Seasonal Variation on the Yield of *Lippia dulcis Trev*. Extract Obtained by Supercritical CO_2

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Lippia dulcis Trev. is an intensely sweet herb originary from tropical America. Its major components, (+)-hernandulcin and (-)-*epi*-hernandulcin, have been investigated. According to literature data, the season and even for how long the plant receives sunlight might influence the phytochemistry of the plant, since some compounds may be accumulated at a particular period to respond to environmental changes. In a previous work we investigated the extraction yield and composition of *L.dulcis* extract obtained by supercritical fluid extraction process. Those extracts were obtained from plants collected during winter time. In the present work, a comparison between the products obtained by supercritical fluid extraction process from the vegetable matrix collected during winter and summer time is described. The main purpose of this work was to verify if there is a difference in the global yield of the extracts according to harvest season and to evaluate the composition of the extract of each season. Extraction assays were performed varying harvest season, extraction pressure and temperature, keeping the other parameters constant. Gas chromatography analyses were performed to evaluate extracts composition. Results indicated that higher yields were reached when applying 120bar/35°C and 140bar/40°C as extraction parameters. GC-MS and HPLC assays indicated the presence of hernandulcin in the products.

Introduction

Lippia dulcis Trev. is a medicinal plant from the Verbenaceae family. It is strongly aromatic, with leaves and flowers of strong sweet taste [1]. The presence of a sweetener compound (hernandulcin) in the extract is mentioned in most of the published researches regarding this vegetable matrix. The constituents of this plant were previously investigated and isolated. Many compounds were identified in this plant extracts, such as camphor, limonene, terpineol, α-pinene, α-copaene, (+)-4β-hydroxy-hernandulcin, (+)-hernandulcin and its stereoisomer (-)-*epi*-hernandulcin, and so on [1].

In a previous work we investigated the extraction yield and composition of *L.dulcis* extract obtained by supercritical fluid extraction process [2]. Those extracts were obtained from plants collected during winter time. According to literature data, in the different seasons of the year, depending on rain distribution and incidence of sunlight, there might be different compositions for the same essential oil [1].

Some authors have investigated the composition of *Rosmarinus officinalis* essential oil depending on different locations and time intervals [3]. The authors found that for the region with the hottest climate the content of the major compound in the essential oil was higher than for regions with moderate hot and cool climate.

Also, an evaluation of the yield of *Salvia* species essential oil according to the season of the year was carried out [4]. It was found that the highest yield was obtained from plants collected in late winter and spring time.

Another work reported that the highest yield of *Salvia officinalis* essential oil was from plants collected in summer time in Yugoslavia [5].

A research was conducted to isolate (+)-hernandulcin and characterize this compound [6]. Using the extract from the leaves and flowers of *L.dulcis* collected in Mexico, obtained by extraction with petroleum ether, the authors found that the amount of hernandulcin was 0.004% w/w in relation to the dry plant. The authors also identified its isomer, (-)-*epi*-hernandulcin, present in the oil.

The sweetener compound was also investigated in the extract from *L.dulcis* collected in Panama [7]. In this survey, the authors found a higher yield of hernandulcin (0.154% w/w) in comparison to that published by [6].

Authors evaluated the composition of *Lippia dulcis* extract obtained by solid-liquid micro extraction [8]. The concentration of hernandulcin was found to be 36% in the extract. The research group also identified the isomer of this compound, epi-hernandulcin, this one corresponding to 22% of the extract.

In the present work, a comparison between the *Lippia dulcis* extracts obtained by supercritical fluid extraction process from the vegetable matrix collected during winter and summer time in Brazil is described. The main purpose of this work was to verify in which season the highest yield of extract could be obtained, using the same process parameters. The composition of the extracts was evaluated and the presence of the sweetener compound, hernandulcin, confirmed.

