COEI-1-TANINS Oenological tannins

INS n° :181

1. Object, origin and field of application

Oenological tannins are extracted from nutgalls, or a wood rich in tannin: chestnut trees, oak, exotic wood, skin or seeds of the grape. Tannins are made up of a mixture of glucosides either from gallic acid (gallotannins), or from dilactone, ellagic acid (ellagitannins) (hydrolysable tannins) or from a mixture of proanthocyanidines (condensed tannins). Tannins are used to facilitate the clarification of wines and musts. Tannins must not change the olfactory properties and the colour of wine.

2. Labelling

The nature of the extraction solvent (water or alcohol), the botanical origin and an estimation of the total phenols contained must be clearly labelled.

3. Characteristics

Oenological tannins range in colour from pale-yellow to reddish brown, with an astringent taste. Tannins are partially soluble in ethyl acetate, water-soluble, ethanol and methanol for condensed tannins and insoluble in most organic solvents, with the exception of ethanol and methanol for hydrolysable tannins.

4. Identitifying characteristics

4.1. The aqueous solution of tannins produces, along with iron (III) salts, a blue/black precipitation between pH 3 and 5. This precipitation disappears with the addition of small quantities of strong acids.

4.2. The aqueous solution of condensed tannin precipitate gelatine, egg whites, blood serum, etc. with a pH level between 3 and 6. Tannins precipitate alkaloids (quinine, strychnine) with a pH level between 4 to 6.

5. Characterisation

It is possible to characterise the botanical origin with the aid of the following criteria: ultraviolet absorption spectrum, flavanol content, proanthocyanidines,
digallic acid, and scopoletine. (see appendix)

6. Test trials

6.1. Foreign matter

Tannin must be almost completely water-soluble and the content of insoluble substances should be under 2%, after shaking for 15 minutes 10 g of tannin in one litre of water.

6.2. Loss during drying

Determine the weight loss in an incubator at 100 – 105°C for 2 hours, of 2g of test solution. The weight must be constant and weight loss must be under 10%.

The limits below refer to the dry product.

6.3. Ashes

Incinerate progressively without going over 550 °C, the residue left over in the determination of loss during drying. The weight of the ashes should be under 4%.

6.4. Preparation of test solution

Take the ashes from 2 g of tannin by 1 ml of diluted hydrochloric acid (R) and one drop of concentrated nitric acid (R). Heat in 100°C water a little to dissolve. Pour this into a 50 ml volumetric flask. Rinse the capsule with distilled water and fill up the line on the flask.

6.5. Arsenic

Take 0.25 g of tannin, and determine arsenic using the method described in Chapter II by atomic absorption spectrometer, after destroying organic matter by the wet method. (Arsenic content must be under 3 mg/kg).

6.6. Iron

Add 2 ml of 5% potassium thiocyanate solution (R) and 1 ml of concentrated hydrochloric acid (R) to 10 ml of test solution prepared according to article 6.4. The resulting colour should not be more intense than the control sample prepared with 2ml of iron (III) salt solution at 0.010 g of iron per litre (R), 8 ml of water and the same volumes of the same reagents. If this is not the case, dilution of the test solution is required.

The iron content must be less than 50 mg/kg, with the exception of the iron content of chestnut-derived tannins, which should be less than or equal to 200 mg/kg and in which case, the test solution prepared according to 6.4 should be diluted as appropriate. It is also possible to measure the iron with the atomic absorption spectrometer.

6.7. Lead

Measure the lead in the solution prepared according to article 6.4 and using the method outlined in the Compendium of International Methods of Analysis of Wine and Musts by atomic absorption spectro-photometer. Content must be less than 5 mg/kg.

6.8. Mercury

Measure the mercury using the method outlined in Chapter II by atomic absorption spectrometer. Content must be less than 1 mg/kg.

6.9. Estimation of richness in total phenols

Total phenol richness is estimated according the method described in Annex 3.

For total phenols the results must be greater than 65%.

6.10. Nature of tannins

6.10.1. Proanthocyanidic tannins are estimated by the DMACH method: mix 5 ml of reagent (100 mg of dimethylaminocinnamaldehyde + 10 ml of 12 M HCl solution; after bring to 100 ml with methanol) to 1 ml of aqueous tannin solution (1g/l). Wait 10 minutes; take a reading of the absorbency at 640 nm on 1 mm optical path. The results are given in equivalent catechin. The result for condensed tannins must be greater than 10 mg/g.The nitrous acid method is used to estimate ellagitannins. Mix 1 ml of aqueous tannin solution (1 g/l), 1 ml of methanol and 160 µl of 6% acetic acid (m/v). Displace the oxygen by nitrogen sparging for 10 minutes, add 160 µl of 6% sodium nitrite (m/v) followed by a brief nitrogen sparging (1 mn), the tube is vacuum sealed and its reaction takes in 60 mn in water bath at 30°C. The intensity of the colour is measured by absorbency at 600 nm. The results are estimated in mg/g in equivalents of castalagine (ε_{600nm}: 983 g-1). For hydrolysable tannins and ellagic type, the result must be greater than 20 mg/g.

6.10.2. Gallic like hydrolysable tannins correspond to other categories of products, and test negatively to 6.10.1 and 6.10.2.

6.11. Extraction process

6.11.1. IS solubility indicator

It is expressed in % of solubility for 5 g of tannin in 100 ml of diethylether/ethanol (9/1, v/v) mixture. For tannins extracted from water, the indicator must be less than 5.

6.11.2. Iex extractability indicator:

$$IEx = (D.O_{370NM} \text{ X } 2) - (D.O_{350nm} + D.O_{420nm}$$

When lEx is greater than 0.05, the products come solely from extraction by water.

6.12. Colouring properties

Without prejudice to the provisions of paragraph 1, the use of oenological tannins changes the colour of wines to some extent, depending on their inherent colouring properties. Definitions are therefore required for yellow colouring properties on the one hand ($E^{420}_{1‰}$), corresponding to the absorbance at 420 nm of an oenological tannin trial solution of 1‰ dry matter (1g/l). The higher the index, the greater the yellow colour will influence the colour of the wine.

Red colouring properties on the other hand (E^{520}-$E^{420}_{1‰}$).) correspond to the difference in colouration between the yellow, measured at 420 nm, and the red, measured at 520 nm, of a 1‰ oenological tannin solution: the tannin is colouring agent when the index becomes positive ($E^{520} > E^{420}$).

Oenological tannins are solubilised in a water/ethanol mixture (50/50 v/v). Absorbences are measured at a 1 cm optical thickness. The measurements are taken immediately after solution treatment. Under these conditions, an oenological tannin should give a clear solution.

The limits of these indices for a oenological tannin not to be considered as a colouring agent are:

- + 1.5 for yellow colouring properties ($E^{420}_{1‰}$) and
- + 0.05 for red colouring properties (E^{520}- $E^{420}_{1‰}$).

7. Storage conditions

Oenological tannins must be kept in sealed closed packages.

