Industrial Crops & Products 139 (2019) 111502

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Purification of industrial tannin extract through simple solid-liquid extractions

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ARTICLE INFO

Keywords:
Hydroxyl-aromatic
Flavonoid
Fractionation
Separation
Simple method
Carbohydrates

ABSTRACT

Acacia Mimosa or black wattle (*Acacia Mearnsii*) industrial tannin extracts are used as such for leather tanning, water flocculants, adhesives and many other applications. However, when higher purity of the polyphenols is requested, these extracts are not suitable anymore. Therefore, feasible strategies have to be developed in order to improve the purity of the extract. Solid-liquid extractions with common solvents were performed and the soluble and insoluble fractions of the tannin extracts in methanol and acetone were quantified and characterized through chemical analysis of phenolics, FT-IR, Py-GC/MS, LC-UV/ESI-MC and GPC. It was observed that the solvent soluble fractions were enriched of polyphenols, the insoluble residue after methanol extraction was the one presenting higher amount of hydrophobic compounds and the fraction soluble in acetone resulted purified of proteins. However, these encouraging findings suggest that this strategy does not allow to separate the phenolics from the saccharidic components which appear to be intimately interconnected.

1. Introduction

Very often the expression “tannin extract” is perceived as a synonym of “polyphenolic extractives”. However, the tannin extract is just the soluble fraction coming from the extraction process of wood and bark chips and it contains principally, but not exclusively, polyphenols. Therefore, the tannin extract contains also considerable amounts of non-phenolic components (20–30%), which are supposed to be mainly constituted of saccharides (easy sugars and hydrocolloids) (Venter et al., 2012). Further, the polyphenolics in the tannin extract can strongly differ depending on the bio-resource fraction extracted and on the extraction conditions applied. In general, a differentiation can be made between hydrolyzable tannins (derived from oak and chestnut), traditionally used in Europe and condensed tannins (derived from acacia mimosa and quebracho), mostly used in the austral hemisphere (Brazil, Argentina, Tanzania and Australia). Between them, the Acacia Mimosa, also named Black wattle (*Acacia mearnsii* formerly *mollissima*, *de Wildt*) is the most commonly extracted tannin and it is also extensively used, principally in the leather tannery row but also for water treatments (Sánchez-Martín et al., 2009; Sepperer et al., 2019), adhesives (Kain et al., 2014; Luckeneder et al., 2016), wood preservatives (Fukushima et al., 2009; Schnabel et al., 2014) and for manufacturing advanced materials like aerogels and lightweight foams (Grishechko et al., 2013; Link et al., 2011; Tondi and Pizzi, 2009).

When the plant extract is used for chemically advanced applications, such as pharmaceuticals or also for enhanced material synthesis, a more defined and purer source of polyphenols is needed.

Various chromatographic techniques, ranging from HPLC to UPLC, have been used to separate phenolic extracts. Several researchers succeeded in separating specific fractions from different bio-resources like grape seeds (Gabetta et al., 2000), Montgomery fruits (Bystrom et al., 2008) and Caesalpinia ferrea (Wyrepkowski et al., 2014). These studies highlighted the specificities for achieving a good separation between the retention time and some detection limitations. A review about polyphenols purification through liquid chromatography techniques coupled with different detection instruments suggests that every analyte requires a particular set up to be properly separated and identified (de Villiers et al., 2016). Other scientists have considered to apply solvent-based pre-treatments on the polyphenolic fraction in order to facilitate the characterization. Through selective extractions it was possible to separate the protein fraction from tannin extracts (Hagerman and Butler, 1980) and to obtain fractions enriched in polyphenols (Missio et al., 2017).

Further common procedures for crude plant extract purification

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https://doi.org/10.1016/j.indcrop.2019.111502
Received 12 February 2019; Received in revised form 14 June 2019; Accepted 22 June 2019

include counter current chromatography (Putman and Butler, 1985), column chromatography with a Sephadex LH-20 column (Roux and Maihs, 1960; Strumeyer and Malin, 1975) or silica (Khallouki et al., 2007) and Soxhlet extraction with different organic solvents (Missio et al., 2017). However, all these techniques are cost and time intensive and lead to contained yields. Therefore, a more efficient method for purifying tannin extracts has to be found.