Material and Methods

Supercritical Fluid Extraction Equipment

Supercritical fluid extraction was conducted in a pilot unit schematically represented in Figure 1. Carbon dioxide (99.9% purity) was supplied to the system through a cylinder (Linde, Brazil) connected to a gas booster (Maxpro Technologies, Germany). The unit was composed by a jacketed extraction vessel (l.8cm internal diameter, 58cm height) pressurized by the gas booster. A jacketed surge tank was placed between the gas booster and the extractor vessel, in order to perform a temperature conditioning of the CO_2 and to avoid eventual pressure overshoots, allowing a better pressure control. The temperature of the surge tank, as well as the extraction vessel was controlled by a thermostatic water bath. In the surge tank, a thermocouple (type J, Consistec) was installed to monitor the CO_2 temperature. The extraction pressure was monitored by a pressure transducer (AEP, Italy). The samples were collected in a flash vessel maintained at 30bar and 35^{o}C, in which each sampling was made possible by reducing the pressure so as to separate CO_2 from the extract. The solvent flux was measured at the separator outlet by a flow meter (Key Instruments, USA).

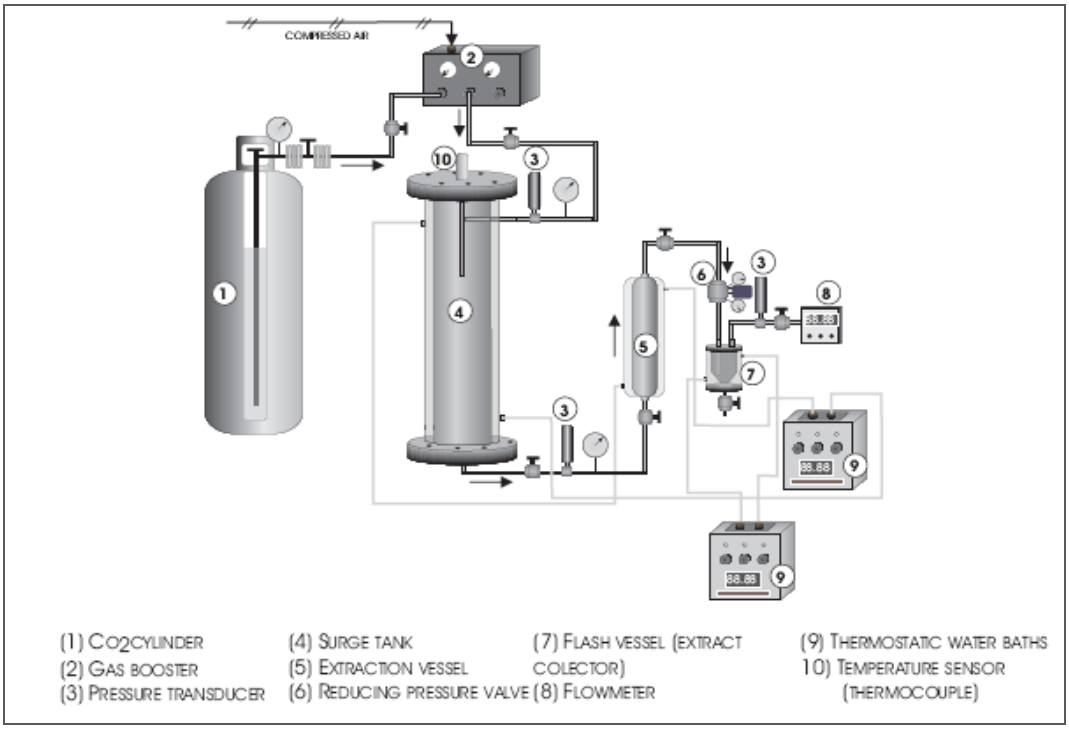

Figure 1 : Supercritical fluid extraction pilot unit.

Extraction procedure

The vegetable matrix was identified as *Lippia dulcis (Trev.)* Moldenke, from the Verbenaceae family, with the voucher specimen FLOR 36.457 deposited at the herbarium of the Botanical Department at UFSC, SC, Brazil. The plant was collected, air dried and its leaves and flowers were milled in a knife grinder (Marconi, Brazil). The powdered plant was screened and classified according to its particle size, using a set of sieves with openings varying from 0.044 to 0.295mm. About 96% of the particles were retained in sieves with openings from 0.212 to 0.295mm (Sauter average diameter – d_{32}=0.280mm).

