UNIVERSITY
CAMPUS BIO-MEDICO OF ROME

Ph.D. in
Bioengineering and Biosciences
Food Technology and Science of Nutrition
XXX cycle - a.y. 2014-2015

Biologically active phenolic compounds in fruits and leaves of *Cyclanthera pedata* (L.) Schrab (caigua) and in related plant-derived dietary supplements

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May 7th 2018

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Dedicated to my parents:

to my mother,
for her sacrifices and
for the strength she transmitted to me;

to my father,
who could physically accompany me only half way,
but I believe he never really abandoned me...

«You never know how strong you are, until being strong is your only choice.»
(Bob Marley)

«We all die. The goal is not to live forever. The goal is to create something that will.»
(Chuck Palahniuk)

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Abstract

Cyclanthera pedata (L.) Schrad., known as caigua, is an edible plant belonging to the *Cucurbitaceae* family and native to South America, which is also used for therapeutic purposes. The local folk medicine recommends the daily intake of fruits and leaves of caigua for the treatment of several diseases, e.g. diabetes, high blood pressure and LDL-cholesterol. Recently, food supplements produced from caigua fruits are available on the European market. They would be associated with anti-hypercholesterolemic and anti-hypertensive properties, also reported in literature. Recent scientific studies relate the anti-hyperglycaemic properties of this plant to the high content of phenolic compounds, the most abundant class of specialized metabolites, and in particular to the glycosylated flavonoids' subclass.

Currently, high performance liquid chromatography (HPLC) is the technique of choice for the qualitative and quantitative analysis of phenolic compounds extracted from caigua. Nevertheless, high performance thin layer chromatography (HPTLC) is widely employed for the initial examination of plant extracts before HPLC analysis, because of well-known advantages, such as short separation times, amenable to detection reagents, possibility of running several samples simultaneously, and suitability to be hyphenated to mass spectrometry (HPTLC-MS).

The aim of this work has been to investigate the occurrence and content of phenolic compounds in a commercial food supplement in comparison to those determined in leaves and fruits of caigua. As part of the research, an HPTLC-MS method was developed for the preliminary screening of phenolic compounds occurring in the above samples. Further studies were performed in order to investigate the influence of pedo-climatic conditions and vegetative state on the accumulation of phenolic compounds in the leaves and fruits of plants grown in different geographical areas, with the purpose to select the best growth conditions to get raw materials with a greater amount of beneficial molecules for food supplements manufacture. More in-depth analysis were performed by developing an HPLC-MS method and the resulting data were compared with previously obtained results.

A total of ten glycosylated flavonoids were identified, nine of which already reported in literature, while one was never identified before. Both HPLC and HPTLC data converge in hypothesizing a strong influence of plant vegetative state on the accumulation of phenolic compounds in caigua leaves, while both techniques reveal the presence of more preserved phenolic patterns in the caigua fruits.

Moreover, a simple hydrolysis procedure, having the advantages of miniaturisation (low sample and solvent consumption), simultaneous hydrolysis of multiple samples, use of inexpense glassware, and no need of instrumentation, was developed, in order to confirm the phenolics characterization in the studied plant. The redox activity and the levels of vitamin C in leaves and fruits of *C. pedata* were determined as well. All obtained results are reported and discussed in this thesis.

Chapter 1:

State of Art

1.1 Introduction

Several scientific reports have pointed out the health-promoting effects of a variety of edible plants. That should not be such a surprising information if we think that both traditional and modern medicine systems are based on the use of plants. Nowadays, with the progress of pharmaceutical industries, the market of plant food supplements is expanding worldwide as well [1]. A wide diversity of plants come from Andean mountains and the Amazon rainforest of South America [1, 2]. *Cyclanthera pedata* Schrad. is a well-fitting example of edible plant native of South America with healthy properties, since it has been used for centuries in folk medicine because of its hypo-glycaemic, hypo-tensive and hypo-cholesterolemic effects. Because of these abilities, *C. pedata* constitution has attracted few research groups and quite a big number of its compounds has been studied and identified until now. Phenolic compounds, and in particular the flavonoids subclass, represent one of the most studied category of molecules of this plant. At least part of their beneficial roles are supposed to be related to the presence of OH-groups in their chemical structure that makes them have a strong redox activity.

However, a herbal product contains multiple constituents that might be responsible for its therapeutic effects, it is thus necessary to define as many of the constituents as possible in order to understand and explain its bioactivity. The term "phyto-therapy" refers to the use of plants for medical purposes. Moreover, we should take into account the "synergistic nature" of the huge amount of helpful compounds contained in a herbal product, since natural products usually exert more benefits than pharmaceutical products because of that. It should be noted that the presence of some molecules can enhance the beneficial effect of other ones, so isolated compounds could display less or even no bio-activity anymore. According to both "phyto-therapeutical" and "synergistic" concepts, deeper knowledge of the flavonoid content of plant-based foods is of paramount importance to understand their role in plant physiology and human health.

Various analytical methods exist for flavonoid analysis, ranging from thin-layer chromatography (TLC) to capillary electrophoresis (CE). With the introduction of hyphenated HPLC and CE techniques, the analytical potential has been dramatically extended. Gas chromatography is generally impractical, due to the low volatility of many flavonoid compounds and the necessity of preparing derivates.

1.2 *Cyclanthera pedata* (L.) Schrab

1.2.1 Botanical classification and description

Cyclanthera pedata (L.) Schrad., commonly known as caigua, is a Peruvian edible plant belonging to *Dicotyledonae* class, to the *Violales* order and to the *Cucurbitaceae* family, as well as *Cucurbita pepo*, C*ucurbita moschata* or *maxima Duch.*, *Cucumis sativus*, *Citrullus lanatus* and *Cucumis melo*, whose fruits, very common on our tables, are respectively zucchini, pumpkin, cucumber, watermelon and melon. The main characteristic of the family is to have angular drums, rough, climbing or creeping, with branching tendrils placed adjacent to the leaves. Indeed, the term "*Cucurbitaceae*" derives from the Sanskrit "corb", which means "to twisting", "to crawl" [3,4].

1.2.1.1 Flowers

Like the other plants includible in the same family, *C. pedata* is an annual, herbaceous (i.e. not has woody stem) and a flowering plant (Angiosperm). In the Mediterranean climatic condition it flowers from August to September and the flowers are monoecious, or rather, individual flowers are either male or female, but both sexes can be found on the same plant, even if usually ripen at different times to avoid self-fertilization. They are pollinated by insects. The flowers (**Figure 1**) are gamo-petals (i.e., with the petals joined together), whose corollas, star-shaped, are always formed by five petals, little and yellow, with a mild odour.

Figure 1: Flowers of *Cyclanthera pedata*.
The flowers of caigua have a light yellow colour, include 5 petals and present the shape of a star. Because of the presence of two different flowers (male and female) on the same plant, *C. pedata* is considered a monoic plant.

1.2.1.2 Leaves

As well as the flowers, also the leaves, hairless and long 12 cm, have their odour. Their main characteristic is that to have five-seven tips, similarly to hemp leaves (**Figure 2**).

Figure 2: Leaves of *C. pedata*.

The leaves of caigua present a light green color and a peculiar shape with seven tips. The last two tips below are, in turn, partially divided in two parts.The dimensions go from 5 cm (apex leaves) until 12 cm (leaves at the maximum maturation stage, pre-senescence stage).

1.2.1.3 Fruits

Caigua produces a semi-flattened fruit (6-15 cm long, and 3-5 cm wide) resembling cucumber for flavour, while recalling a pepper for the appearance (**Figure 3, a**).

This fruit, which is called "pepònide", is sincarpico (with carpels in a single ovarian cavity) and is a berry, a type of fleshy fruit [5]. In botanical terminology, berries are characterized by high water content and are, for this reason, very appetizing for animals. Moreover, the pericarp is divided into three layers:

- the exocarp, the outer part that we call peel;
- the mesocarp, namely the pulp;
- the endocarp, the innermost part, which usually protect the seeds.

As regards exocarp, we can say that fruits are coated with a more or less hard rind with an irregular surface, soft spines and longitudinal grooves. Its colour varies from dark green to white.
The mesocarp (edible part) is thin and succulent. The endocarp is white and fluffy. The latter two parts are not readily distinguishable from each other and, at the end of the maturation, the pulp is reabsorbed to form a large central cavity, as in the case of large pumpkins (**Figure 3, b-e**).

In the fleshy pulp there are numerous seeds (see later).

1.2.1.4 Seeds

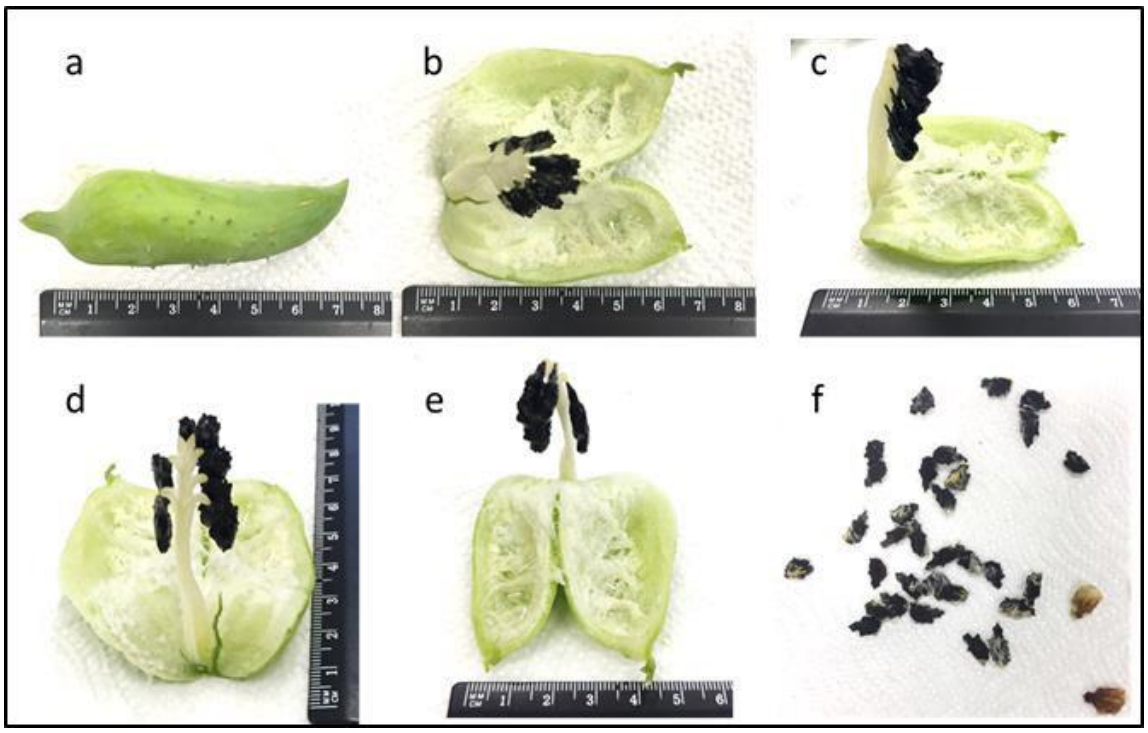


Figure 3: Pictures of one ripe fruit of caigua cultivated in Italy from different perspectives and its seeds. b-e) longitudinal section of caigua fruit. **a)** entire caigua fruit; **b)** transversal plan; **c)** sagittal plan; **d)** frontal plan, length dimension; **e)** frontal plan; wide dimension; f) seeds.

Each fruit contains approximately twelve seeds, united from a single placenta. These seeds are roughly quadrangular and rough black (**Figure 3, f**). They are able to survive to the digestion of the animals that feed on berries. Therefore, the animal becomes the vehicle of distribution, since releases the unchanged seeds at the time of defecation. Both plant and animal benefit from this mode of interaction: the animal gets the nourishment, while plant ensures the propagation of the species. If the fruit comes to maturity on the plant, it opens literally throwing the seeds over long distances.

1.2.2 Distribution and ideal habitat

Caigua is believed to be native to the Andean region or "Sierra" and was cultivated by the Incas who used its fruits as food [6,7,8]. The countries involved in promoting the diffusion of this species are Peru, Ecuador (in particular the southern part), Bolivia, Colombia, and Venezuela; in addition, its presence has also been reported in the southern part of Mexico and in the Caribbean area [9].
Although the relatively limited geographical diffusion, *C. pedata* Schrad. adapts itself to a wide range of environmental conditions [6]. It does not need special crop management techniques apart from proper staking and supports. However, the optimal growth temperatures are comprised between 12 and 18 Celsius degrees. It prefers deep and moist soil, so the field has to be well-drained, with a pH of 6,0 - 7,0. The plant does not tolerate salinity [10].
Caigua can grow in semi-shade (woodland). For its tendency to invade all the ground as possible, it is also called "wild melon" and it needs a big space to expand itself. Its growth cycle extends for 3-4 months in its native environment. In the Peruvian regions, the crop is harvested in June-July and starts to produce fruits 100 days after crop establishment.
Before our study, the cultivation of *C. pedata* in Italy has been documented only by Macchia M. *et al.*[10]. The scientific bibliography on its agronomic, biological and reproduction characteristics is extremely poor.

1.2.3 Uses of *Cyclanthera pedata*

1.2.3.1 *C. pedata* as edible plant

The use of caigua in the human nutrition date back to many centuries, as evidenced by ancient phytomorphic ceramics from Peru which artistically represents its fruits (**Figure 4**).

Figure 4: Ancient ceramic representing the fruits of caigua
[http://www.wikiwand.com/en/Cyclanthera_pedata].

In Brazil, caigua is known as "maxixe do reino" (Portuguese), but elsewhere also as "slipper gourd", "lady's slipper", "sparrow gourd", "pepino de rellenar" (in Spanish, Colombia), which mean "stuffed-cucumber", in English [11]. Indeed, the large cavity in the fruit can be filled with other ingredients to make many dishes. In particular, young fruits are eaten raw or cooked and have a similar taste to cucumbers even if they are not crisp. Older fruits are usually cooked, they can be stuffed in much the same way as marrows. Furthermore, caigua's leaves are eaten either cooked or raw as a salad. Leaves and tender young shoots are cooked and used as greens.

1.2.3.2 *C. pedata* as folk medicine remedy

In South America caigua is not just a traditional food, as a matter of fact, its therapeutic aspect is appreciated as well as its culinary one. Peruvians have been used its seeds, fruits, leaves and roots for curative purposes for years [12]. Each part of *C. pedata* can be used in various ways depending on the disorder and/or the pathology you want to heal. Infusions of seeds are used to control hypertension, whereas dried and minced seeds are eaten in the quantity of 1 gram/day to treat intestinal parasitosis. Both seeds and fruits are recommended in case of gastro-intestinal disorders. Fruits can be boiled in the milk and used as a mouthwash for tonsillitis or boiled in olive oil for anti-inflammatory and analgesic purpose. The fruit juice is recommended two times a day to treat high levels of cholesterol, hypertension, atherosclerosis, blood circulation problems, diabetes, but is simply used also as a diuretic. The decoction prepared from leaves is believed to have hypoglycemic effect. Moreover, the roots are used for the hygiene and cleanness of teeth [13].

1.2.3.3 *C. pedata* as source of plant-derived dietary supplements

Nowadays, it is clear that diet and health are deeply related. Healthy dietary habits are thought to influence many bodily functions, with a preventive action against numerous pathologies. The main result of this cultural and social awareness is the increasing demand for natural food supplements. The plant kingdom includes lots of species which seem to exert a beneficial role, lending to be used for medicinal, herbalist and nutraceutical purposes.
With the term "nutraceuticals" it is meant foods or food supplements intended for human ingestion and thought to have a beneficial effect on human health [14]. Several nutraceuticals are recognized as preventing or providing relief from specific diseases or ailments, and are commonly used for their medicinal qualities. Some of these nutraceuticals may comprise of a single compound, or, alternatively, a complex combinations of substances, which explicate a synergistic effect.

Caigua fruits and leaves can play an important economic role for its nutraceuticals characteristics. Especially because of its anti-cholesterolemic activity, *C. pedata* has a commercial interest in the phytopharmaceutical market of South America and, more recently, has attracted attention also in the European market. It is available in Herbalists' shops in the form of various preparations like pills or powder (**Figure 5**).
The dry and concentrated extracts obtained from the fresh fruits warrant a major steadiness and improve the bio-availability and the half-life of the active compounds. Another advantage could be that the capsules, having no taste, could mitigate possible unpleasant flavour characteristic of the fruit ingested as such [15].

Figure 5: Caigua supplements commonly found in italian herbalist's shop. These kind of supplements come in the form of capsules and are produced from caigua fruits. On the packaging are reported beneficial effects on LDL-cholesterol levels and high blood pressure.

1.2.4 Health benefits of *C. pedata* reported in scientific literature

C. pedata Schrad. has been subjected to biological studies designed to investigate its anticholesterolemic, anti-inflammatory and hypoglycemic properties, handed out by popular tradition. The plant administered dehydrated and capsulated, has been shown to lower harmful LDL cholesterol levels in humans, while raising the level of HDL cholesterol [16]. The effectiveness of the treatment has also been tested in post-menopausal women, who have a greater risk of presenting dyslipidemia due to the cessation of ovarian function. In particular, the treatment with six capsules of Caigua daily for twelve months (six capsules correspond to one fruit of caigua) reduces the rate of LDL cholesterolemia from 75 to 12,5% and improve the hypo-HDL cholesterolemia condition from 62,5 to 12,5% [17].

An *in vitro* study has been carried out by Ranilla L.G. *et al.* [18] to prove the putative inhibitory activity of medicinal plants (among which *C. pedata* was included) on key-enzymes linked to hyperglycaemia and hypertension. Drugs or natural substances able

to inhibit the activity of the α-glucosidase of the α-amilase, enzymes involved, respectively, in the hydrolysis of maltose (disaccharide) and oligosaccharides, polysaccharides and starch (from carbohydrates ingested with the diet) to molecules of glucose, may be able to control the post-prandial hyperglycaemia by reducing the bioavailability and the absorption of the glucose. Similarly, the inhibition of the ACE (Angiotensin Converting Enzyme) represents the therapeutic approach for the treatment of hypertension, in diabetic as well as non-diabetic patients. The study in question asserts that extracts from caigua fruit do not exert any inhibitory effects on α-glucosidase and α-amilase enzymes, but exert a strong inhibitory activity on ACE, suggesting the possible use of this plant in the hypertension treatment [19]. The hypoglycemic properties ascribable to caigua fruits are probably due to another mechanism of action.

1.2.5 Phyto-chemical composition of *C. pedata*

Caigua is extensively formed by water (94 %), whereas the total carbohydrate and reducing carbohydrates are very low (0,52 % and 0,57 %, respectively), so caigua fresh fruits show a low level of calories and may be incorporated in the hypocaloric diets. It contains a large amount of vitamins exhibiting antioxidant properties, such as the vitamins belonging to the B group (thiamine, riboflavin, niacin), ascorbic acid or vitamin C, and retinol. The content of ascorbic acid reported by Rivas M. *et al*. (122, 82 mg/100g of dry weight) is higher than that reported for fruits like kiwi [20]. Respect to others cucurbits (pumpkin, cucumber, chayot, watermelon, melon), caigua fruits are rich in minerals, especially potassium, calcium, phosphorus, magnesium, and iron, while are poor in sodium [21, 22]. The daily mineral requirements of the Andean population can be satisfied by caigua fruits intake.
Also, the presence of vegetal sterols, particularly phytosterols, is very interesting since they are able to compete with the cholesterol for the absorption at the intestinal level. The fruits are also rich in soluble fiber (pectins and mucilage) which, together with water, forms a sort of gel that contributes to increase the sense of food satisfaction.
Several Cucurbitacin Glycosides (triterpenoids) have been isolated from seeds of *C. pedata*. These compounds have long been known to display a moltitude of interesting biological activities which at least partly account for the extensive use of *Cucurbitaceae* in the food and folk medicine of most tropical or semitropical regions [23]. The presence of cucurbitacins has been reported frequently in fruits of *Cucurbitaceae*, and variation in the amount present in different cultivars is one of the criteria for selection of these fruits since these compounds are responsible for the bitter taste of some cultivars [24].
As regards the antioxidant activity, apart molecules with antioxidative capablity of the primary metabolism, both fruits and leaves contain phenolic compounds, in particular, flavones glycoside, such as chrysin and apigenin glycosylated with fucose, glucose, rhamnose units [25]. Some of these compounds are peculiar of fruits and not of leaves

and vice versa [26]. It is important to underline that the phenolics identified in *C. pedata* fruits grown up in Italy are the same of phenolics identified in the fruits grown up in the native regions [27]. That is an important aspect, as well as Italian products, could be used for health purposes, as usual.

1.3 Phenolic compounds

1.3.1 Chemical structure and classification

Phenolic compounds are the wider class of secondary metabolites, or, with a more appropriate term, specialized metabolites. They originate from the pentose phosphate, shikimate, and phenylpropanoid pathways in plants.
Structurally, phenolic compounds comprise an aromatic ring with one or more hydroxyl substituents. In relation to their structure, phenolics can be subdivided into simple phenols and polyphenols. The former are substances containing only one aromatic ring and bearing at least one phenolic hydroxyl group and possibly other substituents, whereas polyphenols contain more than one phenolic ring. Phenols and polyphenols may occur as unconjugated aglycones or, as conjugates, frequently with sugars or organic acids, but also with amino acids, lipids, etc [28].
Besides, phenolic compounds can basically be categorized into several classes, like it is showed in **Table 1**.

Table 1: Classes of phenolic compounds respect to the structure

Classes of phenolic compounds	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proanthocyanidns or flavolans)	$(C_6$-C_3-$C_6)_n$

Of all these classes, phenolic acids, flavonoids and tannins are regarded as the main dietary phenolic compounds.
Phenolic acids are subdivided into two subgroups: one derived from the hydroxybenzoic acid and the other one derived from the hydroxycinnamic acid.

Flavonoids account for over half of the eight thousand naturally occurring phenolic compounds, constituting the largest group of phenolic compounds [29]. They are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6-C3-C6 carbon structure. Essentially the structure consists of two aromatic rings (A and B), joined by a 3-carbon bridge, usually in the form of a heterocyclic ring (C) (**Figure 6**).

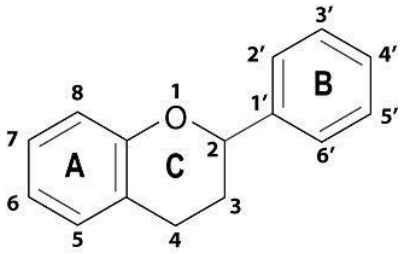


Figure 6: Generic structure of flavonoids.

The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway [30]. Variations in substitution patterns to ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (**Figure 7**) [31], of which flavones and flavonols are the most widely occurring and structurally diversified [32]. Substitutions to rings A and B may include oxygenation, alkylation, glycosylation, acylation, and sulfation. These molecular modifications give rise to the different compounds within each class of flavonoids.

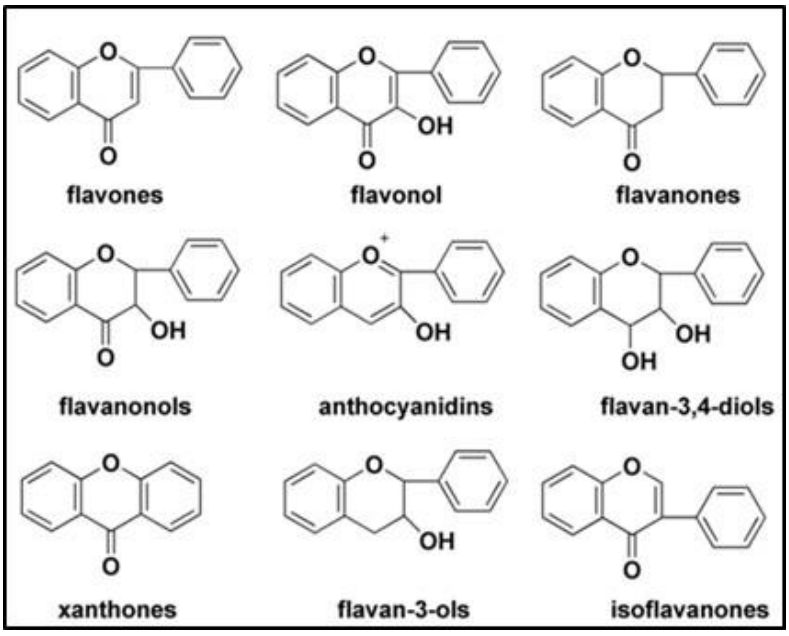


Figure 7: Generic structures of main classes of flavonoids

Tannins are relatively high molecular weight compounds and may be subdivided into hydrolysable and condensed tannins. The former are esters of gallic acid (gallo- and ellagi-tannins) and are commoner in some animal feeds than in humans' diets, while the latter (also known as proanthocyanidins) are derived from flavonoids, being polymers of polyhydroxyflavan-3-ol monomers [33].

