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SUPERCritical FLUIDS AND ULTRASOUND ASSISTED EXTRACTIONS APPLIED TO SPRUCE BARK CONVERSION

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Abstract

Supercritical fluid and ultrasound assisted extractions were applied as a first step in a complex processing of spruce bark in order to recover polyphenols compounds. The aim of the study was to compare the efficiency of these "green" processes with a conventional extraction technique (ethanolic extraction). Supercritical fluid extraction was carried out in two steps: (i) static extraction for 15 min at 1000 psi with pure CO₂ and (ii) dynamic extraction for 45 min at 35, 40 and 50 °C, 1200, 2000 and 2500 psi, with CO₂ and 70% ethanol as co-solvent. UAE was carried out in an ultrasonic bath at 45, 50 and 60 °C for 5 to 75 minutes. The ethanolic extraction was performed using ethanol (70%) in a closed oven for 13 days. The extracts were characterized using Folin-Ciocalteu method for total phenolic content and quantified by high liquid performance chromatography (HPLC). The study recommend SFE and UAE instead of traditional ethanolic techniques since these provide high extraction yields, pure extracts, with a large number of polyphenolic compounds extracted and are environmentally friendly.

Key words: ethanol extraction (Eth E) spruce bark, polyphenols, supercritical fluid extraction (SFE), ultrasound assisted extraction (UAE)

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1. Introduction

One of the great challenges in the 21st century addresses the transition from an economy largely based on fossil origin raw materials to one based on renewable resources, processed according to sustainable development goals (Gravitis et al., 2008). Among all sustainable resources, only biomass represents an accessible source for chemicals, biomaterials, derivatives and products with energetic value (Herrero et al., 2013; Sheldon, 2011). The application of renewables based on biomass, the development of clean production and smart materials may provide the required solution for sustainability.

The main challenge is to develop innovative methods for the efficient use of biomass as it is or as wastes (livestock, industrial, agri-food, agricultural and non-recyclable fraction proceeding from waste treatment plants), (Hidalgo et al., 2014). In order to

be sustainable, biomass use will depend heavily on the successful deployment of innovative, green chemistry so as to avoid the use of toxic and/or hazardous reagents and solvents or eliminate wastes (Sheldon, 2011).

From this point of view, the biorefining concept embraces a wide range of technologies able to fractionate biomass resources into their building blocks (carbohydrates, lignin, extractives, proteins etc.), which can be converted to value added products (Cherubini, 2010). Many compounds resulting in complex biomass processing are recognized by their valuable biological properties (Bodîrlău et al., 2009; Popa, 2011; Popa, 2013). That is why, the extraction and purification processes of some biobased compounds resulted in this fractionation are essential, when they follow to be used in the preparation of dietary supplements, nutraceuticals, functional food ingredients (Volf et al., 2013), food additives,

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pharmaceutical and cosmetic products, allelochemicals (Popa et al., 2008), chelating agents (Volf et al., 2012), modulators in heavy metal bioaccumulation (Stingu et al., 2011) and plant growth regulators (Tanase et al., 2013; Tanase et al., 2014). The biomass extraction results depend on various factors such as, solvents, procedures, extraction time etc., to obtain bioactive components of high quality and quantity in the crude extracts. In addition, the nature of the sample matrix and the compounds to be extracted also substantially affect the efficiency of extraction. The optimal extraction method should be simple, rapid, economical and with a large applicability (Vongsak et al., 2013)

Conventional separation techniques such as solvent extraction and distillation have the drawback of leaving trace amounts of solvents or to cause thermal degradation (Ahluwalia et al., 2013). Non-conventional methods, which are more environmental friendly due to decreased use of chemicals, reduced operational time and better yield and quality of extracts, have been developed during the last years. Thus, ultrasound, pulsed electric field, enzyme digestion, extrusion, microwave heating, ohmic heating, supercritical fluids and accelerated solvents have been studied as nonconventional methods. The ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE) and supercritical fluid extraction (SFE) are probably the most applicable at pilot or industrial scale and were critically reviewed by several researchers (Azmir et al., 2013; Khoddami et al., 2013; Martins et al., 2011; Roberto et al., 2010; Vilku et al., 2008). The conventional extraction methods, such as Soxhlet is still considered as one of the reference method to compare the success of newly methodology (Azmir et al., 2013).