The average CO_2 flow rate was 3.0 L/min (25^{o}C, atmospheric pressure), and the following pressures and extraction temperatures were tested: 100/120/140 bar and 35/40^{o}C, maintaining the other parameters constant. Each extraction experiment was carried out using, approximately, 60g of the powdered plant. The assays lasted from 100 to 260 min, until there was no more available extract to be collected. Extract samples were collected at time intervals, being the first sample collected always after a static time of 60 min (period of time in which supercritical CO_2 remains in contact with the vegetable matrix). The total yield was determined from the mass of extract obtained in the separator in comparison to the initial mass fed into the extraction vessel.

Extracts composition evaluation

Extracts composition evaluation were carried out performing gas chromatography-mass spectrometry (GC-MS), gas chromatography with flame ionization detector (GC-FID) and high performance liquid chromatography (HPLC). Assays used samples of the extract obtained from plants collected during winter (August) and summer (January), in Brazil. The

main purpose of these analyses was to identify the main compounds present in the extracts. The gas chromatography analyses conditions are shown in Table 1.

Table 1: GC-FID and GC-MS analytical parameters.

Parameters	Winter		Summer	
	GC-FID	**GC-MS**	**GC-FID**	**GC-MS**
Equipment	Shimadzu GC14BDIC	Varian CP3800 coupled with MS Saturn 2000	Shimadzu GC2010AF	Shimadzu GCMS QP2010 Plus
Capillary column	OV 5 (30m x 0.25mm id x 0.25µm film thickness)	CP-Sil 8 CB Low Bleed/MS (30m x 0.25mm id x 0.25µm film thickness)	Rtx-5 (fused sílica 5% diphenyl/95% dimethyl-polysiloxane), Restek (30m x 0.25mm id x 0.25µm film thickness)	Rtx-5MS (fused sílica 5% diphenyl/95% dimethyl-polysiloxane), Restek (30m x 0.25mm id x 0.25µm film thickness)
Carrier gas	Nitrogen	Helium	Nitrogen	Helium
Detector temperature	300°C		300°C	
Injection temperature	250°C	250°C	250°C	250°C
Initial temperature	50°C for 1min	50°C for 1min	50°C for 1min	50°C for 1min
Temperature program	5ºC/min until 270ºC for 15min	3°C/min until 240°C	5°C/min until 270°C for 10min; 20°C/min until 300°C for 5min.	5°C/min until 270°C for 10min; 20°C/min until 300°C for 5min.
Software	Peakwin 3D	Saturn GC/MS Workstation 5.1	GC Solution v.2.3, Shimadzu Corporation	GCMS Solution v.2.5, Shimadzu Corporation

HPLC analyses were carried out to confirm the presence of hernandulcin in the essential oil from the plants collected during summer without the risk of degradation due to the analysis temperature. Synthesized (±)-hernandulcin was used as an external standard. The analyses were performed in a HPLC Shimadzu LC-10AT VP, using a PDA (photodiode array) detector (Varian Pro Star) operating at 205 and 254nm wavelengths. The analyses were conducted in isocratic mode, using acetonitrile and distilled water (80:20, v/v) as mobile phase. A pre-column C18, with particle size of 5µm (Alltech), and a column C18, with particle size of 5µm (Varian Pursuit XRs) were used as stationary phase. The flow was set to 1ml/min and the volume injected was 20µl.

Results

Table 2 presents the supercritical fluid extraction conditions used (pressure and temperature) and the extract yield (%, w/w) obtained for each one, according to the harvest season.

Table 2: Supercritical fluid extraction results.