1.3.2 Biological function and distribution in plant kingdom

Phenolic compounds are ubiquitous in plants, even if plants' phenolics contents depend on a number of intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling and storage) factors.
No other class of specialized metabolites has been credited with so many and diverse key functions in plant growth and development. Many of these tasks are critical for survival, such as attraction of animal vectors for pollination [34] and seed dispersal, stimulation of *Rhizobium* bacteria for nitrogen fixation (legumes nodulation) [35, 36] and promotion of pollen tube growth [37]. Other provide a competitive edge to plants that grow in suboptimal environments. Flavonoids, for example, are known to enhance tolerance to a variety of abiotic stressors, like ultraviolet radiation [38] or temperature stress [39]. Moreover, they are employed as agents of defence against herbivores [40] and pathogens [41], and they form the basis for allelopathic interactions with other plant species. The flavonoids are evidently extremely useful to plants, and it is not surprising, therefore, that species from all orders of the plant kingdom, from the basal liverworts to the most advanced angiosperms, invest significant amounts of metabolic energy into the production of these compounds.
Most of their functions depend on their structure, thanks to which they exhibit a high antioxidant activity [42, 43]. In fact, they are able to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations. Their scavenger activity usually increases with increasing degree of hydroxylation, but also with the position of the hydroxyl groups.
As all foods of plant origin potentially contain phenolic compounds, they contribute to the intake of natural antioxidants in the human diets. The intake of phenolics derives especially from fruits, vegetables and beverages, like fruit juices, tea and wine. However, it is important to take into account that the amount of phenolics may be affected by commercial processing procedures and storage. Because of the rising enthusiasm for the beneficial properties of these phytochemicals on human health, the interest for phenolics-rich by-products generated by agricultural industries is also rising up in the last few years [44].

1.3.3 Effects on human health

Toward the end of the 20th century, epidemiological studies and associated meta-analyses suggested strongly that long-term consumption of diets rich in plant foods offered some protection against chronic diseases, especially cancer [45, 46]. Because uncontrolled production of free radicals was thought to be significantly implicated in the aetiology of cancer [47, 48], these observations focused attention on the possible role of radical scavenging and radical suppressing nutrients and non-nutrients in explaining the apparent benefit of such diets [49]. The realization that free radicals were similarly implicated in the aetiology of many other chronic diseases [50], along with the recognition of the "French Paradox" [51], immediately focused attention on flavonoids and the foods and beverages rich therein.

In the context of vascular disease, numerous studies have focused on the ability of phenolic compounds, as pure aglycones and as glycosides, to delay the oxidation of LDL *in vitro* [52]. These works have been paralleled by studies investigating the propensity with which the consumption of dietary phenols/polyphenols/tannins-rich foods and beverages reduce the oxidation of LDL *ex vivo* [53]. However, the decrease in LDL oxidability is not necessarily connected with the reductions in cardiovascular disease (CVD). Phenols, polyphenols and tannins are proposed to act on some atherosclerotic and thrombotic signalling pathways [54].

Phenolic compounds have recently been supposed to have a prebiotic and antimicrobial capability. For example, several studies indicate that regular consumption of green tea, rich in (poly)phenols, influences the composition of the gut microflora in humans, pigs, and sheep; for example, lowering the colonic pH value, suppressing *Clostridium perfrigens*, and increasing the proportion of bifidobacteria without inhibiting lactic acid bacteria [55].

There is a growing body of evidence suggesting that diets rich in phenolics may also influence the absorption and metabolism of glucose, resulting in a lower glycemic index [56]. Red wine [57], coffee [58], and apple juice [59] have all been shown in controller volunteer studies to slow glucose absorption and reduce the postprandial surge in plasma glucose, an event known to be an independent risk factor for coronary heart disease [60]. Studies in which volunteers consumed normal portions of phenols/polyphenols/tannins-rich foods have also produced reductions in the postprandial concentrations of plasma insulin and glucose-dependent insulinotropic polypeptide (GIP) and increase in the concentration of glucagon-like polypeptide-1 (GLP-1), and a polyphenol-enriched diet has been reported to reduce the incidence and severity of nephropathy in type II diabetics [61]. The reduced glycemic index has been attributed to phenols/polyphenols/tannins-mediated inhibition of alfa-amilase [62], maltase [63] or alfa-glucosidase (sucrase) [64], even if it is better explained by an effect on the active glucose transporter (SGLT1) in

the duodenum. Some dietary phenols/polyphenols/tannins have been shown *in vitro* to dissipate the sodium gradient essential to the function of SGLT1 [65], and several quercetin glucosides have been shown to interact with it and thus to have the potential to interfere in glucose transport [66, 67].

Such effects might partially explain the reduced incidence of chronic diseases, especially type II diabetes and the metabolic syndrome, in later life associated with diets rich in fruit and vegetables. Other beneficial properties of phenolics are summarized in **Figure 8** here below.

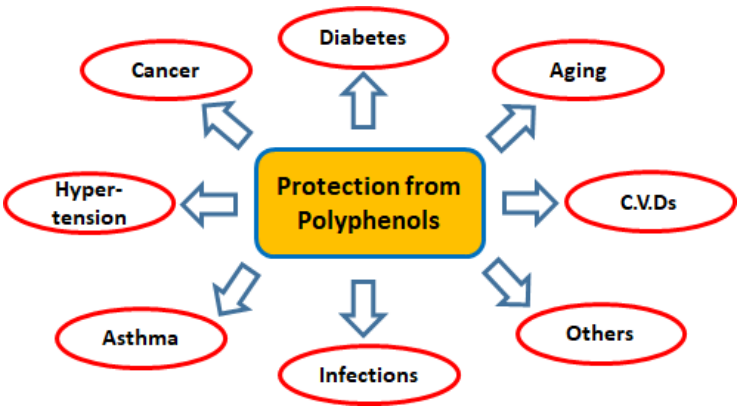


Figure 8: Beneficial functions of Polyphenols.
*C.V.Ds = Cardio-Vascular Diseases.

1.4 High performance liquid chromatography

1.4.1 General aspects

The term "chromatography" is made of two Greek words: "cròmos" = colour and "grafia" = writing. Nowadays, it is universally used to indicate all the separation techniques based on the different distribution of the analytes between two phases, one of which is fixed (stationary phase) and the other one, which flows continuously through the stationary phase (mobile phase), can be a liquid (liquid chromatography) or a gas (gas chromatography). In liquid chromatography, the stationary phase, which is generally solid, may be constituted by a layer of material deposited on a glass support (thin layer chromatography, see paragraph 1.5) or packed in a column. The method of choice for the qualitative and quantitative analysis of flavonoids is High-Performance Liquid Chromatography (HPLC). Since its introduction in the 1970s, HPLC has been used for all classes of flavonoids and hundreds of applications have been published.

HPLC is based on the general principles of chromatography and the "high performances" depend on the dimensions and the characteristics of the material packed into the column. Greater uniformity and smaller size of the material packed into the column correspond to higher efficiency and lower permeability of the column to the liquid phase, resulting in increased backpressure. For this reason, the acronym HPLC was also interpreted as "High-Pressure Liquid Chromatography". HPLC makes use of columns packed with an extremely homogeneous material of reduced particle size (between 2-10 µm), or column made of a monolith. The low permeability of the HPLC columns requires the mobile phase to be pushed through the column by the action of appropriate pumps, which need to be compatible with the backpressures exercised by the chromatographic column (up to about 42000 kPa).

1.4.2 Instrumentation

A schematic representation of HPLC system is shown in **Figure 9**, with emphasis on the flow path of the solvent (solid red arrows) as it proceeds from the solvent reservoir to the detector (the solvents usually referred to as a *mobile phase* or *eluent*). After injection of the sample, a separation takes place within the column, and separated sample components leave (are *eluted* or washed from) the column.

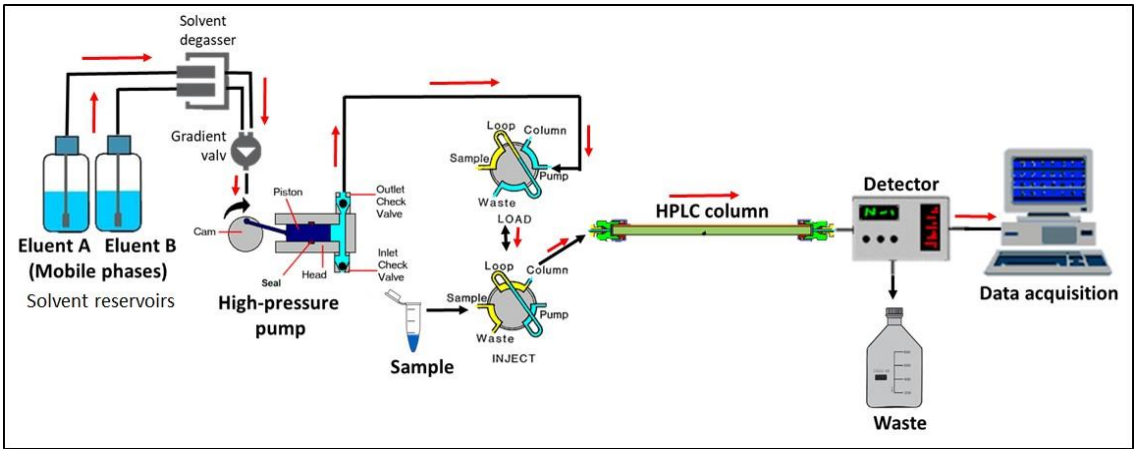


Figure 9: Schematic representation of HPLC instrumentation.
Main HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs. The red arrows indicate the flow path of the mobile phase along the main components of the system.

1.4.2.1 Solvent reservoir

The liquids used as the mobile phase are contained in suitable reservoirs. The mobile phase in RP-HPLC (see 1.4.3 paragraph for further informations) is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

1.4.2.2 Pump

A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, the particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

1.4.2.3 Sample injector

The injector can be manual or an automated system. An injector for analytical HPLC system should provide an injection of liquid samples within the volume range of 0.005 to 1-2 mL with high reproducibility and under high pressure (up to 4000 psi).

1.4.2.4 Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm. Columns with internal diameters smaller than 2 mm are often referred to as microbore and nanobore columns. The temperature of the mobile phase and the column should be kept constant during an analysis.

1.4.2.5 Detector

The HPLC detector, located at the end of the column detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

1.4.2.6 Data collection devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

1.4.3 Separation modes

Separations in liquid chromatography are based on the selective distribution of the analytes between the stationary and the mobile phase, which depends on the physical-chemical properties of the two phases, and on the strength of the interactions that the analytes may establish with them during their elution through the column. The use of stationary and mobile phases of different physical-chemical properties influences type and strength of the interactions established by the sample during the elution, determinig the different chromatographic separation modes summarized in **Table 2**.

The components of the sample that interact more strongly with the stationary phase travel more slowly compared to those displaying weaker interactions. The strength of these interactions are regulated by the composition of the mobile phase, which remain the same throughout separation in the so-called *isocratic elution* or are deliberately changed during the chromatographic run in the case of *gradient elution*.

More in details, the column consists of a cylindrical tube that is typically filled with small porous spherical particles, consisting of either silica or polymeric material. Column consisting of a monolith formed from a single piece of porous silica or polymeric material are also used. Usually, both spherical particles and monoliths are chemically modified by covalently binding at their surface a chemical compound bearing the functional group(s) capable to establish the interactions requested by the selected separation mode, for example hydrophobic or ionizable groups in the case of reversed phase or ion-exchange stationary phase, respectively. The mobile phase surrounds each particle or the monolithic skeleton and the sample molecules, which enter the pores by diffusion and are selectively distributed between the two phases according to the separation mechanism operating for that column.

Table 2: HPLC Separation Modes

Chromatographic Mode	Main characteristics of stationary and mobile phase
Reversed-phase chromatography (RPC)	The stationary phase is non-polar (e.g., hydrocarbons bonded on silica particles, i.e. C_{18} bonded silica), and the mobile phase is polar and consists of a mixture of water and an organic solvent (e.g., acetonitrile, methnol, 2-propanol, etc.); RPC is the most widely used separation mode, especially for water-soluble samples.
Normal-phase chromatography (NPC)	The column is polar (e.g., unbonded silica), and the mobile phase is a mixture of less-polar organic solvents (e.g., hexane plus methylene chloride). It is used for water-insoluble samples, isomer separation, and preparative HPLC.
Non-aqueous reversed-phase chromatography (NARP)	The stationary phase is non-polar (e.g, C_{18} bonded silica), and the mobile phases is a mixture of organic solvents (e.g., acetonitril plus methylene chloride); NARP is used for very hydrophobic, water-insoluble samples, preparative HPLC, and the separation of isomers.
Hydrophilic interaction chromatography (HILIC)	The stationary phase is polar (e.g., silica or amide-bonded phase), and the mobile phase is a mixture of water plus organic (e.g., acetonitrile); HILIC is useful for sample that are highly polar and therefore poorly retained in RPC.
Ion-exchange chromatography (IEC)	The stationary phase contains charges groups that can interact with sample ions of opposite charge, and the mobile phase is usually an aqueous solution of a salt plus buffer; IEC is useful for separating ionizable samples such as acids or bases, and especially for the separation of large biomolecules (e.g., proteins and nucleic acids).
Ion-pair chromatography (IPC)	RPC conditions are used, except that an ion-pair reagent is added to the mobile phase for interaction with sample ions of opposite charge; IPC is useful for the separation of acids or bases that are weakly retained in RPC.
Size-exclusion chromatography (SEC)	It is performed using an inert stationary phase, acting as a molecular sieving, with either aqueous or organic mobile phase; SEC provides separation on the basis of molecular size and is used mainly for large biomolecules or synthetic polymers.
Affinity Chromatography (AC)	It is based on the specific highly selective interactions that certain biomolecules can establish with other substances, such as those between antigen and antibody or enzyme and substrate.

Reversed-phase HPLC is the most commonly used separation mode of HPLC [68]. Differently from normal-phase HPLC, the silica is modified to make it non-polar by covalent binding of long hydrocarbon chains to its surface, typically with either 8 or 18 carbon atoms, or alkyl chains with phenyl substituents [69]. Non-polar analytes are more strongly interacting with the stationary phase than the polar ones, which are weakly retained by the stationary phase and, therefore, can be eluted using polar mobile phases, i.e. hydro-organic mixtures containing an organic solvent at low concentration. The more retained non-polar (hydrophobic) analytes are selectively separated by each other by gradient elution mode, performed by increasing, usually linearly, the concentration of the organic solvent in the hydro-organic mobile phase during the chromatographic run.

The time interval between the injection of a given analyte and its detection is known as the **retention time (t_R)**. This time is measured from the instant at which the sample is injected into the column to the time at which the chromatogram shows a maximum peak height for that compound. Different compounds have different retention times. The main factors that influence the retention time are:

- the nature of the stationary phase (not only what material it is made of, but also particle size, which is related to steric effects);
- the exact composition of the mobile phase (it may modify its elution strength);
- the temperature of the column (t_R decreases at higher temperature);
- the pH of the solvent (affect the charge of ionizable analytes, as well as that of the silanol groups present on the surface silica based stationary phases);
- the flow rate of the mobile phase.

All these parameters have to be carefully controlled in order to obtain repeatable separations.

1.4.4 Hyphenation with UV spectrophotometry

The most frequently used detection method for HPLC is UV spectrophotometry. Routine detection in HPLC is typically based on measurement of UV absorption or visible absorption in the case of anthocyanins. No single wavelength is ideal for all classes of flavonoids since they display absorbance maxima at distinctly different wavelengths. The most commonly used wavelength for routine detection has been 280 nm, which represents a suitable compromise.

In the1980s, the introduction of diode-array technology, provided the possibility of an additional dimension because coupled LC-UV with diode array detection (DAD) allows the chromatographic eluent to be scanned in the UV-visible spectral region. The obtained data are stored and then be compared with a library for peak identification.

This technology improves the power of HPLC analysis because with the information from the UV spectrum, it is possible to identify the compound subclass and in the best of the cases the compound itself. LC-UV with DAD enables simultaneous recording of chromatograms at different wavelengths. The quantification efficiency is improved because detection can be performed at the wavelength maximum of the compound of interest. These are typically found at 270 and 330 to 365 nm for flavones and flavonols, at 290 nm for flavanones, at 236 or 260 nm for isoflavones, at 340 to 360 nm for chalcones, at 280 nm for dihydrochalcones, at 502 or 520 nm for anthocyanins, and at 210 or 280 nm for dihydrochalcones, at 502 or 520 nm for anthocyanins, and at 210 or 280 nm for catechins [70].

Identification of the peaks in a LC-UV chromatogram is made through the comparison of retention times and UV spectra with standard samples or a databank, but wrong conclusions might be drawn for compounds with closely related structures. Besides identification, the coupling of HPLC with DAD allows online quantification of flavonoids in analyzed samples.

1.4.5 Hyphenation with mass spectrometry

Coupled HPLC-MS is one of the most important techniques of the last decades of the 20th century. The combination offers the possibility of taking advantage of chromatography as a separation method and MS as an identification tool. The amazing number of applications and the rapid drop in price (and size) of MS instruments has meant that the use of LC-MS is now extremely widespread. MS is one of the most sensitive methods of molecular analysis. Due to its high power of mass separation, very good selectivities can be obtained. However, the coupling between HPLC and MS has not been straightforward since the normal operating conditions of the mass spectrometer (high vacuum, high temperature, gas-phase operation, and low flow rates) are diametrically opposed to those used in HPLC, namely liquid-phase operation, high pressures, high flow rates, and relatively low temperatures.

1.5 High Performance Thin-Layer Chromatography

1.5.1 General aspects

Paper chromatography and Thin-Layer Chromatography are the two main branches of planar chromatography. Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids [71], but those techniques are superseded by TLC, which now represents the method of choice for simple and inexpensive analytical runs. Although this technique follows the same basic principles of paper-chromatography, it uses a different material as bed or plane for the stationary phase of the process: a plate coated with a sorbent is used, instead of using a cellulose paper.

The advantages of this technique are well known: short separation times, amenability to detection reagents, and the possibility of running several samples at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis.

1.5.2 Instrumentation

Thin-Layer Chromatography is a relatively cheap technique. It makes use of few main components: a device for the sample application, a plate, a chamber, a developing solvent, a detector (**Figure 10**).

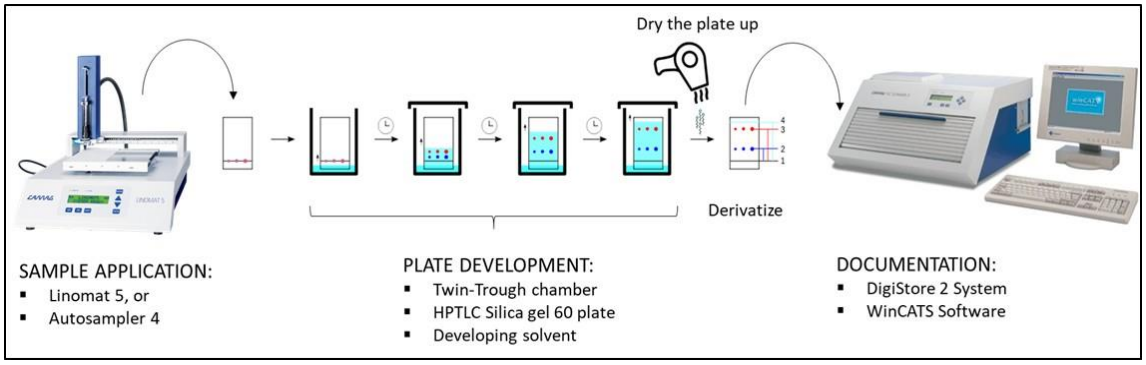


Figure 10: Scheme of main HPTLC steps.
After the application of the sample/s on the plate with the special device, the development of the plate occurs in the appropriate chamber. When the developing solvent reach the set distance, the run is stopped and the plate is dried up and eventually derivatized for subsequent sample detection. The plate image is taken at the opportune visible or UV wavelength.

1.5.2.1 Devices for samples application

The application of the sample test solutions on the plate can be performed manually, in a semi-automatic way or by means of an automatic sampler.

A simple instrument for a precise manual sample application is the Nanomat (Camag, Muttenz, Switzerland) [72]. It allows the application of samples as spots. The instrument is usually used for quick qualitative work, for initial trials during method development and whenever cost of instrumentation has to be kept very low. When handled with care, the Nanomat is also well suitable for quantitative work.

One of the most widely used sample applicators is the Linomat (Camag) [72], an affordable semi-automatic device that introduced all advantages of spray-on technique to planar chromatography. Precise volume dosage and exact positioning combined with flexibility and convenient handling are among the outstanding features of the instrument. The user only loads the sample manually into a syringe and selects the y-position of the application, while the instrument manages all the other parameters of the application process. The most advanced, versatile, and powerful system on the market is the Automatic TLC Sampler 4 (ATS 4; Camag) [72]. Up to 66 samples from vials or 96 samples from well plates can be applied fully automated using either the spray-on technique or spot application by contact transfer. Any x- and y- position on the TLC plate can be selected for application. Samples can be applied as rectangles, a feature that is very useful for large volumes of samples containing the analyte in very low concentration. Prior to chromatography, the rectangles are focused to narrow bands with a solvent of high strength. A special feature of the ATS 4 and also the Linomat 5 is "overspotting". Onto a given position (spot or band) more than one sample can be applied. Spiking a sample, application of several references compounds from different vials onto the same track, or pre-chromatographic derivatization can easily be accomplished.

1.5.2.2 The plate

The plate is a flat, inert plane (often glass-made) coated with a sorbent such as silica gel, cellulose or allumina. Amino bonded layers can also be found. Among them, the first stationary phase is the most commonly used. The plate represents the place on which the separation of the samples' constituents occurs.

1.5.2.3 The chamber

The chamber is a closed and reasonably tight container where four partially competing processes occur:

1- Establishment of equilibrium between components of developing solvent and their vapour phase;

2- Absorption of polar molecules from the gas phase onto the stationary phase;

3- Interaction between the layer of the plate already wetted with mobile phase and the gas phase;

4- Possible separation of the components of the mobile phase by the stationary phase (eventual formation of secondary fronts).

The processes 1 and 2 can be experimentally affected by:

- fitting the chamber more or less completely with filter paper soaked with developing solvent;
- waiting a certain time between the introduction of developing solvent into the chamber and beginning of chromatography (chamber saturation);
- allowing the plate to interact with the gas phase prior to chromatographic development, i.e. without contact to the developing solvent (pre-conditioning).

1.5.2.4 The developing solvent

The developing solvent is the solution used for the development of the plate. Its composition is chosen according to the separation mode and the chemical properties of the compounds object of the study. The change of its composition is one of the most significant factors for the optimization of the separation method. Unfortunately the terms "developing solvent" and "mobile phase" are often used as synonyms. Indeed, with the exception of single component liquids (neat solvents), developing solvent and mobile phase are, strictly speaking, not the same. Moreover, only the composition of the developing solvent at the time when it is placed into the chamber is positively known because then vapours are emitted from it and thus its composition continuously changes. Simply, more volatile solvents evaporate and their percentage in the liquid state decrease, forming the gas phase, while more stable solvents stay in the liquid form.

1.5.3 Mode of separation

Planar chromatography differs from all other chromatographic techniques in the fact that a gas phase is present in addition to stationary and mobile phase. This gas phase can significantly influence the result of the separation.

To test a substance using TLC chromatography, a small dot or band/line, similar to an ink blot, is placed onto a strip of chromatography plate. The plate with the sample upon it is then placed inside a sealed container (the chamber) which contains a sufficient amount of developing solvent. The lower end of the plate should be immersed several millimetres. Driven by capillary action the developing solvent moves up the layer until

the desired running distance (called also "front" or "developing distance") is reached and chromatography is interrupted (**Figure 11**).

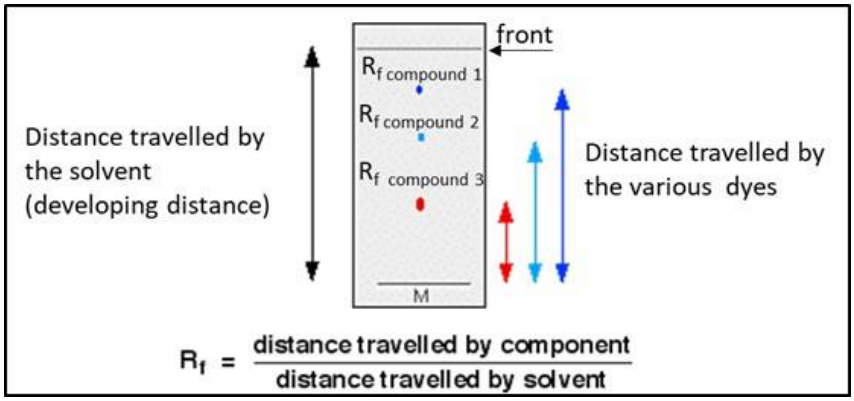


Figure 11: Schematic representation of the separation of the components of a mixture. The solvent is allowed to rise until it almost reaches the top of the plate. That will give the maximum separation of the dye components for this particular combination of solvent and stationary phase.The retention factor (R_f) for each dye is defined as the distance travelled by the compound divided by the distance travelled by the solvent.

