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Study of the bioactive compounds variation during yerba mate (*Ilex paraguariensis*) processing

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ABSTRACT

Ilex paraguariensis St. Hil. (Aquifoliaceae) (yerba mate) is one of the most used plant species in South America due to its nutritional and medicinal properties. The industrial processing involves different stages (green leaves, zapecado, drying, forced or natural aging) which can modify the qualitative and quantitative composition and the pharmacological activities. In this work, the main compounds, caffeoyl derivatives (caffeic acid, mono- and dicaffeoylquinic acids), methylxanthines (caffeine and theobromine) and flavonoids (rutin, quercetin and kaempferol) were studied by HPLC in extracts obtained by decoction of samples of a large amount of *I. paraguariensis* during its industrial process stages. The comparative quantitative analysis of all the samples indicated that those obtained after the zapecado, drying and aging stages possess higher content of biologically active principles when compared with green leaves. No differences were found between the natural and forced aging processes. This is the first complete report on the quantitative variation of the bioactive compounds of *I. paraguariensis* during each step of the industrial processing. The results obtained in this work provide a guideline for the obtention of extracts enriched in biological principles for the pharmaceutical, food and cosmeceutical industry.

1. Introduction

Ilex paraguariensis St. Hil. (Aquifoliaceae) (yerba mate) is one of the species in South America most employed as a decoction or infusion due to its nutritional and medicinal properties (tonic, choleretic, diuretic, antirheumatic, etc.) (Blumenthal, Goldberg, & Brinckman, 2000, Heck & de Mejía, 2007). The main countries which produce yerba mate are: Argentina (northeast of Corrientes and Misiones provinces), Brazil (states of Paraná and Santa Catarina) and Paraguay. It is also consumed in Uruguay (Filip & Ferraro, 2003). This species is currently being exported to Europe, US, Syria and Japan, where it is commercialised as a vegetal drug and extracts to be used in phytotherapeutical products for the treatment of overweight and obesity as well as in dietary supplements due to its vitamins and minerals content (Carducci, Dabas, & Muse, 2000; Ramallo, Smorcewski, Valdez, Paredes, & Schmalko, 1998) and due to its energising proterties given by methylxanthines, caffeine and theobromine (Filip, López, Coussio, & Ferraro, 1998). Nowadays, yerba mate is also considered functional food (Zuin, Montero, Bauer, & Popp, 2005).

Phytochemicals, especially the phenolic compounds found in fruits and vegetables, have been regarded as the major bioactive compounds which provide the health benefits associated with plant-foods based diets. Polyphenols are reducing agents that protect body tissues against oxidative stress (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). Oxidative damage has been implicated in aging processes and in the pathogenesis of several degenerative diseases such as cancer, cardiovascular diseases, inflammation and others (Ames, Shigenaga, & Hagen, 1993). Epidemiological studies have shown that plant-foods based diets significantly reduce the incidence and mortality rates associated with degenerative diseases caused by oxidative stress (Tibble, 1998). On the other hand, the prevention of chronic diseases is a subject of great interest for scientists, consumers and the food industry (Liu, 2003). Although data on polyphenol bioavailability and its metabolic fate are scarce and controversial (Karakaya, 2004), many studies involving humans have provided evidence that these compounds undergo absorption and urinary excretion after the intake of phenolic-rich foods (Pedersen et al., 2000; Rechner, Spencer, Kuhnle, Hahn, & Rice-Evans, 2001). Beverages made from yerba mate are also recognised as a source of antioxidant substances, such as phenolic acids (Filip, Lotito, Ferraro, & Fraga, 2000), which are readily absorbed by the body (Bravo, 1998; Olthof, Hollman, Buijsman, van Amelsvoort, & Katan, 2003).

Flavonoids and hydroxylated derivatives of the cinnamic acid have antioxidant activities, a mechanism by which they exert their hepatoprotective (Thabrew, Huges, & McFarlane, 1998) and probably their antiinflammatory and antispasmodic effects. Previous works have suggested the possible application of chlorogenic, caffeic and ferulic acids for cancer chemoprophylaxis in tongue and other tissues such as skin, lung, liver and esophagus (Tanaka

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et al., 1993). Dicaffeoylquinic acids (DCA), 3,5 DCQ and 4,5 DCQ have displayed antiinflammatory activity *in vitro* and a hepatoprotective effect in rats (Basnet, Matsushige, Hase, Kadota, & Namba, 1996). Flavonoids have been proved to have antitumoral activity related to the inhibition of the enzymatic pathways involved in cell proliferation and differentiation and the generation of free radicals. The latter reactive species would be involved in inflammatory diseases and cancer (Halliwell, Gutterdge, & Cross, 1992).