Annex: Identification of the botanical origins of oenological tannins

1. Materials and methods

1.1. Principle

The recognition of the botanical origin of oenological tannins requires the formulating of the following observations in order:

1°) The presence of condensed tannins taken from grapes,

2°) The presence of tannins from nutgalls,

3°) The presence of tannins from exotic wood,

4°) Differentiating the tannin from oak and the tannin for chestnut wood.

- Tannins from grapes is characterized by high content of flavanols, as expressed in (+) catechin.
- Nutgall tannins have a high content of digallic acid.
- The ultraviolet spectrum for tannins from exotic wood has a specific peak.
- Tannins from oak trees are richer in coumarines, in particular scopoletine, than chestnut tannins.

1.2. Equipment and analytical conditions

- Laboratory glassware.
- Magnetic mixer.
- UV/visible absorption spectrophotometer double beam.
- 1 cm optical pathway glass cuvette
- 1 cm optical pathway quartz cuvette,
- 100° C water bath (optional)
- Heated rotating evaporator
- Composed chromatographic system (as an example):
    - pressure gradient pump for binary mixtures
    - an injector equipped with a 20- µl loop
    - a spectrophotometer detector with wave length 280 nm
    - a fluorimetric detector
    - An reversed phase column (C18) diameter of particles 5 µm, dimensions of the column: 20 cm X 4.6 mm to measure the gallic acid and the scopoletine.
- pH meter.

1.3. Reagents and reference solutions

- para-dimethylaminocinnamaldehyde

- concentrated hydrochloric acid solution(R)
- (+) catechin
- digallic acid
- absolute ethanol
- ethyl acetate
- concentrated sodium hydroxide solution(R)
- methanol
- ethyl ether
- acetonitrile
- acetic acid
- scopoletine
- umbelliferone
- distilled water or demineralised or ultra filtered water.

1.4. Preparation of reagents

p-dimethylaminocinnamaldehyde (p-DACA) solution

100 mg of p-DACA are put into a solution of 10 ml 12 M hydrochloric acid and 90 ml of methanol

Elution solvents for digallic acid

- solvent A: pure methanol
- solvent B: perchloric acid solution in water at pH 2,5

Elution solvents for scopoletine

- solvent A: distilled water containing 3% acetic acid
- solvent B: acetonitrile containing 3% acetic acid

1.5. Preparation of reference solutions

- (+) catechin solution
- Dissolve 10 mg of (+) catechin in 1 l of distilled water
- Digallic acid solution at 100 mg/litre of distilled water

- Scopoletine solution at 20 µg/litre of distilled water.

1.6. Operating methods

There are 2 methods for identifying the presence of grapes tannins*:*

- Measuring total flavanols.

5 ml of p-DACA reagent are added to 1 ml of aqueous solution at 200 mg/l of tannin.

After 10 mn measure the absorption of the mixture at 640 nm in a glass cuvette with an optical path of 10 mm.

The absorbance values are then read from the calibration curve obtained from an increasing concentration range in (+) catechin analysed under the same conditions.

- Measuring proanthocyanic tannins.

Add 2 ml of distilled water and 6 ml of 12 M hydrochloric acid to 4 ml of solution of 200 mg/l of tannin in a hydrolysis tube. This tube is heated to 100 °C for 30 mn and cooled in a cold bath.

A second tube containing the same mixture stays at room temperature for the same amount of time.

Then, 1 ml of ethanol is placed in both tubes and the absorbance values are measured at 550 nm.

The difference between the 2 absorbance values is multiplied by 380 to give the Proanthocyanic tannin content.

Identification of tannins from nutgall

20 ml of aqueous tannin solution at 50 mg/l is brought to pH 7 with the aid of a concentrated sodium hydroxide solution (R).

An initial series of extractions carried out 3 times 20 ml of ethyl acetate to eliminate neutral substances.

Secondly, the aqueous state is brought to pH 2 by the addition of concentrated hydrochloric acid solution (R). and then followed by a new series of 3 extractions with ethyl acetate.

After the evaporation of the ethyl acetate, the residue is taken by 20 ml of methanol then analysed by chromatograph under the following conditions: (as an example):

- injected volume: 20 µl of extract or standard digallic acid solution
- Detection at 280 nm
- Composition of an elution gradient:

- from 10 to 20% of solvent A in 35 mn
- from 20 to 40% of solvent A in 15 mn
- from 40 to 98% of solvent A in 20 mn

- Mobile phase flow: 0.8 ml/mn.

Identification of tannins from exotic wood

Prepare an aqueous solution of tannin so that when placed in a 1 cm optical pathway quartz cuvette. The solution has an absorbency measured at 280 nm between 1 and 1.5.

Carry out a continuous absorbency readings between 250 and 300 nm.

Note the presence or the absence of a maximum absorption peak.

Identification of tannins from oak or chestnut

Scopoletine contained in the 20 ml aqueous solution of tannin at 5 g/l is extracted 3 times with 20 ml of ethylic ether.

After the total recuperation and evaporation of the ether phase, the extract is taken from 50 ml of water and then analysed by chromatography under the following conditions: (as an example):

- Injected volume: 20 µl of extract or scopoletine reference solution
- fluorimetric detection:
  - excitation wavelength: 340 nm,
  - emitting wavelength: 425 nm
- Composition of an elution gradient:
  - 94% of solvent A during 10 mn
  - from 94 to 85% in 20 mn
  - from 82 to 67% in 5 mn
  - from 37 to 42% in 5 mn.
- Mobile phase flow: 1 ml/mn