Both solvent and plate are chosen on the basis of their polarity, which is related to the affinity with the compounds present in test solutions. In other words, the final distribution of the compounds on the plate is the result of the differential interaction that occurs during development between the different substances present in the analysed sample and the sorbent/solvent selected.

1.5.4 Application to the study of phenolics

Many different solvent systems have been employed for the separation of flavonoids using TLC. Highly methylated or acetylated flavones and flavonols require nonpolar solvents such as chloroform-methanol (15:1). Widely distributed flavonoid aglycones, such as apigenin, luteolin, and quercetin, can be separated in chloroform-methanol (96:4) and similar polarity solvents. One system that is of widespread application for flavonoid glycosides is ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26). By the addition of ethyl methyl ketone (ethyl acetate-ethyl methyl ketone-formic acid-glacial acetic acid-water, 50:30:7:3:10), rutin and vitexin-2''-*O*-rhamnoside can be separated [73]. Careful choice of solvent system also allows separation of flavonoids glucosides from their galactosidic analogs. This is especially important for the distinction of *C*-glucosides from *C*-galactosides. For example 8-*C*-glucosylapigenin (vitexin) can be separated from 8-*C*-galactosylapigenin with the solvent ethyl acetate-formic acid-water (50:4:10) [74].

With regard to detection, as stated by Markham [75], flavonoids appear as dark spots against a fluorescent green background when observed in UV light (254 nm) on plates containing a UV-fluorescent indicator (such as silica gel F254). In 365 nm UV light, depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence, which is intensified and changed by the use of spray reagents. The colors observed in 365 nm UV light are as follows:

- Quercetin, myricetin, and their 3- and 7-*O*-glycosides: orange-yellow
- Kaempferol, isorhamnetin, and their 3- and 7-*O*-glycosides: yellow green
- Luteolin and its 7-*O*-glycoside: orange
- Apigenin and its 7-*O*-glycoside: yellow-green.

For quantitative analysis, scanning of TLC plate with a densitometer provides good results. The flavonoids, both aglycones and glycosides, in *Vaccinium myrtillus* and *V. vitisidaea* (*Ericaceae*) were determined after TLC and densitometry at 254 nm [76].

Better resolution is obtained by chromatographing flavonoids on high-performance TLC (HPTLC) plates. Silica gel 60 F245, RP-18 or, less frequently, Diol HPTLC plates are used for separation purposes. Methanol-water eluents are indicated for HPTLC or RP-18 chemically bonded silica gel but some acid is generally added to avoid tailing. Polar glycosides require eluents containing a high percentage of water. Special HPTLC plates have been designed for this purpose, since normal plates can only accommodate aqueous methanol mixtures with up to 40% water.

1.5.5 Hyphenation with mass spectrometry

Until few decades ago it was possible the connection between TLC and MS techniques just in an off-line way. So, unknown substances were scraped off from the TLC/HPTLC plate, eluted into a tube and transferred into the mass spectrometer (MS). All these fragmented steps were operator-dependent, as well as inefficient from the time point of view.

Now a very convenient and universal TLC-MS Interface (**Figure 12**) designed by CAMAG is available which can semi-automatically extract zones of interest and direct them online into any brand of HPLC-MS system. In more detail, the pioneering device is a versatile instrument for elution of unknown compounds previously separated on a suitable TLC/HPTLC plate. The elution from the TLC/HPTLC plate and the transfer into the mass spectrometer for identification or structure elucidation in an online mode is possible because the instrument is equipped with a probe, which allows the isolation of a specific band corresponding to a specific compound. CAMAG TLC-MS Interface can be quickly and easily connected to any brand of LC-coupled mass spectrometer (ESI-MS, APCI-MS, APPI-MS) without adjustments or mass spectrometer

modifications. Questioned substances are directly extracted from a TLC/HPTLC plate and sensitive mass spectrometric signals are obtained within a minute per substance zone. The interface extracts the complete substance zone with its depth profile and thus allows a detection comparable to HPLC down to the pg/zone range.

Figure 12: CAMAG® TLC-MS Interface 2

TLC-MS Interface by Camag includes oval elution head 4 x 2 mm (mounted), for elution of substances from TLC/HPTLC layers, semi-automatic instrument involving automatic elution head movement, cleaning of the elution path with compressed air, manual positioning and switching.
Dimensions and weight: W x D x H: 275 x 425 x 275 mm; 14.5 kg. Laser: 5mW, class 2M, battery operated (two batteries 1.5 V, AA or LR6), operating time on batteries: up to 100 hours.
Elution head: made of passivated stainless steel, resistant to all common solvents.
Requirements: Gas connection: compressed air or Nitrogen 4–6 bar; Solvent flow rate: 50–300 µL / min; Pressure of elution head onto HPTLC plate: max 400 N.

Chapter 2:

Aim of the thesis

The food market trends reveal the need for high-quality foods and an increasing interest for nutraceutical products and functional foods. These requirements together with recent medical-nutritional findings concerning *C. pedata* benefits on different widespread clinical conditions, have led us to investigate the content of beneficial molecules in commercial food supplements produced from the fruits of *C. pedata*, in comparison to the raw material, which has been extended to the leaves, in addition to the fruits of the plants grown to perform this study.

The aim of the research project has included the development of novel HPTLC-MS and RP-HPLC-ESI-MS methods, suitable for the identification and quantification of the target compounds. The development of HPTLC-MS methods has been finalized to the preliminary screenings of the samples extracted from the plant materials and the food supplements, whereas RP-HPLC-ESI-MS methods has been aimed at obtaining accurate information on occurrence and abundance of the compounds with potential beneficial effects on human health.

Moreover, the dissertation has been proposed to enlarge the knowledge on the qualitative and quantitative accumulation of phenolic compounds in leaves and fruits of *C. pedata* according to pedo-climatic conditions and plant vegetative state. Agronomic factors and harvesting time are already known to exert an influence on metabolites accumulation in plant, but modifications of phenolics in *C. pedata* according to these parameters have never been studied before. For this reason, the experimentation required the collection of leaves and fruits of caigua from plants grown in two different geographical areas (Maribor in Slovenia and Rome in Italy) and in different crop years, 2014 and 2016.

The comparative survey of fingerprints, obtained by both HPTLC and HPLC, has been performed with the purpose to select the most suitable environmental conditions and harvesting time in order to guarantee a desirable bio-accumulation of molecules with health properties, also in view of their potential use in the formulation of food supplements.

Chapter 3:

Materials & Methods

3.1 Plant material

3.1.1 Plant growth and development

The cultivation was performed during the spring season in the second half of April, in two different years, 2014 and 2016. *Cyclanthera pedata* seeds were from ripe fruits of the plant grown up in Perù, from ripe fruits collected in Slovenia and some seeds were bought from an online shop. The seeds were separately sowed in small jars filled with universal soil. Few weeks after germination, when, over the cotyledons, there were around four small leaves, the plants were transferred to an open field in the centre of Italy near Rome (plants obtained by seeds from Perù and from the online shop) and in a small land in the north-east of Slovenia close to Maribor (plants obtained by seeds from Slovenia). Both fields in Italy and in Slovenia were equipped with a special support (a metallic grid) to allow the plant climbing. The plants were watered daily in the early morning and in the late afternoon. In the month of June, all plants started to blossom except the plant obtained from 'online' seeds. The fruits started to appear at the end of July in all plants except the ones bought on the website.

In order to make a comparison between plants from different years of cultivation, the seeds from Perù were sowed in 2014 as well. The leaves and fruits collected from the plants grown up in that year were lyophilized and stored at -20°C. The stability of the phenolic compounds extracted from the leaves and fruits of these plants was confirmed by comparing the HPLC chromatograms obtained in the following years 2015, 2016 and 2017. The comparison was carried out by using the same chromatographic method, separation column and instrumentation.

3.1.2 Fruit harvesting and leaf sampling

In order to evaluate how (poly)phenolic profiles change according to the degree of the ripening of the entire plant, the leaves of caigua were collected in two different moments of the plant maturation: the first set of leaves were sampled after few months from the germination (young leaves) and the other after flowering and fruiting, when the plant completes its life development (old/ripe leaves). To get an easy and fast impression of the samples to our disposition examine the **Table 3** below.
To evaluate putative modifications of the phenolics composition related to the sun exposition, some leaves were collected in different plant portions: apex or base and east or west orientation. These different samplings were possible for some plants grown in Italy, but not for the Slovenian one.
Once the fruits of caigua reached the maturity, they were collected. The fruits are considered ripe when the pulp is reabsorbed to form a large central cavity. It is possible to know when the fruits reach the maturative stage just touching them: if they are still

unripe, they will be hard to the touch; if ripe, the exocarp (external skin) will be soft, easily changeable. During this stage, once the fruit is opened, is also possible to collect the mature seeds for eventual next sowing.

In order to evaluate differences in phenolics composition related to the degree of ripening, some fruits were collected during the unripe stage. For further information about harvested fruits see the **Table 3** below.

Table 3: Scheme of available samples for analysis and their main features.

The leaves collected in Italy during 2016 year were also divided on the basis of sun exposition as follow: base west, apex west, base east, apex east, flowered base, flowered apex. The last two samples of leaves were from plants obtained from peruvian seeds, while all the others were from plants obtained from seeds bought on an on-line shop. Only flowered plants fructified. All these additional samples were analysed just by HPLC, while for HPTLC analysis only samples in the table were used. Since no important differences were detected in leaves sampled according to sun exposition, these data were left out from the thesis and only samples reported in the table were taken into account, also for HPLC.

	Plant organ	Notes	Location	Date of collection
a		Complete growing stage		2014 October
b		Half growth stage		2016 June
c		Complete growing stage		2016 September
d		Complete growing stage		2016 October
e		Ripe/Unripe		
f		Ripe		2016 August
g		Unripe		2016 September
h		Unripe		2014 September

3.1.3 Sample storage and handling

After harvesting, the samples were immediately transferred at University Campus Bio-Medico (UCBM) of Rome in a refrigerated bag and frozen at -80°C, after a sudden freezing in liquid nitrogen. For the transfer to the Institute of Chemical Methodologies (IMC) of the National Council of Research (CNR) in Montelibretti, the samples were wrapped in aluminium foils and put in a polystyrene refrigerated box, filled with dry ice. Upon arrived at the laboratory of IMC, the samples were weighted using an analytical balance, transferred to glass tubes closed with parafilm on the top and frozen at -20°C. Once frozen, the parafilm on the top of the tubes were holed by a needle and positioned inside a lyophilizer for 24 h. After 24 h, the samples were weighed again to evaluate the water loss and then lyophilized for others 24 h to be sure the process was complete. The lyophilized samples obtained were pulverized and stored at -20°C until the analysis.

Part of the frozen samples was transported in thermal boxes to the National Institute of Chemistry in Ljubljana for further analysis. Leaves and fruits of *C. pedata*, sampled in

the nord-eastern side of Slovenia were cut into smaller parts, frozen with liquid nitrogen and lyophilized (Micro Modulyo, IMAEdwards, Bologna, Italy) for 48 h at -50°C. Freeze-dried samples (leaves and fruits of caigua, sampled both at nord-east side of Slovenia and in the south part of Rome) were frozen again with liquid nitrogen, crushed and subsequently pulverized by Mikro-Dismembrator S (Sartorius, Göttingen, Germany) at the frequency of 1700 min^{-1} for 1 min.

The freeze-drying procedure, technically known as lyophilization or cryodesiccation, is a dehydration process typically used to make a biological material or foodstuff more preservable during the time. The basic principle is connected to the loss of water, which is the main source of deterioration, since it provides the ideal substrate for bacterial multiplication as well as biochemical changes of any biological material. Freeze-drying is performed by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to the gas phase (**Figure 12**).

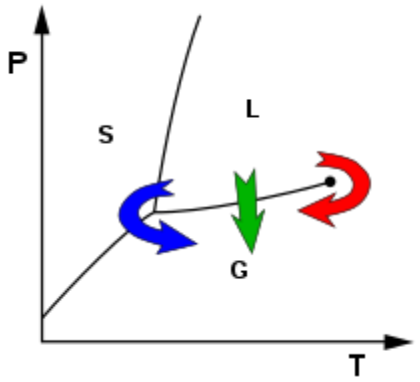


Figure 12: Phase diagram.
In a typical phase diagram, the boundary between gas and liquid runs from the triple point to the critical point. Freeze-drying (blue arrow) brings the system around the triple point, avoiding the direct liquid-gas transition seen in ordinary drying time (green arrow). Cartesian axes: P = pressure; T = temperature. Phases: S = solid; L = liquid; G = gas.

3.2 Chemicals

3.2.1 Chemicals for HPLC-MS analysis

All the reagents used during the experimentation are of purity grade "pure reagent for analysis" such as 99% formic acid (Carlo Erba Reagenti, Milan, Italy), while the solvents used for the preparation of the mobile phases are of purity grade "solvent for HPLC", such as acetonitrile, methanol, ethanol. Deionized water was produced by Milli-Q deionizer (Millipore, Bedford, MA, USA). The isovitexin (or apigenin-6-*C*-glucoside) and apigenin-7-*O*-glucoside standards were purchased from Extrasynthese (Genay, France), while 4-p-hydroxybenzoic acid was purchased from Sigma Aldrich (Milan, Italy).

3.2.1.1 Preparation of standard solutions

All standard solutions were prepared by solubilizing weighed quantities of the compounds in methanol. The prepared solutions were stored at -18 °C in the dark and were stable during the study period (October 2015 – March 2016).

3.2.2 Chemicals for HPTLC-MS analysis

All chemicals were at the purity level of analytical grade or better. Acetic acid, ethyl acetate, n-hexane, formic acid and hydrochloric acid (37%) were purchased from Merck (Darmstadt, Germany), while ethanol, acetonitrile and methanol were from Sigma-Aldrich (St. Louis, MO, USA). LC–MS grade acetonitrile and methanol from Fluka (Buchs, Switzerland) were used for MS analyses. Milli-Q 18.2 MΩ water was used as well. Flavonoid standards apigenin-7-*O*-glucoside, apigenin-6-*C*-glucoside or isovitexin, and apigenin were obtained from Sigma-Aldrich.

Sugar standards rhamnose, fucose, fructose, mannose, glucose, galactose, sucrose, maltose, gentiobiose were purchased from Sigma Aldrich (St. Germany).

3.2.2.1 Preparation of standard solutions

Stock solutions (0,1 mg/mL) of flavonoid standards were individually prepared in methanol and were stored in amber glass storage vials at -80°.

Stock solutions (0,1 mg/mL) of sugar standards were solubilized in 2 mL of ddH_2O and then diluted in MeOH up to 10 mL V_f (final volume: solution of 80% aqueous methanol).

3.3 Instrumentation

3.3.1 RP-HPLC-ESI-MS

Herewith is described the instrumentation we have employed for the separation and identification of the glycosylated flavonoids extracted from the leaves of Caigua by RP-HPLC-ESI-MS. The instrumentation used in Montelibretti (Rome) consisted of a Shimadzu Model LCMS-2010 unit, comprising a SCL-10Avp system controller, two pumps model LC-10ADvp Solvent Delivery Module, a SPD-M10Avp UV/VIS Photodiode Array Detector, a single quadrupole mass analyzer Model 2010 equipped with an electrospray (ESI) interface with nitrogen as the nebulizing and drying gas.
The MS acquisition was performed with the ESI interface in either the negative or positive ionization mode at the following conditions: nebulizing gas nitrogen at flow rate of 4.5 L/min; temperature of block heater, 200°C; temperature of the curved desolvation line (CDL), 225°C; probe voltage (+), 4.5 V; probe voltage (-) -3.5 V; CDL voltage, 25 V; Q-array voltages (+), 50, 15, 60 V; Q-array voltages (-), 0, -15, -60 V; Q-array RF, 150.

3.3.2 HPTLC-MS

HPTLC was performed on the 20 cm x 10 cm glass backed HPTLC silica gel 60 plates (Merck, Art. Nº. 1.05641.0001, Batch: HX604153). Standard solutions and sample test solutions (STS) were applied on the plates by an automatic TLC Sampler 4 (Camag, Muttenz, Switzerland) as 8 mm bands, 9 mm from the bottom edge, 15 mm from the left edge, a 10 mm apart. Sample test solutions were applied bya semiautomatic Linomat 5 (Camag, Muttenz, Switzerland) by using roughly the same dimensions used for standards. The plates were developed up to 9 cm (for phenolics) or 8 cm (for sugars) using 5 or 10 mL of developing solvent (according to the chamber dimensions), which was added only in one through of a 10 min-saturated twin-trough chamber (Camag).
The device used for the MS acquisition was a TLC-MS Interface (Camag) coupled to an LCQ ion trap mass spectrometer (Thermo, Finnigan). The ionization source (ESI) was set out in negative mode using 70% aqueous methanol as the elution solvent. Other conditions are reported below: flow rate, 0.2 mL/min; sheath gas, 90 a.u.; auxilary gas, 38 a.u.; spray voltage, |5kV|; capillary temperature, 240 °C; capillary voltage, - 34 V; normalized collision energy, 30-35%.

3.4 Extraction methods

3.4.1 Extraction of phenolic compounds for HPLC analysis

First of all, it should be said that, for practical matters, we chose to conduct the optimization of the extraction and analysis methods (explained later), using another plant, *Arabidopsis thaliana*, elected the model organism of the Plant Biology, since it presents innumerable advantages from an experimental point of view. Among the advantages, for the study in question, certainly stand out the small size and short life cycle, which allow the growth of a large number of plants in a small environment and in a relatively short time (6 weeks, against the 12-14 weeks of *C. pedata*, which also requires a large space for its growth).

The choice of the extractive method was a key point in the experimentation. For the optimization of the extraction, the type of solvent (pure or in a mixture), the extraction ratio (sample weight/extracting solvent volume) and the number of extractions necessary for the elimination of the compounds of interest from the pellet, were taken into account. Regarding the extraction solvent, simultaneous extractions were carried out with different solvents (pure or mixed solvents; hydro-alcoholic solutions) and the extractive efficiency was evaluated by subjecting the obtained extracts to a chromatographic separation and then to a quantitative estimation of the principal compounds, identified in the extract.
In order to optimize the number of extractions required for the optimal recovery of the phenolic compounds, five subsequent extractions were conducted. After each extraction, the mixtures were spin-dried and aliquots of the obtained supernatant were taken and subjected to the chromatographic separation.

3.4.2 Extraction of phenolic compounds for HPTLC analysis

The choice of extraction conditions is of pivotal importance for research of natural phenolic compounds [77]. The extraction method used for the HPTLC analysis was a simple single step-method routinely applied to biological or food matrices in the Department of Food Chemistry at the National Institute of Chemistry (NIC) of Ljubljana (Slovenia). The choice of the extraction solvent was guided by literature research and by previous extractions tests carried out at the National Council of Research (CNR) located in Montelibretti (Rome, Italy).

Preparation of STS for TLC and HPTLC-MS method development was prepared by dispersing 250 mg of powdered lyophilized samples in 5 mL of 80% aqueous methanol or pure methanol. The suspension was vortexed (2 minutes at 2800 rpm) and then centrifuged at 4500 rpm for 5 minutes. The supernatant was collected and filtered through a 0.45 µm polyvinylidene fluoride (PVDF) membrane filter (Millipore,

Billerica, MA, USA). STS were stored in an amber glass storage vial at -20°C until being applied on HPTLC plates undiluted. All the experimental steps performed for the preparation of the samples before the analytical separation are displayed in **Figure 13**.

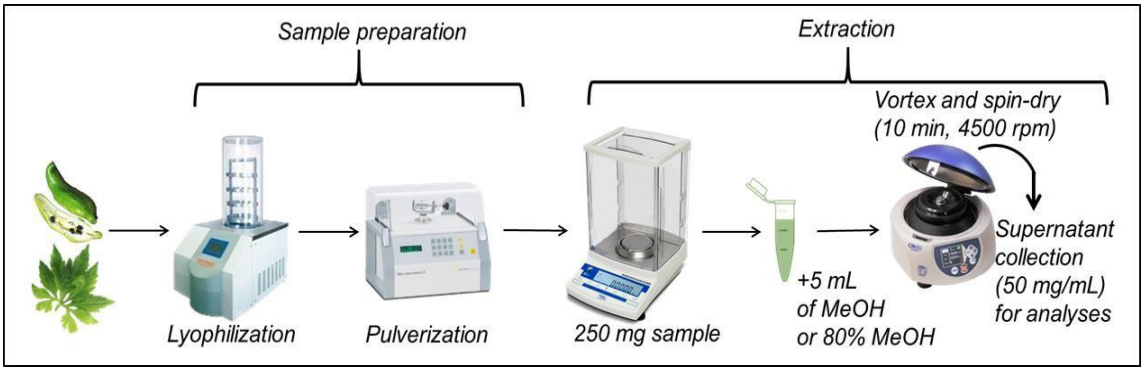


Figure 13: Pre-treatment of italian and slovenian samples, carried out before HPTLC analysis. Before analysis, the obtained extracts were filtrated by 0.45 µm filters to eliminate putative contaminants. At the National Institute of Chemistry in Ljubljana the pulverization was performed by Mikro-Dismembrator S (Sartorius, Göttingen, Germany) at the frequency of 1700 min^{-1} for 1 min.

3.5 Separation and identification of phenolic compounds

3.5.1 Separation and identification of phenolics by HPLC-MS method

The extracts were analyzed by RP-HPLC-ESI-MS. The optimization of the experimental conditions has been crucial in identifying the most suitable composition of the mobile phases and the best gradient to be used for qualitative analysis. After performing a series of preliminary tests under experimental conditions previously developed with different plant extracts (*Arabidopsis thaliana*), it was decided to optimize the chromatographic conditions for separations of phenolics by defining an experimental design and subsequent processing of the data using a commercial software called DryLab® 4.

For this purpose, mobile phase A consisted of water and formic acid 0.2% (v/v), while mobile phase B was acetonitrile. Chromatographic analyses were conducted with a Polaris C-18 reversed phase column, equipped with C_{18} pre-column, and eluted by an increasing gradient of the acetonitrile concentration in the hydro-organic mobile phase at a flow rate of 0.2 mL/min. (**Figure 14**).The last gradient segment displayed in Figure 14 is required to bring the mobile phase composition back to the initial chromatographic conditions. The experimental design was constructed by performing linear gradient separations of 30 and 90 minutes and varying the acetonitrile concentration from 15 to 45% (v/v).

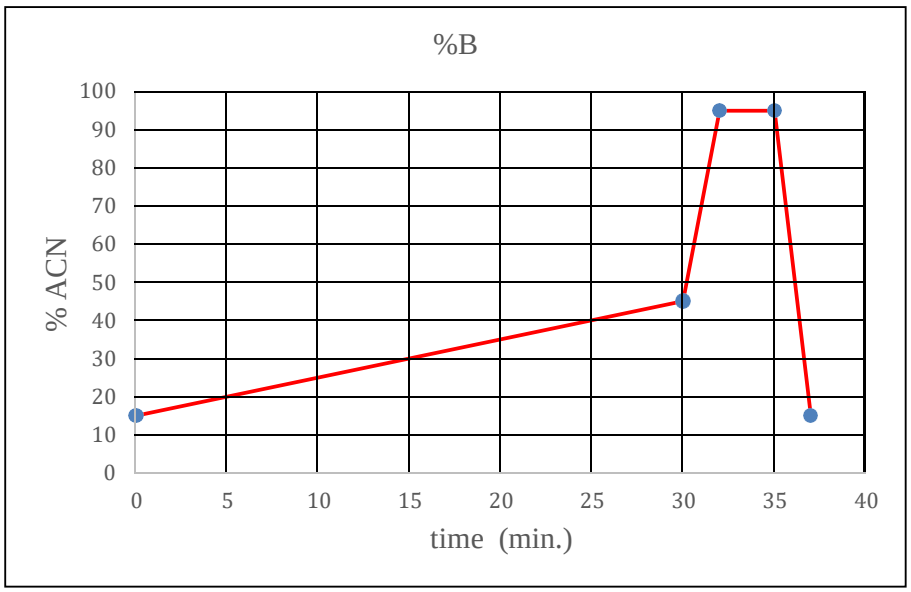


Figure 14: Gradient profile. Eluent composition, A: water containing 0.2% (v/v) formic acid, eluent B: acetonitrile. Flow, 0.2 mL / min.