The objective of this study was to investigate if easy, sustainable and industrially-viable solid-liquid extractions of commercial mimosa tannin performed with common solvents at room temperature allows the separation of tannin fractions with higher phenolic content or with specific purification grade.

2. Material & methods

2.1. Raw material and chemicals

Industrial tannin extract Weibull AQ was supplied by Tanac S.A. (Brazil). According to the manufacturer, Weibull AQ is a natural extract, rich in tannin colloidal structure and phenols. It is obtained by hot-water extraction of black wattle (*Acacia mearnsii*) bark. Solvents used were methanol (HPLC grade), acetonitrile (HPLC grade), technical acetone and ethyl acetate purchased from VWR (Germany), ethanol (96%), dichloromethane and 2-propanol from Roth (Germany). Reactants used were, sulphuric acid (96%) and sodium carbonate from Roth (Germany), vanillin (99%), aluminum chloride (99%) and gallic acid were from Alfa Caesar (USA), sodium nitrate and Folin-Ciocalteu's phenol reagent from Merck (Germany) and (+)-catechin monohydrate from Cayman Chemicals (USA).

2.2. Solid-liquid extraction

Sample preparation was performed by solid-liquid extraction at room temperature (20 °C). The general procedure is illustrated in Fig. 1. A total of seven solvents of different polarity were chosen for separation. The solvents were: dichloromethane, 2-propanol, ethyl acetate, ethanol, methanol, acetone, and acetonitrile. 1 g of the original tannin extract was dissolved in 100 ml of the solvent and magnetically stirred for one hour at room temperature for each fractionation. Using this weight/liquid ratio it was assured to avoid saturation of the solvent and therefore get a complete separation. The solutions were filtered (pore size 12-15 µm) and both, the soluble and insoluble fractions, were collected. The insoluble powders were dried at 103 °C for 48 h, the liquid soluble fractions were recovered by rotary evaporation. Finally, the weight was registered and the solubility was calculated for each solvent.

2.3. Total phenolic content

Determination of total phenolic content (TPC) was performed using a modified version of the Folin-Ciocalteu assay described by Ozturk et al. (Ozturk et al., 2018). In brief, 200 µl of the aqueous tannin solution (1 mg/ml) were mixed with 3 ml of deionized water and 500 µl of Folin-Ciocalteu's phenol reagent. The mixture was shaken for 3 min, then 2 ml of 20% sodium carbonate solution were added and shaken again. The mixture was left in the dark for 60 min. to incubate. Afterwards absorbance was read at 765 nm using Shimadzu UVmini 1240 spectrophotometer. Measurements were performed in triplicate. TPC was expressed as µgGAE/mg (GAE = Gallic acid equivalent). Calibration curve was created by stepwise dilution of a 1 mg/ml aqueous gallic acid solution into 500, 200, 100 and 50 µg/ml. R^2 of the calibration curve was 0.9990.

2.4. Total flavonoid content

A slightly modified version of the assay described by Xi and Yan (Xi and Yan, 2017) was used to evaluate the total flavonoid content (TFC). 250 µl of aqueous tannin solution (1 mg/ml) was mixed with 75 µl of 5% sodium nitrate solution and thoroughly mixed for 6 min. Afterwards 150 µl of 10% aluminum chloride solution were added and incubated for another 5 min. Finally, 500 µl of 1 N sodium hydroxide solution were added and the volume was adjusted to 2.5 ml using deionized water. Absorbance was measured at 510 nm and results were expressed as mgCE/mg (CE = Catechin equivalent). Quantification was performed in triplicate. Calibration curve was created by using a stepwise diluted aqueous catechin solution with concentrations of 30, 20, 10, 5 and 2.5 mg/ml. R^2 was 0.9999.