Previous studies have demonstrated that *I. paraguariensis* has choleretic (Gorzalczany et al., 2001) and antioxidant activities (Anesini, Ferraro, & Filip, 2006; Filip et al., 2000) which could be related to the presence of caffeoyl derivatives and flavonoids (Filip, López, Giberti, Coussio, & Ferraro, 2001).

The production process of yerba mate involves the harvest of the older leaves of *I. paraguariensis* which are dried over fire, milled, stored and packed for their commercialisation. The industrialisation process may vary among industries although the procedure is basically the same and can be described as follows (Barchuk, 1998; Valduga, Finzer, & Mosele, 2003):

1. Harvesting (H): green leaves and small stems are cut manually or mechanically, put into 100 kg sacks and then carried to the processing plant.
2. Roasting or zapecado (Z): this process is also known as "predrying". The green yerba is exposed to direct fire at temperatures between 250 °C and 550 °C during 2–4 min. The inactivation of oxidising enzymes which occur in this step preserves the colour, flavour and aroma of the leaves. Approximately 25% of moisture is lost in this stage.
3. Drying (D): the leaves are exposed to a current of hot air until a 3% of moisture is reached. The drying process can be "barbacuá" style or continuous belt system. The "barbacuá" style system is constructed of wood or brick, either round or square, with a vault made of wooden slats or merely poles, upon which the yerba mate, which has undergone the zapecado, is piled. Hot air is piped in from outside. The process lasts from 12 to 18 h and the leaves must be overturned – also manually – every 3–4 h. The continuous belt system consists of a tunnel of 25–35 m long, 3–4 m wide and 7–10 m high through which the continuous belts run horizontally. The belts transport the leaves slowly through the tunnel, which is supplied with hot air from outside. This process lasts from 3 to 6 h.
4. Milling: the product is triturated to large particles to ease manipulation and transport.
5. Aging: the period of time necessary for the product to acquire the adequate flavour, aroma and colour. In the natural aging (NA) process, the product is stored under natural conditions of temperature and humidity for about 9–12 months. In the forced aging (FA) process, the product is stored under controlled temperature, humidity and air circulation conditions during 30–60 days in order to acquire the desired taste, aroma and colour.
6. Final preparation: yerba is finally ground to smaller particles and then sieved in order to eliminate the powder and the remaining stems – known as "palo". Finally the yerba is mixed, blended and packed.

During the industrial process stages, specially harvest, roasting, drying, and aging, some changes in the profile and concentration of bioactive compounds of *I. paraguariensis* may be produced which could modify its pharmacological activities (López, Isolabella, Anesini, Ferraro, & Filip, 2006).

Only a few recent studies have been carried out to assess the changes that occur in yerba mate during is processing. Over the last years, variations in the physicochemical parameters, caffeine content, chlorophylls and minerals have been investigated in the yerba mate subjected to different conditions during the industrial processing (Esmelindro, Toniazzo, Waczuk, Dariva, & de Oliveira, 2002; Giulian et al., 2009; Schmalko & Alzamora, 2001) and no investigations comparing the process of natural aging with the forced aging with regard to the chemical composition have been carried out. Bastos, Fornari, Queiroz, and Torres (2006) have studied the variation of caffeine, chlorogenic acid (5-caffeoylquinic acid) and caffeic acid in three stages of the industrial processing (green leaves, zapecado and drying), however, no updated and thorough studies have been performed on the qualitative and quantitative composition of yerba mate in all the stages of the industrial processing.

The use of plants and herbs as antioxidants in processed foods is acquiring of increasing importance in the food industry as an alternative to synthetic antioxidants (Proestos, Boziaris, Nychas, & Komaitis, 2006).

The study of the changes occurring during the industrialisation process of yerba mate would help improving the quality of the final product. On the other hand, this information could allow selecting plant material with the highest amount of active principles for the preparation of *Ilex* extracts to be used as antioxidants in the food, cosmeceutical and/or pharmaceutical industries.