N°	**Season**	**P (bar)**	**T (°C)**	**ρ_{CO2} (g/cm^3)**	**y (%)**
1	winter	100	35	0.714	0.8
2	winter	100	40	0.630	1.2
3	winter	120	35	0.768	1.3
4	winter	120	40	0.719	1.1
5	winter	140	35	0.802	1.5
6	winter	140	40	0.764	2.7
7	summer	100	35	0.714	1.6
8	summer	100	40	0.630	2.2
9	summer	120	35	0.768	2.7
10	summer	120	40	0.719	1.9
11	summer	140	35	0.802	2.6
12	summer	140	40	0.764	4.1

P: pression; T: extraction temperature; ρ_{CO2}: density of carbon dioxide calculated in the process conditions; y: extract yield (%, w/w).

According to Table 2 it is possible to observe that the extract yield was higher for the plant harvested in summer, for all conditions applied (pressure and temperature), in comparison to the same parameters used for the plant harvested in winter. Figures 2 and 3 show the supercritical fluid extraction curves (mass yield vs. extraction time), for winter and summer, respectively, plotted with the data presented in Table 2. The results are expressed as extraction yield (%, w/w).

The behavior of the curves obtained under different pressures and temperatures is similar for both winter and summer. The maximum extraction yield was achieved with 140bar and 40°C, obtaining 2.7% and 4.1% for winter and summer, respectively. However, good results were also obtained at 120bar and 35°C, 1.3% and 2.7% for winter and summer, respectively.

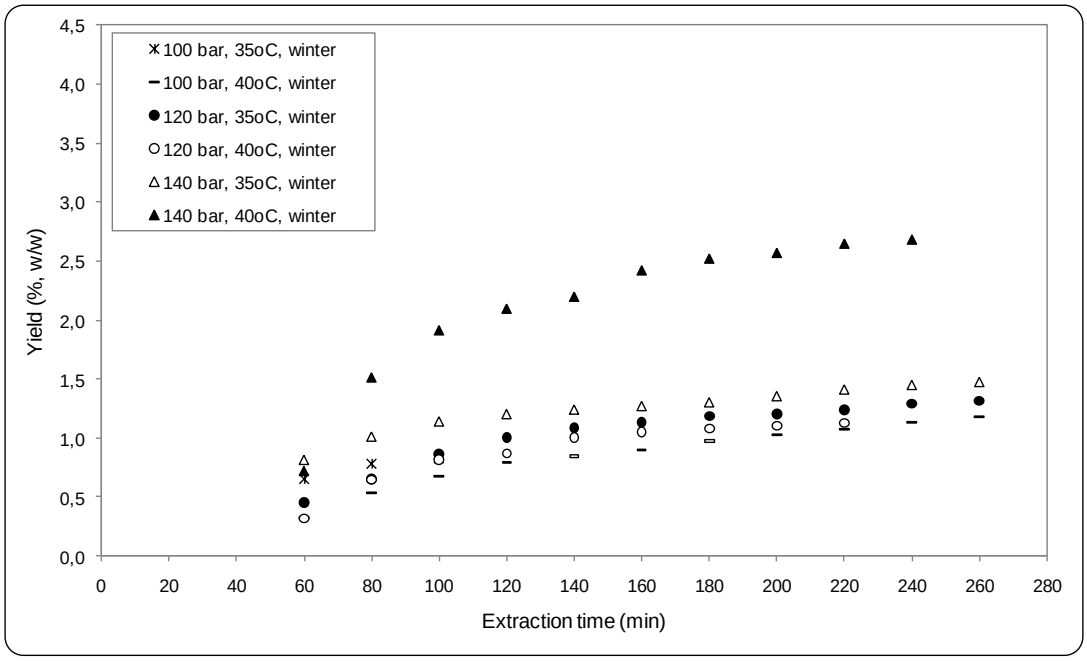

Figure 2: Supercitical fluid extraction curves for winter.