2.5. Total condensed tannin

Content of total condensed tannins (TCT) was evaluated by spectrophotometric determination of the transformation of condensed tannins into anthocyanidols under the presence of sulphuric acid and vanillin, as described by Sun et al. (Sun et al., 1998). In brief, 50 µl of the methanolic tannin solution (1 mg/ml) (in case of methanol insoluble, water was used) were mixed with 2 ml of 4% methanol vanillin solution and 450 µl concentrated sulphuric acid. After 15 min, absorbance was read at 527 nm and results were expressed as mgCE/mg. Measurements of the sample were repeated three times. The calibration curve was created with catechin methanol solution for 1, 0.5, 0.1 and 0.05 mg/ml. R^2 was 0.9994.

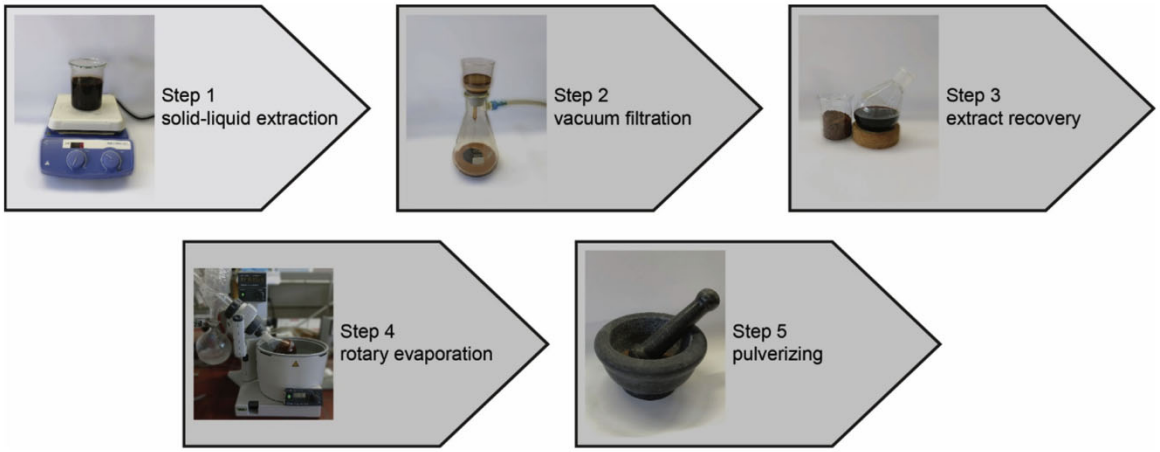

Fig. 1. General procedure of tannin fractioning.

2.6. ATR FT-IR analysis

Vibrational infrared spectroscopy was conducted for both, soluble and insoluble fractions, of each solvent as well as the original industrial extract using an ATR FT-IR spectrometer Frontier (Perkin-Elmer) and software PerkinElmer Spectrum 10.5.1. The powders were put in direct contact with the diamond. 32 scans of the spectral region between 4000 and 600 cm^{-1} were collected with a resolution of 4 cm^{-1}. The measurements were performed in triplicate. The spectra were then baseline corrected and area normalized with the software Unscrambler (CAMO software) and the spectra were investigated in the fingerprint region 1800–600 cm^{-1}.

2.7. Py-GC/MS

The tannin samples were subjected to pyrolysis-gas chromatography (GC)-mass spectroscopy (MS), to degrade them and break them down to specific lower bonding energy points forming smaller volatile fragments, under inert atmosphere. From these fragments, useful structural information of tannins can be provided. The analysis was performed with a 5150 Pyroprobe pyrolyzer (CDS Analytical Inc., Oxford, PA). Samples in the range between 400–800 µg were pyrolyzed in a quartz boat at temperatures of 500 °C for 15 s using a heating rate of 20 °C/ms and being the interface at 260 °C. The pyrolyzates were purged from the interface to the GC injector employing helium gas to achieve inert conditions. The GC was equipped with a 30 m x 0.25 mm x0.25 µm film thickness HP-5MS ((5%phenyl)-methylpolysiloxane) column. The GC oven program began at 50 °C (2 min) and increased to 120 °C (5 min) at a heating rate of 10 °C/min. Then the temperature was raised to 280 °C (8 min) and finally to 300 °C (10 min) both at 10 °C/min heating rate. Mass-charge ratio *m/z* was set to 200–1000. The identification of the pyrolysis products was achieved using a gas chromatograph coupled to a mass spectrometer (GC–MS) (Agilent Techs. Inc. 6890 GC/5973 MSD). The different compounds were identified comparing their mass spectra with the National Institute of Standards Library (NISL) and with those compound reported in the literature.