Taking into account these considerations, the purpose of this research was to investigate the variations in the most relevant active principles (methylxanthines, caffeoyl derivatives and flavonoids) of one lot of *I. paraguariensis* during the different stages of the industrial processing.

2. Materials and methods

2.1. Chemicals and reagents

Standards of caffeic acid, chlorogenic acid, cynarin (1,3-dicaffeoylquinic acid) and rutin were purchased from Carl Roth Gmbh, Germany. Standards of caffeine and theobromine were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetic acid were purchased from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). HPLC grade water (18 mX) was prepared employing a Mill-X50 purification system (Millipore Corp., Beddford, MA, USA).

2.2. Plant material

Leaves of fresh and processed yerba mate (*I. paraguariensis* St. Hil.), obtained from a local factory in Gobernador Valentín Virasoro, Corrientes, Argentina, were used. The collection and the storage of the material were carried out under strict controlled conditions. The test material was mechanically harvested in March and belonged to the same lot during the following industrialisation steps: (1) green leaves: fresh leaves just harvested; this material was dried in our laboratory in a stove with forced air circulation at a temperature below 40 °C for 24 h (sample labelled "H"), (2) zapecado (Z): the green yerba was exposed to direct fire at temperatures between 250 °C and 300 °C during 2–4 min, (3) Drying (D): was performed employing a continuous belt system at 90–110 °C, (4) Forced aging (FA): the product was placed in a chamber and subjected to a temperature 60 ± 5 °C and 40 ± 10% humidity for 60 days and (5) natural aging (NA): the material was stored for 12 months in a warehouse where the average temperature varies during the year, reaching 80 °C on the top in summer and 60 °C in winter. Due to this temperature variation the internal air in the warehouse was subjected to a forced circulation.

All the samples were ground separately to fine powder with a cutter mill with 1 mm pore mesh.

2.3. Preparation of plant extracts

Decoctions extracts were prepared according to the Farmacopea Nacional Argentina (6th edition) (1978). Briefly, 10 g of each sample were boiled with 200 ml of water for 20 min and left to cool at room temperature to 40–45 °C. After filtration through filter paper (Whatman No. 1) the extracts were lyophilised using a flexi-dry FTS System. Then, 50 mg of the lyophilised extract were solubilised in 10 ml of water and filtered for HPLC analysis. Moisture content of plant material and lyophilised extracts were determined as mass loss at 105 °C up to constant weight (European Pharmacopoeia (3rd edition), 1997). The weight percentage of aqueous crude extracts obtained after lyophilisation was calculated.

2.4. High Performance Liquid Chromatography

A Varian™ series 9000 equipment with a Varian 9012 binary pump was used. Quantification of caffeoyl derivatives, flavonoids and methylxanthines was carried out by validated HPLC external standard methods (Filip et al., 1998, 2001). A reverse phase Luna IB-SIL RP 18 (5 μm, 250 × 4.6 mm I.D.) Phenomenex column and an elution gradient consisting of solvent A: water:acetic acid (98:2); solvent B: methanol:acetic acid (98:2) was used. For caffeoyl derivatives the elution gradient was: from 15% B to 40% B, 30 min.; 40% B to 75% B, 10 min.; 75% B to 85% B, 5 min. The flow rate was 1.2 ml/min. For methylxanthines the elution gradient was: from 17% B to 20% B, 10 min; 20% B (isocratic), 5 min; 20% B to 23% B, 10 min, 23% B to 100% B, 5 min with a flow rate of 1.0 ml/min. Identification and quantitation was carried out by simultaneous detection with a UV Varian 9050 UV detector and a Varian 9065 Photodiode-Array Detector at 325 nm for caffeoyl derivatives, 254 nm for flavonoids and 273 nm for methylxanthines. Samples were injected with a Rheodyne injector fitted with a 100 μl loop.

2.5. Statistical analysis

Data were expressed as means ± standard error of the mean of three independent experiments carried out by duplicate. A one-way ANOVA with the *a posteriori* Bonferroni's test was used to evaluate the significancy of results. A probability (P) value <0.05 was considered significant.