The sample, after being eluted from the column, is first detected by a diode array detector (DAD) at different wavelengths (254 nm, 280 nm, 320 nm, 350 nm) and then conveyed to the mass spectrometer (MS) using an electrospray ion source (ESI). The experimental parameters are reported above (paragraph 2.1.3.1). The acquisition of the chromatogram in HPLC-MS was performed by the following detection modes:

1) Total-ion current (TIC): the signal resulting by the sum of the separated ion currents, carried out by the different ions generated in the ion source, and detected by the MS within a selected mass/charge range, which is scanned during detection.

2) Selected-ion monitoring (SIM): the mass spectrometer is set to measure only a specified value of mass, corresponding to the signals generated by ions with a selected value of the mass / charge ratio.

3.5.2 Separation and identification of phenolics by HPTLC-MS method

HPTLC was used as a screening technique and as a HPLC-complementary technique, capable to analyze different caigua extracts simultaneously. With this approach all the analyzed test solutions are subjected to the same experimental conditions and parameters, thus a quick and direct comparison is obtained.

Thin-layer chromatography was performed on 10 cm × 20 cm or 10 cm × 10 cm silica gel 60 HPTLC plates (Merck, Germany). All standards and samples were applied by means of Autosampler IV or Linomat V (Camag, Muttenz, Switzerland). Plates were developed in saturated twin trough developing chamber up to 9 cm using ethyl acetate–water–formic acid (85:15:10, v/v/v) [78]. In order to detect the phenolic compounds, the visualization of the HPTLC bands (documentation) was performed at 366 nm (natural fluorescence) after spraying with diphenylboric acid 2-aminoethyl ester (NST reagent). The antioxidant capability was instead observed after spraying with 2,2-diphenyl-1-picrylhydrazyl (DPPH reagent) 0.02% solution in methanol. The sugar profiles for each extract were also studied. In the latter case, the developing solvent was constituted by NST solution-water-methanol (17:3:0.25 v/v/v) and the development was carried out until 8 cm. The detection reagent used for sugars was diphenylamine-aniline-phosphoric acid (DAP reagent) and the documentation was performed in the visible light spectrum (white transmittance, white T). In all the cases, documentation of TLC plates was performed by Camag DigiStore 2 Documentation System.

After the chromatographic development, the obtained bands were analyzed by means of a mass spectrometer (MS) with ESI ion source, coupled to the HPTLC technique through a Camag TLC-MS Interface. The MS conditions were optimized by changing the following parameters: sheath gas, auxiliary gas, spray voltage, capillary temperature and voltage. The acquisition was carried out in negative mode.

All performed chromatographic steps and optimized MS-details are reported in **Table 4**.

Table 4: Summary of HPTLC-MS methods for separation and identification of phenolic compounds.

HPTLC methods			
HPTLC plate (Merck)	Silica gel 60		
Chamber (Camag)	Twin-trough, saturated 15 min		
Application device (Camag)	Linomat 5, Autosampler 4		
Documentation (Camag)	DigiStore 2 Documentation system		
	Phenolic compounds		**Sugars**
Deactivation	30 min		/
Developing solvent (v/v)	Ethyl acetate: H_2O: HCOOH (17: 3: 2)		NST[a] solution: H_2O: MeOH (17: 3: 0.25)
Developing distance	9 cm		8 cm
Detection	NST[a] after heating the plate up 3 min at 105 °C	0.02% DPPH[b] + 30 min in dark	DAP[c] detection reagent + heat the plate up 4 min at 150 °C
Tuned HPTLC-ESI-MS method			
Device: TLC-MS Interface (Camag) coupled to LCQ ion trap mass spectrometer (Thermo, Finnigan) **Elution solvent**: 70% aqueous methanol; **flow rate**: 0.2 mL/min **Sheath gas**: 90 a.u.; **auxillary gas**: 38 a.u., **spray voltage**: |5kV|; **capillary temp.**: 240 °C; **capillary vol.**: - 34 V **Ionization source**: ESI (negative mode); **normalized collision energy**: 30-35%			

[a] 2-aminoethyldiphenylborinate; [b] 2,2-diphenyl-1-picrylhydrazyl; [c] Diphenylamine-aniline-phosphoric acid reagent

3.6 Quantification of phenolic compounds

The identified flavonoids in the samples were quantified by the internal standard method, based on the areas of the chromatographic peaks revealed by a spectrophotometric detector set at 280 nm, using 4-parahydroxybenzoic acid as internal standard. Apigenine-6-*C*-glucoside (isovitexin) was used as a standard compound for the construction of a single calibration graph for the quantification of all identified flavonoids. The internal standard was added to the samples at the initial stage of their preparation and extraction and to the isovitexin solutions prepared at five different concentrations, used to construct the calibration graph.

3.7 Biological tests

3.7.1 *In vitro* Model of Digestion

The human digestive process of caigua leaves was simulated *in vitro* by using some modified steps of the method described by Sayar *et al*., 2005 [79]. Briefly, the method was based on 3 subsequent incubation steps with different digestive enzyme solutions at specific pH values. Leaves of caigua (100 mg) were initially subjected to a cooking step in boiling water for 4 minutes. After cooling to room temperature, the solution was incubated with 50 mM phosphate buffer (pH 6.9) for 15 minutes at 37 °C. Leaves solution was subjected to the first enzymatic incubation with human salivary α-amylase (5 mg/mL in 3.6 mM $CaCl_2$) for 15 minutes to simulate mouth conditions. In order to simulate the gastric digestion, the second step of incubation was carried out at pH 2 with porcine pepsin (0.5 mg/mL in 0.9% NaCl) for 30 minutes. The pH 2 was reached adding sequentially few drops of 1 M HCl to the solution containing the leaves of caigua. Finally, the third step of incubation at pH 6.9 was carried out adding pancreatin from porcine pancreas (0.5 mg/mL in 50 mM Phosphate Buffer, pH 6.9) and bile acids standard mixture (containing 1.35 µM of sodium cholate, sodium deoxycholate, sodium glycocholate and sodium taurocholate) for 90 minutes in order to simulate duodenal digestion. All incubation steps were performed at 37°C, to simulate the human body temperature, and in gentle agitation conditions (90 rpm) in an orbital shaker.

3.7.2 DPPH assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colourless ethanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry [80], so it can be useful to assess various products at a time.

In our case, the DPPH-based assay for the evaluation of the antioxidant capacity was performed directly on the silica gel plate after chromatographic separation. Following the chromatographic steps for the separation of the phenolic compounds, the plate was submerged in 0,02% DPPH solution (see **Table 4**). Then, the plate was dried up and stored in the dark for 30 minutes. During this time, in presence of antioxidant compounds, the above-mentioned reaction occurs. Thus, the site of the plate in which molecules displaying redox activity are located became pale yellow. In order to identify the most reactive compounds present in *C. pedata* fruits and leaves, the mass spectrometry analysis was used and its results were compared with the results previously determined.

3.7.3 Determination of Vitamin C as Ascorbic acid

L-Ascorbic acid (L-AA) and dehydroascorbic acid (DHA) are respectivelyreduced and oxidized forms of vitamin C, which are naturally present in most fruits and vegetables. Vitamin C, is one of the most important water-soluble vitamins for human health, known by its antioxidant activity, but human body is unable to synthesize it. Fruit and vegetables are the major sources for the human diet. For these reasons it is of paramount importance to evaluate its content in foodstuffs and food plants. However, the heterogeneity of food samples and high sensitiveness of this nutrient to degradation conditions potentially occurring during its analysis, needed a special attention for the development of analytical methods for vitamin C quantification [81]. The review by Spìnola V. *et al.* (2014) [81], summarizing all the high-performance liquid chromatography methods for vitamin C analysis in food commodities during the period 2000-2014, was taken into account for the development of the analytical method used in our study.

We adopt a simple and quick extraction procedure in order to ensure the extraction efficiency with minimal sample loss [82]. For what it concerns extraction solvent, previous works have already demonstrated higher samples stability in metaphosphoric acid (MPA) in comparison to other acids such as trichloroacetic acid (TCA) [82]. Therefore, the extraction was carried out by adding 1 mL of 5 % MPA to 5 mg of lyophilized and pulverized fruit or leaf sample. The obtained solution was vortexed for around 3 minutes and then spin-dried at 4500 rpm for 10 minutes. The surnatant was collected and passed through a Millipore 0.45 µm membrane before injection in HPLC system. The injection occurs immediatelyafter extraction in order to minimize samples degradation (L-AA). To protect light-induced vitamin C degradation, the extracts were also wrapped in aluminium foils. To quantify the total concentration of vitamin C, a solution of DL-1,4-dithiotreitol (DTT) 20 mg/mL was prepared and an aliquot of 100 µL was added to 0.5 mL of vacuum-filtered sample, used for the AA analysis. The mixtures were kept in the darkness for 2 h. Then they were passed through a Millipore 0.45 µm membrane and injected into the HPLC system. DHA was calculated by subtracting the value found for AA to the total one.

AA and DHA belong to the group of very small polar molecules that are difficult to retain in conventional reversed-phase (RP) chromatographic systems and separate from the dead retention volume. To get sufficient retention, a very high percentage of water, usually in inorganic/organic acid or inorganic buffer (sometimes even 100%), must be applied in combination with low pH. Because of that 0,5 % HCOOH in water and MeOH were selected respectively as A and B mobile phases. The separations were carried out by a Luna C_{18} column (250 x 4,6 mm I.D. 5 µm) at 25 °C and by increasing linearly the percentage of B mobile phase from 2 to 10 % in 20 min. The mobile phase flow rate was 0,2 mL/min and the wavelenght of detection 245 nm.

Chapter 4:

Results and Discussion

4.1 Optimization of the sample pre-treatment method

A sample pre-treatment method was optimized in order to obtain representative samples of material extracted from leaves and fruits of caigua plants grown in Rome and in Maribor (Slovenia). Collected leaves and harvested fruits were stored in a freezer at a temperature of -80 °C after frozen in liquid nitrogen. Then the samples were grounded in liquid nitrogen and subsequently freeze-dried for 48 hours. The grounding was carried in liquid nitrogen to make leaves and fruits fragile and thus easier to break down, while ensuring that the breakage of the cell walls occur with concomitant inhibition of any enzymatic processes that could lead to a variation in the sample composition. The lyophilization process, on the other hand, was conducted to eliminate the aqueous component of the plant matrices and to avoid any unwanted enzymatic reactions during the subsequent sample treatment and storage. Prior to proceeding with the extraction of phenolic compounds, precision weighing measurements were performed to determine the percentage of lost weight, and thus of the water eliminated by the lyophilization process. These measurements were carried out for the first time after 24 hours of lyophilization and a second time after 48 hours of freeze-drying in order to ascertain the total loss of water. For each sample, water loss after 48 hours was around 90%, practically comparable to the loss of water obtained after 24 hours of freeze-drying (see Table 5). This result suggests that the lyophilization conducted for 24 hours was sufficient to completely remove the water present in the plant matrices.

Table 5: Loss of weight after sample 24h- and 48h-lyophilization.

	Samples	Weight of pulverized samples (g)	Weight of 24h-lyophilized samples (g)	Loss of weight after 24h-lyophilization (%)	Weight of 48h-lyophilized samples (g)	Loss of weight after 48h-lyophilization (%)
Leaves	a	3,64	0,36	90,11	0,35	90,38
	b	2,89	0,29	90,00	0,28	90,31
	c	7,57	0,68	91,02	0,68	91,02
	d	3,55	0,35	90,14	0,35	90,14
Fruits	e	3,03	0,27	91,09	0,26	91,42
	f	2,57	0,26	89,88	0,26	89,88
	g	6,39	0,64	89,98	0,64	89,98
	h	8,33	0,83	90,04	0,83	90,04

4.2 Screening of caigua fruits and leaves and nutraceutical product by HPTLC

The HPTLC analysis allowed the rapid and cost-efficient comparison, also in terms of time, among leaves, fruits and supplements extracts.

As it is possible to see from the chromatogram detected at the wavelength of 366 nm (**Figure 15**), the leaves are certainly richer in terms of phenolic compounds followed by the fruits. While the food supplement, produced from the fruits of the same plant, is the poorest in both qualitative and semi-quantitative terms. It is important to note (**Figure 15**) that the post-chromatographic derivatization with a specific reagent for the class of

compounds of interest (in this case the 2-aminoethyldiphenylborinate, NST) allows their display in a highly specific manner at the wavelength appropriately selected.

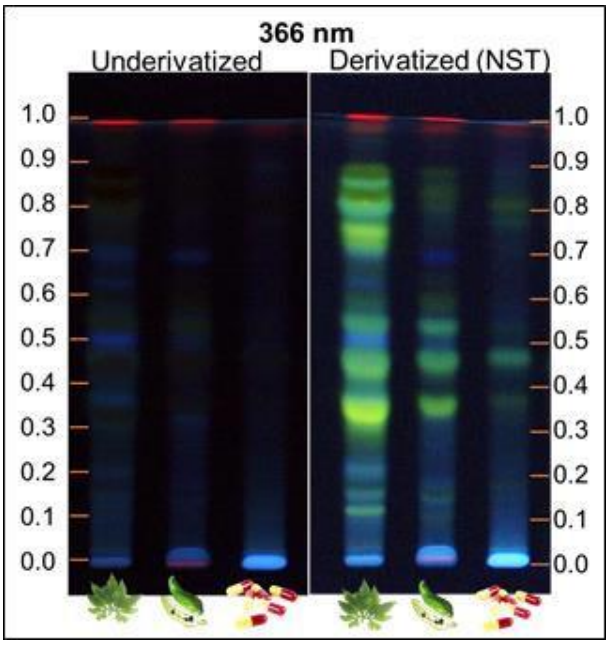


Figure 15: HPTLC analysis of leaves, fruits and supplement from caigua plant. Leaves and fruits of caigua are from Slovenia. Application volume for each sample: 15 µL (50 mg/mL). 2-aminoethyldiphenylborinate (NST) is the derivatization reagent that allow the selective detection of the phenolic compounds. Leaves, fruits and supplement of caigua are, in the mentioned order, proportionally rich in phenolic compounds.

From the obtained results, we can speculate the extraction procedure of the compounds of interest from the fruits of caigua in order to produce the supplement we analysed, is probably not that efficient. In order to get a supplement richer in phenolics content, it would be great to produce a supplement from leaves of caigua, since they are already more concentrated in phenolic compounds compared to fruits extracts. Moreover, it is of paramount importance to investigate the impact of growing conditions on the accumulation of phenolics in plants.

4.2.1 Identification of phenolics by HPTLC/MS method

After a first fast screening, a HPTLC-ESI-MS method was tuned in order to identify as much compounds as possible from caigua supplement in comparison to leaves and fruits of caigua. The method optimization has been performed on available standards belonging to flavonoids and phenolic acids subclasses. Information about the instrument and the tuned HPTLC-ESI-MS conditions are summarized in **Table 6** below.

Table 6: Tuned HPTLC-ESI-MS method.

Device: TLC-MS Interface (Camag) coupled to LCQ ion trap mass spectrometer (Thermo, Finnigan) **Elution solvent**: 70% aqueous methanol; **flow rate**: 0.2 mL/min
Sheath gas: 90 a.u.; **auxillary gas**: 38 a.u., **spray voltage**: |5kV|; **capillary temp.**: 240 °C; **capillary vol.**: - 34 V
Ionization source: ESI (negative mode); **normalized collision energy**: 30-35%

The functioning of the performed procedure is summarized in the **Figure 16**.

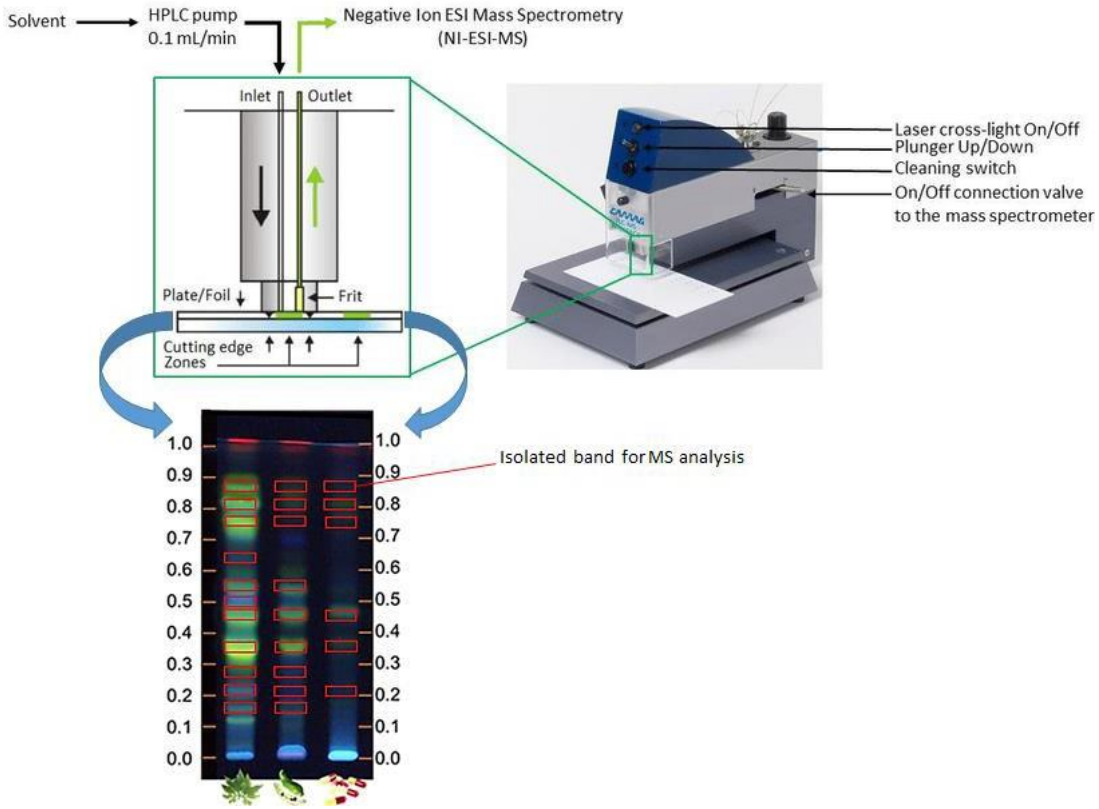


Figure 16: Schematic explanation of the HPTLC-ESI-MS functioning.
The TLC-MS Interface by Camag allowed the isolation of zones of interest (red) and their transfer into MS system for high specific and selective analysis. Red squares indicate the position where it was possible to obtain a clear *m/z* signal. Leaves and fruits of caigua were from Slovenia.

The developed method allowed the separation of 11 phenolics in total (at least in leaves) and the identification of 5 of them. The identification occurred by comparing the obtained mass spectra with the mass spectra found in the scientific literature. The obtained data and the respective scientific references are summarized in the **Table 7**.

Table 7: Summary of the main data obtained by HPTLC-MS analysis.

Tentative identification	R_F (min) (average of 3 analysis) ± standard deviation	*m/z*	Fragment signals $[M-H]^-$	Ref.
Chrysin 7-*O*-gentiobioside	0,18 ± 0,01	577	MS^2 [577]: 413, 457, 415, 311, 293, 225 MS^3 [577→ 413]: 293, 335	[83]
Unknown	0,22 ± 0,02	399	MS^2 [399]: 295, 325 MS^3 [399→295]: 267, 295	--
Unknown	0,26 ± 0,02	441	MS^2 [441]: 295, 381, 307, 337 MS^3 [441→295]: 267,295	--
Luteolin 8-*C*-glucoside (orientin)	0,32 ± 0,03	447	MS^2 [447]: 327, 357 MS^3 [447→327]: 299, 327	[84, 85]
Apigenin 6-*C*-glucoside (isovitexin)	0,45 ± 0,01	431	MS^2 [431]: 311, 341 MS^3 [431→ 311]: 283, 311	[86]
Unknown	0,50 ± 0,01	473	MS^2 [473]: 311, 413, 341, 395 MS^3 [473→311]: 283	[87]
Unknown	0,53 ± 0,01	457	MS^2 [457]: 295, 325, 397 MS^3 [457→295]: 267, 295	--
Apigenin 6-*C*-fucopyranoside	0,65 ± 0,02	415	MS^2 [415]: 311, 341 MS^3 [315→311]: 283	[86]
Chrysin 6-*C*-fucopyranoside	0,74 ± 0,02	399	MS^2 [399]: 295, 325 MS^3 [399→295]: 267, 295	[86]
Unknown	0,80 ± 0,01	441	MS^2 [441]: 295, 381, 307, 337 MS^3 [441→295]: 267, 295	--
Unknown	0,84 ± 0,03	441	MS^2 [441]: 295, 381, 307, 337 MS^3 [441→295]: 267, 295	--

According to the preliminary HPTLC screening, only six out of eleven/nine phenolics detected respectively in leaves and fruits were also detected in caigua supplement by HPTLC-MS method.

The identified molecules mostly belong to the flavonoid subclass of phenolic compounds. In nature, flavonoids generally occur as glycosides or, most commonly, single units or polymers of hexose, pentose, rhamnose, arabinose and/or their combinations [88]. These glycosidic moieties are mostly attached in *O*-position to free hydroxyl groups in the A and C rings via a β-glycosidic bond. Flavonoid-*C*-glycosides may also occur in plant tissues but they are not as common as their *O*-glycoside counterparts and, for this reason, have received much less attention in the literature [89], despite their common occurrence in major cereal crops and medicinal species has

reported for years [90]. As for other medicinal plants, in *Cyclanthera pedata*, flavonoids seem to mostly occur as *C*-glycosides, wherein the glycoside moiety is directly attached to the aglycone backbone via an acid-resistant C-C bond. *C*-glycosylation mostly occurs at the C_6 and C_8 positions. Flavonoids *C*-glycosides are biologically active both *in planta* and as dietary components. Activities ascribed to these plant specialized metabolites include them functioning as antioxidants [91], insect feeding attractants [92], antimicrobial agents [93], and UV-protective pigments. From a dietary perspective, these compounds have also been ascribed positive biological activities, such as the counteraction of the tissue oxidation [94], inflammation and cancer development [95].

The *C*-glycoside flavonoids saponarin (isovitexin 7-*O*-glucoside) and isovitexin have been identified in other three species of the *Cucurbitaceae* family [96] and are proposed as good markers for chemotaxonomic investigations of this family, since their rare dissemination in the plant kingdom.

4.3 *In situ* hydrolysis of *O*-glycosylated flavonoids from leaves and fruits of caigua on HPTLC silica gel plates

In order to support further characterization of glycosylated flavonoids in caigua leaves and fruits by HPTLC image analysis, densitometry and HPTLC-ESI-MS, *in situ* hydrolysis method of *O*-glycosylated flavonoids on HPTLC silica gel plate in a twin-trough chamber was developed. The method includes eight experimental steps, carried out directly on the silica gel plate. The optimization of the hydrolysis method and its validation are discussed in **Appendix I**, while in **Figure 28** and **29** below results are discussed, respectively for sugars and flavonoids.

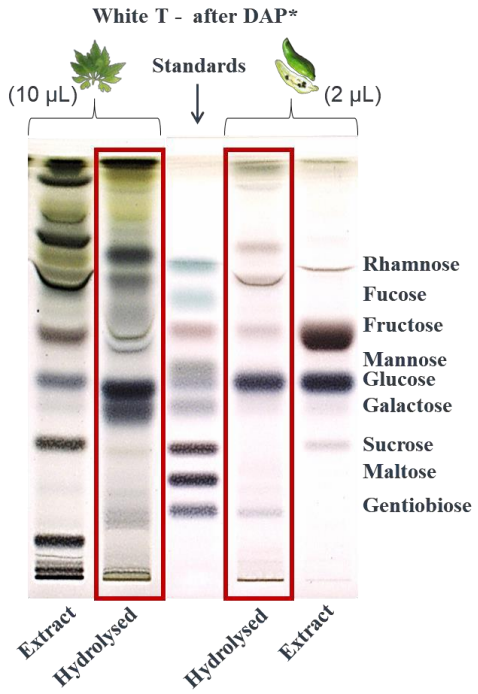


Figure 28: Comparison of sugars profiles of leaves and fruits of caigua from Slovenia before and after hydrolysis. *DAP=Diphenylamine-aniline-phosphoric acid reagent.

In both leaves and fruits, a small amount of gentiobiose appears after hydrolysis supporting the presence of *O*-gentiobiose flavonoids.
Galactose, present in small quantities in both leaves and fruits before hydrolysis rise up in leaves after HCl exposure, suggesting its presence in the extract bound to some aglycones. Nothing can be said about rhamnose and fucose, since they occur in the upper part of the plate where flavonoids occur as well, preventing their clear detection.