2.8. Protein analysis

For protein detection, the original industrial extract and the pulverized fractions soluble and insoluble in acetone and methanol were dissolved directly in 3 M Urea + Laemmli buffer containing beta-mercapto ethanol. A concentration of 50 µg/µl was obtained. To linearize potential proteins and to remove secondary structures, the samples were incubated at 95 °C for 10 min. Thereafter, they were cooled on ice and centrifuged to remove any insoluble particles. Protein analysis was performed by SDS-Page electrophoresis using an Any kDTM Mini-PROTEAN TGX Stain-FreeTM gel (Bio-Rad, Germany) by loading an amount of 0.75 mg tannin extract of unknown protein content onto the gel. The detection of the proteins was performed using the ChemiDocTM XRS + Imaging System (BioRad, Germany). The Precision Plus Protein™ Dual Color Standard (Bio-Rad, Germany) ranging from 250 to 10 kD was applied as molecular weight marker.

The volume intensity measurements of the protein were performed by Image LabTM Software (Bio-Rad, Germany).

2.9. LC-UV/ ESI-MS

The analysis of the five fractions were carried out using a UPLC Acquity (Waters) equipped with an Acquity C18 column (100 × 2.1 mm; 1.7 µm) at 40 °C. Gradient program was used with 0.1% formic acid (A) and methanol (B) and a flow rate of 300 µl/min. The samples were solubilized with concentration of 500 µg/ml and the injection volume was10 µL. Detector of the apparatus was a LCT Premier XE (Waters) mass spectrometer with Electrospray positive mode / W mode *m/z* range 100–2000 where capillar and cone volt 1000 and 100 respectively were set.

2.10. Gel permeation chromatography

Gel Permeation Chromatography (GPC) was performed to evaluate tannin average molecular weight (M_w), and molecular weight distribution (M_{WD}) using a JASCO instrument equipped with an interface (LC-NetII/ADC) and a refractive index detector (RI-2031Plus). Two PolarGel-M columns (300 mm x 7.5 mm) and PolarGel-M guard (50 mm x 7.5 mm) were employed. The flow rate was 700 mm^3 min^{-1} at 40 °C and N,*N*-dimethylformamide (DMF) was used as mobile phase. Calibration was made using polystyrene standards (Sigma-Aldrich) ranging from 70,000 to 266 g/mol.

2.11. Statistical analysis and data processing

ANOVA and Tukey's post hoc test were performed at a significance level of $\alpha = 0.05$ using OriginPro 2016 Software as well as IBM SPSS 25 statistics processor.

3. Results & discussion

Solid-liquid extraction processes were applied on Acacia Mimosa industrial tannin extract to obtain purified fractions. Common, inexpensive technical solvents were selected and the extraction yields are reported in Fig. 2.

The solubility of the industrial extracts generally increases by increasing solvent polarity. This result was expected because of the

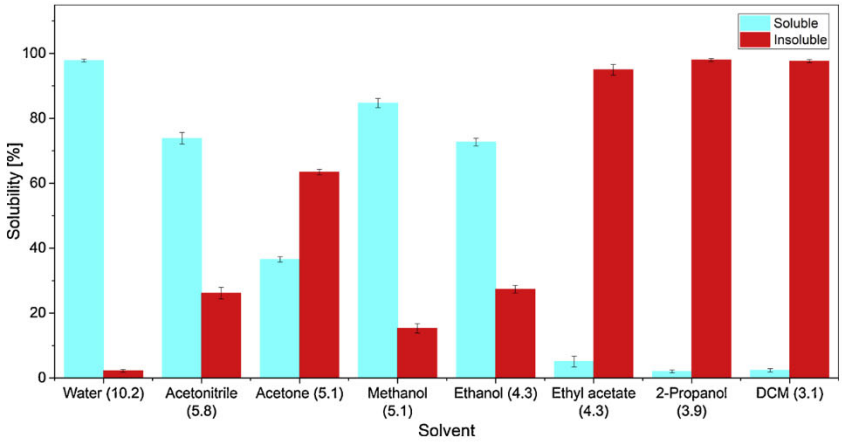

Fig. 2. Solubility of industrial tannin extract in common organic solvents.