2. Conclusion

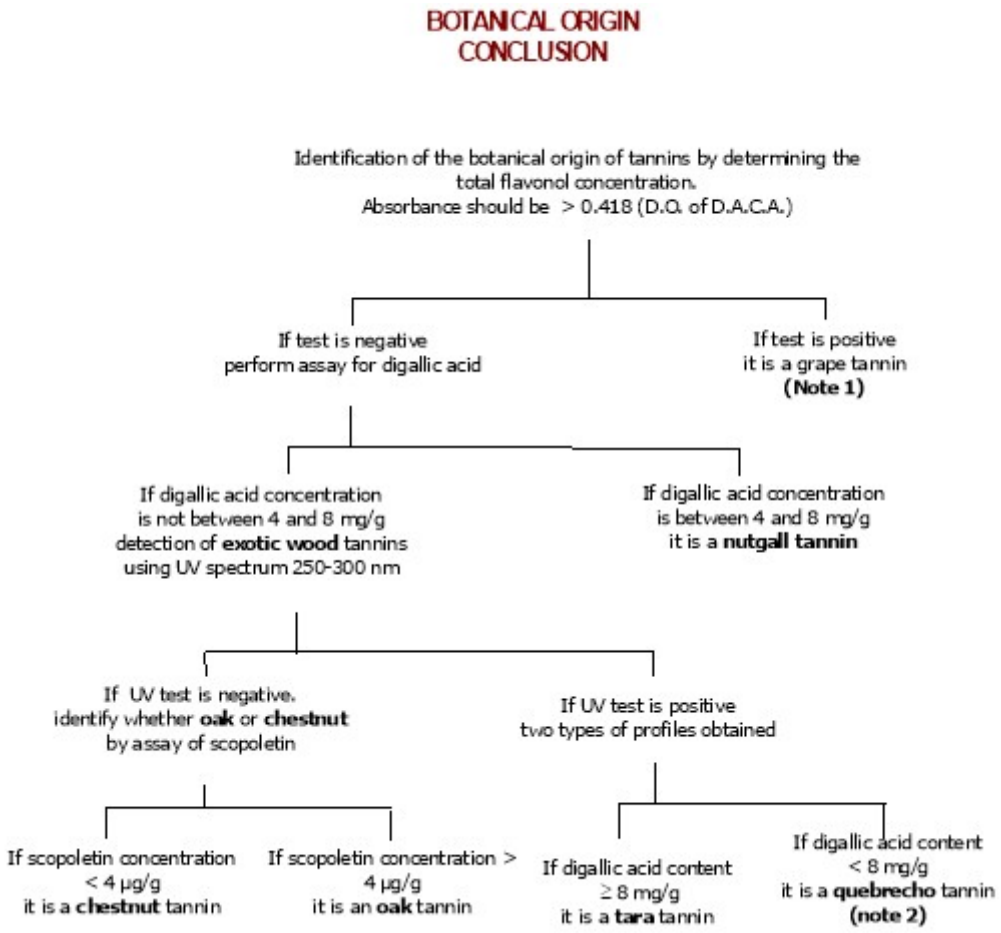
Tannin is recognised as being from grapes when the total flavanol content, expressed as (+) catechin is over 50 mg/g or its proanthocyanic tannin content is over 0.5 mg/g.

Tannin is recognized as coming from nutgall when digallic acid content is between 4 and 8 mg/g.

Tannin is recognized as coming from exotic wood when its spectrum reveals an absorption peak between 270 and 280 nm.

Tannin is recognized as coming from oak when scoploletine content is over 4 µg/g

Tannin is recognized as coming from chestnut trees when its scopoletine content is equal to or less than 4 µg/g and if it is not identified as coming from another origin.



Note 1

Grape tannins are formed from 3-flavonol units, which can be released by thiolytic cleavage of the flavonol intermonomer linkages in proanthocyanidols under heat in an acid medium. The monomers thus released are then separated and assayed using HPLC. This means that the procyanidols and prodelphinidols can be quantified

separately. This method is used to identify tannins from grape skins, stems and seeds. Under these conditions, Quebracho tannin does not produce a peak (see method and diagram below).

3. Differentiation Method for proanthocyanidin tannins by HPLC

3.1. Definition

Identification of Quebracho, grape skin and grape seed tannins

3.2. Apparatus and methods

Apparatus and test conditions

- 1 ml straight-sided pipette with 0.05 ml calibrations
- 10 ml volumetric flask
- HPLC system

Must be equipped with: a pump with the capacity for extremely precise constant or programmmed flow-rate or, and a 20 µl sample loop.

A C18 type reversed-phase column, with a particle diameter of for example 10 µm.

Length: 250 mm; internal diameter: 4.6 mm.

A UV/visible detector.

- Oven
- 10 ml teflon-stoppered hydrolysis tubes
- Cellulose ester filters, pore diameter 0.45 µm
- Vacuum filtration system
- 1000 µl automatic pipette
- Analytical balance to 1 mg

3.3. Reagents and calibration solutions

- HPLC grade methanol
- Distilled water
- Toluene- α -thiol (CAS 100-53-8) 99%
- Hydrochloric acid (12M) 37%

- Phosphoric acid 84%

3.4. Preparation of reagents

- Preparation of solvents for HPLC:

Solvent A: into a 1l volumetric flask, introduce 1ml phosphoric acid and bring up to volume with distilled water which has been previously filtered in a vacuum filtration system.
Solvent B: into a 1l volumetric flask, introduce 1ml phosphoric acid and bring up to volume with methanol that has been previously filtered in a vacuum filtration system.

- Methanol containing 1.7% HCl: into 10 ml methanol, introduce 140 µl hydrochloric acid, using a 1000 µl automatic pipette.
- Thioacidolysis reagent = 5% toluene- α-thiol solution: into 10 ml of the solution, introduce 470 µl toluene- α-thiol using a 1000 µl automatic pipette.
- Oenological tannins (commercial preparations)
- Tannin solutions at 1 g/l: 10 mg tannins are introduced into 10 ml methanol.

3.5. Procedure

0.5 ml of tannin solution and 0.5 ml of the thioacidolysis reagent (5% toluene-□-thiol solution) are introduced into a hydrolysis tube. The mixture is stirred and heated at 60°C for 10 min. The tube is then cooled and 0.5 ml distilled water added.

The sample is analysed using HPLC on a C18 reversed-phase column. The eluents used are solvents A and B. The elution sequence is as follows: from 70% (for 5 min.) of solvent B to 10% in 40 min., then from 10 to 70% (for 5 min.) in 10 min. (return to initial conditions). The flow-rate of 1ml/min is constant for the whole sequence and the wavelength used is 280 nm.

The peaks are identified and respectively quantified according to the data provided by *Vivas et al.* (2004)*.

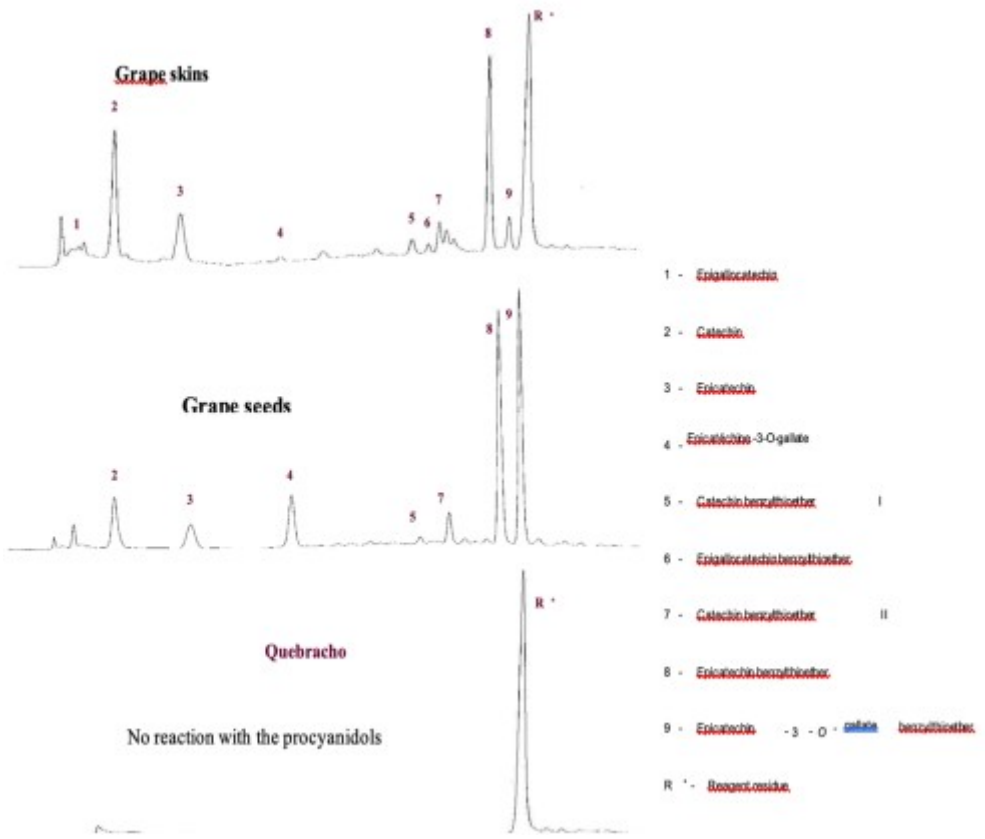
Tannins from seeds, skins and Quebracho have different profiles. Grape seed tannins are composed exclusively of procyanidols, and are identified by a high galloylation level, a high epicatechin content and a low mean degree of polymerisation (MDP). Skin tannins are identified by a combination of procyanidols and prodelphinidols, with a predominance of procyanidols, a low level of galloylation, a significant quantity of epicatechin and a variable MDP. Quebracho tannins do not produce any 3-flavonols. It is therefore possible to determine their composition in terms of proanthocyanidol tannins.