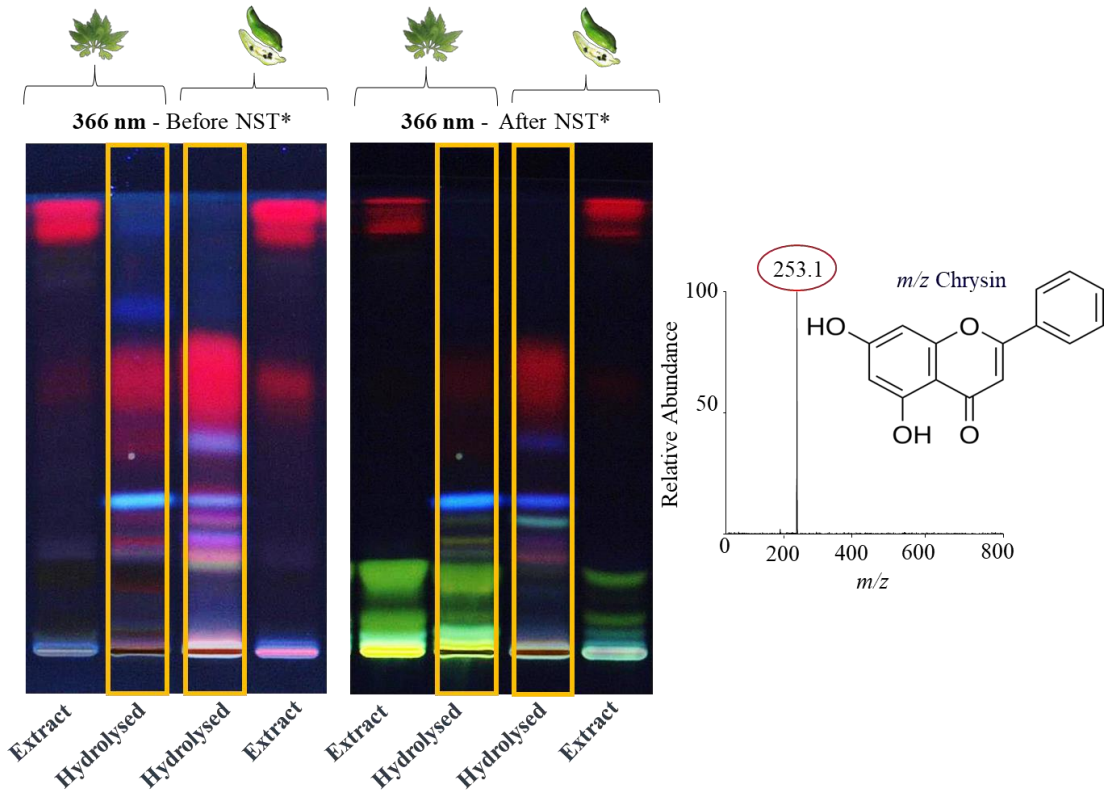


Figure 29: Flavonoid profiles in leaves and fruits of caigua from Slovenia before and after hydrolysis. *NST=2-aminoethyldiphenylborinate.

HPTLC-ESI-MS analysis on hydrolysed leaves test solution, allow the identification of aglicone chrysin (*m/z* 253), supporting the presence in leaves of chrysins *O*-glycosilated, such as Chrysin 7-*O*-gentiobioside previously identified. The presence of that molecule in our extracts would be also supported from the releasing after hydrolysis of the sugar gentiobiose, as explained before. Since we did not detect *m/z* value corresponding to chrysin in fruits, we can speculate the presence of a bigger amount of *O*-glycosilated chrysins in leaves test solution than in fruits extract.
Some detected *m/z* values confirm the identity of *C*-glycosilated flavonoids, assigned previously. In particular, apigenin-6-*C*-fucopyranoside (*m/z* 415), apigenin 6-*C*-glucoside (*m/z* 431), luteolin 8-*C*-glucoside (*m/z* 447) and chrysin 6-*C*-fucopyranoside (*m/z* 399).

4.4 Screening of phenolics and their redox activity in different samples of caigua leaves and fruits

To date, many works demonstrated that common postharvest processing treatments have a strong influence on phenolic metabolites. The impact of growing conditions on the accumulation of phenolics in plants has been studied as well. Environment is supposed to be one of the main causes of plant specialized metabolites compositions, since these

compounds are implied in stress responses. In nearly all multi-year studies considerable variation exists in phenolic development and accumulation. The same can be said for multi-cultivar studies within the same species.

In order to better elucidate the mechanisms at the base of plant specialized metabolites accumulation, phenolic fingerprints from caigua leaves and fruits harvested in different years, at different maturation stages and in different locations were compared by means of HPTLC method.

Since plant-based phenolic metabolites are of special interest due to their potent antioxidant activity, the redox capability has been tested as well by HPTLC-DPPH method. The phenolics and redox fingerprints obtained are displayed in **Figure 30**.

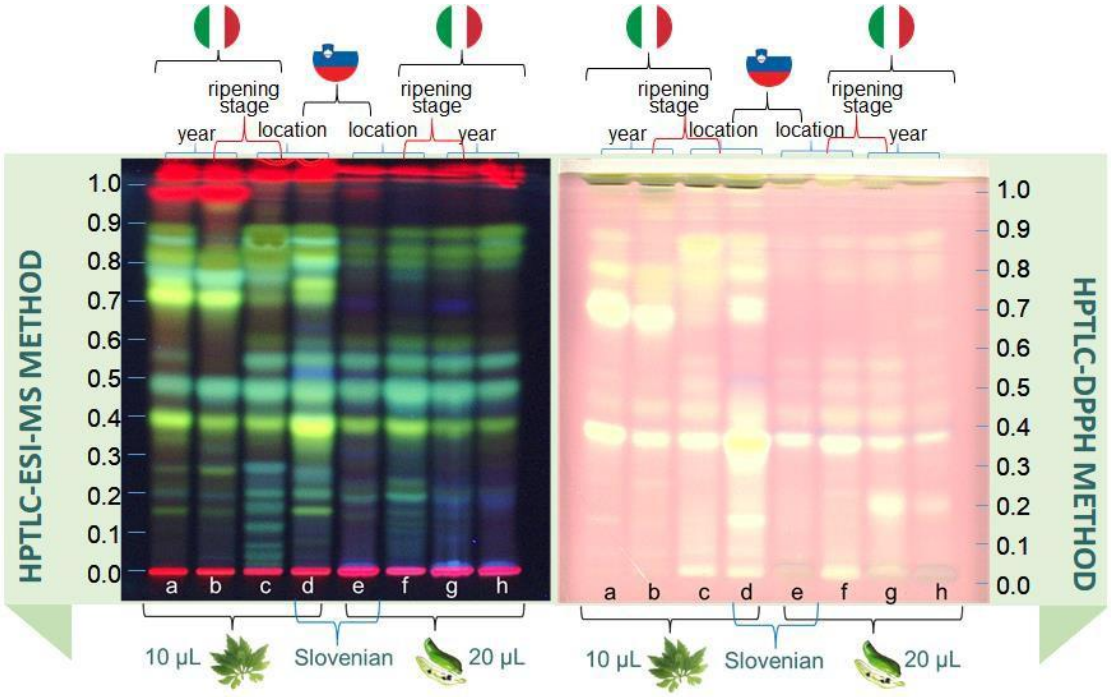


Figure 30: Phenolics (left plate) and redox (right plate) fingerprints of different caigua leaves and fruits. For simplicity, samples which differ for just one characteristic were coupled with braces and labelled with the writing 'year', 'ripening stage' or 'location' in order to emphasize the main characteristic for which each pair of compared samples differs between each other. For example, leaves identified with letter 'a' differ from leaves named 'b' for the year of cultivation, rspectively 2014 and 2016. The same applies for samples of fruits labelled with 'g' and 'h', while samples 'b' and 'c' of leaves differ for the ripening stage, as well as samples 'f' and 'g' of fruits. Samples 'c' and 'e' vary for the location of cultivation in comparison to samples 'd' and 'f' respectively. Differences according to the place of cultivation are also emphasized by rounded italian and slovenian flags (on the top).
As highlighted in the image above, the volume of leaves extracts applied is twice (20 µL) the amount of fruits extracts (10 µL).

Managing phenolic development in plants is important from a food science perspective. Obtaining fruits and vegetables with higher level and number of beneficial molecules is not just important for agronomic industries but also for the industries producing food supplements.

Isovitexin (R_T = 0,45 ± 0,01) is easily identified in all samples analysed, supporting its presence in *Cucurbitaceae* species and thus its possible importance as taxonomic character [97].

Further analysis are needed to deeper investigate the factors which may influence the accumulation of phenolics.

The redox activity of almost all the studied compounds was confirmed by means of the HPTLC-DPPH method. Regardless of quantitative analysis, luteolin 8-*C*-glucoside or orientin (R_T = 0,32 ± 0,03) is one of the most reactive molecule from the redox point of view. This is in agreement with investigations by Zielinska D. and Zielinski H. (2011) [98], who found out an higher redox activity of orientin and homoorientin in comparison to vitexin and isovitexin.

In order to deeper highlight differences among samples of caigua leaves and fruits, a more accurate method of extraction and an HPLC-ESI-MS method were optimized (see later).

4.5 Sugars composition in leaves and fruits of caigua

Qualitative sugars composition has been evaluated in leaves and fruits of caigua by HPTLC method. Results about this topic are displayed in **Figure 31**. Sucrose, glucose and fructose are the main sugars found in fruits of caigua. In addition, great amount of galactose is found in leaves collected in Italy, while a small amount in leaves collected in Slovenia, suggesting the influence of this sugar by place of cultivation. Levels of sucrose are influenced by pedo-climatic conditions and ripeness degree, since its levels are really low in both leaves collected in Italy in 2014 and leaves collected in 2016 during unripe stage in comparison to leaves collected in Italy or Slovenia in 2016 at the complete maturative stage.

As for the fruits, it is interesting to note that the content of sugars in fruits decrease during the ripening, unlike what happens in most of the fruits from other plants.

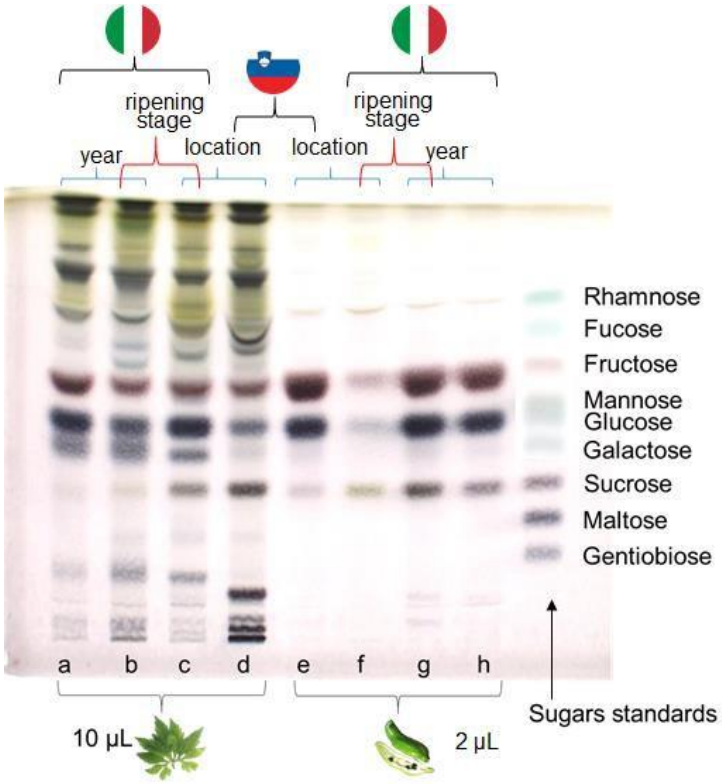


Figure 31: Sugars composition in leaves and fruits of caigua according to ripening stage, year and place of cultivation.
For simplicity, samples which differ for just one characteristic were coupled with braces and labelled with the writing 'year', 'ripening stage' or 'location' in order to emphasize the main characteristic for which each pair of compared samples differs between each other. For example, leaves identified with letter 'a' differ from leaves named 'b' for the year of cultivation, rspectively 2014 and 2016.The same applies for samples of fruits labelled with 'g' and 'h', while samples 'b' and 'c' of leaves differ for the ripening stage, as well as samples 'f' and 'g' of fruits. Samples 'c' and 'e' vary for the location of cultivation in comparison to samples 'd' and 'f' respectively. Differences according to the place of cultivation are also emphasized by rounded italian and slovenian flags (on the top).

4.6 Optimization of the extraction method of phenolic compounds before their identification by HPLC-DAD-ESI-MS

The optimization of the extraction method regarded both the choice of the best solvent and the number of extractions necessary to extract almost all compounds of interest from the pellet. As it can be seen from the histograms displayed in **Figure 32**, the extraction with methanol (MeOH) produced the optimal extraction yield for all compounds, better than that obtained with ethanol. The tested hydro-organic (methanol-water) mixtures are still able to provide better extractive yields than ethanol, but not as well as the extraction efficiency obtained with MeOH alone. For these reasons, MeOH was selected as extraction solvent even because it is easily evaporated in order to concentrate the extract.

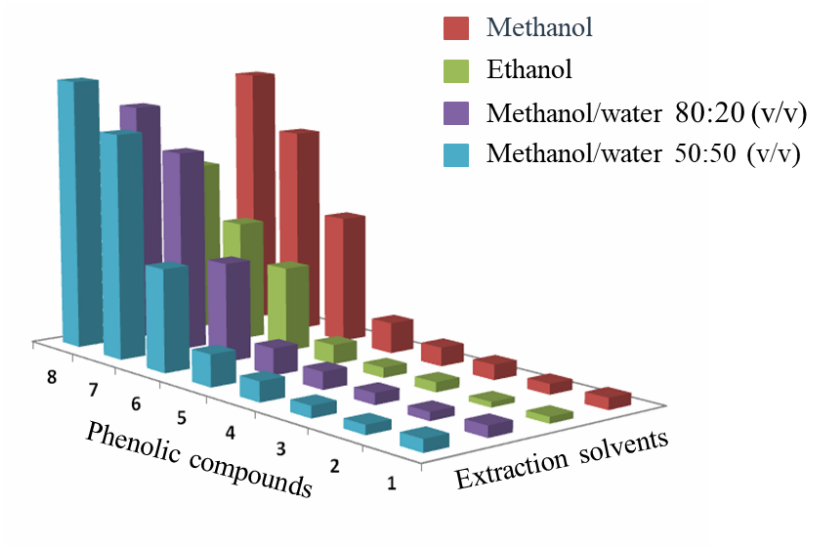


Figure 32: Optimization of the extraction conditions - Choice of the best extraction solvents. with "hydro-organic" is intended "methanol-water".

In order to optimize the number of extractions required for the optimal recovery of the phenolic compounds, five subsequent extractions were conducted, and, after each of them, aliquots of the resulting supernatant were collected and subjected to chromatographic separation using a 30 minutes linear gradient of increasing concentration of acetonitrile in the mobile phase, consisting of water containing 0.2% (*v/v*) formic acid. Four peaks with retention times homogeneously distributed within the above gradient time (and thus representative of phenolic compounds of different polarity) were selected to evaluate the necessary number of subsequent extractions necessary to maximize the extraction yield.

The four plots displayed in **Figure 33** show that, for three analytes over four, after three extractions the amount of the selected analytes remaining in the pellet is minimum and does not decrease significantly in the further extractions. Therefore, we decided to proceed with five extractions to maximize the extraction yield of all compounds of our interest and the optimized extraction method was carried out according to the scheme displayed **Figure 34**. Briefly, a known amount (25 mg) of lyophilized leaves was subjected to five successive ultrasound-assisted liquid-solid extractions. Each extraction lasted 30 minutes and was carried out at 40 ± 1 °C with 1 mL of extracting solvent (methanol). At each addition of the extraction phase, the sample was centrifuged at 13.000 rpm for 10 minutes in order to separate the pellet from the supernatant, which was harvested and recovered in a 5 mL flask. The obtained extract was concentrated under reduced pressure and at 35 °C by means of a rotary evaporator and subsequently solubilized with 500 µL of the same extraction solvent.

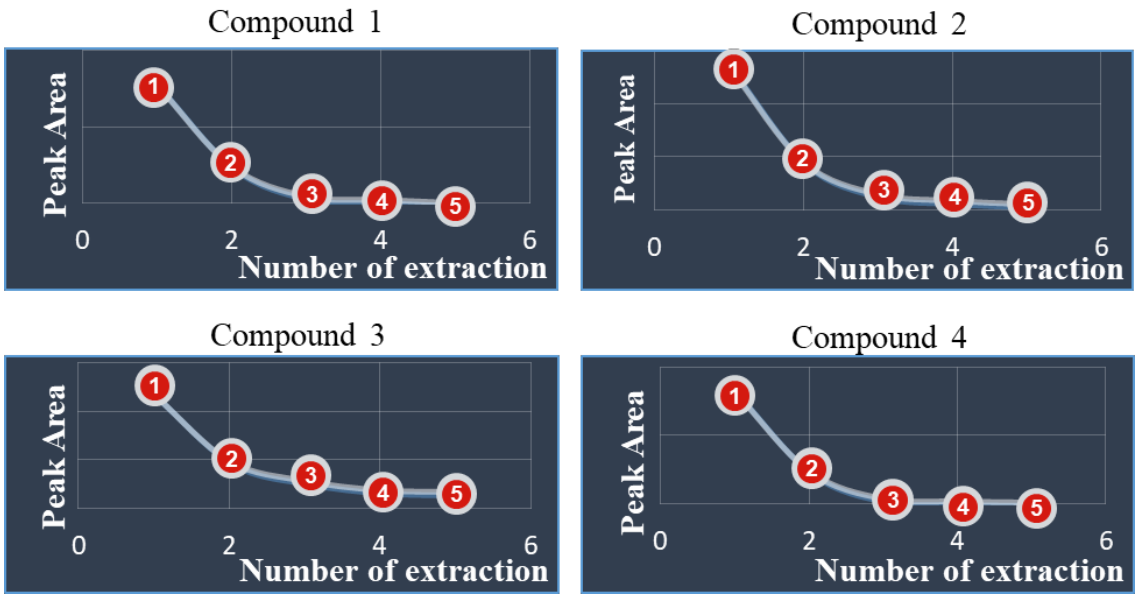


Figure 33: Optimization of the extraction conditions - Number of extractions necessary to minimize the content of four representative analytes in the pellet.

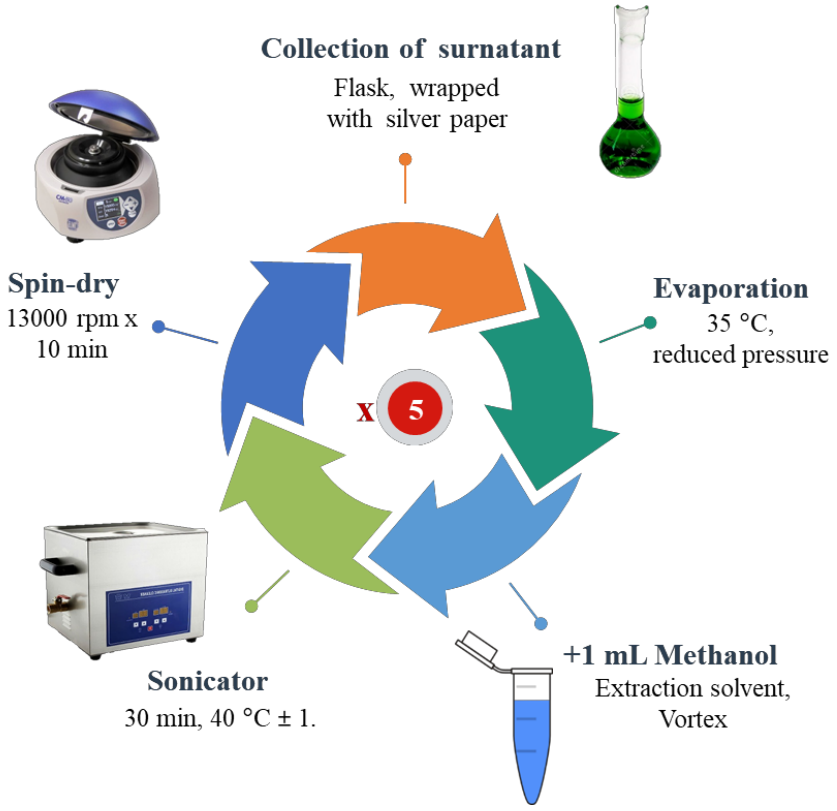


Figure 34: Scheme of the optimized extraction method of phenolic compounds from caigua leaves.

4.7 Optimization of RP-HPLC analysis using a *Quality-by-Design* approach

The optimization of the chromatographic method for the separation of the extracted phenolic compounds was carried out using a "*Quality-by-Design*" approach, using a commercially available software, named DryLab® 4. This software allowed the investigation of the influence of the main experimental parameters influencing the RP-HPLC separation of phenolic compounds extracted from the caigua leaves and fruits. A mixture of standard phenolic compounds, used as model substances, was used to carry out a limited number of experiments, requested by the software to build up a so called "*Design Space*", describing the influence of column temperature, duration of the elution gradient, and composition of the mobile phase on the resolution of the considered analytes.

The chromatographic separations were carried out by eluting the mixture of selected standards by two linear gradients of increasing concentration of the organic solvent in the hydro-organic mobile phase, with the column maintained at two different values of temperature, and by setting the operative conditions requested by the software DryLab® 4. The effect of each of the investigated parameters was evaluated by executing the chromatographic separations at two different times of the elution gradient, that is the time requested to change linearly the composition of the mobile phase from its initial composition A1 + B1 to the final one A2 + B2. Where "A" indicates the aqueous component of the mobile phase and "B" indicates the organic solvent or a mixture of organic solvents. In all experiments and for both gradients, the initial and final concentrations of the two components of the mobile phase were the same, whereas the duration of the second gradient was three times longer of the first one (see **Figure 35**).

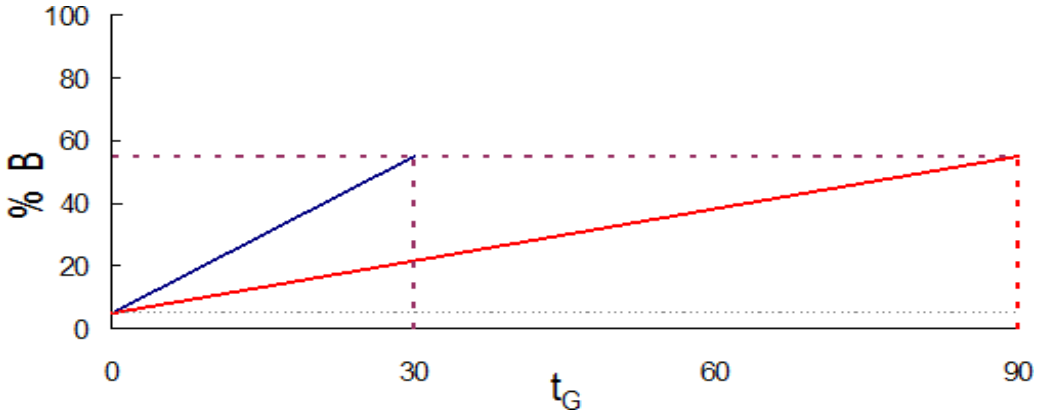


Figure 35: Linear gradients employed for the generation of the in-put data for the optimization of the RP-HPLC method by DryLab® 4. The graph expresses the variation in the percentage of solvent B (acenotrile,% in A + B) in function of the gradient time (t_G, minutes). Both shorter gradient (blu line) and longer one (red line) were performed starting from 5% to 55% of eluent B (acetonitrile). The latter one last 3 times more of the first one.

The results obtained from the elaboration of the experimental data by the simulation software DryLab® 4, allowed the optimization of the experimental conditions requested to obtain the complete separation of the considered analytes, necessary for their subsequent identification and quantification in leaves and fruits of caigua. As it is displayed in **Figure 36**, the main phenolic compounds extracted from the leaves of caigua were completely resolved eluting the samples by a multi-segment linear elution gradient, which was run according to the following program: time 0, % B 15; time 30 min, % B 45; time 32 min, % B 95; time 37 min, %B 15; time 50 min, % B 15.

