-$ ion
All the elemental formulas are correspondent to one or more records found in the Dictionary of Natural Products

GC-MS tentative assignment of compounds present in the crude extract of *S. vulgaris* before (A) and after hydrolysis (B)

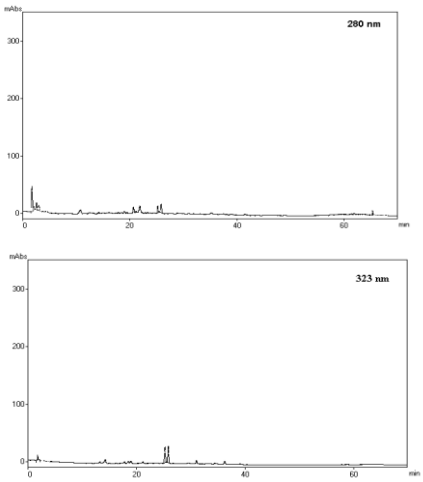
Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*
S. vulgaris	9.03	L-valine	*S. vulgaris*	10.41	Butanedioic acid
	10.42	Butanedioic acid		13.33	Malic acid
	10.74	Propanoic acid		13.96	Pyroglutamic acid
	20.3	Glucopyranose		17.13	Tartaric acid
	22.03	D-galactose		17.33	Gluconic acid
	23.39	Caffeic acid		19.22	Xylitol
	23.98	9,12- octadecadienoic acid (Z,Z)		21.37	3,4 dihydroxyhydrocinnamic acid
	24.06	9,12,15 octadecatrienoic acid (Z,Z)		22.33	Galactose
				22.49	Caffeic acid
				23.39	9,12- octadecadienoic acid (Z,Z)
				24.00	9,12,15 octadecatrienoic acid (Z,Z)
				23.15	Octadecanoic acid
				24.06	βsitosterol
				24.27	
				32.55	

Spirea aruncus

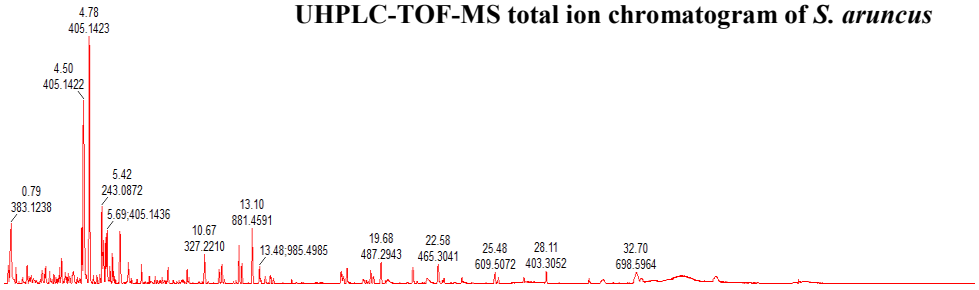
Yield of extraction, colorimetric results and characteristic HPLC-UV profiles

Yield of extraction (%)	Phenolic content (mg GAE/g DW ± SD)[a]	Flavonoid content (mg rutin/g DW ± SD)[b]
24.99%	4.06 ± 0.98	1.47 ± 1.3

Data expressed as milligrams of gallic acid (GAE) equivalents per one gram of dry weight (DW) sample; [b] Data expressed as milligrams of rutin equivalents per one gram of dry weight (DW) sample. Mean and standard deviation, *n*= 5



UHPLC-TOF-MS total ion chromatogram of *S. aruncus*



No	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Fragments (CV 50 V)	Acc. Mass found[a]	Elemental composition	Tentative assignment
1	3.4	-	666 (405)	-	666.2195 405.1425 328.0581 306.0743	405.1417	$C_{17}H_{26}O_{11}$	Not identified
2	5.43	216	243	-	423.1278 243.0685	243.0873	$C_{11}H_{16}O_6$	Not identified
3	5.62	218 329	503 (341)	-	525.1059 503.1223 341.0901 179.0356 161.0249	341.0680	$C_{15}H_{18}O_9$	Caffeic acid glucoside
4	29.71	225	-	481	481.3519 284.3316	481.3564	$C_{28}H_{48}O_6$	Not identified

a. Found accurate mass for $[M-H]^-$ ion

All the elemental formulas are correspondent to one or more records found in the Dictionary of Natural Products