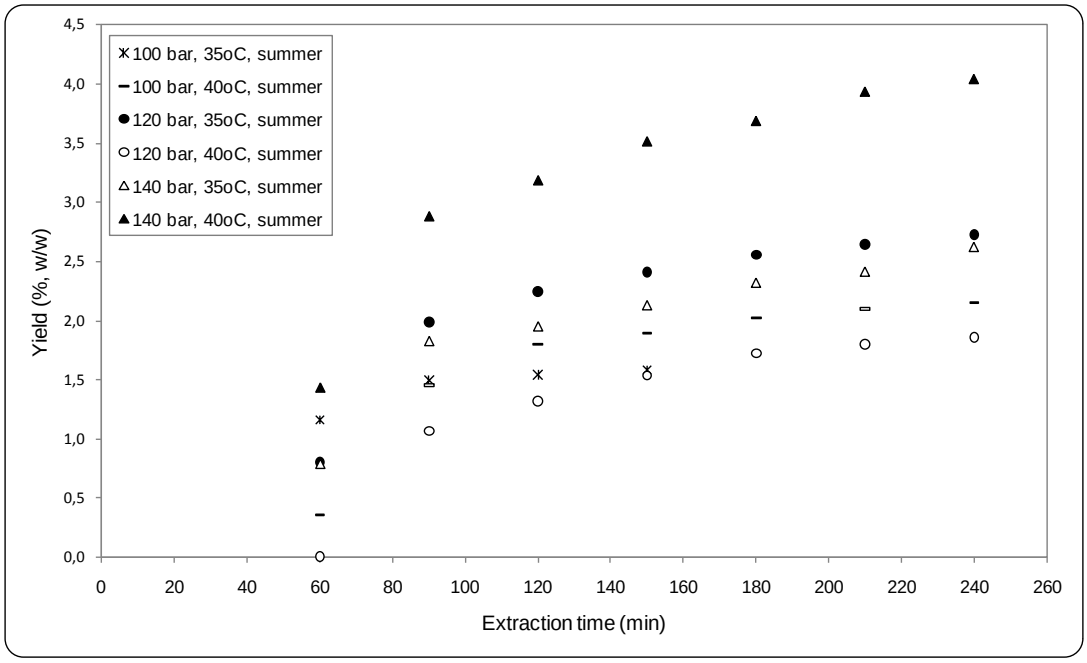

Figure 3: Supercitical fluid extraction curves for summer.

It is possible to observe that the behavior of the extraction curves is not always related to the density of CO_2, whereas for the highest density value the product yield is not maximum. In general, increasing pressure at a constant temperature causes a raise in the yield, since CO_2 density is higher.

Because this plant has its origin in Central America, with a tropical climate, having the incidence of constant sunlight and high temperatures, the results observed for global yield are consistent with that, since during summer time in the region of cultivation is when these

characteristics are closer to the region from where this plant is native. Table 3 presents GC-MS compounds identification and GC-FID results for the extracts from winter and summer.

Table 3: GC-MS and GC-FID results.

Component	Chromatogram data			
	Winter		Summer	
	Rt (min)	Area (%)	Rt (min)	Area (%)
5-hepten-2-one, 6-methyl	8.911	3.894	9.248	20.948
2-cyclohexen-1-one,3-methyl	11.780	3.325	11.393	16.600
Copaene	25.789	7.614	21.036	8.800
Caryophyllene	27.618	7.753	22.306	10.998
Cedrene	29.052	3.533	*n.i.*	*n.i.*
Naphthalene	31.687	7.956	23.944	4.136
α-bisabolol	38.157	4.565	28.928	5.431
(+)-hernandulcin (identified by	43.889	19.215	*n.i.*	*n.i.*
comparison with the literature)	43.933	12.365	*n.i.*	*n.i.*
Total (%) =		70.220		66.913

n.i. : not identified; Rt: retention time

In the analyses conducted for the sample of summer there was, apparently, thermal degradation of hernandulcin in 5-hepten-2-one, 6-methyl and 2-cyclohexen-1-one, 3-methyl, also known as precursors for the synthesis of the sweetener. This was probably because the injection temperature was higher than the temperature of degradation of this compound. According to the literature [6] when heated above 140^{o}C hernandulcin dissociates into these substances. In the analyses of the winter extract the same compounds peaks can be observed, but in smaller proportions. This may be related to the use of a linear programming of temperature for the injection of the winter sample, so that the compound does not dissociate before entering the column. The absence of a peak corresponding to the sweetener compound in the chromatogram of the summer extract supports this hypothesis.