abundance of hydroxyl groups in the polyphenols that allow to establish strong secondary forces (e.g. H−bonds, strong dipolar interaction) with polar solvents. Indeed, the only major exceptions to this trend were represented by ethanol and ethyl acetate. For the first one, despite the contained nominal polarity, the capacity of alcohols to establish H-bonds allowed higher solubility (72.7%) (Tessensohn et al., 2015). For the latter, the absence of hydroxyl groups and the lower relative polarity limits the solubilization of polyphenols (Missio et al., 2017).

Methanol and acetone were selected to prepare 4 fractions following the principles: i) average solubility (allowed to obtain consistent amount of the 2 fractions), ii) their chemical difference (we thought different molecules could be separated), iii) the contained solvent costs, iv) lower boiling points (< 65 °C)- lower solvent recovery costs and vi) other studies about polyphenol purification used these solvents(Kalt et al., 1999; Scalbert et al., 1989; Tasioula-Margari and Tsabolatidou, 2015; Zhang and Lin, 2008).

The four fractions, namely: Methanol soluble (MeS), methanol insoluble (MeI), acetone soluble (AcS) and acetone insoluble (AcI) and the original industrial extract (OIE) were chemically characterized by total phenolic content (TPC), total flavonoid content (TFC) and total condensed tannin (TCT) as well as through FT-IR, Py/GC–MS, LC-UV/ESI-MC and GPC. In Table 1 the findings of the lab-chemical analysis are summarized.

Considering the values of the OIE as reference, the purified fractions always presented opposite values, in particular when considering the soluble fractions (MeS and AcS) that always contained higher values for each parameter (TPC, TFC and TCT) suggesting that the hydroxyl-aromatic part concentrates mostly in the soluble fractions. The difference of these fractions was also confirmed by the mean comparison as they are all statistically significant different. The AcS fraction, in particular, presented the highest values for every analysis, while the MeI fraction showed the lowest values for TPC and TFC which means that these fractions are the more and the less hydroxyl-aromatic ones, respectively. These fractions, indeed, are the ones having lower yield (AcS 36.5% and MeI 15.3%) suggesting an accumulation of molecules of similar kind (more and less hydroxyl-aromatic). In terms of TCT there was no difference observable between OIE and the fraction insoluble in methanol.

The FT-IR spectra analysis (Fig. 3) showed that there is a similarity between the soluble fractions and the insoluble fractions. Major distances are observed in the regions 1230–1130 cm^{-1} and 1100–1000 cm^{-1}. In the former, the soluble fractions are more intense, while in the latter they are weaker. According to previous studies these two regions belong principally to aromatic C−O the former, and to aliphatic C−O and C−C and aromatic C−H the latter (Tondi and Petutschnigg, 2015). This confirms that the soluble fractions are richer in phenolics, while the insoluble fractions contain more aliphatic compounds.

The five fractions were analyzed also through Py/ GC–MS and the results are summarized in Table 2.

The pyrolysed components are relatively similar except for the fraction insoluble in methanol (MeI) which presents lower amounts of phenolics and more aliphatic acids as well as slightly less furanics and significant amount of other compounds such as squalene, styrene, toluene and high molecular mass residues. This suggests that methanol is a good solvent for separating the fraction of hydroxyl-poor residuals (e.g. plant oils, fatty acids, waxes, terpenes) from the raw mixture (Alén et al., 1996; Fabbri et al., 2005). The fraction soluble in methanol (MeS) concentrates the phenolics and slightly the furanics, meaning that the polyphenols and the carbohydrate fractions remain mostly together. This can be observed also for the acetone soluble and insoluble fractions suggesting that the carbohydrate and the phenolic fractions are intimately interconnected, possibly also through stable covalent bonds.