** N. VIVAS, M.F. NONIER, N. VIVAS de GAULEJAC, C. ABSALON, A. BERTRAND, M. MIRABEL, "Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (Vitis Vinifera) and heartwood of Quebracho (Schinopsis balansae) by MALDI-TOF/MS and thioacidolysis/LC/methods", Analytica Chimica Acta, 2004, 513, Issue 1, 247-256.*

Note 2

Identification of Quebracho as the botanical origin of a tannin is achieved by a process of elimination. Formal identification of the presence of Quebracho-derived tannin can be made using HPLC in combination with mass spectrometry (MALDI-TOF). The latter shows that the monomer constituents of this tannin are obtained from fisetinidol and robinetinidol, which have no hydroxyl in 5- position on the atomic nucleus (in other words grape-derived tannins are formed from monomers which have a trihydroxyl nucleus (phloroglucinol) whereas Quebracho-derived tannins are formed from monomers with a dihydroxyl nucleus (resorcinol).

Fig .1 Chromatograms of proanthocyanidols from grape skins and seeds and Quebracho, obtained by HPLC after thiolysis



Annex 2: Differentiation of commercial enological tannins by GC-MS analysis of monosaccharides and polyalcohols

1. Introduction

According to the International Enological Codex of the O.I.V., the enological tannins should be extracted from gall nuts (of Quercus, such as Aleppo galls, and of Tara, also called Caesalpina Spinosa), oak wood (Quercus sp.), grape seeds and skins (Vitis vinifera) and the wood of certain trees such as quebracho (Schinopsis balansae) and chestnut (Castanea sp.).

2. Scope

The method described here is suitable for the differentiation of commercial enological tannins from different origins (plant galls, seed and skin grape, oak wood, chestnut and quebracho).

3. Principle

The concentration of monosaccharides (arabinose, xylose, fructose and glucose) and

polyalcohols (arabitol, quercitol, pinitol, chiro-inositol, muco-inosotol, scyllo-inositol and meso-inositol) in tannin samples was determined by gas chromatography-mass spectrometry (GC-MS) after their previous derivatization into their trimethylsilyl ethers.

4. Reagent and materials

Reagents

- Trimethylsilylimidazole (TMSI) 97 % pure
- Trimethylchlorosilane (TMCS)
- Dried pyridine 99.5 % pure
- High purity water produced in a Milli-Q synthesis A10 system

Standards

- Phenyl- β-glucoside (internal standard): 1 mg/mL prepared in 70 % methanol

Preparation of the standard solutions (of monosaccharides and polyalcohols)

Standard solutions of glucose, fructose, arabinose, xylose, arabitol, pinitol, meso-inositol, scyllo-inositol, muco-inositol and chiro-inositol were disolved in methanol: water 30:70 at concentrations varying between 0.05 and 0.5 mg/mL of each standard. As quercitol and bornesitol are not commercially available, aqueous extracts were prepared from oak acorns of Quercus sp. and from leaves of Echium vulgare. The extracts were evaporated at low temperature under vacuum, silylated and injected as described below. Carbohydrate composition (in triplicate, RSD 5 %) of oak extract was 68 % quercitol, 20 % fructose and 18 % glucose and 20 % fructose, 33 % glucose, 27 % bornesitol, 2 % meso-inositol and 19 % saccharose for the Echium extract.

Note: All standard solutions have to be prepared working daily and preferably stored cold in a refrigerator prior to injection. All samples have to be derivatised and analysed in the day.

5. Samples

Twenty eight samples of different commercial tannins, including oak wood (O; n=4), grape seed (S; n=6), grape skin (H; n=2), plant galls (G; n=6), chestnut (Ch; n=3), quebracho (Q; n=3), gambier (GMB; n=1) and mixtures of grape+quebracho (GQ; n=1), quebracho+chestnut+plant gall (QChG; n=1) and chestnut+quebracho (ChQ; n=1) tannins, were directly purchased in the market or supplied by the manufacturers.

6. Apparatus

- Fume cupboard
- Laboratory glassware: beakers, vessels, etc.
- Micropipets
- Rotaevaporator
- Vortex
- Domestic mill
- Centrifuge
- Gas chromatograph equipped with a flame ionisation detector (FID)
- Gas chromatograph coupled to a quadrupole mass spectrometry detector operating in electronic impact (EI) mode at 70 eV. MS data were registered from 40 to 700 m/z.
- Column: 25 m x 0.25 mm i.d. x 0.25 µm film thickness fused silica column coated with crosslinked methyl silicone.

7. Procedure

Derivatization procedure

50 mg of tannins are dissolved in 5 mL of deionized water and filtered through Whatman No. 1 or similar filter paper. 1 mL of the sample is mixed with 1 mL of phenyl-□-glucoside, as internal standard. This mixture is evaporated under vacuum and trimethylsilyl derivates were formed by addition of 100 µL of anhydrous pyridine, 100 µL of TMSI and 100 µL of TMCS, shaking after each addition. Extraction of the trimethylsilyl (TMS) derivatives is carried out using 100 µL of hexane and 200 µL of water.

GC analysis

1 µL of the hexane upper layer is injected on the GC. Identity of each compound is confirmed by comparison of their retention times and mass spectra using GC-MS method with those of standards. The typical chromatographic profile of each tannin origin is shown in Figure 1.