In this perspective, the paper presents a comparative analysis regarding technical aspects related to the two innovative and environmental friendly techniques: ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) in comparison with a classic solvent extraction process (EthE). These techniques were applied to isolate, from spruce bark, bioactive compounds included in polyphenols group, which are one of the most widely occurring groups of phytochemicals, described in details elsewhere (Ignat et al., 2011; Ignat et al., 2013).

The analysis takes into account the total yield in polyphenolic concentration and the qualitative extracts content highlighted by high liquid pressure chromatographic profile. The approach is appropriate even more because in Romania large amounts of bark are released as residues from forestry and woodworking processes, mainly burnt for energy recovery, without considering that as an important source of valuable chemicals. More than this, even if the presented techniques are increasingly used at laboratory and industrial level, a comparative study regarding the polyphenolic compounds extraction from spruce bark doesn't exist.

2. Materials and methods

2.1. Materials

Spruce (*Picea abies*) bark of industrial origin was provided as a waste from a wood processing Romanian company. The spruce bark was washed several times with doubly distilled water to remove impurities, dried at room temperature under normal aeration conditions and milled in a GRINDOMIX GM 2000 mill for 0.15-1.25 mm particle size. The material was stored in desiccators and was directly used without any pre-treatment. The raw material humidity was determined using a RADWARG MAX 5011 thermo-balance. The FT-IR spectrometry was used to characterize the functional groups present in biomass. The FT-IR spectra were recorded with a Bio Rad FT-IR spectrometer in 400 – 4000 cm^{-1} spectral domain, with a 4 cm^{-1} resolution and 32 scans, by KBr pellet technique.

Ethanol, Folin Ciocalteu's phenol reagent, phenols standards and other chemicals used in experiments were provided by Sigma-Aldrich and Fluka. Carbon dioxide 5.3 for supercritical extraction procedure was purchased by Linde Group. Distilled water was used for all experiments.

2.2. Ethanolic extraction (Eth E)

A classic ethanolic extraction was conducted using 50 mL ethanol/water mixture (70:30 mL, v/v) as solvent and 5 g of milled spruce bark. The extraction was performed at 40 $^{\circ}\text{C}$ in a closed reaction vessel during 13 days. The polyphenolic extraction was monitored every 24 hours in order to relieve the kinetics profile and to achieve the equilibrium (full extraction of polyphenols from biomass). The experiment was carried out in triplicate.

2.3. Ultrasound assisted extraction (UAE)

The ultrasound assisted extractions were performed in a ultrasonic bath, SONOREX RK type 100 H, (35 kHz, 320 W) produced by Bandelin Electronic GmbH & Co.KG, Berlin, Germany using 5 g of biomass in a glass vessel and 50 mL ethanol/water mixture (70%), biomass/solvent ratio of 1:10 w/v. Extractions were carried out at 45, 50 and 60 $^{\circ}\text{C}$ for 5, 10, 20, 30, 45, 60 and 75 minutes. The obtained extracts were centrifuged at 4000 rpm for 4 min in a centrifuge Hettich Rotofix 32 A type. The supernatant was collected and analyzed and all experiments were carried out in triplicate.

2.4. Supercritical fluid extraction (SFE)

Extractions were performed using a SFT 100 XW supercritical fluid extractor system in two steps: (I) static with pure CO_2 , 6 mL/min flow rate at 1000 psi constant pressure for 15 min to allow contact between the samples and the supercritical solvent;

(II) dynamic for lipophilic extraction (in the first 10–15 min at 1000 psi) followed by polyphenols extraction (30 min) with CO₂ and ethanol (70%) as co-solvent with 10:1 mL/min flow rate ratio.

The investigated conditions for the polyphenols extraction were 1200, 2000, 2500 psi at 35, 40 and 50°C. An amount of 2 g of spruce bark was used for all the experiments that were carried out in triplicate. Some inert glass balls (4 mm in diameter) were added to fill the extraction reactor.

2.5. Total phenolic content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu method, based on the oxidation/reduction proprieties of phenols occurring in contact with Folin-Ciocalteu reagent. For analyses, 1 mL of diluted extract (1%) was mixed with 0.5 mL Folin-Ciocalteu reagent, 2 mL sodium carbonate for pH adjustment (10% concentration) and 5 mL distilled water.