A considerable amount of pyridine is equally divided between the two fractions MeS and MeI. Pyridine is one of the pyrolysis product of proteins and this would suggest the presence of tannin-protein complexes in the extract (Fukushima et al., 2009).

The extraction with acetone differentiates principally for the strong presence of pyridine in the insoluble fraction highlighting that the protein fractions are selectively concentrated in the AcI fraction (Crowell et al., 2013).

To elucidate this aspect, SDS-Page electrophoresis was performed (see supplementary data). The detection of well-defined bands exclusively in the region < 10 kDa indicates the presence of low molecular mass proteins, whereas the highest intensity is observed in the AcI fraction.

As the method used for protein determination is based on UV-induced fluorescence detection after the interaction of a trihalo compound with tryptophan residues, these findings give evidence for tryptophan-containing proteins. Conversely, the lane of the AcS fraction shows just a faded band indicating that this fraction has no or very low amount of proteins. The lanes of MeS and MeI fractions show intermediate staining, also suggesting the presence of low molecular mass proteins. These findings corroborate the results obtained with py/GC–MS.

The LC-UV/ESI-MS analysis study confirms that the five fractions are very difficult to be separated. The five chromatograms are similar and present one major multiple peak and some smaller peaks at a higher retention time (Supplementary annexes). The LC-UV/ESI-MS spectra of the major retention peaks of OEI, MeS, AcS and AcI are similar and they lead to evident flavonoid fractions. Fig. 4 shows the mass spectra of the AcS fraction, some identified peaks are marked with a corresponding structure. With exception of the first peak (288 Da, proribotinoidin), catechin (290 Da) is always the starter unit for dimers with fisetinidol (274 Da) and robinetinidol (290 Da) as extender (Pasch et al., 2001). Furthermore, trimers with again catechin as initiator and robinetinidol or fisetinidol as extenders are observed.

Dimer, trimer, tetramer, pentamer and hexamer can be identified with predominant abundance of dimers and trimers for all four extracts. In these experiments, the settings were configured for optimizing the detection of polyphenols therefore the more aliphatic part contained in the MeI fraction could not be detected. MeS, MeI and AcI present also three small side peaks in the chromatogram that can be attributed to: Gallocatechin (Fabbri et al., 2005); Procyanidin dimer (Bystrom et al., 2008) and cyanidin-pentoside (Crowell et al., 2013).

GPC analysis of the fractions were performed and the weight average molecular weight (M_w), the number average molecular weight (M_n) and the polydispersity (M_w/M_n) registered are listed in Table 3.

The fraction insoluble in methanol (MeI) is not soluble in DMSO which confirmed the strong chemical difference observed also by wet chemistry experiments and Py-GC/MS. The extract soluble in methanol (MeS) presented a slightly higher molecular mass than the original

Table 1
Total phenolic content, total flavonoids and total condensed tannins of selected fractions.

Solvent	OIE	MeS	MeI	AcS	AcI
TPC [μgGAE/mg]	680.3[a] (7.473)	723.0[b] (4.545)	345.8[c] (3.318)	736.5[b] (3.473)	581.8[d] (7.918)
TFC [mgCE/mg]	20.326[a] (0.289)	22.953[b] (0.429)	16.216[c] (0.196)	27.099[d] (0.186)	18.047[e] (0.103)
TCT [mgCE/mg]	0.784[a] (0.001)	0.855[b] (0.006)	0.767[a] (0.005)	0.865[b] (0.001)	0.742[c] (0.019)

Note: Same superscript letter means no significant difference, Tukey (< 5%).

