

ANTIOXIDANT ACTIVITY OF SUPERCRITICAL EXTRACTS OF *POPULUS* BUDS

A. M. Mainar^{1,*}, E. Langa¹, B. Berrueco¹, C. Maestro², J. S. Urieta¹

¹ Group of Applied Thermodynamics and Surfaces (GATHERS). Aragon Institute for Engineering Research (I3A) University of Zaragoza. Faculty of Sciences, c/. Pedro Cerbuna, 12- 50009-Zaragoza (Spain)

² Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) ammainar@unizar.es

In the last years the supercritical fluid extraction has been frequently used to obtain volatile oils from aromatic plants. In this work we have applied this technique to a different vegetable matrix: poplar (*Populus nigra* and MC) buds as it is known that they contain antioxidant compounds. The results of the supercritical extraction have been compared with those of the conventional soxhlet extraction. Besides the activity of the natural antioxidants in the poplar bud extracts has been assessed by the spectroscopic method of the DPPH radical and the effective concentration (EC₅₀) in an hour has been calculated. The chemical composition of the extracts was determined using GC-MS chromatography.

INTRODUCTION

From the ancient times the healing properties that offer the buds, leaves and bark of poplar are known. In the case of the buds those properties could be attributed, in part at least, to the antioxidant activity of substances that they contain. The antioxidants are compounds that are currently being used to preserve food but they are increasingly demanded for health treatments. Then a possibility is open to use the residues generated in the pruning of poplars. In this frame, this work is aimed to study and compare the antioxidant activity of extracts obtained from buds of two varieties of poplar, *Populus nigra* and the hybrid known as MC. They will be also compared the extracts from buds of *Populus nigra* obtained with different techniques and solvents. Thus, soxhlet extraction with hexane and ethanol as well as supercritical fluid extraction with CO₂ have been used.

MATERIALS AND METHODS

Plant material

Buds of two different species of poplar, namely, *Populus nigra* and the hybrid variety MC have been used in this study. The buds were obtained from pruned branches of trees cultivated (controlled crops) in the territory of Zaragoza (Spain) by the CITA (Centro de Investigación y Tecnología Agroalimentaria de Aragón). The buds were let to dry at room temperature between sheets of “drying” paper (filter paper).

Chemicals

All solvents were of analytical grade: hexane ($\geq 99.0\%$) from Panreac, ethanol (99.9%) and dichlorometane ($\geq 99\%$) from Scharlau. 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma and Aldrich, respectively.

The CO₂ gas used for the supercritical fluid extraction was from Abelló Linde (purity 99.995%).

Equipment and methods

Soxhlet extraction

A mass of approximately 6 grams of buds is crushed at a low temperature reached by adding liquid nitrogen over the material. Then the product, placed in a little bag of inert material, is introduced in the body of the soxhlet extractor. Extractions were carried out during a period of 4 hours using either ethanol or hexane as solvents. Later the solvent was removed from the extract using a rotatory evaporator (Büche) at 40 °C and pressure below the atmospheric one. The dry extract was solved in 25 mL ethanol and stored at 4 °C in a phial hermetically sealed and covered with aluminium foil to avoid the attack of light.

Supercritical Fluid Extraction (SFE)

The SFE was made in a pilot plant built by the GATHERS Group [1] and optimised by E. Langa. Its main elements are the following: a compression pump (Milton Roy) with a filter (HiP), a cylindrical extraction vessel of 1 L (HiP), and two separators of 180 mL (Hoke). The pilot plant is completed with a 0.5 L cylindrical refrigerating jacket (Hoke) a heat exchanger (Haake), a back pressure valve (Tescom Corporation), and a flowmeter-totalimeter (Aalborg). Several valves HiP, and needle valves from Hoke, are used, as well as a pressure transducer (Omega), manometers (WIKA), thermopars (Kosmos), and temperature controllers (Omron). The conditions of the extraction process were a pressure of 180 bar, a temperature of 50 °C and a flow rate of 7 L/min. The pressures in the first and second separators were around 80 and 20 bar, respectively.