The reaction mixture was left at rest protected from light, at room temperature for 90 min. The absorbance was measured at 765 nm using a GBS AVANTA UV-Vis spectrophotometer.

TPC was determined based on the calibration curve using gallic acid, the results being expressed as milligrams of gallic acid equivalents (GAE) per spruce bark gram (mg GAE g⁻¹), using a CINTRA software.

2.6. HPLC procedure

The extracts were fractionated by liquid-liquid extraction using ethyl acetate, then mixed with 5 mL of ultra pure methanol and filtered by a 0.45 µm diameter filter before injection in the column. The phenolic acids separation was carried out using a DIONEX Ultimate 3000 chromatographic system equipped with a DDA UV-VIS detector, at 280 nm, on a Zorbax RX C18 (4.6×250 mm, particle size 5 µm) column, using 1% acetic acid in ultra pure water as mobile phase (A) and ultra pure methanol as stationary phase (B).

The operation conditions were 30°C temperature, 1.2 mL min⁻¹ flow rate with a gradient of 10–40% B in 40 minutes. Compounds were identified by comparing their fragmentation profiles with references, run under the same experimental conditions.

2.7. Statistical analysis

All the results are expressed as mean ± standard error where $n = 3$. Comparison of the means was performed by the Fisher least significant difference (LSD) test ($p \leq 0.05$) after Statistica (Statsoft version 10.0) analysis. Sampling and chemical analyses were examined in triplicate, in order to decrease the experimental errors and to increase the experimental reproducibility.

3. Results and discussion

3.1. Chemical characterization of the biomass

Spruce bark biomass was characterized from the dry mater content, humidity and structure point of view. FTIR spectroscopy was used as tool and the spectrum (Fig. 1) displays a large number of absorption bands indicating the complex structure of the investigated biomass.

According to Chupin et al. (2013) the bands at 3400–3300 cm⁻¹ could be assigned to OH stretch vibration in phenolic and aliphatic structures. Small peaks at 2930 and 2850 cm⁻¹ originate from CH stretch vibration in aromatic methoxyl groups and in methyl and methylene groups of side chain. Peaks between 1400 and 2000 cm⁻¹ show the aromatic nature of the structure.

The wavelengths situated between 1300 and 1450 cm⁻¹ corresponds to the stretching vibrations of carbonyl groups and the deformation vibration of the C–C bonds in the phenolic groups absorb in the region 1500–1400 cm⁻¹. Signals between 1500 and 1700 cm⁻¹ may be assigned to aromatic nucleus vibrations. The signals from 1070 and 1092 cm⁻¹ are indicative for alcohol functional group, while the 1338 cm⁻¹ absorption band can be attributed to the O–H in plane deformation in polyphenols. It is a good concordance between this result and other structural characterization of spruce bark of other origin made in our research group (Ghitescu et al., 2015). The biomass used in all experiments had 10.27 % humidity.

3.2. TPC assessment of extraction procedures

3.2.1. Eth E extraction

Ethanol extractions were performed in a closed oven at 40°C for 13 days. The equilibrium concentration was reached after 6 days when a maximum concentration of TPC by 87.15 mg GAE g⁻¹ was attend (Fig. 2). After that, a slight decrease (11.2 %) of the total polyphenolic content was recorded. The decrease can be explained by the fact that after attending the equilibrium between phases, polyphenols are re-adsorbed by the biomass.

3.2.2. UAE extraction

Ultrasound assisted extraction is one of the upcoming extraction techniques offering high reproducibility in shorter times, simplified procedure and reduced energy and solvent consumption (Khan et al., 2010) based on the cavitation process involved during sonication which intensify and improve mass transfer, solvent penetration into plant tissue and capillary effects (Da Porto et al., 2013). In recent studies UAE has been applied to obtain bioactive compounds from different materials (Adam et al., 2012; Chavan et al., 2013; Dey et al., 2013; Sun et al., 2011). Time and temperature are the most important factors that have influence on UAE processes.