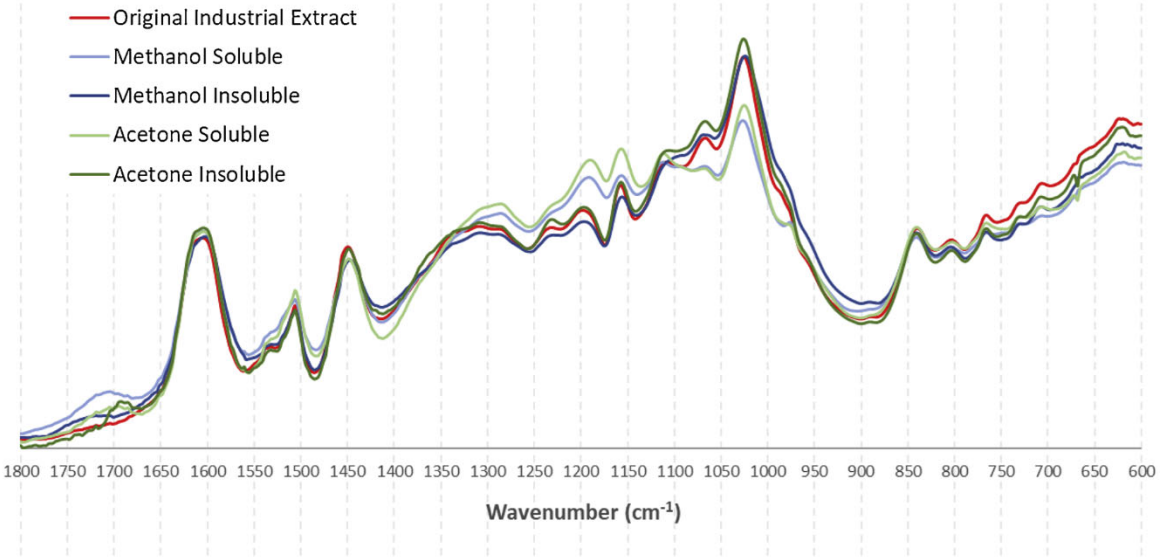

Fig. 3. ATR FT-IR spectra of the five extracts (OIE, MeS, MeI, AcS, AcI).

Table 2
Normalized relative percentage grouped by class of chemicals of the pyrolysis peak areas obtained by Py/GC–MS at 600 °C.

	OIE	MeS	MeI	AcS	AcI
Furanics (%)	10.94	13.98	11.41	14.47	14.17
Phenolics (%)	64.22	59.83	28.86	69.26	64.66
Alyphatic acid (%)	18.42	18.60	44.85	10.82	10.86
Pyridine (%)	6.42	7.58	7.17	0.00	8.98
Others (%)	0.00	0.00	7.71	5.45	1.33

Table 3
$\bar{M}_w$, $\bar{M}_n$, y $\bar{M}_w/\bar{M}_n$ of the global samples.

Sample	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
OIE	2541	763	3.328
MeS	3238	856	3.782
MeI[a]	–	–	–
AcS	2292	767	2.989
AcI	3218	838	3.841

[a] The fraction insoluble in methanol (MeI) was also insoluble in dimethylsulphoxide (DMSO).

extracts, which suggests that the fraction of MeI should have a lower molecular mass.

The fraction soluble in acetone was lighter and less poly-dispersed than the insoluble fraction. This means that the eventual presence of protein does not affect the molecular masses of the whole fraction.

In Table 4 the summary of the findings is reported.

4. Conclusions

A simpler method compared to known lab techniques (e.g. gel filtration) to purify the industrial tannin extracts has been presented and the soluble and insoluble fractions in methanol and acetone were investigated through analysis of phenolics, FT-IR, Py/GC–MS, protein tests, LC-UV/ESI-MS and GPC analysis. Results are summarized in Table 4. The fractions MeS, AcS and AcI presented high amount of polyphenols with majority of pro-robinetinidin units and also a significant amount of carbohydrates, which suggest a strong interaction of the latter with the polyphenols. Conversely, the MeI fraction was significantly enriched of aliphatic compounds.

In particular, the fraction soluble in methanol (MeS) presented higher amount of polyphenols with high molecular mass. This fraction might result suitable for polymer synthesis because it would require less hardener to be cured.

Conversely, the fraction insoluble in methanol (MeI) contains high amount of hydroxyl-poor molecules and significantly less phenolics.