Figures 4 and 5 correspond to the total ion chromatogram for winter extract and the mass spectrum of the peak that corresponds to that of the compound of interest, hernandulcin. The chromatogram of the summer extract is shown in Figure 6.

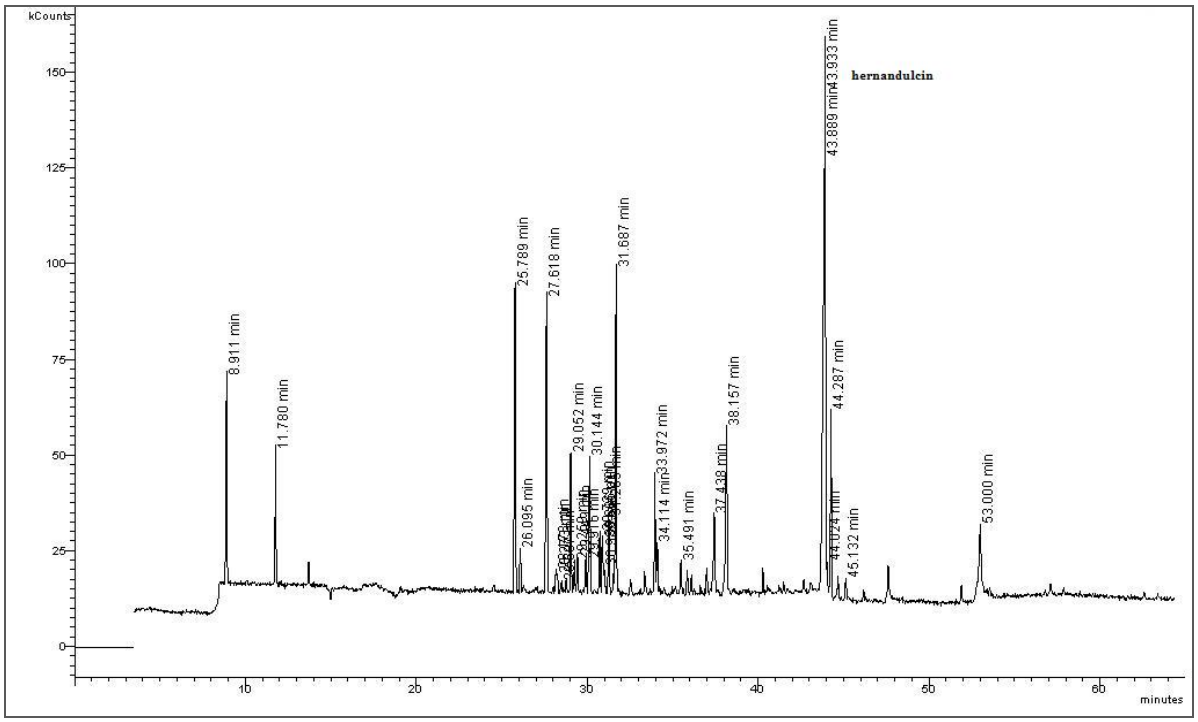

Figure 4: Total ion chromatogram for the winter extract.