In this context preliminary tests were carried out at different temperatures (45, 50 and 60°C) for 5, 10, 20, 30, 45, 60 and 75 min to establish the effect of time and temperatures on polyphenols extraction. The data show a very clear influence of temperature on the extraction of polyphenols from spruce bark (Fig. 3). At 60°C the TPCs were greatly increased (42.5 mg GAE g⁻¹) comparing to the TPCs obtained at 45°C (33.48 mg GAE g⁻¹). This behavior can be explained by the higher solubility of polyphenols in the solvent, the higher diffusivities of the extracted molecules and the improved mass transfer at higher temperatures.

A rise in extraction temperature could also break the phenolic matrix bonds and influence the membrane structure of plant cells and therefore facilitate the extraction process (d'Alessandro et al., 2012). Also, it was observed that the initial rate of polyphenols extraction was faster in the first 5 to 20 min, followed by slower extraction rate approaching to an equilibrium concentration. The experiment was carried out in triplicate.

3.2.3. SFE extraction

SFE was applied for some advantages such low temperatures use, reduced energy consumption and higher product quality due to the absence of solvent in final solute phase. SFE uses the properties of fluids over their critical points to selectively extract soluble components from biomass. Supercritical carbon dioxide is recognized as an ideal solvent to extract bioactive compounds being nontoxic, non-explosive, readily available, easy to remove from the final extract, does not cause major disruptions in bio-compounds, and its biological properties can be preserved (Espinosa-Pardo et al., 2014).

A two-step SFE was performed, in triplicate, at 35, 40 and 50°C and at 1200, 2000 and 2500 psi. Experiments carried out at 35°C help us to clarify the fact that under near critical conditions lower content of polyphenols were achieved (Fig. 4). At 1200 and 2000 psi, a raising extraction temperature produced a decrease on the TPC extracted, which varied from 122.41 mg GAE g⁻¹, at 40°C, 60.12 mg GAE g⁻¹

at 50°C, due to the reduction in solvent density. On the other hand, at higher pressures 2500 psi, TPC slowly increase at 35 and 50°C despite of solvent density reduction. This behavior could be caused by the enhancement in the solute (spruce bark) of the vapor pressure with temperature, which was more significant than the reduction in the solvent density, increasing consequently the overall extraction yield as Benelli et al. (2010) reported.

3.3. Chromatographic profile of the polyphenolic extracts

Spruce bark polyphenolic extracts obtained by all three procedures were analyzed by HPLC in order to achieve a qualitative and complete characterization. The method allowed identification of seven phenolic acids (Table 1) as: catechin, gallic, vanillic, syringic, p-coumaric, ferulic and synapic acids. Their presence in spruce bark extracts was also reported in works of our research group (Hainal et al., 2011). The chromatographic profiles of spruce bark are presented in Fig. 5. The most important compounds identified in spruce bark extracts, based on their recovered amount were *p*-coumaric acid with values of 260.4073 mg L⁻¹ of extract in the case of SFE extraction and 99.4731 mg L⁻¹ of extract in UAE, followed by catechin with 171.1765 mg L⁻¹ of extract (SFE) and 112.084 mg L⁻¹ of extract (UAE), and synapic acid with 35.0534 mg L⁻¹ of extract for SFE and 13.3618 mg L⁻¹ of extract for UAE respectively. In relation to the extracts composition the retention times, peak areas and the recovered amount of each identified compound varies from an extraction technique to other.

It can be notice that by SFE and UAE extractions the numbers of the identified compounds in spruce bark tested extracts were higher compared with EthE. However, it can be observed that the extracts obtained by SFE present larger amounts of phenolic compounds in some cases (260.4073 mg L⁻¹ of extract compared to 99.4731 mg L⁻¹ of extract for *p*-coumaric acid) and lower in other (12.9180 mg L⁻¹ of extract compared to 5.1224 mg L⁻¹ of extract for syringic acid) comparing to UAE.