The experimental procedure is similar to the one described by Reverchon [2]. A mass of 77.8162 g of crushed buds was introduced in the extraction vessel and embedded symmetrically by inert porous materials, that allows a homogenous CO₂ flow to be achieved. The bed height of the vegetable material was 12.3 cm. Once the temperature in the vessel and separators was reached, the pump compressed the CO₂ up to the desired pressure, and by the action of the heat exchanger, temperatures above the critical one were obtained. When this supercritical fluid in the selected pT working conditions fills the reaction vessel, the extraction can be started. The conditions in both separators were different, so that the heavy compounds precipitated in the first separator, letting the volatiles to precipitate on their own in the second separator. In the first separator, pressures of 80 bars and temperatures ranging from -10 to 0°C were reached. In the second separator, pressures of 20 bars and temperatures ranging from 10 to 20 °C are used. The pressure in these separators was controlled by manipulating different valves and needle valves, and the low temperatures were reached due to the action of the refrigerating jacket. Waxes in the extracts are avoided by precipitating in the first separator. The extract in the second separator was collected and weighed (Mettler Toledo) at different time intervals in order to obtain an extraction curve.

Evaluation of the antioxidant activity of the extracts.

The spectroscopic method of the DPPH radical, according to the procedure described by Brand-Williams et al. [3] was used to evaluate the antioxidant activity of the extracts. In this method the decrease of the absorbance at 515 nm of a solution of the radical when adding an antioxidant substance is observed. The method employed determines the ability of the antioxidant to donor a proton to the DPPH. This neutralization can be monitored by means of visible spectrophotometry because it implies a change of color from purple to yellow, change that leads to a fall in the absorbance at 515 nm. The absorbance data were acquired through an UVIKON 941 UV-Visible spectrophotometer (Kontron Instruments). The first step was to determine the measurement conditions. After carrying out a study concerning the stability of the radical, it was decided to work with cuvettes thermostated at 25 °C. To reach this temperature the cuvette was maintained for 15 minutes into the measurement cell before adding the antioxidant. In all cases fresh solutions of DPPH were used.

Several previous works were carried out with solutions of Trolox (a commercially available antioxidant) in ethanol in order to verify the accuracy of the measurements obtained by comparing the results with those reported in the literature. Then the study continued with the extracts of poplar buds. The solutions of the extracts were prepared with ethanol as solvent for the extracts from the soxhlet method whereas dichloromethane was the solvent selected for the extracts from SFE because they were not solvable in a more innocuous liquid.

A suitable series of 4 dilutions of each of the extracts was prepared while 5 dilutions were used in the case of Trolox. The procedure for the measurements is the following: 50 µL of a dilution are added on 3.9 mL of a solution of 0.03 g/L of DPPH thermostated in the spectrophotometer, then the solution is stirred, introduced again in the instrument and the absorbance at 515 nm is continuously measured during the reaction.

The initial and final concentrations of DPPH in the cuvette were calculated from the calibration line: $A(515\text{ nm}) = 29.299 \cdot x(\text{g/L}) - 0.0075$ ($R^2 = 0.9996$) that was obtained through a linear regression of data corresponding to 5 different concentrations of the radical.

For each sample the remaining DPPH was defined as: $\text{DPPH}_{\text{rem}} = [\text{DPPH}]_{\text{final}} / [\text{DPPH}]_{\text{initial}}$. The amount of antioxidant is given as mg antioxidant/mg DPPH and, in the case of Trolox, it was also calculated as mol Trolox/mol DPPH. All the values of remaining DPPH obtained for each series of dilutions were plotted in a graphic versus the amount of antioxidant/extract and through interpolation the activity of the antioxidant expressed by the parameter EC_{50} (Efficient concentration) was estimated. EC_{50} is the amount of either extract or Trolox necessary to decrease a 50 % the initial concentration of DPPH in the steady state. The reaction reached the steady state in the case or Trolox but for the extracts the time needed to achieve the steady state exceeded 12 hours in some of the cases. For this reason and taking into account both the stability of the radical and the time available, measurements of 1 hour duration were carried out, that is, the EC_{50} for 1 hour was determined.