3. Results and discussion

3.1. Weight percentage of aqueous extracts

Aqueous extracts were obtained by decoction and subsequent lyophilisation. The weight percentage of aqueous crude extracts (g lyophilised material/100 g dry vegetal drug) obtained were: H: 32.17 ± 0.38, Z: 35.98 ± 0.43, D: 36.03 ± 0.33, FA: 34.87 ± 0.45, NA: 34.37 ± 0.29. The results showed that the solid content of the extract prepared with samples H was lower than that of the extract prepared with the other samples. This phenomenon has also been observed by other authors. For example, Bastos et al. (2006) reported that the soluble solid content of the extraction prepared with dried yerba mate leaves was approximately fourfold higher than that of the extract prepared with fresh leaves. According to the authors, this could be due to cell disruption and mechanical impact during the processing stages.

3.2. Caffeoyl derivatives

The following caffeoyl derivatives were identified and quantified: caffeic acid, chlorogenic acid and isochlorogenic acids. The isomers of the isochlorogenic acid: 3,4 dicaffeoylquinic (DCQ), 3,5 DCQ and 4,5 DCQ were quantified and expressed as cynarin (1,3 DCQ).

The analysis of the chromatographic profile of each sample showed a similar quali–quantitative pattern. Moreover, the isomers of the isochlorogenic acid had a constant concentration relationship being the concentration of the isomer 4,5 DCQ > 3,5 DCQ > 3,4 DCQ (Fig. 1C). It was found that the chlorogenic acid and the 4,5 DCQ were the most abundant components. The concentration of caffeic acid was the lowest in all the samples analysed.

When different samples were compared to one another, it was observed that the green leaves had a significantly lower concentration of active principles. Those samples corresponding to the zapecado and drying had significant differences in the 3,5 DCQ isomer acid and did not display significant variations in the drying process when compared to the forced aging stage. Differences were not found between forced and natural aging either. The levels of caffeic acid were found to be constant throughout the process (Fig. 1).

3.3. Methylxanthines

Regarding the analysis of methylxanthines, an increase in the content of caffeine and theobromine was observed in the zapecado when compared to the green leaves, followed by a decrease during the drying process, a value which was sustained during the forced aging (Fig. 2A). These results are in line with those of Colombo and Nuñez (1983) who postulated that the increase in the levels of caffeine might be due to degradation reactions involving nucleic acids during the zapecado, where the necessary purines for the biosynthesis of caffeine are released. Moreover, Ashihara and Takeo (2004) have demonstrated that caffeine is synthesised from the xanthosine, via 7-methylxanthosine, 7-methylxanthine and theobromine. Theobromine is methylated to caffeine which would be the final metabolite.

The influence of the industrial processing in the physicochemical parameters and in the content of caffeine has been studied by other authors demonstrating that a loss in the amount of caffeine is produced during the zapecado and the drying processes. This loss was shown to be directly related to the industrial process (Esmelindro et al., 2002). The most considerable loss of caffeine (roughly a 20%) would occur during the drying process (Schmalko & Alzamora, 2001). The results obtained herein agree with those published previously, however, the loss of caffeine was found to be lower in our study (8.7%). These quantitative differences could be attributed to the different conditions employed by the producers of yerba mate, mainly in temperature and time span of the different industrialisation processes (Esmelindro et al., 2002).

3.4. Relationship between methylxanthines and caffeoyl derivatives

An increase in the content of methylxanthines was observed in the zapecado when compared to the green leaves, followed by a decrease during the drying process. The same behaviour was observed for the caffeoyl derivatives (chlorogenic acid and the isomers of the isochlorogenic acid: 3,4 DCQ, 3,5 DCQ and 4,5 DCQ) which content was found to be significantly higher in the zapecado, as compared to the green leaves. The caffeic acid was the only compound which levels were found to be constant throughout the process (Fig. 1).