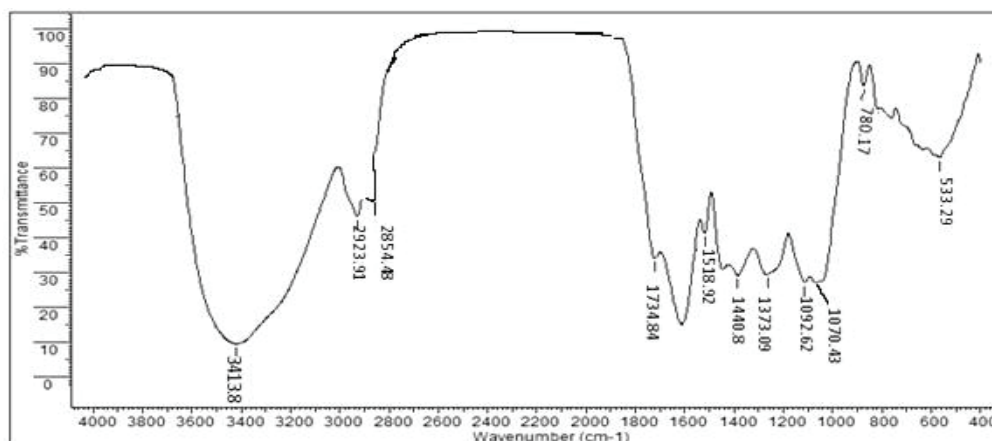


Fig. 1. FTIR spectrum of spruce bark

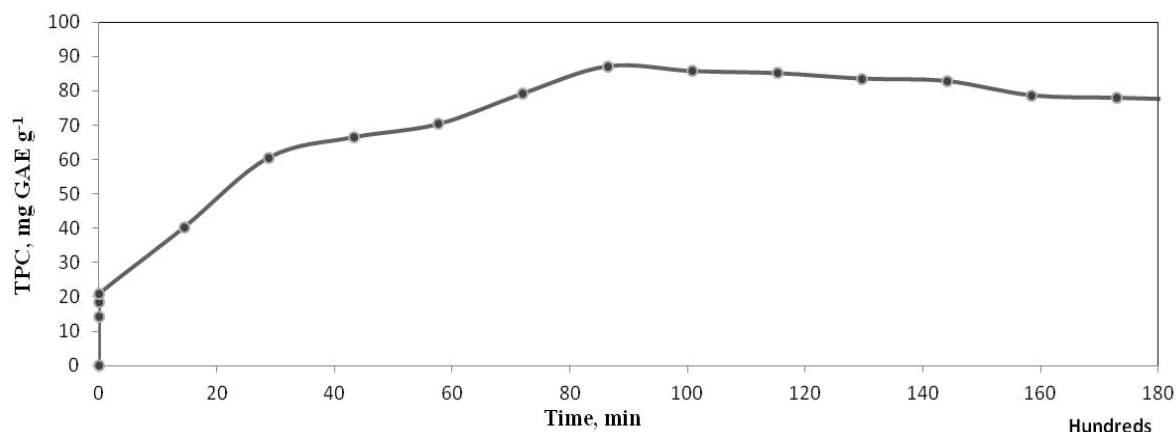


Fig. 2. Total polyphenolic content obtained by Eth E

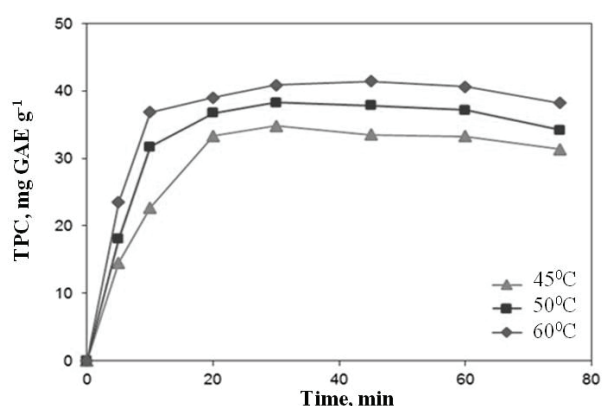


Fig. 3. Total polyphenolic content obtained by UAE

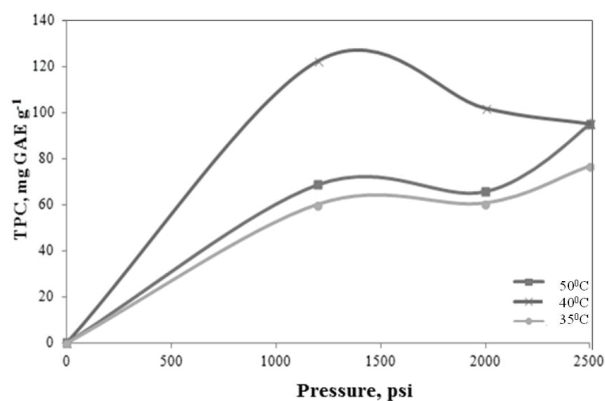


Fig. 4. Total polyphenolic content obtained by SFE

3.4. Comparative analysis

The different extraction processes applied in this study had significant effect on the TPCs extracted from spruce bark. As it was reported, the ethanolic extraction has the biggest disadvantage of a longer extraction time. To improve performances of the Eth E, ultrasounds waves and supercritical pressure of CO₂ were used to intensify the extraction process. Using UAE the total polyphenolic content increase from 14.38 mg GAE g⁻¹ spruce bark

(ethanolic extraction, 60 min) to 33.48 mg GAE g⁻¹ spruce bark and to 122.41 mg GAE g⁻¹ spruce bark in case of supercritical extraction (Fig. 6).