GC-FID analysis: chromatographic conditions

Injections are made in splitless mode. Injector and detector temperature are 300 °C. Oven temperature is maintained at 100 °C for 1 min, then programmed with a heating

rate of 30 °C/min up to 200 °C kept for 15 min and finally programmedat a heating rate of 15 °C/min up to 270 °C maintained for 20 min. Carrier gas is nitrogen.

GC-MS analysis: chromatographic conditions

Injections are made in splitless mode. The injector is at 300°C and the oven temperature is maintained at 100 °C for 1 min, then programmed with a heating rate of 30 °C/min up to 200 °C kept for 15 min and finally programmed at a heating rate of 15 °C/min up to 270 °C maintained for 20 min. Carrier gas is He at 1 mL/min.

8. Calculation (Results)

Quantitative analysis is carried out using the response factor (RF) of each standard relative to phenyl- □-D-glucoside (internal standard) over the expected range. Reproducibility of the method is evaluated analyzing one sample on five different days. However this method does not allow to distinguish quebracho tannins from those of skin grape.

For example the limits of detection (LOD) and quantification (LOQ) (Tables 1 and 2) are calculated for each compound according to Foley and Dorsey (1984). Mean

values of 0.42 ng and 1.41 ng injected were obtained for LOD and LOQ, respectively. Concentrations of polyols and monosaccharides in tannins analysed are respectively in tables 3 and 4.

This method allows the classification of tannins according to the scheme suggested in Figure 2. The presence of quercitol is indicative of tannins from oak wood, whereas pinitol is mainly indicator of tannins from tara galls and bornesitol of tannins from gambier. The absence of arabinose and xylose in gall tannins can also help to the characterization of these samples. Therefore, bornesitol, quercitol, pinitol, arabinose and xylose could be used to unequivocally differentiate these products, and furthermore, to distinguish these tannins from the rest of the products analyzed. Tannins from galls and grapes can be easily differentiated from tannins of other origins due to the absence of arabinose and xylose in their monosaccharide composition. Referring to grape tannin samples, fructose could be observed in seed grape tannins, whereas it was absent in skin grape tannin. The presence of muco- and chiro-inositol could be useful to distinguish tannins from chestnuts from those of quebracho or grape skin.

9. Bibliography

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Table 1. Repeatability of the GC method for the determination of carbohydrates in tannins (sample Q3).

	Mean value	Standard deviation
Xylose	0.17	0.01
Arabinose	0.43	0.03
Arabitol	0.04	0.00
Quercitol	0.00	0.00
Fructose	0.32	0.04
Glucose	0.60	0.02
Muco-inositol	0.02	0.00
Chiro-inositol	0.00	0.00
Scyllo-inositol	0.00	0.00
Meso-inositol	0.05	0.00

Table 2. Limit of detection (LOD) and of quantification (LOQ) of the GC method for the determination of carbohydratesand of plyols in oenological tannins samples by means of gas-chromatography (expressed in injected ng)

	LOD (ng)	LOQ (ng)
Xylose	0.50	1.66

Arabinose	0.66	2.21
Arabitol	0.21	0.70
Fructose	1.11	3.70
Glucose	0.51	1.70
Muco-inositol	0.16	0.52
Chiro-inositol	0.22	0.74
Scyllo-inositol	0.20	0.68
Meso-inositol	0.24	0.80

Table 3. Concentration of polyols (mg/100g) in commercial tannins mg/100g

a) Arabitol, ***b)*** Quercitol, ***c)*** Pinitol, ***d)*** Bornesitol, ***e)*** Muco-inositol, ***f)*** Chiro-inositol, ***g)*** Scyllo-inositol, ***h)*** Meso-inositol

	a	***b***	***c***	***d***	***e***	***f***	***g***	***h***
Oak wood								
O1	0.06	6.92	-	-	0.10	0.10	0.52	0.49
O2	0.06	4.49	-	-	0.11	0.11	0.57	0.55
O3	0.05	1.57	-	-	0.04	0.02	0.13	0.12
O4	0.09	3.14	-	-	0.14	0.17	0.17	0.30
Gall plant								
G1	-	-	0.73	-	-	-	-	-
G2	-	-	0.26	-	-	-	-	tr
G3	-	0.03	0.07	-	-	-	0.03	tr

G4	-	0.06	0.06	-	-	-	0.04	-
G5	-	-	1.35	-	-	-	-	0.02
G6	-	-	-	-	-	-	-	-
Seed grape								
S1	-	-	-	-	-	-	tr	0.16
S2	-	-	-	-	-	-	tr	0.01
S3	-	-	-	-	-	-	0.38	2.34
S4	-	-	-	-	-	-	tr	0.01
S5	-	-	-	-	-	-	-	0.01
S6	0.64	-	-	-	-	-	tr	0.25
Skin grape								
H1	-	-	-	-	-	-	-	-
H2	-	-	-	-	-	-	-	tr
Chestnut								
Ch1	0.08	-	-	-	0.14	0.55	-	0.62
Ch2	0.04	-	0.49	-	0.03	0.33	-	0.05
Ch3	0.07	-	-	-	0.19	0.52	-	0.49
Quebracho								
Q1	tr	-	-	-	-	-	-	0.01
Q2	0.02	0.05	0.09	-	-	-	-	tr

Q3	0.03	-	-	-	0.02	-	-	0.05
Gambier								
GMB	0.01	-	tr	0.02	-	-	-	0.03
Grape+quebracho								
GQ	0.10	-	0.19	-	0.02	0.06	-	0.07
Quebracho+chestnut+gall								
QChG	0.03	-	0.19	-	0.03	0.12	-	0.12
Chestnut+quebracho								
ChQ	0.05	-	-	-	0.13	0.56	-	0.53

tr= traces

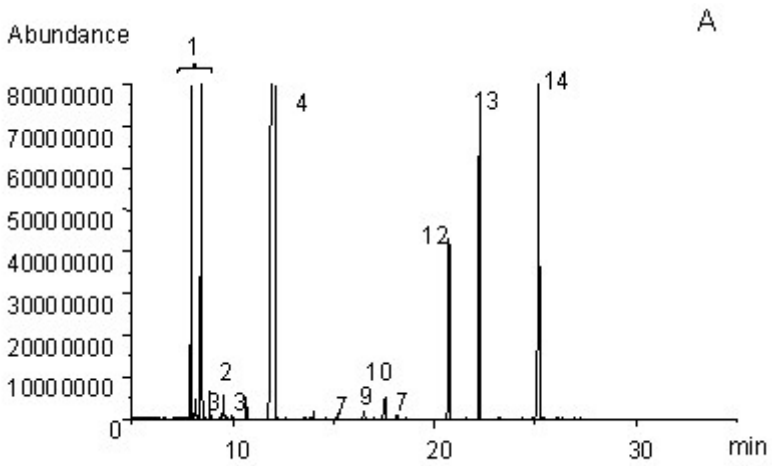
Table 4. Concentration of monosaccharides (mg/100g) in commercial tannins mg/100g