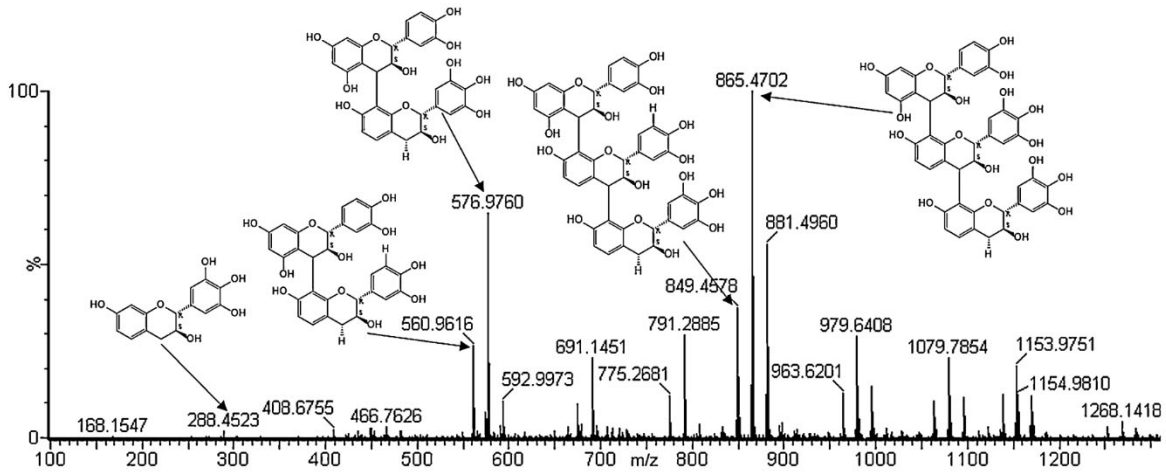

Fig. 4. Mass spectrum of major retention peak of AcS fraction.

Table 4
Summary of the findings per every analysis.

Fraction	Phenolic analysis	FT-IR	Py GC-MS	Protein	LC-UV/ ESI-MS	GPC	Description
MeS	+ Phenolic + Flavonoids + Cond. Tannin	+ C-O = > + Phenolics	+ Phenolics	Low amount of protein	Majority flavonoids	Higher Molecular weight	Phenolic rich fraction partially purified of aliphatics (oils, resins)
MeI	– Phenolic – Flavonoids - Cond. Tannin	+ C-H = > - Phenolics	–Phenolics, + + aliphatics	Low amount of protein	Presence of flavonoids	Insoluble, different chemistry	More aliphatic fraction, rich of resins, Poorer of phenolics
AcS	+ Phenolic + + Flavonoids + Cond. Tannin	+ C-O = > + Phenolics	No or very low amount of proteins	No or very low amount of proteins	Majority flavonoids	Lower Molecular weight	Low MW Phenolic rich fraction purified of protein
AcI	- Phenolic – Flavonoids - Cond. Tannin	+ C-H = > - Phenolics	+ Proteins	Certain presence of low MM proteins	Majority flavonoids	Higher Molecular weight	Higher Mw fraction enriched of the low MM proteins

"+" represents an increase, "-" indicates a decrease.

This residual has lower molecular mass, which may be an advantage for further purification to obtain aliphatic compounds such as terpens and wood resins. The fraction soluble in acetone (AcS) showed increased phenolic content and no or very low amount of protein suggesting that this fraction might have higher antioxidant activity and hence it could be suitable for cosmetic and pharmaceutical purposes. In contrast, the acetone insoluble fraction (AcI) contains higher amount of proteins with low molecular mass (< 10 kDa).

These easy separation processes did not carry to selective chemical separation, but the soluble fraction obtained by acetone can be appropriately selected for tailored applications due to their overall increase in phenolic structures and removal of proteins. Furthermore, technical acetone is a cheap solvent widely used and hence the fraction obtained by this separation method represent an added value for industrial process of tannin extraction and purification.

Acknowledgements

The authors would like to acknowledge the support from European funding INTERREG Italy-Austria for the project InCIMa ITAT 1023.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111502.

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