Increasing of phenolic compounds recover degree using ultrasound assisted extraction was also reported in the literature. d'Alessandro et al. (2012) reported a positive effect of ultrasounds on the polyphenols extraction from chokeberry (d'Alessandro et al., 2012). Also Wang et al. (2013) notice that ultrasounds are important in improving the extraction yields of polyphenols from *Sparganii rhizoma* (Wang et al., 2013).

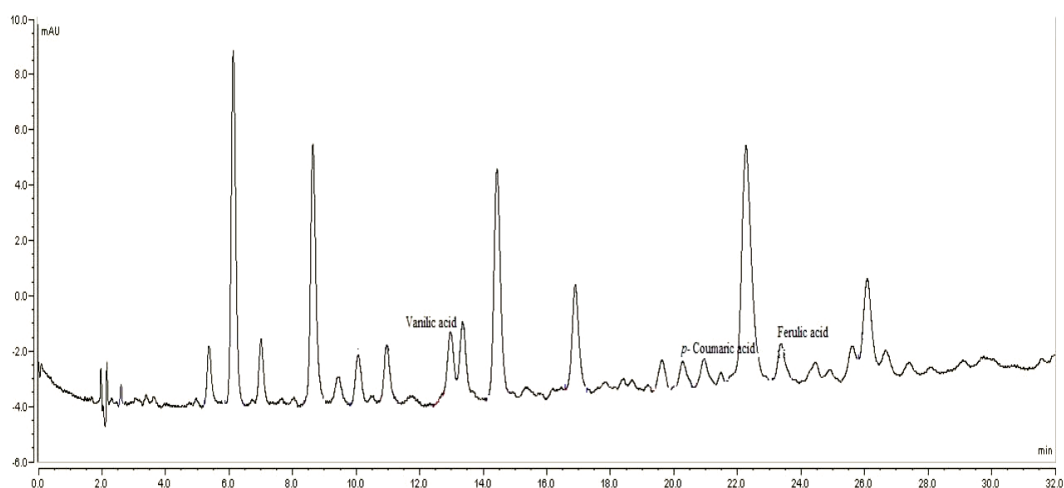
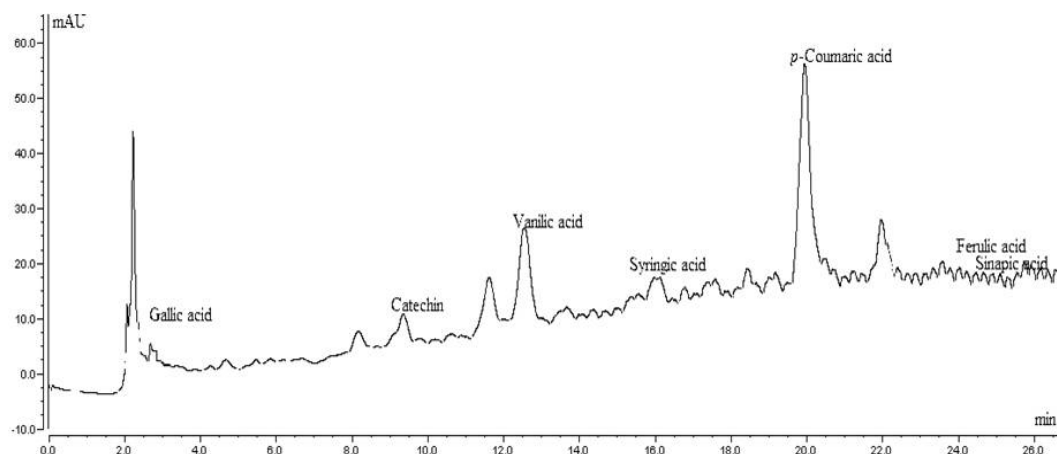
Beyond being a green technique, supercritical extraction provided extracts with better activity than the conventional extraction methods (Kazan et al., 2014) and even ultrasound assisted extraction (Roseiro et al., 2013). Data from literature sustain our research results which show that SFE offer higher total polyphenolic content extracted from spruce bark, compared with ethanolic and ultrasound assisted extraction. This can be due to the fact that SFE processes use critical points of fluids to increase the extraction yields of polyphenols from biomass (Roberto et al., 2010).