	Xylose	Arabinose	Fructose	Glucose
Oak wood				
O1	0.29	1.18	-	0.22
O2	0.57	2.53	-	0.07
O3	0.37	0.85	0.12	0.58
O4	0.41	1.84	1.82	2.69
Gall plant				
G1	-	-	0.26	0.42
G2	-	-	0.07	0.17

G3	-	-	0.05	0.05
G4	-	-	0.11	0.16
G5	-	-	0.50	0.63
G6	-	-	-	-
Seed grape				
S1	-	-	10.01	9.59
S2	-	-	0.64	0.50
S3	-	-	45.23	32.46
S4	-	-	0.61	0.46
S5	0.13	-	-	0.03
S6	-	-	1.22	tr
Skin grape				
H1	-	-	-	0.07
H2	0.31	0.48	0.30	0.67
Chestnut				
Ch1	0.50	1.46	1.15	0.78
Ch2	0.41	1.04	0.95	0.91
Ch3	0.65	1.55	0.28	0.69
Quebracho				
Q1	0.30	0.44	0.22	0.20

Q2	0.07	0.10	0.05	0.10
Q3	0.16	0.42	0.32	0.59
Gambier				
GMB	0.02	-	0.42	0.12
Grape+quebracho				
GQ	0.07	0.11	0.25	0.28
Quebracho+chestnut+gall				
QChG	0.04	0.07	0.17	0.30
Chestnut+quebracho				
ChQ	0.29	1.29	1.34	1.46

tr= traces



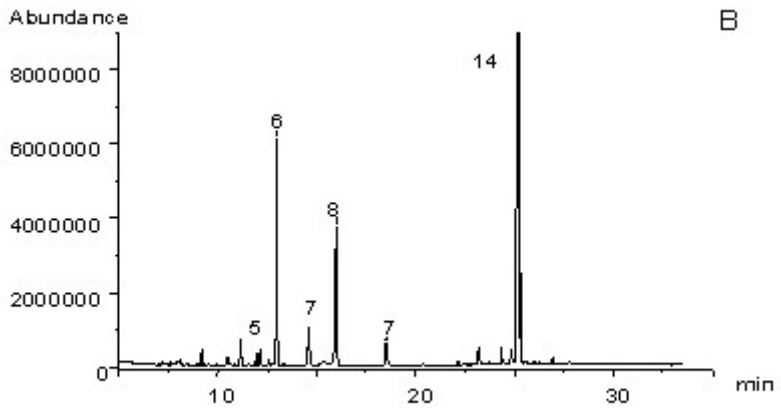
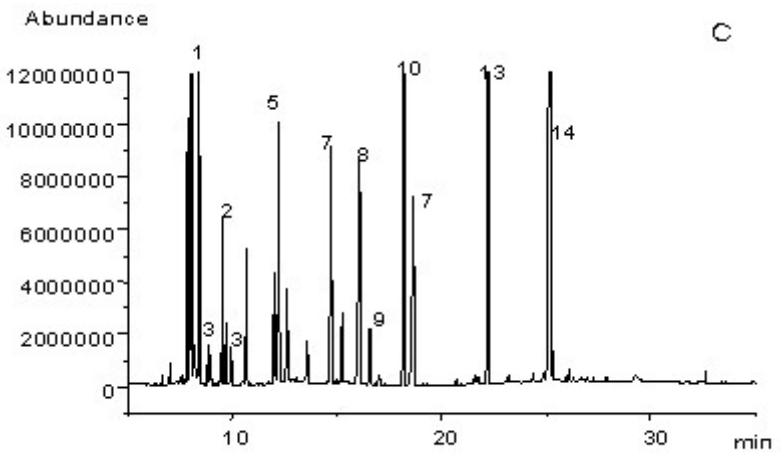
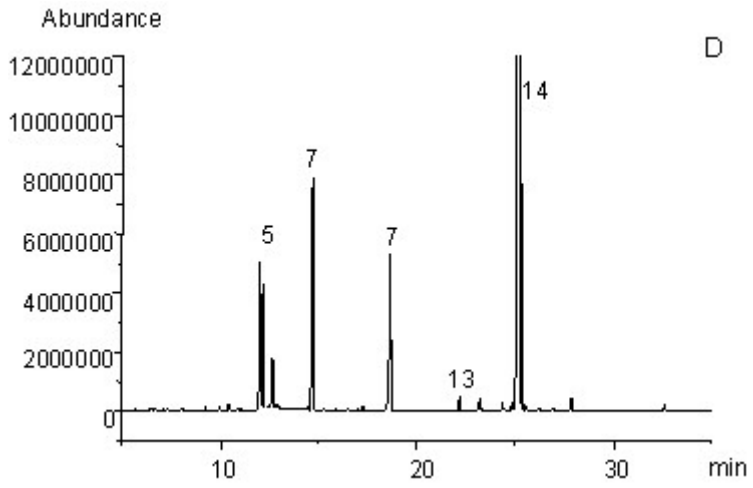


Figure 1. Gas chromatographic profiles of polyalcohols and carbohydrates in commercial tannins of A) oak wood, B) plant gall, C) chestnut wood, D) seed grape, E) skin grape, F) quebracho wood, G) Gambier. 1-Arabinose, 2-Arabitol, 3-Xylose, 4-Quercitol, 5-Fructose, 6-Pinitol, 7-Glucose, 8-Gallic acid, 9-Muco-inositol, 10-Chiro-inositol, 11-Bornesitol, 12- Scyllo-inositol, 13-Meso-inositol, 14-Phenyl-α-D-glucoside (i.s.)