Chromatography

The chemical analysis of the extracts was done in Instituto Química Orgánica General, CSIC. A gas chromatograph Agilent 6890 (Palo Alto, CA, USA) coupled to a Mass Spectrometer with Quadrupole Analyzer (Agilent 5973) was used. An HP-1 methyl silicone column (25m x 0.2 mm i. d., 0.33 µm film thickness) (Supelco Inc, Bellefonte, PA, USA) was temperature programmed from 70 to 300 °C (at 4 °C/min, final hold time 20minutes). Helium at 0.6 ml/min was used as carrier gas. A total flow of 20.5 ml/min was reached, with a split flow of 17.6ml/min.

Injector temperature was 270 °C, with a 30:1 split relation. Mass spectra were recorded in the

electron impact (EI) mode at 70eV, scanning the m/z 35-450 range. Interface, source and quadrupole temperatures were 280, 230 and 150 °C respectively. Peaks in the TIC (total ion current) profiles were characterized or tentatively identified from their mass spectral data by using Wiley mass spectrometry libraries [4].

RESULTS

Soxhlet extraction

The yields in extracts obtained from the pretreated samples of buds after four hours of soxhlet extraction are gathered in table 1

Table 1. Yields of the soxhlet extractions

Variety	Solvent	g buds	g extract	yield
P.N.	Hexano	5.9681	0.4277	7.2%
P.N.	Etanol	5.9891	3.0254	50.5%
MC	Hexano	6.0920	0.7200	11.8%
MC	Etanol	6.0880	3.0185	49.6%

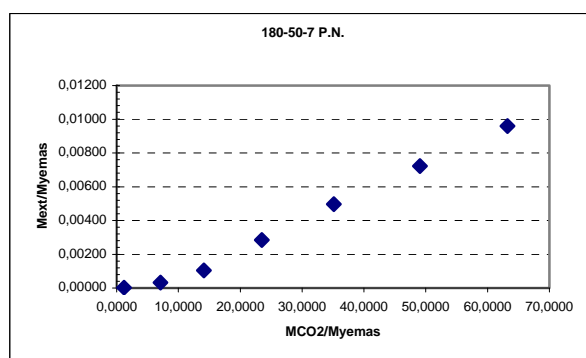


Figure 1. Extraction curve for volatile compounds of *Populus nigra*

Supercritical fluid extraction

As explained when treating of equipments and methods the extracts in the second separator was collected and weighed (Mettler Toledo) at different time intervals carrying out a fractional extraction that leads to extraction curve shown in figure 1.

Table 3. CO₂ volumes, masses extracted and yields for SFE

	Fraction	V (l) of CO ₂	M _{extracted} (g)	Yield
C2	1	52	0.0012	
	2	253	0.0234	
	3	302	0.0561	
	4	404	0.1405	
	5	500	0.1646	
	6	599	0.1760	
	7	610	0.1844	
	TOTAL	4222	0.7462	0.96 %
C1	TOTAL	4222	0.3475	0.53%

The volume of CO₂ used along with the masses of extract obtained in each fraction in the second separator (C2), the total masses extracted in the first (C1) and second separators and the global yield are shown in table 2. The total mass obtained in the two separators amounts to 1.49 % of the initial mass of buds.

Evaluation of the antioxidant activity of the extracts

The value of EC₅₀ for Trolox is compared with literature values in table 3

Table 3. EC₅₀ for Trolox. Comparison with literature values

	EC ₅₀ (mol Trolox / mol DPPH·)
Experimental	0.26
Literature ⁵	0.26
	0.24
	0.19

The values of EC₅₀ for the extracts of poplar buds are gathered in the following table:

Table 4. EC₅₀ for the different extracts along with the regression coefficients of the lines used for the interpolation

Sample	EC ₅₀ (g antioxidant/g DPPH·)	Regression coefficient (R ²)
P.N. soxhlet ethanol	0.32	0.9923
P.N. soxhlet hexane	1.65	0.9783
MC soxhlet ethanol	0.64	0.9921
MC soxhlet hexane	3.18	0.9930
P.N. SFE heavy	3.43	0.9947
P.N. SFE volatile	9.22	0.9914

The sample name indicates the species of poplar where the buds come from, the extraction method, the solvent used in the case of soxhlet and the separator where the extract was obtained in the case of SFE. The heavy fraction is the extract precipitated in the first separator and the volatile fraction is the extract obtained in the second separator. The regression coefficients for the the lines used in the interpolation are also included in the last column.