The caffeine of yerba mate (named mateine in old works) would be mostly combined with organic acids, the caffeoylquinic ones. For instance, caffeine was demonstrated to bind to chlorogenic acid (Ashihara & Takeo, 2004). The latter phenomenon has also been observed in *Coffea* spp, where chlorogenic acid is trapped by caffeine to form caffeine chlorogenate (Campa, Dobleau, Dussert, Hamon, & Noirot, 2005). The temperature and humidity conditions

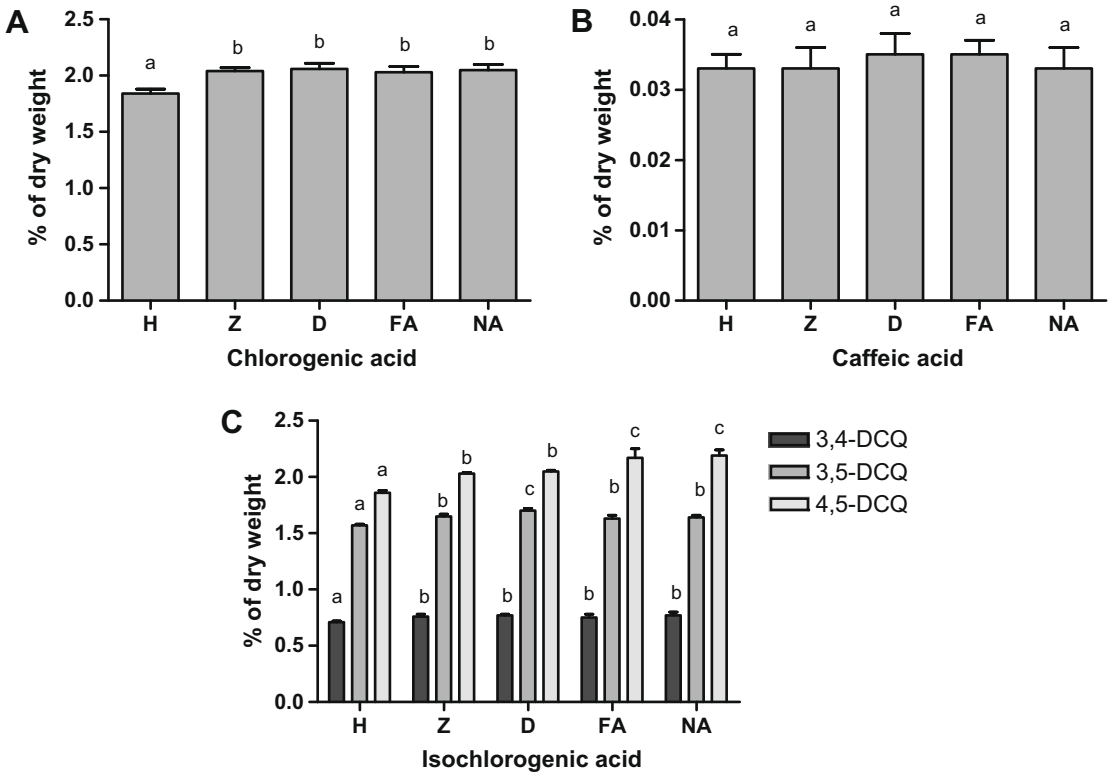

Fig. 1. HPLC quantitative determination of polyphenol compounds in yerba mate in the different stages of the industrial processing: (A) chlorogenic acid, (B) caffeic acid and (C) isochlorogenic acid. Bars represent the mean ± SEM of three independent experiments carried out by duplicate and are expressed as percentage on dried weight. Different letters indicate significant differences between samples. One-way ANOVA and *a posteriori* Bonferroni's test, $p < 0.05$. Detection limit: 0.2 ppm. Quantification limit:1.0 ppm. DCQ: dicaffeoylquinic acid, quantified and expressed as cynarin (1,3-DCQ). Stages of the industrialisation process: H: harvest; Z: zapecado; D: drying; FA: forced aging; NA: natural aging.