Figure 1. continue

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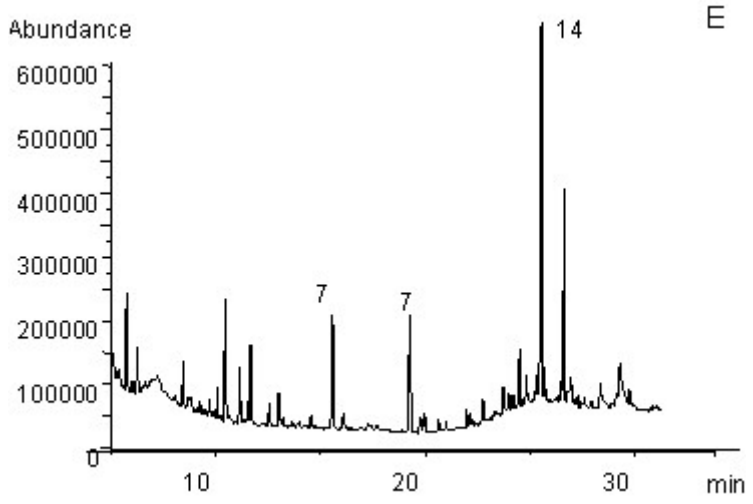
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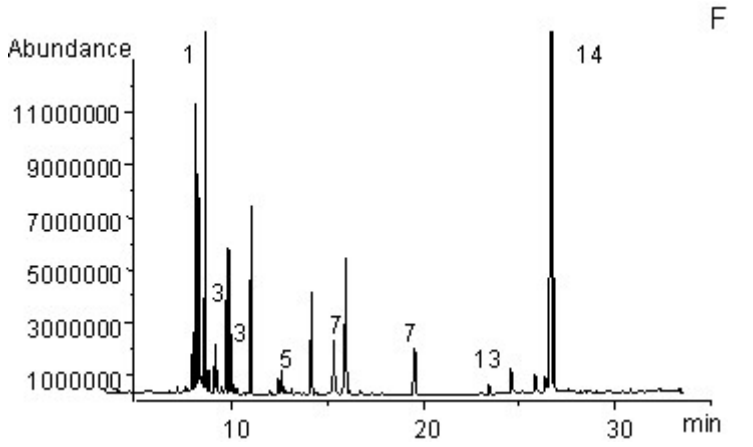
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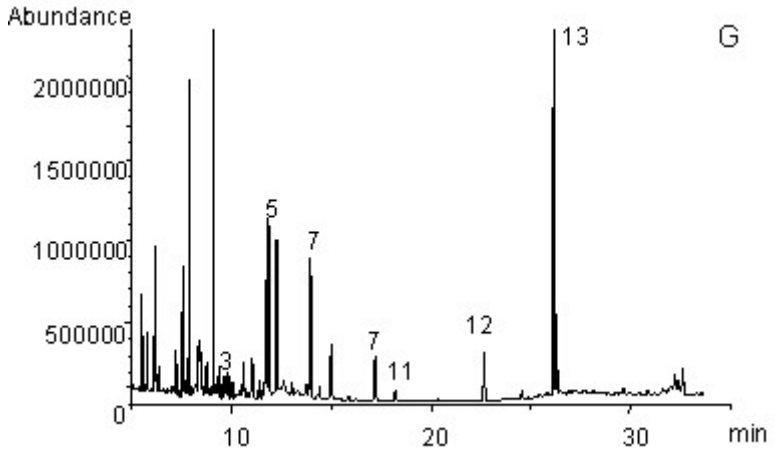
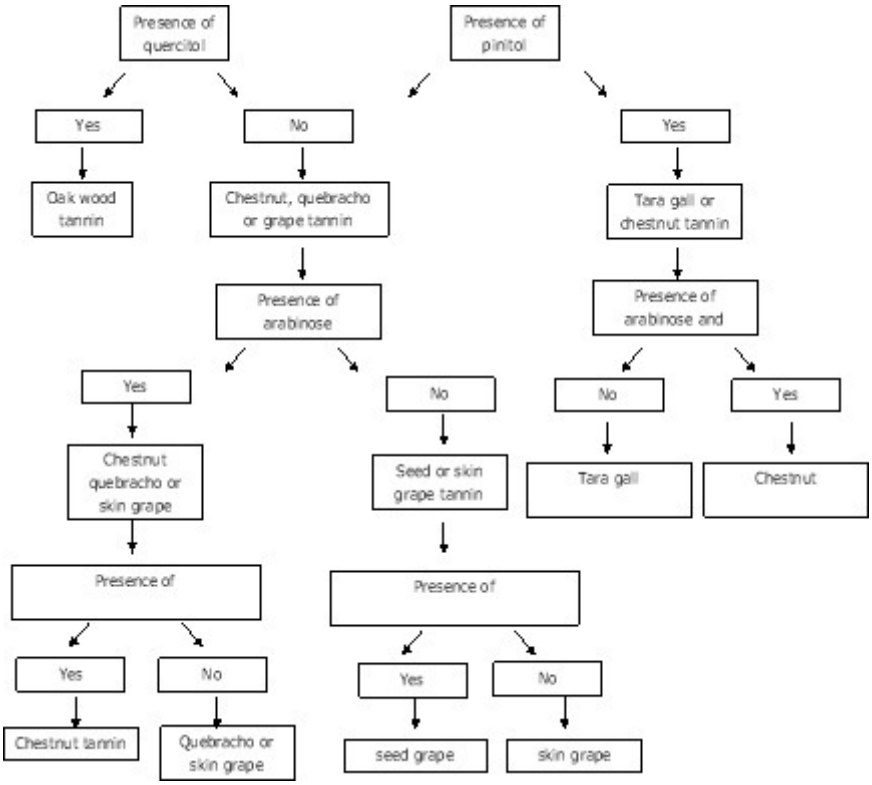


Figure 2. Scheme of tannins classified according to their monosaccharide and polyalcohol composition

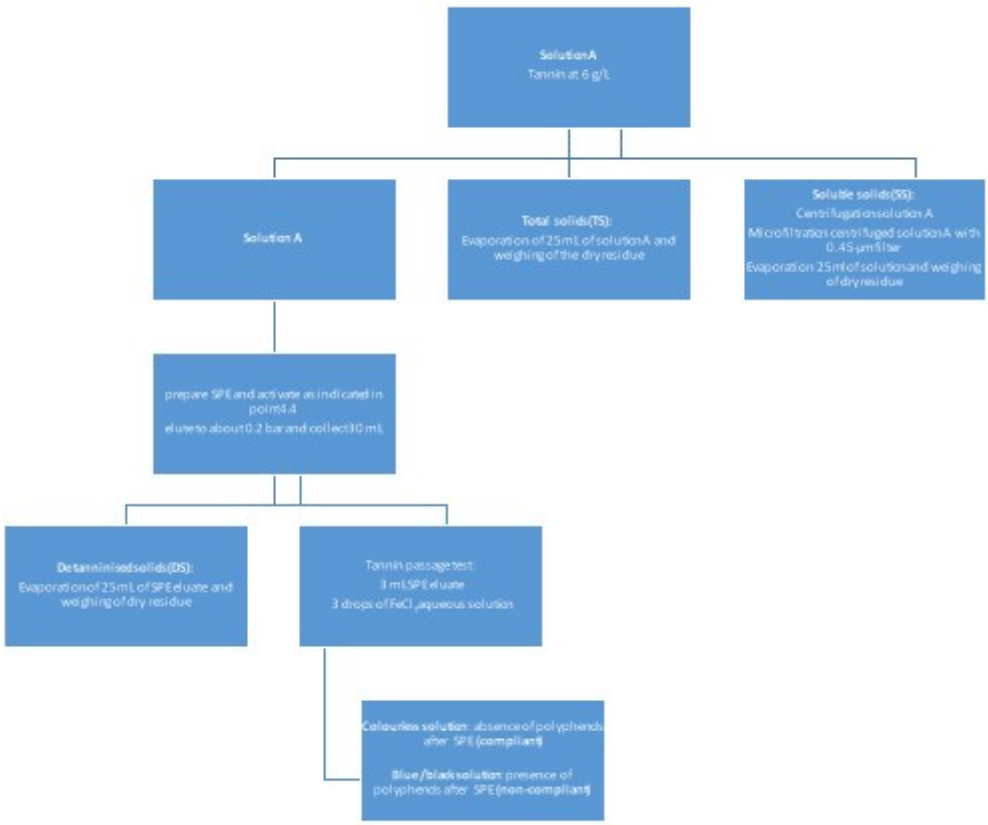


Annex 3: Method for the estimation of the total polyphenols content

1. Principle

This method will measure the polyphenol concentration of preparations of oenological tannins and is based on gravimetric analysis using solid-phase extraction (SPE). Tannins in aqueous solution are adsorbed onto a polymer in a SPE column – polyvinylpolypyrrolidone, in this case – able to retain the polyphenols. The substances not retained by the PVPP are non-phenolic compounds that were present in the original sample.