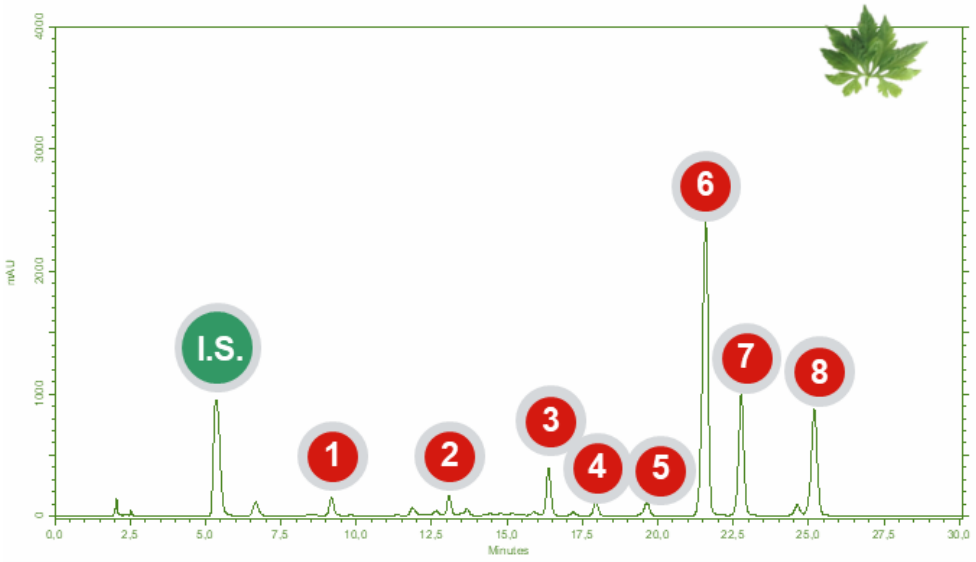


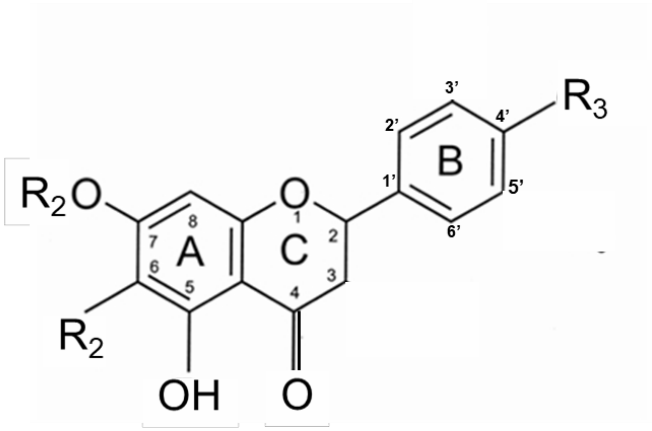
Figure 36. Optimized separation of phenolic compounds extracted from caigua leaves.

Details about RP-HPLC method validation are discussed in **Appendix II**.

4.8 Identification of flavonoids by HPLC with DAD and ESI-MS detection

The identification of the phenolic compounds extracted from *C. pedata* leaves samples was conducted by analysing, for each analyte separated by HPLC, MS spectra collected by the ESI-MS detector and the UV-Vis absorption spectra collected by the spectrophotometric detector, and by comparing the retention time relative to each peak with that of standard compounds (if available). The mass spectrum analysis, produced by ESI-MS was carried out both in positive and negative scan mode, and consisted in the recognition of pseudomolecular ions and characteristic fragmentation ions, which were compared with those of the pure compound (if available). Alternatively, the comparison was performed with ESI-MS spectra reported in the literature for glycosylated flavonoids extracted from fruits and leaves of *C. pedata*.

The RP-HPLC method described above allowed to identify in the leaves of caigua 8 glycosylated flavonoids, seven of which are already reported in scientific literature and one of them never identified before (compound identified with the number 2). These compounds differ according to the composition or position of the glycidic component and / or about the position of the acyl substituent, which is constituted by malonate (when present), while for all glycosylated flavonoids the aglycone is constituted by apigenin (3,7,4' tri-hydroxyphenone) or chrysin (5-7-dihydroxyphenone) (**Figure 37**). The **Table 8** reports the summary of the data obtained by HPLC-MS analysis. Mass spectra obtained in positive ionization mode are reported in **Figure 38**.



N°	Flavonoids	R1	R2	R3
1	Apigenin 6-*C*-glucoside (isovitexin)	H	Glu	OH
2	Apigenin 7-*O*-glucoside (apigetrin, cosmosiin)	Glu	H	OH
3	Apigenin 6-*C*-fucopyranoside	H	Fuc	OH
4	Apigenin 4-malonil-6-*C*-fucopyranoside	H	Fuc-Ma	OH
5	Apigenin 3-malonil-6-*C*-fucopyranoside	H	Fuc-Ma	OH
6	Chrysin 6-*C*-fucopyranoside	H	Fuc	H
7	Chrysin 4-malonil-6-*C*-fucopyranoside	H	Fuc-Ma	H
8	Chrysin 3-malonil-6-*C*-fucopyranoside	H	Fuc-Ma	H

Figure 37: Chemical structure of flavonoid glucosides identified in leaves of C.pedata. (Glu= glucose, Fuc= fucose, Ma= malonil).
The identified compounds differ for the composition or the position of the glucidic component and/or for the position of the acyl substituent, represented by malonate (if present), while for all the glycosylated flavonoids the aglicone is constitued by apigenin (3,7,4'-tri-hydroxiflavone) or by chrysin (5-7-di-hydroxyflavone).

Table 8: Mass spectra obtained by means HPLC-MS method. The separation-identification was performed by HPLC coupled to spectrophotometric detector with photodiode series (DAD) or mass spectrometer (MS) with ion source for electro-evaporation (ESI). A narrow-bore inversion phase column was used with a multi-step gradient elution.

N°	Flavonoids	T_R average ± DS (min)	UV λ max (nm)	$[M-H]^-$ (relative intensity)	$[M+H]^+$ (relative intensity)	Fragmentation $[M+H]^+$ (relative intensity)
1	Apigenin 6-*C*-glucoside (isovitexin)	9,173 ± 0,007	213 336 269	431 (100%)	433 (99,35%)	[M+H-120] + 313 (100%); [M+H-2H_2O] + 397 (31,47%); [M+H-H_2O] + 415 (42,29%); [M+Na] + 455 (28,49%).
2	Apigenin 7-*O*-glucoside	13,086 ± 0,014	214 270 336 333	431 (100%)	433 (100%)	[M+H-H_2O] + 415 (71,30%); [M+H-2H_2O] + 397(30,38%).
3	Apigenin 6-*C*-fucopyranoside	16,390 ± 0,016	213 336 270	415 (100%)	417 (100%)	[M+H-104] + 313 (85,53%); [M+H-44-2H_2O] + 337(19,36%); [M+H-2H_2O] + 381(49,77%); [M+H-H_2O] + 399 (71,49%); [M+H+Na] + 439 (36,34%).
4	Apigenin 4-malonile-6-*C*-fucopyranoside	17,976 ± 0,018	213 335 270	501 (16,32%)	503 (100%)	[M+H-2H_2O-104] + 363(4,97%); [M+H-H_2O-104] + 381(30,43%); [M+H-H_2O] + 485 (12,32 %).
5	Apigenin 3-malonile-6-*C*-fucopyranoside	19,661 ± 0,025	213 336 269	501 (5,81%)	503 (100%)	[M+H-2H_2O-104] + 363(19,51%); [M+H-H_2O] + 485 (14,96%).
6	Chrysin 6-*C*-fucopyranoside	21,589 ± 0,013	269 213 248 315	399 (100%)	401 (100%)	[M+H-104] + 297 (65,46%); [M+H-44-2H_2O] + 321 (38,51%); [M+H-2H_2O] + 365 (61,36%); [M+H-H_2O] + 383 (70,94%); [M+Na] + 423 (32,05%).
7	Chrysin 4-malonil-6-*C*-fucopyranoside	22,799 ± 0,020	266 217 213 248 314	485 (3,80%)	487 (92,77%)	[M+H-44-2H_2O] +321(6,08%); [M+H-2H_2O-104] +347(28,80%); [M+H-H_2O-104] + 365 (100%); [M+H-104] + 383(5,98%); [M+H-H_2O] + 469 (51,44%).
8	Chrysin 3-malonil-6-*C*-fucopyranoside	25,169 ± 0,033	266 269 218 213 211	485 (2,20%)	487 (100%)	[M+H-2H_2O-44] + 321 (6,81%); [M+H-H_2O] + 347 (20,50%); [M+H-H_2O-104] +365 (14,90%); [M+H-H_2O] + 383 (5,40%); [M+H-H_2O] + 469 (20,24%).

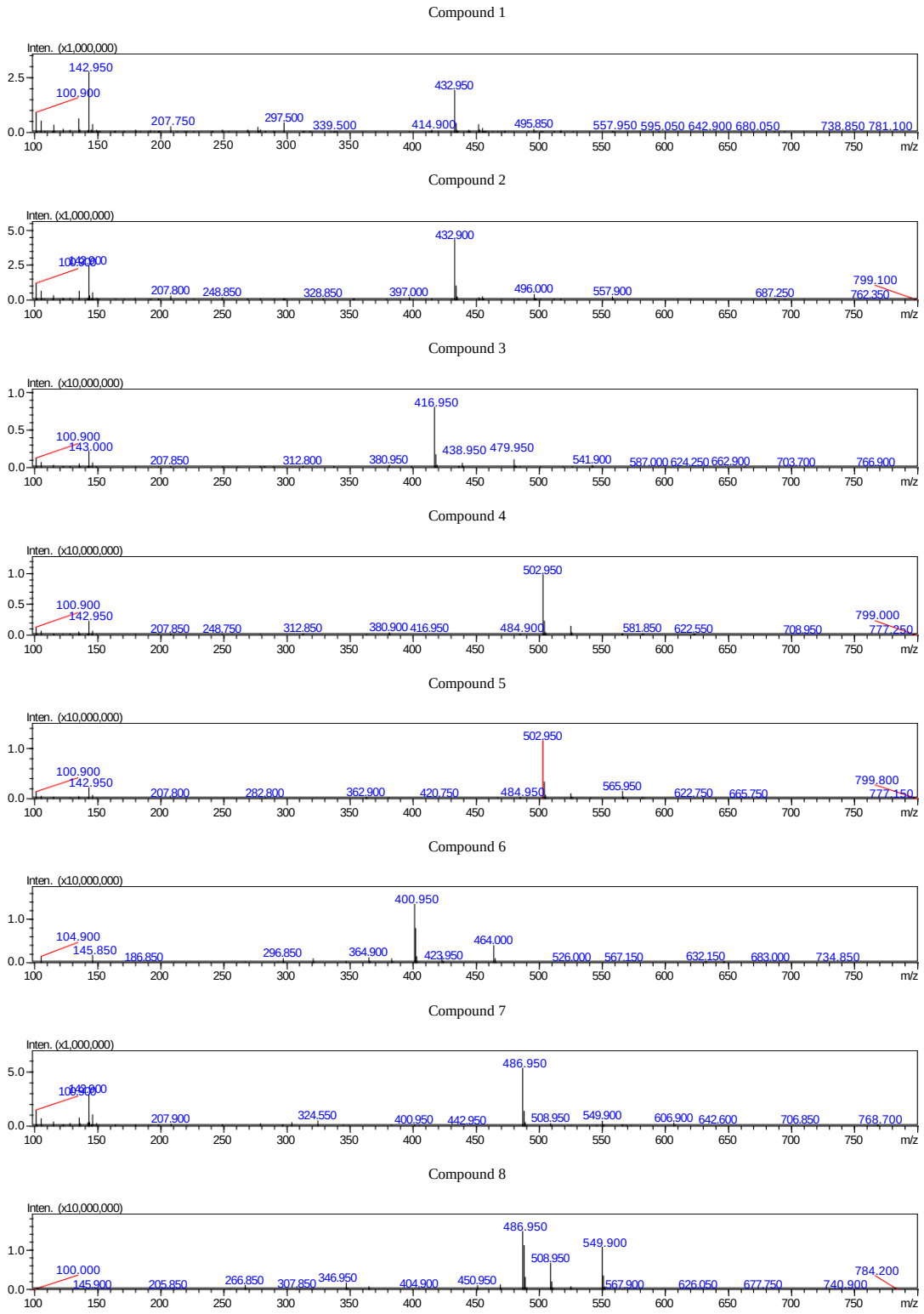


Figure 38: MS detection in positive ionization mode of eight compounds identified in caigua leaves extracts.

4.9 *In vitro* Model of Digestion - Effect of enzymatic hydrolysis on phenolics

Before continuing with further analysis, it was decided to first ensure that flavonoids identified in the plant matrix being studied survive to the digestion process and are absorbed by the intestine, thus the phenolics extracted are bioavailable. The term bioavailability refers to the degree and rate at which a substance (such as a drug or a biomolecule) is absorbed into a living system and is made available at the site of physiological activity.

To test the flavonoids bioavailability, a digestive system was simulated according to Sayer *et al*. [99]. For that purpose, only one extract of the caigua leaves was used, since leaves of caigua contains more phenolics in an higher amount compared to fruits. The chosen extract was subjected to the action of the enzymes that attend in the human gastro-intestinal tract. After the three incubations with salivary alpha-amylase (1st incubation - mouth), pepsin (2nd incubation - stomach), pancreatin and bile acids (3rd incubation - intestines), the digested extract was spin-dried and the supernatant was collected. Following the precipitation of the protein fraction by adding particles of ammonium sulphate, the chromatographic separation was performed. The performed *in vitro* digestion and the chromatogram obtained after the digestive process are represented in the figure below (**Figure 39**).

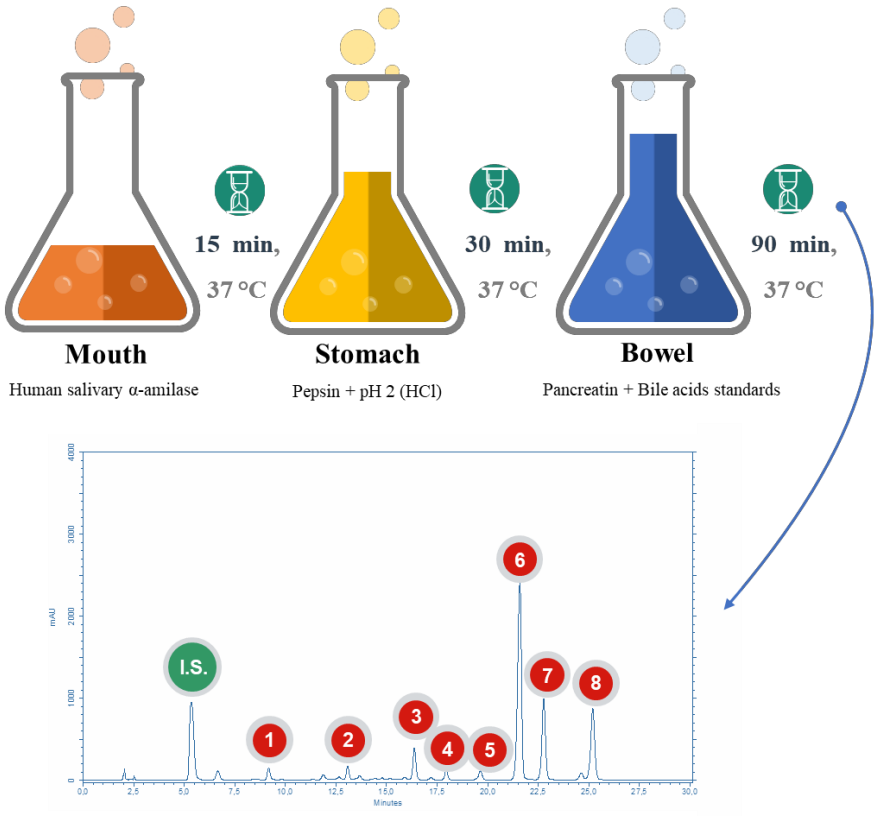


Figure 39: Fingerprint of leaves of caigua after digestive process. Main phenolics identified before *in vitro* digestion can be also identified after digestion.

As represented, the chromatographic profile remains unchanged. This suggests that digestion process does not influence the qualitative composition of the phenolic fraction. This result would seem negative for the bioavailability of flavonoids present in *C. pedata* (glycosylates), since *in vitro* studies show greater absorption of aglycone (hydrophobic) forms. Moreover, because passive diffusion is the main absorption mechanism through the lipid bilayer constituting the intestinal cell membrane, glycosylated flavonoids are conventionally believed to be poorly absorbed unless a very efficient mechanism of active transport occurs (unlikely). However, surprisingly, *in vivo* studies would disown this conventional belief, rather greater bioavailability of flavonoids in the glycosylated form or, however, as a result of their increased water solubility, is reported. The possible explanation for this latter assertion was provided by Gonzales G.B. [100] less than a year ago (2016). According to this theory, glycosylated flavonoids (and other soluble compounds) would be more bioavailable, because they can penetrate through the mucus present within the gastrointestinal tract (hydrophilic), unlike the agonists (hydrophobic). Once the mucus layer and thus the intestinal epithelium is reached, the beta-glucosidases present at the level of the bristles would free the aglycone which for its nature, can therefore spread passively through the cells.

4.10 Qualitative and quantitative comparison among caigua leaves, fruits and supplement fingerprints

Fewer flavonoids have also been identified in fruits and nutritional supplements (**Figure 40**). In the case of the fruit and the commercial food supplement, the identification was carried out by comparing the mass spectra and UV spectra of the analytes separated by RP-HPLC with those previously obtained from the extract obtained from the caigua leaves. Leaves of caigua are qualitatively (**Figure 40**) and quantitatively (**Table 9**) richer in phenolics compounds in comparison to caigua fruits and supplement. These data are in agreement with HPTLC-MS data showed and discussed before.

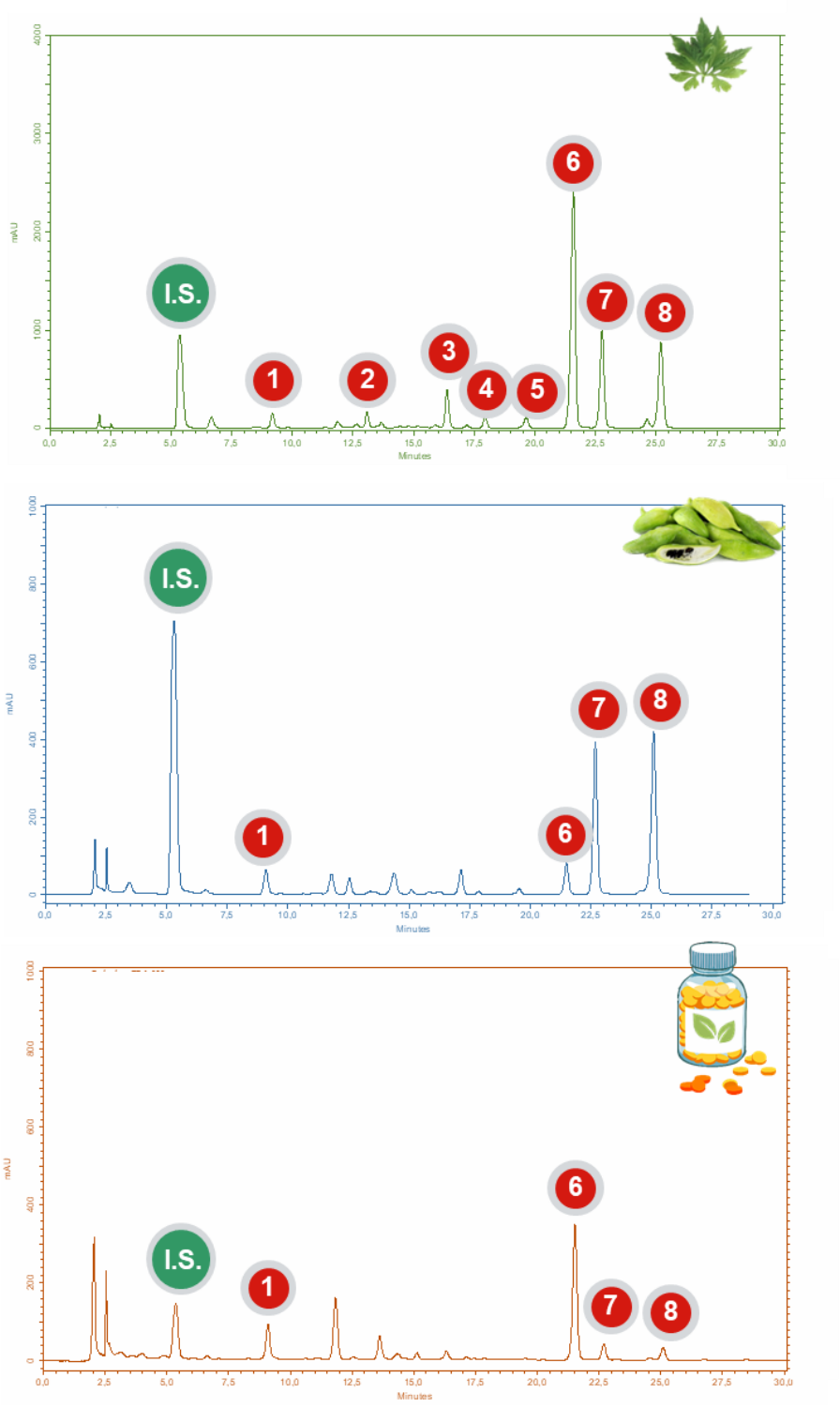


Figure 40: Comparison between leaves, fruits and supplement chromatographic separations. It should be taken into account that the supplement is diluted three times compared to leaves and fruits extracts analysed, as it is deducible by internal standard (I.S.) peak height.

Table 9: Quantitative evaluation of caigua leaves, fruits and supplement.

		Flavonoids	Leaves	Fruits	Food supplement
			mg/g*	mg/g*	mg/g*
Compounds	1	Apigenin-6-*C*-glucoside (isovitexin)	2.51	1.02	1.12
	2	Apigenin-7-*O*-glucoside	2.49	-	-
	3	Apigenin-6-*C*-fucopyranoside	4.93	-	-
	4	Apigenin-4-malonil-6-*C*-fucopyranoside	2.29	-	-
	5	Apigenin-4-malonil-6-*C*-fucopyranoside	2.35	-	-
	6	Chrysin-6-*C*-fucopyranoside	25.87	1.97	4.08
	7	Chrysin-4-malonil-6-*C*-fucopyranoside	13.36	4.23	0.66
	8	Chrysin-3-malonil-6-*C*-fucopyranoside	10.85	4.95	0.59
	Tot.		64.65	12.17	6.45

*dried weight

From a practical point of view, data reported in **Table 9** correspond to around 0,7183 mg/g wet weight for caigua leaves and to around 0,1352 mg/g wet weight for caigua fruits. Considering that one leaf of *C. pedata* weights around 2 g and that a medium fruit weights aroung 40 g, we can assume that each leaf contains around 1,4366 mg of phenolic compounds, while one fruit around 5,408 mg. Because two capsules / day of caigua supplement (around 12,90 mg of phenolics) are thought to explicate the beneficial effects in humans, this means that ten leaves (a salad bowl) or two small fruits can be considered enough to have the same positive properties on human health.

4.11 Factors influencing the biosynthesis of phenolics in *C. pedata*

It is known that management system, environmental and pedo-climatic conditions, genetic origin are expected to influence the production of plant specialized metabolites. Hence, our research has been extended to investigate the occurrence and abundance of the phenolic compounds extracted from leaves and fruits of *C. pedata* of different origin grown in Italy in the years 2014 and 2016, and in *C. pedata* of different origin grown in the same year (2016) in two different geographical areas (Italy and Slovenia). The investigation has been carried out performing metabolomic analysis by the approach of metabolite fingerprinting, profiling and targeted quantification, using RP-HPLC with DAD and ESI-MS detection.

The metabolic fingerprinting approach has been carried out to compare metabolic fingerprints, without the need to carry out compound identification and quantification and has been used for sample screening. The metabolite profiling approach has been

applied for the identification and quantification of the metabolites that has been identified by ESI-MS, whereas the metabolite target analysis has been employed to identify and quantitate a limited number of compounds.

4.11.1 Influence of year of cultivation, vegetative growing phase, and growing region on occurrence of phenolics in the leaves of caigua

The RP-HPLC fingerprints of the phenolics extracted from the leaves of the plant cultivated in Rome in 2016 and collected in June and in September are displayed in **Figures 40, b** and **c**, respectively. The phenolics extracted from the leaves collected in September 2016 and eluting the HPLC column with retention time 12.531 (peak 2), 17.984 (peak 4), 19.835 (peak 5), 22.795 (peak 7), and 25.132 min (peak 8) are missing in the fingerprint of the phenolics extracted from the leaves collected in June 2016 (**Figure 41, b**). It appears that these significant differences between the two fingerprints are related to the different vegetative growth phase of the plants.

This hypothesis can be corroborated by the observation that good similarity is observed between the fingerprints of phenolics extracted from leaves of plant grown in Italy (**Figure 41, c**) and in Slovenia (**Figure 41, d**), extracted at analogous vegetative growing phase in September and October 2016, respectively.