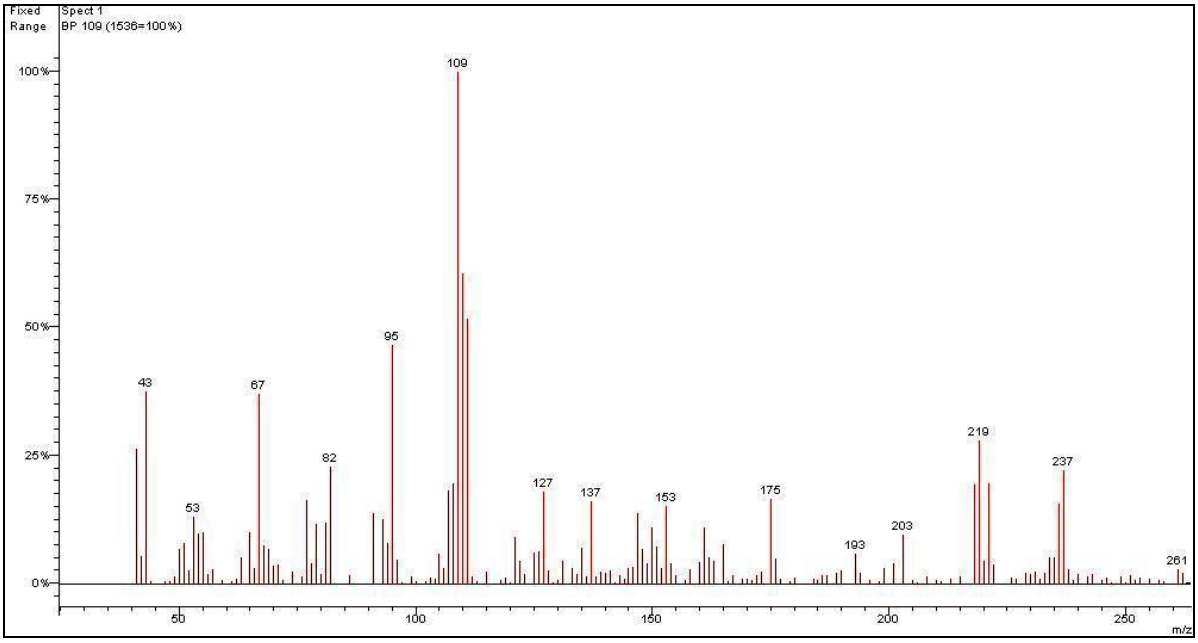

Figure 5: Mass spectra of the hernandulcin peak (Rt = 43.889 min).

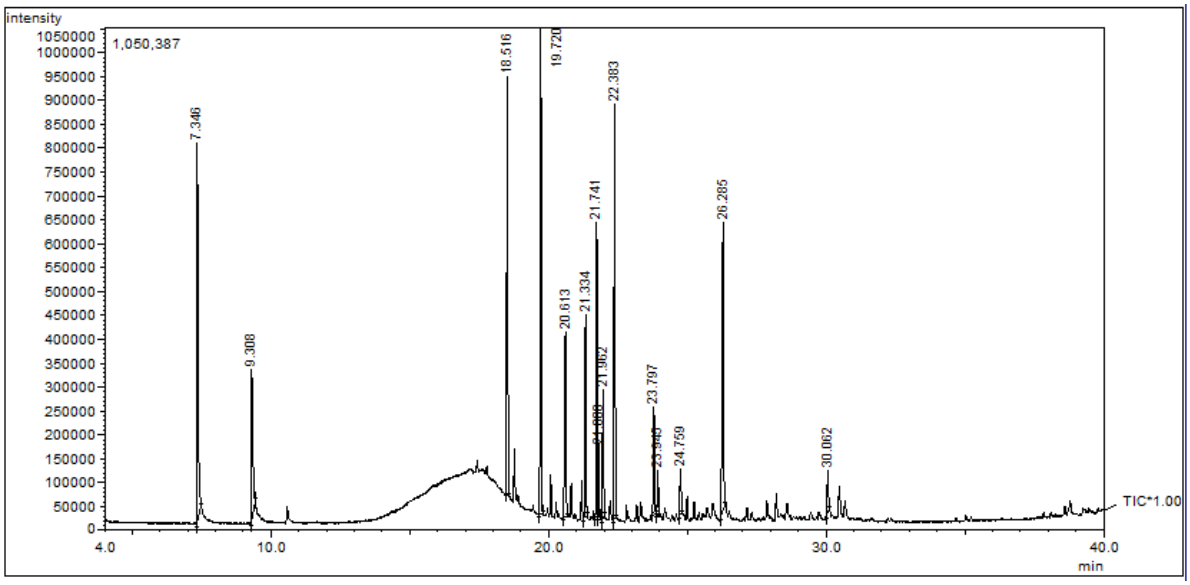

Figure 6: Total ion chromatogram for the summer extract.