Though it is important to underline that working with mixture of solvents, in our case CO₂ - ethanol - water, the supercritical operational conditions change each one having different critical points, so the experiments could be achieved in near-supercritical conditions. Based on this study, a comparative analysis between conventional (EthE) and nonconventional extraction methods (SFE and UAE) reporting to: extraction time, quantity of solvent and biomass, total polyphenolic content and HPLC was done (Table 2). The comparison of yields (reported as mg GAE g⁻¹), times required for the extraction, volumes of solvent and selectivity for the phenolic acids (shown by HPLC) demonstrated that SFE technique is more efficient than Eth E and UAE.

The supercritical CO₂ has a higher diffusion coefficient, lower viscosity and surface tension than the liquid solvents used in UAE and Eth E, leading to easier penetration to sample matrix and favorable mass transfer.

Table 1. HPLC analysis of spruce bark polyphenolic extracts obtained by SFE, UAE and Eth E

Peak name	Parameters	SFE 60 min, 40°C, 1200 psi	UAE 60 min, 40°C	Eth E 60 min, 40°C
Gallic acid	Retention time (min)	3.010	2.998	-
	Area (mAU min)	0.0635	0.0626	
	Amount (mg L ⁻¹)	5.4676	5.454	
Catechin	Retention time (min)	9.528	9.358	-
	Area (mAU min)	3.3499	2.165	
	Amount (mg L ⁻¹)	171.1765	112.084	
Vanillic acid	Retention time (min)	13.512	13.668	12.957
	Area (mAU min)	0.1514	0.532	0.403
	Amount (mg L ⁻¹)	7.8771	15.2019	2.321
Syringic acid	Retention time (min)	15.888	15.775	-
	Area (mAU min)	0.0758	0.7544	
	Amount (mg L ⁻¹)	5.1224	12.9180	
p-Coumaric acid	Retention time (min)	20.290	19.938	20.278
	Area (mAU min)	35.7026	13.2957	0.2142
	Amount (mg L ⁻¹)	260.4073	99.4731	5.5174
Ferulic acid	Retention time (min)	22.898	23.592	23.370
	Area (mAU min)	0.3081	0.5722	0.339
	Amount (mg L ⁻¹)	6.6290	9.7051	6.9942
Synapic acid	Retention time (min)	23.782	23.773	-
	Area (mAU min)	1.1934	0.3361	
	Amount (mg L ⁻¹)	35.0534	13.3618	