Moreover, better similarity is also observed between fingerprints of phenolics extracted from leaves of plants grown in Rome in different years but collected at analogous vegetative growing phase, in September 2016 (**Figure 41, c**) and October 2014 (**Figure 41, a**). Indeed, all the main peaks displayed in the chromatogram 'c' also occur in the chromatogram 'a', with the only exception of the minor peaks with retention time 7.275, 9.696, 12.557 and 17.121 min. that have been detected in the sample extracted from the leaves collected in September 2016 (chrom. 'c') and are missing in the sample extracted from the leaves collected in October 2014 (chrom. 'a').

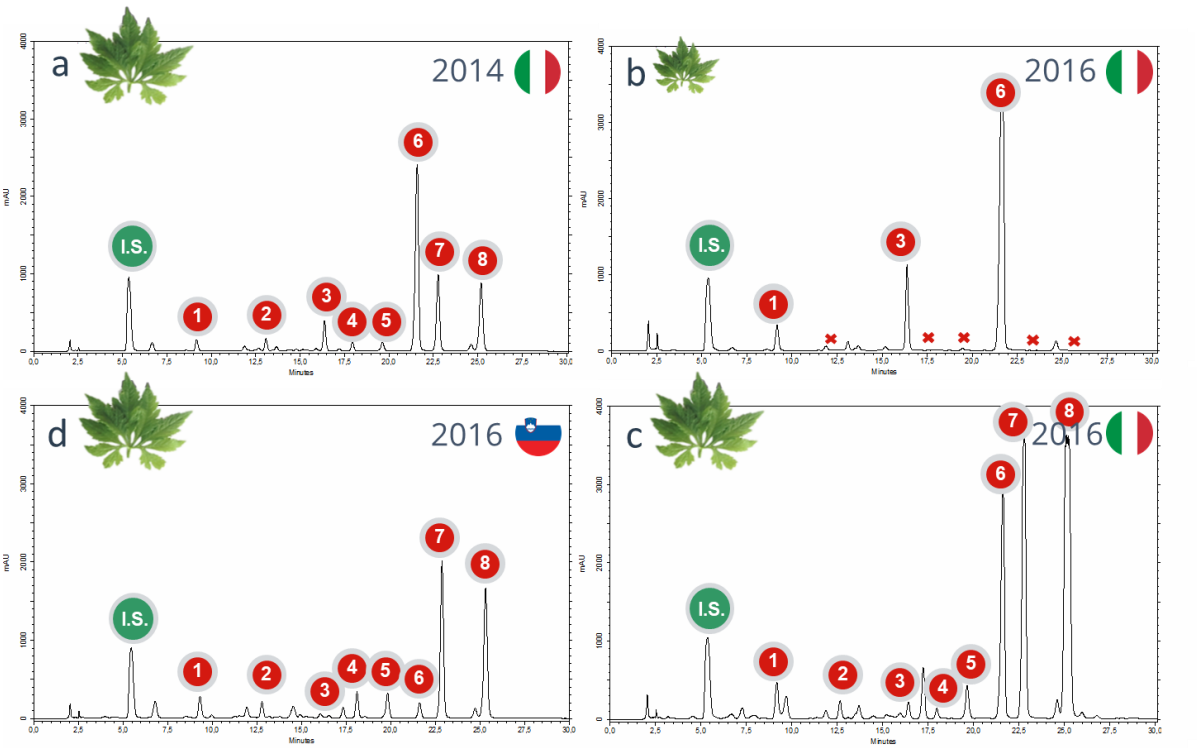


Figure 41: Influence of year of cultivation, vegetative growing stage and growing region on occurrence of phenolics in leaves of caigua.
(a) Leaves of caigua cultivated in Rome (Italy) and collected in 2014; (b) Leaves were sampled on day 60 after sowing, all sampled leaves being the unripe state; (c) Leaves of caigua cultivated in Rome (Italy) and collected in 2016; (d) Leaves were cultivated in North-east of Slovenia (Maribor) and collected in 2016; (a, c) Both set of leaves were collected from plants grown up in Rome (Italy) when the complete maturation was reached, but the collection occurred in different years; (a, c, d) Leaves were collected on day 150 after sowing, all sampled leaves being the mature state; (b, c) Both set of leaves were collected from the same plant cultivated in Rome, but at different vegetative stage of the plant.
I.S. = Internal Standard, 4-para-idroxybenzoic acid.

From the fingerprinting analysis it can be inferred that the vegetative growing phase appear to have a greater influence on the synthesis of phenolics in the leaves of caigua than the year of cultivation and the growing region.
Differences related to pedo-climatic conditions can be due to thermal stress. As discussed in introduction chapter (paragraph 1.2.2), the optimal growth temperatures for *C. pedata* are comprised between 12 and 18 Celsius degrees. These values strongly differ from common temperatures found in Rome (Italy), rather than in Slovenia, but also from temperatures recorded in 2016 rather than 2014 (**Table 10**). 2016 has been considered by NASA (National Aeronautics and Space Administration) and NOAA (National Oceanic and Atmospheric Administration) the hottest year ever recorded before. Thermal stress, id est the exposure to temperatures higher (as in our cases) or lower than optimal for growth, is referred to induce the production of phenolic compounds, such as flavonoids and phenylpropanoids [101, 102]. It is possible that

temperature and humidity values recorded in Rome during 2016 induce an higher bio-accumulation of positive molecules respect to conditions recorded in Maribor (Slovenia) in the same year and in Rome in 2014. Other small differences could also be related to other environmental conditions such as soil characteristics, of which we do not have detailed informations.

Table 10: Values of temperatures and humidity recorded in Rome during the years 2014 and 2016 and in Maribor on 2016 (www.meteo.it). The months taken into account are related to the caigua growing period.

2014 Rome	average T (°C)	T min (°C)	T max (°C)	Humidity (%)
April	16,4	9,7	20,6	69,6
May	19,5	13,7	23,2	61,6
June	24,2	17,9	28	61,3
July	24,8	19,3	28,3	66,7
August	26	19,1	29,9	62,9
September	23	16,4	27,4	68,5
AVERAGE	**22,32**	**16,02**	**26,23**	**65,10**

2016 Rome	average T (°C)	T min (°C)	T max (°C)	Humidity (%)
April	18,4	10,7	22,1	57,7
May	20,5	14,5	23,5	58,1
June	25,1	18,6	28,3	55,1
July	29,2	21,5	32,8	44,8
August	28,3	20,3	31,8	46,1
September	24	16,2	28	59,4
AVERAGE	**24,25**	**16,97**	**27,75**	**53,53**

2016 Maribor	average T (°C)	T min (°C)	T max (°C)	Humidity (%)
April	15	9	19,3	63,9
May	18,1	11,5	22,4	63,1
June	22,4	16,7	26,2	67,7
July	26,1	18,3	30,6	58,8
August	24,8	16,5	29,6	56,5
September	22,1	15,2	27,5	60,2
AVERAGE	**21,42**	**14,53**	**25,93**	**61,70**

4.11.2 Influence of year of cultivation, ripeness, and growing region on occurrence of phenolics in the fruits of caigua

Similar evaluation has been performed for the RP-HPLC fingerprints of the phenolics extracted from fruits harvested from caigua's plants grown in Italy and in Slovenia. The Italian samples were extracted from fruits, harvested in August and September 2016, respectively, from caigua plants grown in Italy. Due to different sun exposition, the ripe fruits were those harvested in August, whereas unripe fruits were those harvested in September. Other Italian samples were extracted from fruits harvested in September 2014 from caigua plants grown in Italy. Slovenian samples were extracted from a mix of unripe and ripe fruits harvested in October 2016 from caigua's plants grown in Slovenia.

The RP-HPLC fingerprint of the phenolics extracted from fruits harvested in October from plants grown in Slovenia is displayed in chromatogram 'e' (**Figure 42, e**), whereas the fingerprints of the phenolics extracted from fruits harvested from plant cultivated in Rome in August and September 2016 are displayed in chromatograms 'f' and 'g', respectively (**Figure 42, f** and **g**). The two fingerprints related to the samples harvested in 2016, either in Italy or in Slovenia, appear to be qualitatively very similar (**Figure 42**). The same can be said for fingerprints obtained for ripe and unripe fruits harvested in Italy in 2016 (**Figure 42, g** and **e**). On the other hand, three minor dissimilarities were observed between the fingerprints produced by the samples extracted from the fruits harvested in 2014 and 2016 from plants grown in Italy (**Figure 42, h** and **g**). The three peaks detected at 8.427, at 13.707 and at 24.897 min in the samples extracted from the fruits harvested in August 2016 were not detected in the samples extracted from fruits harvested in 2014.

Differences related to the year of harvesting, at parity of soil characteristics, can be related to thermal stress, according to what explained in the previous paragraph also for leaves.

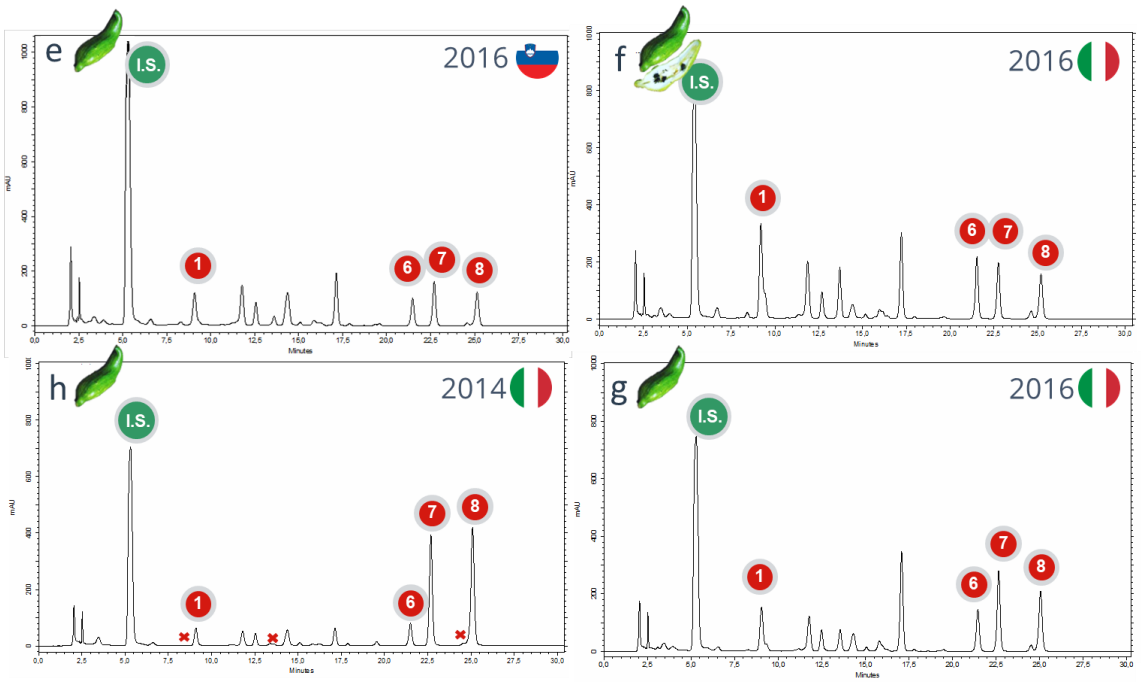


Figure 42: Influence of the pedo-climatic conditions, ripeness degree and year of cultivation on occurrence of phenolics in the fruits of caigua.
(e) Fruits were collected in 2016, in Slovenia, ripe and unripe; (f) Fruits were collected in 2016, in Italy, ripe; (g) Fruits were collected in 2016, in Italy, unripe; (h) Fruits were collected in 2014, in Italy, unripe; (g, f) Both set of fruits were collected from the same plant cultivated in Rome (Italy).
I.S. = Internal Standard, 4-para-idroxybenzoic acid.

4.12 Determination of Vitamin C as Ascorbic acid in leaves and fruits of caigua

Vitamin C is one of the most abundant water-soluble metabolites found in plant. It is important especially for its antioxidant properties. The oxided / reduced ascorbic acid ratio offers a fast picture of the plant redox state and thus of its health conditions. In order to investigate the influence of year of cultivation, ripeness degree and pedo-climatic conditions on the levels of vitamin C, an HPLC method for the quantification of the levels of vitamin C in leaves and fruits of caigua was developed and performed. The quantification was performed according to the calibration curve built by using ascorbic acid by Sigma Aldrich as standard (**Figure 43**).

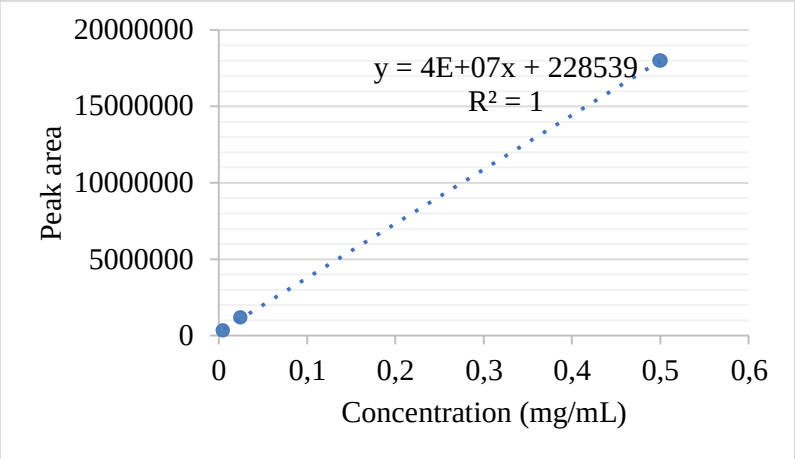


Figure 43: Calibration curve of ascorbic acid.

Vitamin C data obtained are summarized in **Figure 44**. According to phenolics results, ascorbic acids levels increase during maturation in leaves. Leaves collected in Slovenia show the higher amount of vitamin C with the best redox state (red./ox. asc. ratio almost 1), probably due to more suitable climatic conditions for *C. pedata* there. The same trend of leaves is found in fruits according to ripening stage, while no significant differences are found in fruits according to different growing region.

It is important to note that the levels of vitamin C in caigua plant can reach 1,267 mg/g, which is an higher value in comparison to kiwi (0,85 mg/g) or oranges (0,5 mg/g), two of the richest fruits of vitamin C in our diet [103].

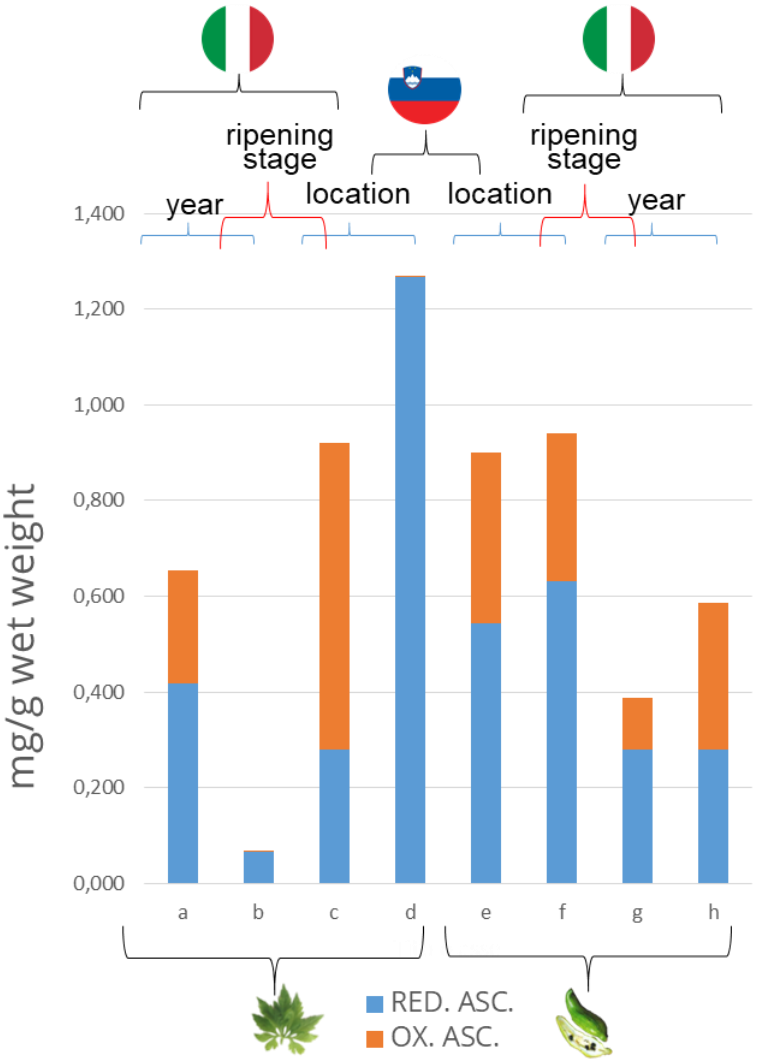


Figure 44: Levels of dehydroascorbic (DHA) and L-ascorbic acids (L-AA) in leaves and fruits of caigua according to ripening stage and year and place of cultivation.
For simplicity, samples which differ for just one characteristic were coupled with braces and labelled with the writing 'year', 'ripening stage' or 'location' in order to emphasize the main characteristic for which each pair of compared samples differs from the other. For example, leaves identified with letter 'a' differ from leaves named 'b' for the year of cultivation, rspectively 2014 and 2016. The same applies for samples of fruits labelled with 'g' and 'h', while samples 'b' and 'c' of leaves differ for the ripening stage, as well as samples 'f' and 'g' of fruits. Samples 'c' and 'e' vary for the location of cultivation in comparison to samples 'd' and 'f' respectively. Differences according to the place of cultivation are also emphasized by rounded italian and slovenian flags (on the top).
DHA is the oxided form of vitamin C (ox. asc.), while L-AA is the reduced one (red. asc).
*wet weight

Chapter 5:

Conclusions and further prospective

The thesis reports the results of a study performed during the three years of doctorate, which was carried out to investigate in deep occurrence and abbundancy of phenolic compounds in leaves and fruits of caigua and in a commercial food supplement produced from this plant. Fruits and leaves of caigua were collected from plants grown in two different geographical areas (Maribor in Slovenia and Rome in Italy) and in different crop years. As part of the investigation, three methods were developed: two complementary chromatographic methods coupled to mass spectrometry, for the characterization of the phenolic compounds extracted from plants, and one *in situ* hydrolisis method performed directly on HPTLC silica gel plates for the innovative simultaneous hydrolysis of multiple samples. The comparative analysis of the chromatographic fingerprints, obtained by both HPLC and HPTLC, are in agreement with each other, pointing out the occurrence of a minor number of beneficial metabolites in caigua fruits and supplements in comparison to those extracted from the leaves of caigua. Moreover, according to HPLC, the leaves of caigua rather than fruits and supplement, contain also apigenin 7-*O*-glucoside, a flavonoid never identified in *Cyclanthera pedata* in previous scientific works.

The thesis also reports the results of a study performed to evaluate the dependence of the synthesis of the considered phenolic compounds on agronomic factors, such as pedo-climatic conditions and plant vegetative state. According to the results of this study, the harvest time is the determinant factor on the variation of the qualitative composition in phenolics of caigua leaves. Interestingly, no important differences were found in caigua fruits fingerprints according to ripeness degree.

On the other hand, the pedo-climatic conditions appear to exert a minor influence on the phenolics profile of both leaves and fruits of caigua. Pedo-climate mainly concerns with soil (*pedo*) and climate characteristics, the latter of which are especially connected with temperature and humidity. We were able to collect average values of humidity and temperatures, which occurred during the plant growing months in Rome, both in 2014 and in 2016, and for the latter year, also in Slovenia. The comparison of these data with those related to the determination of phenolic compounds in the leaves and fruits of *C. pedata* grown in the corresponding years appears to indicate a possible dependence of the bio-accumulation of the considered specialized metabolites on climate. However, data related to more than two different years would be necessary for an accurate evaluation of the correlation between climate and occurrence of phenolic compounds in fruits and leaves of caigua.

On the contrary, we do not have informations on the soil characteristics, neither in Rome nor in Maribor growing areas. Therefore, further investigations are sorely needed to make final conclusions. For example, it would be really interesting to study the qualitative and quantitative patterns of phenolic compounds extracted from fruits and leaves of caigua collected from plants grown in the native territory, such as Peruvian Andes.

Appendix I:
Setup of in situ-hydrolysis method on HPTLC silica gel plates

I.1 Preliminary tests

A method largely employed for the identification and quantification of the individual flavonoid glycosides extracted from plant materials, is based on the acid hydrolysis of the extracted samples before HPLC or HPTLC analysis. Hydrolysis to produce aglycones reduces the number of compounds to be analyzed and made the chromatographic separation easier to achieve. In addition, it facilitates the quantification of flavonoids because standards for a large number of the glycosylated flavonoids are not available. Our study has investigated an innovative approach based on the simultaneous hydrolysis of multiple samples performed directly on the silica plates that are employed for HPTLC analysis. The method is based on the exposure to HCl vapors of the samples applied as a band on the HPTLC plate.

Kaempferol and kaempferol 3-*O*-glucoside standards were selected for the development of the hydrolysis method. kaempferol and kaempferol 3-*O*-glucoside standard methanol solutions (0,1 mg/mL) were applied on the plates as 8 mm bands 15 mm from the side edges and 10 mm from the bottom by Automatic TLC Sampler 4 (Camag, Muttenz, Switzerland) equipped with a 25-µL microsyringe. The plates with standards were placed into a twin-trough chamber (Camag) at different timetables: the first was placed 24 hours before the hydrolysis was supposed to be stopped, the second 8 hours before and so on until the last plate, which was placed into the chamber to stay just one hour in contact with HCl vapours. In this way, all the plates exposed for different periods of time to HCl vapours were taken off the chamber at the same moment in order to develop them all together. For each plate placed into the "HCl chamber", another plate with the same standards was placed into an empty chamber, in order to have a "control plate" halting on silica gel plate for the same period of time.

The exposition to HCl vapours was interrupted 24 h after the first two plates were placed into the respective chambers. All plates were taken off the chambers and a hair-drier was used to remove any HCl vapors residue. Therefore, each plate was developed ascendently at room temperature to a distance of 9 cm in twin-trough chamber (Camag) lined with filter paper and saturated for 15 minutes with 10 mL of developing solvent ethyl acetate: water: formic acid (ratio 17: 3: 2 v/v) in each trough. After development, all plates were dried under a stream of cool air using a hair dryer.

The visual presentation of the chromatogram on each plate was obtained by Camag DigiStore 2 documentation system (Camag) at 366 nm wavelength, before and after derivatization with NST (2-aminoethyldiphenylborinate). Results are showed in **Figure 45** below.

Hydrolysis started immediately after 1 hour of HCl vapours exposure, as it can be noted by comparing the plate exposed 1 hour to HCl vapours with the respective "control"

plate. In fact, with the used method, the glycosilated molecule stays almost at the start position, while the respective aglycone counterpart migrates upper during the development. Thus, the success of the hydrolysis process is deducible from the appearance of an upper band also in the second track, corresponding to kaempferol 3-*O*-glucoside, which, in case of unsuccessful hydrolysis, should stay at the bottom position (see **Figure 45**, second line). The successful result of the method was proved by means of MS analysis.

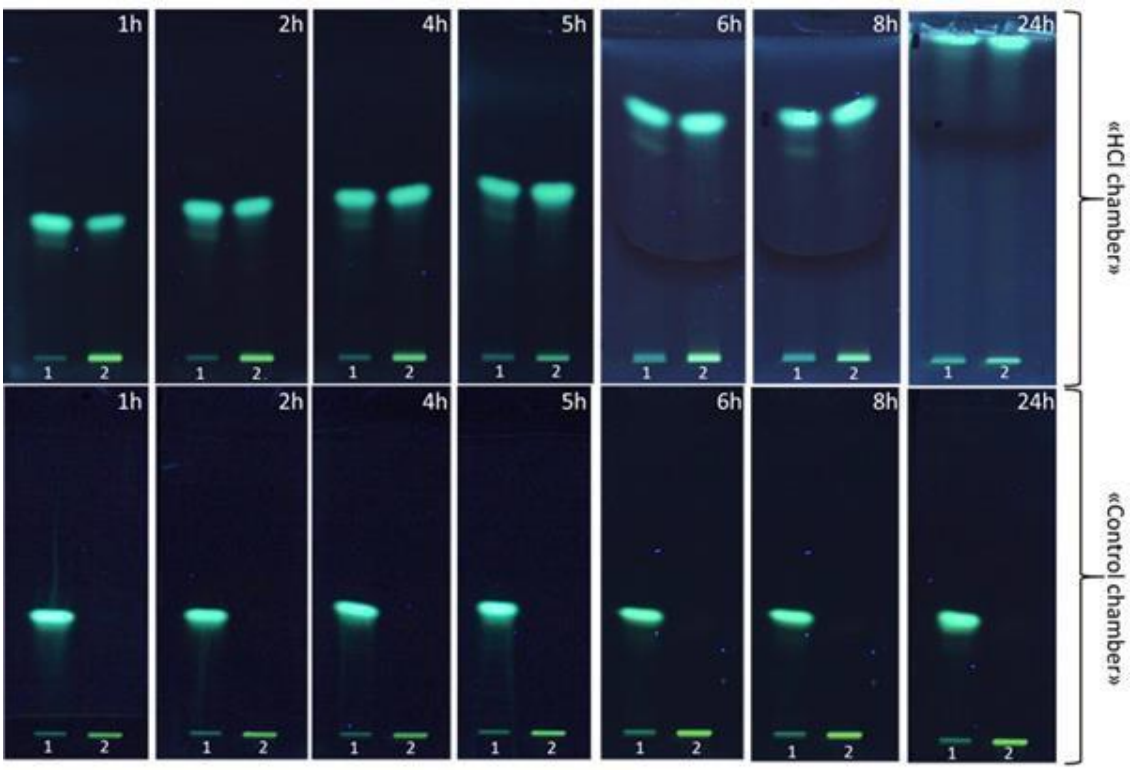


Figure 45: Preliminary results about different span of *in situ* hydrolysis on standards: kaempferol (track 1) and kaempferol 3-*O*-glucoside (track 2). First line. During development, glicosilated kaempferol stay at the bottom of the plate while the aglicone form migrates over it, thus two standards are well recognizable between each others (see down plates). All the "control" plates are similar, that means standards are stable on the plate. Second line. Hydrolysis already starts after 1 h of HCl vapours exposure, as it can be noticed by the appearance of another band in the 2nd position at the same R_F value of the aglicon counterpart. Anyway, the hydrolysis is not complete for expositions shorter than 24 h, as it can be evinced by the colours of the two bands at the start position (they become almost of the same colour only after 24 h). The HCl vapours have an influence on the characteristics of the sorbent and change the standard migration in a way that is directly proportional to the exposure time. The standards exposed at HCl vapours for 24 h migrate with the front.