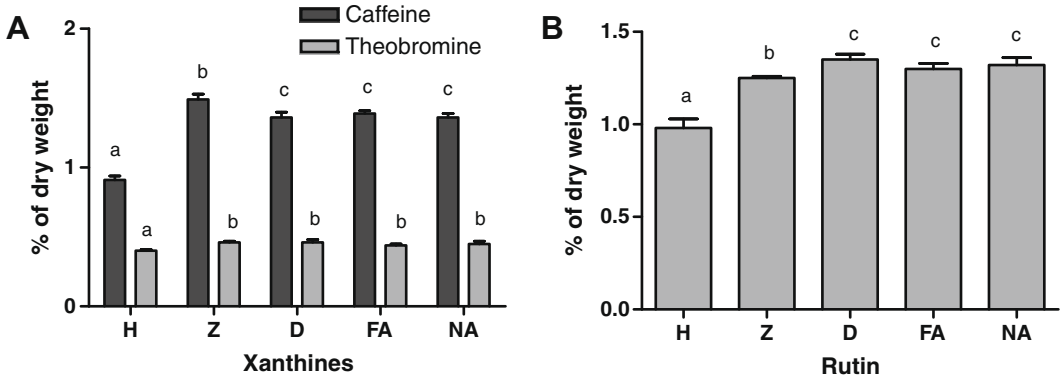

Fig. 2. HPLC quantitative determination of: (A) methylxanthines (caffeine and theobromine) and (B) rutin in yerba mate in the different stages of the industrial processing. Bars represent the mean ± SEM of three independent experiments carried out by duplicate and are expressed as percentage on dried weight. Different letters indicate significant differences between samples. One-way ANOVA and *a posteriori* Bonferroni's test, $p < 0.05$. Detection limit: 0.2 ppm. Quantification limit: 1.0 ppm. Stages of the industrialisation process: H: harvest; Z: zapecado; D: drying; FA: forced aging; NA: natural aging.

employed during the process would release different amounts of caffeine and the organic acids with which it combines, inducing an increase in these metabolites during the zapecado. The latter hypothesis could explain the lower content of caffeoyl derivates found in the green leaves when compared to the content observed after the zapecado (Figs. 1 and 3). Bastos et al. (2006) have reported that the extraction of caffeine and 5-caffeoylquinic acid was found to be more effective when dried processed leaves are used due to the cell disruption and mechanical impact during the processing stages. We propose that both processes would be contributing to the increase in the levels of the major bioactive compounds found in the samples subjected to heat during the manufacturing process, On the other hand, the levels of caffeoyl derivatives were similar after the natural and forced aging processes (Fig. 3).

3.5. Flavonoids

The flavonoids rutin, quercetin and kaempferol were analysed. The results showed an increase in the content of rutin in the zapecado when compared to the green leaves. On the other hand, this flavonoid was found to be significantly higher in the drying as compared to the zapecado. Rutin did not display significant varia-

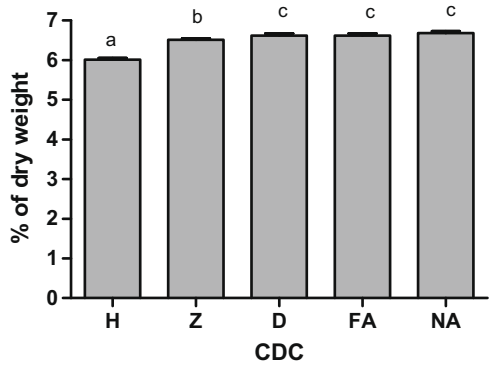

Fig. 3. Total caffeoyl derivatives content in yerba mate in the different stages of the industrial processing. The values were obtained by HPLC and represent the mean ± SEM of three independent experiments carried out by duplicate. The results are expressed as percentage on dried weight. Different letters indicate significant differences between samples. One-way ANOVA and *a posteriori* Bonferroni's test, $p < 0.05$. CDC: Caffeoyl derivative compounds. Stages of the industrialisation process: H: harvest; Z: zapecado; D: drying; FA: forced aging; NA: natural aging.

tions in the drying process when compared to the natural and forced aging stage (Fig. 2). Quercetin and kaempferol were found at very low concentrations (below the quantitation limit, 1 ppm).

This is the first complete report on the quantitative variation of caffeoyl derivatives (caffeic acid, mono and dicaffeoylquinic acids), methylxanthines (caffeine and theobromine) and flavonoids (rutin, quercetin and kaempferol) during all and each one of the steps of the industrial process of yerba mate.

4. Conclusions

The comparative quantitative analysis of all the samples indicated that those obtained after the zapecado, drying and aging processes possess higher content of biologically active principles (total caffeoyl derivatives, caffeine, theobromine and rutin) when compared with green leaves.

The results obtained in this work could provide a guideline for the obtention of *Ilex* extracts enriched in biologically active principles to be used in the pharmaceutical, food and cosmeceutical industry.

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