The complete diagram of the method is shown below:



2. Regents, materials, equipment

2.1. Reagents

2.1.1. PVPP (polyvinylpolypyrrolidone [CAS No. 9003-39-8]

2.1.2. $FeCl_3$ aqueous solution (1 g/L)

2.1.3. Double-distilled water

2.1.4. Ethanol (20% v/v)

2.2. Materials

2.2.1. Aluminium dishes (70 mL)

2.2.2. Disposable tubes with caps (50 mL)

2.2.3. SPE columns (70-mL reservoir,150*29,75 mm)

2.2.4. SPE column frits (27-mm diameter – 20 µm PE)

2.2.5. 1000-mL Pyrex flask

2.2.6. Class A 50-mL cylinders

2.2.7. Cellulose acetate membrane filter 0.45 µm; Ø 47 mm

2.2.8. Plastic syringe; 50 mL

2.2.9. Graduated glass pipettes (2 marks); 25 mL; Class A

2.3. Equipment

2.3.1. Bath thermostated to 20 °C

2.3.2. Technical balances with 0.01 g scale

2.3.3. Analytical balances with 0.1 mg scale

2.3.4. Oven thermostated to 105 °C

2.3.5. Oven thermostated to 80 °C or alternatively a thermostatic water bath

2.3.6. Centrifuge

2.3.7. Vacuum manifold

2.3.8. Q Class A volumetric glassware

2.3.9. Desiccator

3. Preparation of samples

The solution (referred to as solution A) is used for measuring total solids (TS), soluble solids (SS) and detanninised solids (DS).

Weigh about 6 g of tannin on the analytical balance and record the weight. Dissolve the tannin in about 950 mL of warm (60–70 °C) double-distilled water in a litre Pyrex flask and shake well. Leave the flask to stand at room temperature for 30 minutes. Cool the solution in a bath thermostated to 20–22 °C, top up the volume with double-

distilled water and mix well.

4. Operating mode

4.1. Measuring total solids (TS):

Collect and transfer 25 mL of solution A to an aluminium dish (see 2.2.1),

evaporate in an oven thermostated to 80 °C until dry,

move to an oven thermostated to 105 °C to dry until constant weight and weigh the residue (cool the dishes in the desiccator before weighing).

The formula to apply for the calculation of total solids (TS) is as follows:

$$\%TS = \frac{TS_dry_residue(g)}{weight_of_tannins(g)} \cdot \frac{1000}{(mL)solA} .100$$

4.2. Measuring soluble solids (SS):

Centrifuge solution A at 10 000 g during 5 minutes,

microfilter centrifuged solution A through the membrane filter in order to obtain a clear solution, then evaporate 25 mL of solution in an oven thermostated to 80 °C until dry,

move to an oven thermostated to 105 °C to dry until constant weight and weigh the residue (cool the dishes in the desiccator before weighing).

The formula to apply for the calculation of soluble solids (SS) is as follows:

$$\%SS = \frac{SS_dry_residue(g)}{weight_of_tannins(g)} \cdot \frac{1000}{(mL)solA} .100$$

4.3. Measuring insoluble solids (IS):

Calculate the difference between the total solids and the soluble solids as follows:

$$\%IS = \%TS - \%SS$$

4.4. Measuring detanninised solids (DS):

- Prepare the SPE columns: introduce the first frit, 7.0 ± 0.1 g of PVPP previously rehydrated with a 20% hydroalcoholic solution for 15 minutes, and the second frit, then pack the stationary phase well,place the SPE column on the vacuum manifold (as in Figure 1, for example),
- activate the column with three washes (do not dry the PVPP and apply a vacuum of about 0.2 bar to avoid compacting the polymer): first wash with 50 mL

ethanol (20% v/v), second wash with 50 mL double-distilled water and third wash with 20 mL solution A to eliminate water residue from the PVPP,

- add 30 mL solution A to the top of the column and collect the 30 mL of eluate (DS, detanninised solids) in a 50-mL Falcon tube, then stop elution when the liquid reaches the level of the upper frit,
- take 25 mL of eluate and transfer to an aluminium dish,

evaporate in an oven thermostated to 80 °C until dry,
move to an oven thermostated to 105 °C to dry until constant weight and weigh the residue (cool the dishes in the desiccator before weighing).
The formula to apply for the calculation of detanninised solids (DS) is as follows:

$$\%DS = \frac{DS_dry_residue(g) - BK(g)}{weight_of_tannins(g)} \cdot \frac{1000}{(mL)solA} .100$$

where *BK* is the blank value measured after SPE (see 4.5).

Figure 1 – Example of SPE extraction

To ensure there are no polyphenols present in the eluate after passing through the column, add 3 drops of $FeCl_3$ aqueous solution to 3 mL of detanninised solids (DS) solution. If the solution develops a blueish-black hue, then polyphenols have passed through the polymer, so the analysis should be repeated reducing the initial product weight. If the solution remains colourless after this treatment, proceed with the gravimetric analysis.

4.5. Blank measurement (BK)

When performing SPE elution, a blank test is required before starting so as to assess any interference caused by the analytical process. Proceed as follows:

prepare the SPE columns: introduce the first frit, 7.0 ± 0.1 g of PVPP previously rehydrated with a 20% hydroalcoholic solution for 15 minutes, and the second frit, then pack well,

place the SPE column on the vacuum manifold (as in Figure 1, for example),

activate the column with two washes (do not dry the PVPP and apply a vacuum of about 0.2 bar to avoid compacting the polymer): first wash with 50 mL ethanol (20% v/v), second wash with 70 mL double-distilled water,

add 30 mL double-distilled water to the top of the column and collect the 30 mL of eluate (blank for detanninised solids) in a 50 mL Falcon tube, then stop elution when the liquid reaches the level of the upper frit,

take 25 mL of eluate and transfer to an aluminium dish, then evaporate in an oven thermostated to 80 °C until dry,

move to an oven thermostated to 105 °C to dry until constant weight and weigh the residue (cool the dishes in the desiccator before weighing).

5. Expression of results

5.1. Measuring the percentage of total polyphenols (%polyphenols):

The formula to apply for calculating the percentage of tannins is as follows:

$$\%polyphenols = \frac{\%SS - \%DS}{\%TS}.100$$

<u>Measuring PVPP suitability</u>: REFER TO OENO 11/2002 - COEI-1-PVPP: 2007, PARA. 6.