Even if HCl vapours succeed in their "hydrolytic task", it was needed longer time to complete the process than usual hydrolysis methods. In fact, before than 24 hours the hydrolysis is incomplete, as deducible from the colour of the second track at the bottom

position. Moreover, MS analysis revealed no signal corresponding to kaempferol 3-*O*-glucoside in the plate subjected to 24 hours exposure to HCl vapours. For this reasons, the span of 24 hours was selected for further analysis.

As it is possible to see from the above figure, HCl vapours modify the retentive characteristics of the stationary phase, thus changing the migration of the standards over the plate, and so their R_F values. The modification is directly proportional to the exposure time.

This problem has been solved by using two different plates: one for the hydrolysis and another for the development. This implies additional methodological phases to extract the test solutions from the "hydrolysis plate" and re-apply them on a new plate. Each step of the method and its validation are explained more in detail in the following paragraphs.

I.2 Definitive procedure

Each sample of which hydrolysis is desired was applied on the plate as a long band. The plate with the sample has been exposed to the HCl vapours in a Twin-through chamber for 24 h. This was possible by pouring 5 or 10 mL of pure HCl in one of the two containers of the twin-trough chamber using a glass funnel, while the other through of the chamber was left empty for the plate (see **Figure 46**). After 24 h-incubation with HCl vapours, the plate with the sample was taken out of the chamber and a hair-drier was used to accelerate the HCl evaporation. After that, the sample was scratched from the plate by using a small spatula. During this step, the silica gel particles were inevitably scratched as well, thus the sample had to be extracted again from the plate sorbent. The extraction was performed once with an opportune volume of pure methanol, necessary for the complete solubilization of the samples test solutions. To help the extraction procedure, the solutions were repeatedly vortexed and put in a Sonicator for 15 minutes. Finally, the test solutions were spin-dried, in order to separate the pellet, containing the sorbent, from the supernatant, containing the hydrolysed compounds. The latter was collected in a flask. A filtration with 0,45 µm filters was carried out to ensure the complete elimination of contaminants and putative sorbent residues. Subsequently, the extraction solvent was evaporated by means a Rotary Evaporator at 35 °C and at reduced pressure (236 bar). Once dried, the sample was dissolved in methanol to get the concentration of 50 mg/mL. All these steps are summarized in the **Figure 46** below.

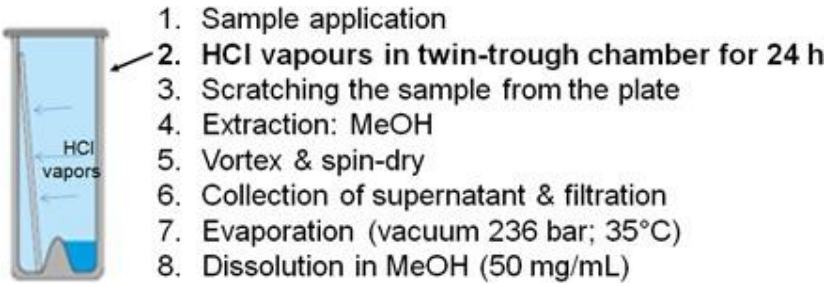


Figure 46: Image of the *in situ* hydrolysis directly on HPTLC silica gel plate and summary of all the steps of the hydrolysis procedure.

I.2.1 Method validation

As long as we know, acid hydrolysis of flavonoid glycosides has never been performed on silica gel before, so some methodological aspects had to be deeper investigated in order to test the robustness of the procedure:

- hydrolysis effectiveness on compounds of interest;
- stability of compounds on HPTLC silica gel for 24 h;
- efficiency of compounds extraction from HPTLC silica gel.

I.2.1.1 *In situ* hydrolysis method of glycosylated flavonoids and sugars standards

The effectiveness of the hydrolytic method has firstly been tested on standards. For sugars, the method has been tested on sucrose standard. Sucrose is a disaccharide consisting of glucose and fructose. Sucrose was subjected to the hydrolysis procedure explained above.

The outcome of the procedure has been tested by applying on a new plate both standards (sucrose and its components fructose and glucose) and the sucrose previously subjected to 24 h-hydrolysis process. The plate has been developed up to 8 cm in a pre-saturated Twin-Through chamber (15 min) by using NST solution: H_2O: MeOH in the following ratio 17: 3: 0.25 as developing solvent. The plate has been detected under white light transmission mode after plate deactivation with DAP (Diphenylamine-Aniline-Phosphoric acid reagent) detection reagent and an heating step at 150 °C for 4 minutes. The densitometric evaluation has been performed as well by scanning the final plate in absorption/reflectance mode at the wavelength of 638 nm. Both chromatographic and densitometric results are displayed in **Figure 47**.

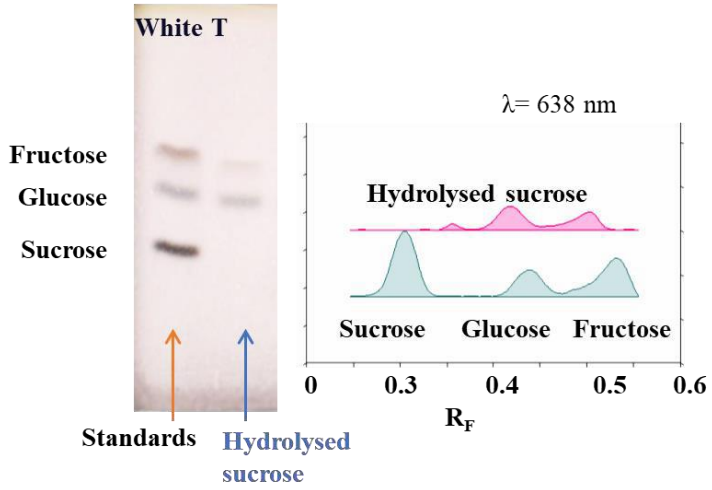


Figure 47: Chromatogram and densitogram of sucrose standard before and after hydrolysis

As shown in the above **Figure 47**, the 24 h-*in situ*-hydrolysis is highly efficient on the tested sugar. In fact, after hydrolysis, the band of sucrose is not visible anymore, while glucose and fructose appear as distinct bands, thus suggesting the success of the procedure. The same result can be evidenced also by HPTLC densitometric analysis.

The same procedure has been performed on glycosides flavonoid standard, in particular on Apigenin-7-*O*-glucoside (or apigetrin or cosmosiin). Apigenin 6-*C*-glucoside (or isovitexin) was also used to test the effect of hydrolysis on *C*-glycosilated flavonoids. The standards has been applied separately on silica gel plates and subjected to the same procedure explained before.

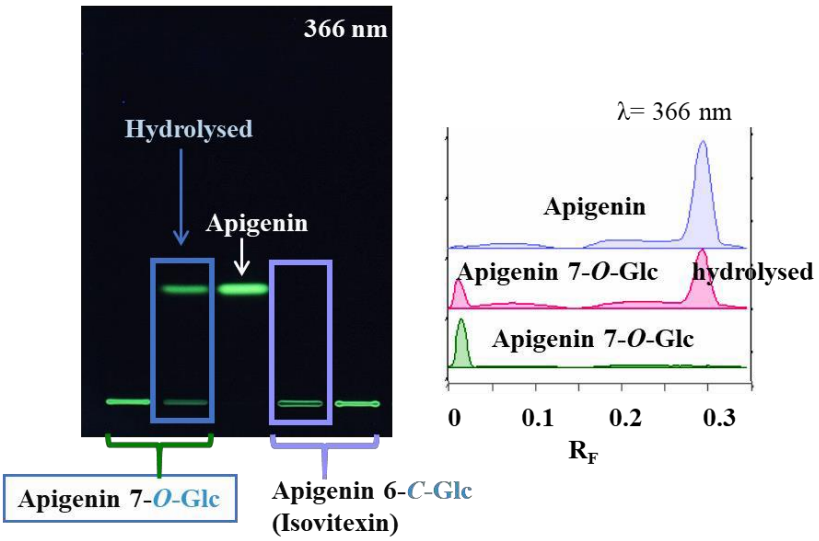


Figure 48: Chromatogram and densitogram of apigenin and glucosides apigenins standards before and after hydrolysis.

HPTLC-image analyses and HPTLC-densitometry (**Figure 48**) show the efficiency of the method on sugars, but partial efficiency on flavonoid *O*-glucosides. As expected, *C*-glucosides (e.g. isovitexin) could not be hydrolysed.

I.2.1.2 Compounds stability and their extraction efficiency from the HPTLC silica gel plate

Standards were applied at time 0 on silica gel plates, taking care of leaving enough space for other two bands. The standards applied at time 0 represent the "control" to test the stability of the compounds on the plate. The same standard was applied on the same plate 24 hours later together with the standard applied on one plate and extracted from it as previously explained. The latter band represents the "control" to test the extraction efficiency from the silica gel plate.

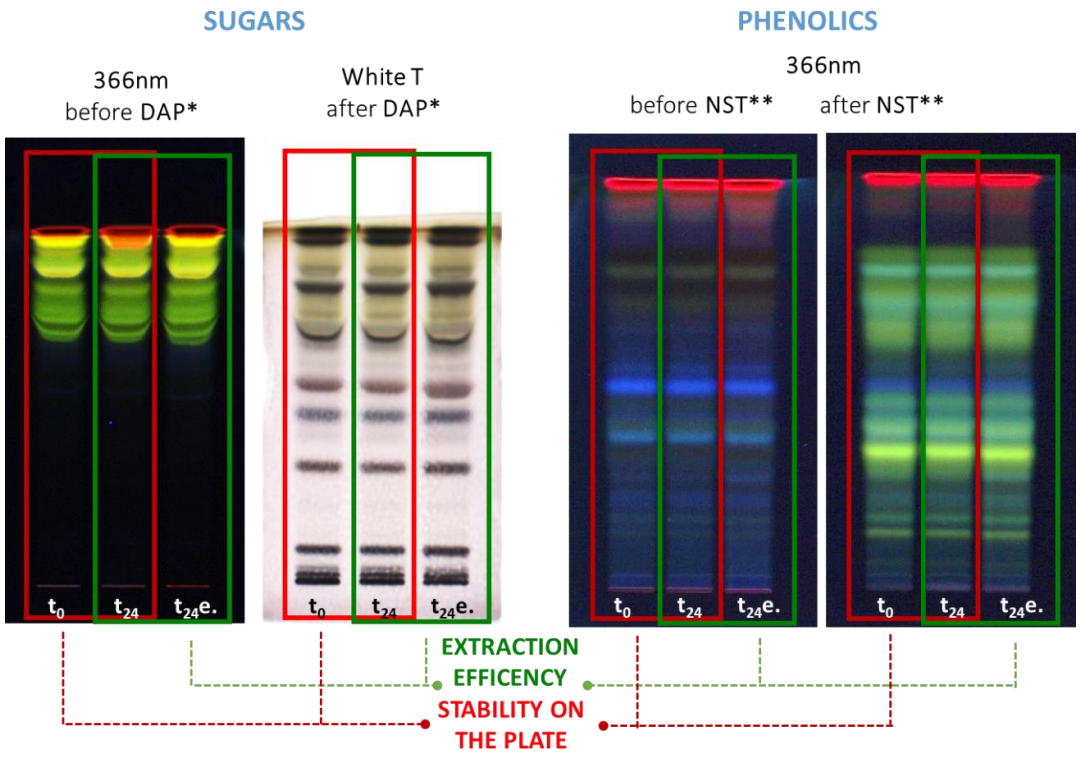


Figure 49: Chromatograms of representative standards to test their extraction efficiency and their stability on the plate.
*DAP = Diphenylamine-aniline-phosphoric acid reagent; **NST = 2-aminoethyldiphenylborinate.

As shown in **Figure 49** above, no difference is detected between the standards and the respective "control" bands, that means both stability of compounds and their extraction from the silica gel is proved. In other words, sugars and flavonoids are stable 24 hours on the silica gel sorbent. The extraction of compounds procedure from silica gel sorbent is efficient.

Appendix II:
RP-HPLC analysis - Quantitative determination and Method Validation

II.1 Calibration curve for quantification of phenolic compounds

To quantitatively determine the phenolic compounds occurring in our studied samples, it was necessary to build up a representative calibration curve by using solutions at different of a standard subjected to the optimized method. Isovitexin was used as the external standard for this aim, since this compound is representative of all studied samples. The calibration curve is the graphic built up by interpolation of the obtained statistical data from generic analysis in which the response of the instrument (signal) is evaluated according to the concentration of the analyte of interest. In our case, the revealed signal is the peak area as a function of the analyte concentration, revealed as chromatographic peak. The linear regression of the data related to the areas of the peaks as a function of the concentration of the added standard, performed by means of Excel, produced the equation of the calibration graph, with its correlation coefficient $\mathbf{R^2}$ (see **Figure 50**).

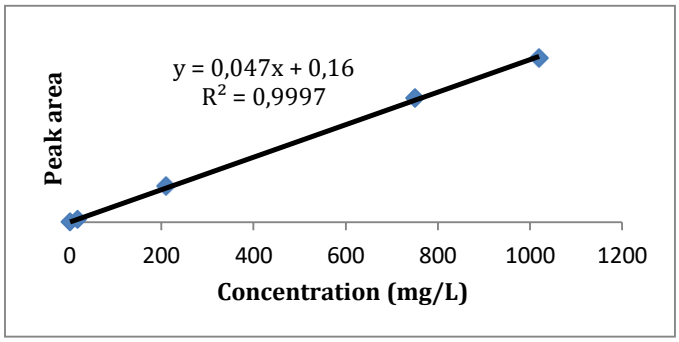


Figure 50: Calibration curve of Isovitexin - Method of Internal Standard. In the table the average (three analysis) of the peak areas for each concentration, with which the calibration curve has been built up, are reported.

Since in the sample an internal standard has been added, the calibration curve was actually built up by using the ratio between the peak area of the analyte taken into account (isovitexin) and the peak area of the internal standard (4-paraidroxybenzoic acid). With this type of curve it is possible to minimize the instrumental intereferences and also the interferences due to the sample manipulations.

II.2 Method Validation

The optimized HPLC method has been validated, according to standards ISO/IEC 17025:2000 describing the general requirements for the competence of testing and calibration laboratories. These standards define a serie of conditions which the method should satisfy to be considered valid in each scientific laboratory. The results of the tested parameters are reported below.

II.2.1 Precision and Robustness

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
- Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.
- Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

The first parameter evaluated was the method **repeatability intra-day** (in the same day) and **inter-day** (during three consecutive days). In order to evaluate the method repeatability, the variation of the retention times (R_T) in terms of standard deviation and relative standard deviation was calculated (see **Table 11** below).

Table 11: Intra-day and Inter-day Repeatability Retention Time.

		Repeatability retention time (min)					
		Intraday (n = 5)			Interday (n = 15 over 3 days)		
Peak nr.	Analyte	Average	SD	RSD (%)	Average	SD	RSD (%)
1	Apigenin 6-*C*-glucoside (isovitexin)	9,173	0,007	0,081	9,241	0,192	2,082
2	Apigenin 7-*O*-glucoside (cosmosiin)	13,086	0,014	0,107	13,105	0,035	0,265
3	Apigenin 6-*C*-fucopyranoside	16,390	0,016	0,099	16,395	0,055	0,337
4	Apigenin 4-malonil-6-*C*-fucopyranoside	17,976	0,018	0,098	17,735	0,419	2,365
5	Apigenin 3-malonil-6-*C*-fucopyranoside	19,661	0,025	0,129	19,672	0,152	0,771
6	Chrysin 6-*C*-fucopyranoside	21,589	0,013	0,061	21,589	0,089	0,412
7	Chrysin 4-malonil-6-*C*-fucopyranoside	22,799	0,020	0,086	22,789	0,116	0,508
8	Chrysin 3-malonil-6-*C*-fucopyranoside	25,169	0,033	0,130	25,242	0,128	0,507

From the reported data it is possible to note that for all the examined compounds both intra-day and inter-day repeatability was better than 0,13 and 2,36%, respectively. The relative standard deviation (RSD) or variation coefficient is an index of the measure precision and thus allow the evaluation of the values dispersion around an average. In

our case, the RSD % never exceed 0,13 % for the intra-day repeatability and 2,36 % for the inter-day repeatability. This means the optimized method is repeatable both for chromatographic analysis performed in the same day and for analysis performed in different days.

The same method has been tested at the National Institute of Chemistry of Ljubljana, Slovenia by using a different instrumentation: UHPLC-DAD-MS system (Accela 1250 coupled to an LTQ Velos MS, Thermo Fisher Scientific). The data collected in Rome, in addition with the data collected in Slovenia, has given the possibility to calculate also the reproducibility of the method (see **Table 12**).

Table 12: Intra-day and Inter-day Reproducibility Retention Time.

		Reproducibility retention time (min)					
		Intraday (n = 5)			Interday(n = 15 over 3 days)		
Peak nr.	Analyte	Average	SD	RSD (%)	Average	SD	RSD (%)
1	Apigenin 6-C-glucoside (isovitexin)	9,096	0,131	1,437	9,152	0,192	2,097
2	Apigenin 7-O-glucoside (cosmosiin)	13,028	0,122	0,939	13,037	0,145	1,109
3	Apigenin 6-C-fucopyranoside	16,375	0,122	0,742	16,396	0,084	0,513
4	Apigenin 4-malonil-6-C-fucopyranoside	18,002	0,087	0,483	17,990	0,108	0,600
5	Apigenin 3-malonil-6-C-fucopyranoside	20,185	0,150	0,744	20,064	0,315	1,572
6	Chrysin 6-C-fucopyranoside	22,282	0,251	1,124	22,171	0,350	1,577
7	Chrysin 4-malonil-6-C-fucopyranoside	23,460	0,313	1,336	23,321	0,368	1,577
8	Chrysin 3-malonil-6-C-fucopyranoside	25,548	0,145	0,568	25,489	0,204	0,800

The RSD % never exceed 1,44 % for the intra-day reproducibility and 2,10 % for the inter-day reproducibility. The inter-laboratory analysis are reproducible, that means the method is robust.

II.2.2 Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several methods exist to calculate the LOD; we chose the method which use the calibration curve according to the following formula:

$$LOD = \frac{3 \times \sigma}{m}$$

where σ is the standard deviation of the analyte concentration and m is the angular coefficient of the calibration curve.

II.2.3 Limit of quantification (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. The LOQ has been calculated as follow:

$$LOQ = 3 \times LOD$$

II.2.4 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The range of linearity has been calculated within the standard concentration used to build up the calibration curve ranging between 1,9 and 1020 mg/L.

The data about LOD, LOQ and linear range are summarized in the **Table 13**.

Table 13: Limit of detection (LOD), Limit of Quantification (LOQ) and Results of Linear Regression Analysis of Calibration Graphs Based on Absorbance at the Reported Wavelength (λ).

Peak nr.	Analyte	λ (nm)	LOD (mg/L)	LOQ (mg/L)	Linear range (mg/L)	Equation*	Correlation coefficient
1	Apigenin 6-*C*-glucoside (isovitexin)	280	0,15	0,9	1,92-1020	y = 0,047x + 0,16	0,9997

*y expresses the detection response (peak area in arbitrary units) and x the concentration for phenolic compounds in mg/L.

II.2.5 Recovery and Accuracy

Recovery is expressed as the amount/weight of the compound of interest analyzed as a percentage to the theoretical amount present in the medium. Thus, recovery allow the determination of putative losses of analyte during the analytic procedure. Recovery represents also a parameter by means express the accuracy. Recovery of isovitexin was determined by adding known amounts of this compound to one sample of caigua and applying the following equation:

$$\text{Recovery } (\%) = \frac{C_2 - C_1}{C_0} \text{ x } 100$$

Where C_2 is the analyte concentration determined in the sample after the addition of the standard at known concentration; C_1 is the analyte concentration determined in the sample before of the addition; and C_0 in the known concentration of the added analyte.

In **Table 14** are reported recovery data for isovitexin, with relative standard deviations. Recovery was determined by adding 80, 100 and 120 % of the analyte to the sample.

Table 14: Recovery calculations for Isovitexin.

	Apigenin-6-*C*-glucoside (isovitexin)		
	Recovery (80 %)	**Recovery (100 %)**	**Recovery (120 %)**
	93,91	105,67	99,65
	97,73	99,76	97,11
	104,79	102,23	100,98
Average	98,81	102,55	99,25
S.D.	5,520	2,968	1,966
RSD %	5,586	2,894	1,981

Recovery data allow to state that the proposed method is sufficiently accurate. Consequently, the method is suitable to be applied for determining the phenolic compounds in *Cyclanthera pedata* extracts.

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Acknowledgements

I would like to thank Prof. Laura De Gara for her courteous readiness for dialogue and listening. She has always been ready to encourage me. To me she represents one of the most discreet and professional people I have met to date and I admire her way of doing and her ability to handle critical situations. Thanks to her I was able to grow humanly as well as professionally during my academic career.
I would also like to thank Dr. Danilo Corradini who has scientifically supported all of my PhD research work and Dr. Isabella Nicoletti who advised and guided me during the daily routine in the laboratory at the Institute of Chemical Methodologies (National Council of Research, Rome). Both of them represented parental figures, ready to support me at every step of my doctoral path. Thanks to their patience and stillness I managed to overcome moments of discouragement and difficulty with my head held high.
A special thanks goes to Dr. Irena Vovk, who welcomed me to her Department of Food Chemistry (National Institute of Chemistry – Ljubljana, Slovenia) in an excellent manner and hosted me for nine months. She supported my research project and helped me to find congenial solutions to the drawbacks encountered during the laboratory practice. She is a smart, strong, courteous and friendly person. I really admire her determination and fortitude in dealing with any inconvenience and her ability to keep her team together. At this point, I take this opportunity to thank all her laboratory team, in particular:

- Dr. Vesna Glavnik, who followed me in the first person with all her sweetness and sensibility;
- Urška Jug, with whom I had the pleasure of sharing the office during my stay abroad and creating a relationship of complicity both inside and outside the workplace;
- Eva Kranjc, who taught me to face everyday problems with the same determination with which she climbs walls in sporting life;
- Andreja Starc, who represents the best example of humility and modesty, always available to help me and others whenever needed;
- Mateja Puklavec, who, with her smiling eyes and her hugs, has softened even the hardest days;
- Dr. Alen Albreht, who, with his technical explanations, has shown me that even the most difficult concepts can become simpler;
- Dr. Mitja Križman, who always understood me… even when I talked just Italian!

All of them have incorporated me into the group making me feel an integral part of the team and never making me feel alone. I am really grateful to them for having made my period abroad more enjoyable and carefree.

I would also like to thank all the people who work at the cooperative Agricoltura Nuova in Rome, in particular Davide Pastorelli, who helped me to take care of the plants needed to carry out my research project.

Finally, I gratefully acknowledge the financial support from the Campus Bio-Medico University of Rome, in particular for the opportunity to obtain the "Erasmus Plus" grant for a training period of nine months at the National Institute of Chemistry (NIC - Ljubljana, Slovenia), and the financial support from the Slovenian Research Agency (research core funding No. P1-0005) that made possible my Ph.D. work in the Department of Food Chemistry in Ljubljana.