To confirm the presence of the compound hernandulcin in the summer sample HPLC analyses was carried out. The confirmation was possible by using an external standard of the sweetener, with which it was built a calibration curve. In order to quantify the sweetener an internal standard, acetophenone, was used. Figure 7 shows the HPLC chromatogram of the extract, in which the peak reached in 7.527 minutes corresponds to the compound of interest. The concentration of hernandulcin in the extract was 0.46mg/ml extract.

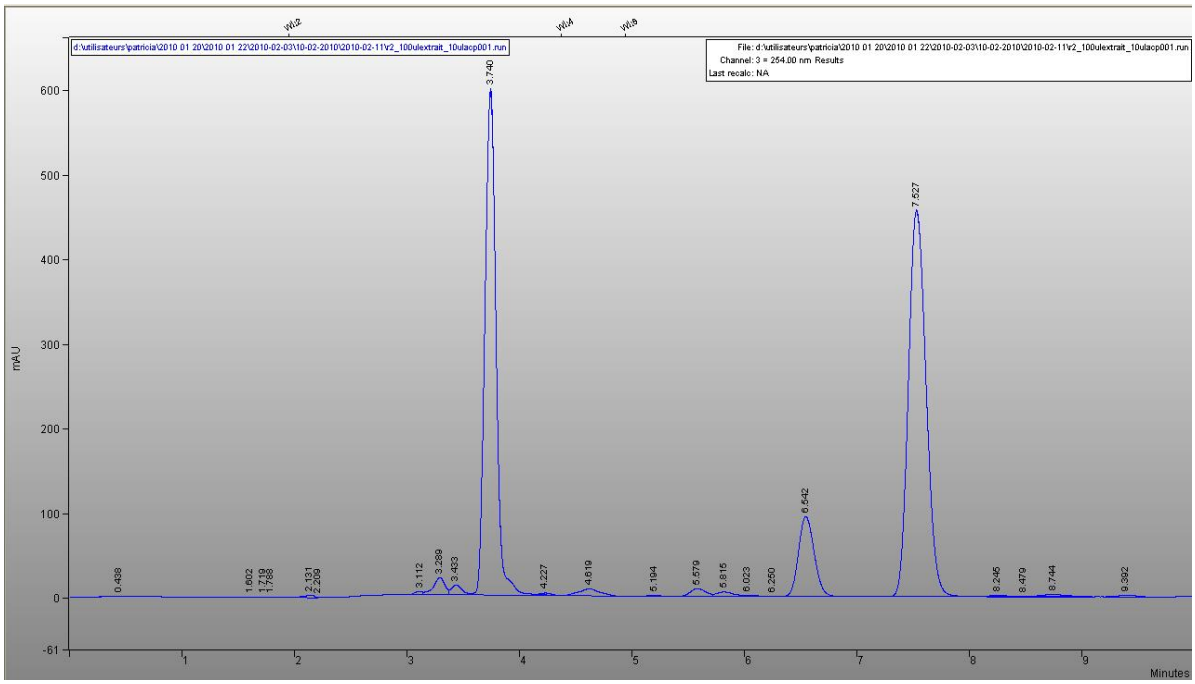

Figure 7: HPLC chromatogram for the summer extract.

Conclusions

From this research it was possible to observe that the highest yields were obtained for the plant collected during summer and also that the operating conditions of 120bar/35^{o}C and 140bar/40^{o}C were those which promoted higher amount of extracts. Regarding the composition of the extracts, they are in accordance with the literature available for these plant extracts, although not all compounds in the extract have been identified. Also, it was observed that there was no change in the composition of the evaluated samples, from winter and summer. However, a more accurate evaluation is needed. The presence of the compound of interest, hernandulcin, was confirmed by GC-MS and HPLC analysis.

Acknowledgments

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