**a)****b)**

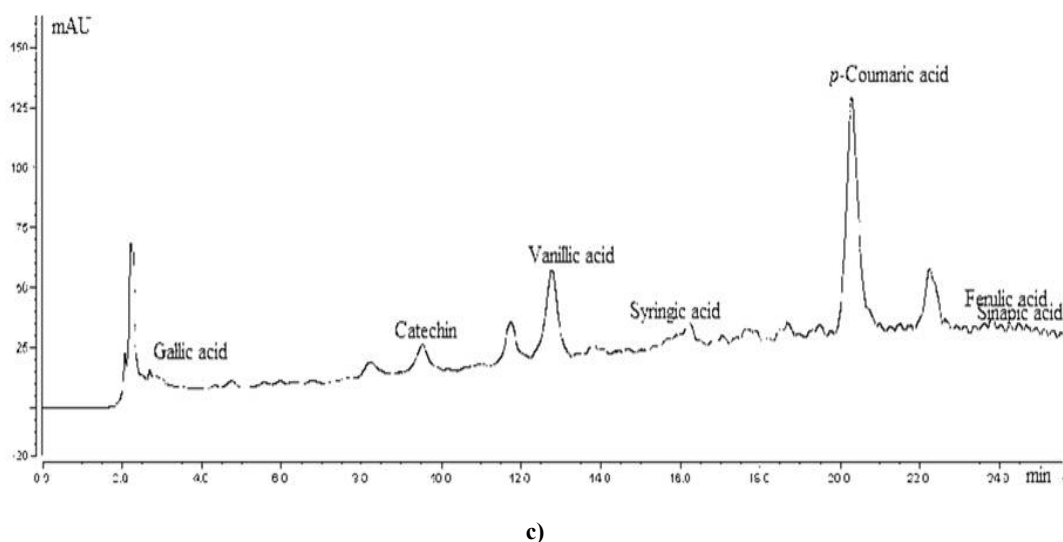


Fig. 5. Chromatographic profile of: a) Eth E polyphenolic extract; b) UAE polyphenolic extract; c) SFE polyphenolic extract

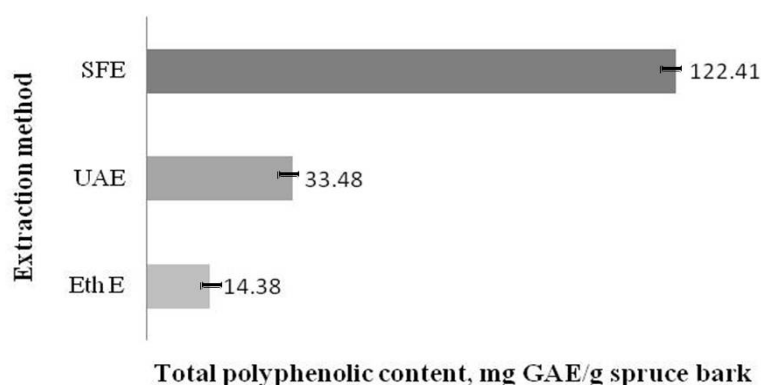


Fig. 6. Total polyphenolic content obtained by conventional and nonconventional extraction methods

Table 2. Comparative analysis between classical and non-conventional extraction techniques applied for polyphenols extraction from spruce bark.

Method Parameters	Eth E	UAE	SFE
Extraction time till maximum concentration (h)	144	1	1
Grams of spruce bark (g)	5	5	2
Volume of solvent (ethanol) ₀ (mL)	50	50	20
Total phenolic content (mg GAE g ⁻¹ spruce bark)	14.38*	33.48	122.41
Total phenolic amount by HPLC (mg L ⁻¹)	14.83	269.20	491.73

* Concentration of TPC after one hour of extraction

4. Conclusions

In this study different extraction techniques were used to extract polyphenolic compounds from spruce bark coming as a waste from forestry industry.

It was reported that applying supercritical CO₂ the total phenolic content extracted has a significant increasing from 14.38 mg GAE g⁻¹ spruce bark (ethanolic extraction, 60 min) to 33.48 mg GAE g⁻¹ spruce bark (UAE extraction) and to 122.41 mg GAE g⁻¹ spruce bark in case of supercritical extraction. Although SFE gives better extraction yields, allows obtaining high purity extracts and rich in terms of

polyphenolic compounds recovered, in a relatively short time extraction. This is a very important factor on operating with this technique because of the high running cost comparing with the conventional techniques and even UAE.

UAE it's also a rapid extraction technique (5-60 min), simple to operate, provides extracts with a composition similar to those obtained by SFE and have the most important advantage of a much lower operating costs.

The study recommend SFE and UAE instead of traditional ethanolic techniques, both at laboratory and pilot scale, since these provide high extraction

yields, pure extracts, with a large number of polyphenolic compounds extracted and are environmentally friendly.

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