From its definition, it is clear that the smaller the value of EC₅₀ for a given substance the higher its antioxidant activity as a minor amount of the substance is required to reduce to the half the initial concentration of DPPH.

Chromatography

Chromatograms have been carried out for each of tthe extractions. In the SFE a chromatogram was carried out for the heavy extract and for the third fraction of the light extract. In all the cases the number of observed peaks that could be related to chemical compounds exceeds one hundred. Besides the major compound in all of the chromatograms is the same that has been identified as 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-(E)-2-propen-1-one by comparison of its mass with those in the Wiley library [4]. The composition obtained for the extracts of *Populus nigra* is similar to that reported by Jerković and Mastelić [6].

Comparing our chromatograms it can be observed that the SFE extracts are more similar to the soxhlet extracts with hexane. The difference arises in that the amount of compounds of

minor molecular weight is nearer the amount of compounds of higher molecular weight in the case of the soxhlet extracts whereas in SFE extracts the heavier compounds prevail.

CONCLUSION

From the exam of the results about the antioxidant activity of the extracts it can be concluded that the buds of *Populus nigra* are a better source of natural antioxidants than those of MC as the amounts of extract (EC_{50} values) of *Populus nigra* required for the same activity are approximately a half of those of MC. On the other hand, if the results of *Populus nigra* are compared with that obtained for Trolox ($EC_{50 (P.N)} = 0.32$ g extracto/g DPPH, $EC_{50 (Trolox)} = 0.16$ g Trolox/ g DPPH) it can be said that the extracts of poplar buds in ethanol are a powerful antioxidant because the same effect produced by a commercial antioxidant of high purity is achieved with a mass of extract that is only twice the mass of that commercial antioxidant and it must be taken into account that the extract has acted only for 1 hour while Trolox has reached the steady state.

Referring to the SFE, the comparison of the results obtained with those of soxhlet for both yield and antioxidant activity shows that the door is open for future works to optimise the extraction process. On the one hand, several variables such as pretreatment, amount of vegetable material, pressure, temperature or use of cosolvents could be modified to improve the yields. On the other hand, as the more active extracts are those in ethanol solutions, this alkanol could be used as cosolvent with the CO_2 to improve the antioxidant activity.

Summing up, it has been proved that the buds of *Populus nigra* are a source of antioxidant products. If the extraction process is optimised and the main antioxidant compounds in the extract are identified and isolated the residues generated in the pruning of those trees could be greatly revalorised.

The authors are grateful for financial support from MEC-FEDER (Project CTQ2006-15597-C02-02) and University of Zaragoza (Project UZ-2006-CIE-11). B. Berruero is grateful for a *Young Scientists Grant* from GATHERS.

REFERENCES :

- [1] LANGA, E. "Extracción con CO_2 supercrítico de aceites esenciales de plantas aromáticas" Ph. D. Thesis. Facultad de Ciencias, Universidad de Zaragoza, **2007**
- [2] REVERCHON, E. Journal of Supercritical Fluids, Vol. 5, **1992**, p. 256
- [3] BRAND-WILLIAMS, W., CUVELIER, M.E., BERSET, C. Lebensmittel Wissenschaft und Technologie, Vol. 28, **1995**, p. 25
- [4] MCLAFFERTY, F.W., STAUFFE, D.B., Wiley /NSB Registry of Mass Spectral Data, Wiley, New York, **1989**
- [5] BORTOLOMEAZZI, R., SEBASTIANUTTO, M., TONIOLO, R., PIZZARIELLO, A. Food Chemistry, Vol. 100, **2007**, p. 1481
- [6] JERKOVIĆ, I., MASTELIĆ, J. Phytochemistry, Vol. 63, **2003**, p. 109