GC-MS tentative assignment of compounds present in the crude extract of *S. aruncus* before (A) and after hydrolysis (B)

Label	R_t (min)	Tentative assignment*	Label	R_t (min)	Tentative assignment*
A *S. aruncus*	13.34	DL Malic acid	**B** *S. aruncus*	12.47	Undecylenic acid
	22.3	D-gluconic acid		21.37	n-hexadecanoic acid
	22.35	Hexadecanoic acid		22.33	Hexadecanoic acid
	23.39	Caffeic acid		23.15	Myo inositol
	23.98	9,12- octadecadienoic acid (Z,Z)		23.27	6-octadecenoic acid
				23.5	Octadecanoic acid
	24.06	9,12,15 octadecatrienoic acid (Z,Z)			
	32.55	β Sitosterol			

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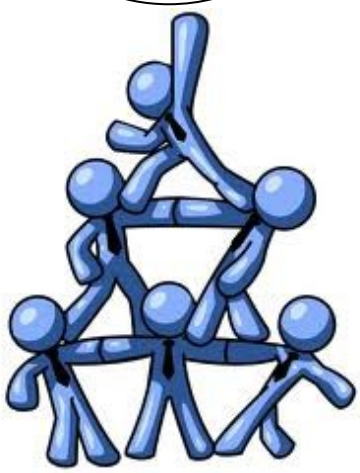
Conclusions and perspectives



V. Conclusions and perspectives

A multitask qualitative workflow strategy was set up and applied to little investigated crude extracts obtained from aerial parts of different wild growing species. The strategies focused on the following key points:

- ✓ The detection of the fraction that resulted in the highest number of compounds that absorb at pre-selected wavelengths by means of a full factorial 2^3 MAE experimental design;
- ✓ Testing various extraction techniques to provide comparative data;
- ✓ Follow up classic characteristic colorimetric approaches based on the previous results;
- ✓ Systematic analytical approaches: from the simplest to the more sophisticated analytical techniques.

The analytical platforms used, namely LC-MS^n, UHPLC-TOF-MS and GC-MS proved to be pivotal for the dereplication process allowing the detection of various classes of compounds with different polarities. Metabolite assignments using LC-MS as a tool for compound identification were obtained by combining accurate mass, isotopic distribution, the algorithm of the seven heuristic rules, fragmentation patterns and any other MS information available. Beside the initial target compounds considered, every decision step was inspired not only by the results obtained every "one step back analysis", but also on the need to extract as much non-targeted metabolite information as

possible. In GC-MS, identification was based on library spectra and fragmentation patterns but the overcome remains the detection of compounds with high molecular weights after derivatisation e.g. chlorogenic acid which is easily analyzed by LC-MS. Nevertheless, due to the complementarity of LC and GC, it is worthwhile to consider merging analyses performed on the same samples to obtain a more complete overview of the metabolites in the samples. The main part of this work consisted in developing multitask strategies meant to overcome the drawbacks originating in the low amount of plant material available and the subsequent screening of crude extracts of *C. alpina*. Secondly, the most representative information generating features were carried out to screen the crude extracts of the other plants. Every crude extract of the various investigated plants was characterized both by compounds previously reported by other studies and unknown compounds. To the unknown compounds was assigned the elemental composition. Additional data such as UV absorbance and retention times is useful for future compound assignments. Therefore, further experiments should be carried out in order to isolate all these constituents for structure elucidation. Once completed the identification task, the decision of the next step is always required by the type of structures that may present interest for future biological bio-guided assays.