Study of the Qualitative and Semi-quantitative Analysis of Grape Seed Extract by HPLC

by
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Abstract

The main aim of this study is to carry out a qualitative and semi-quantitative analysis of tannin extracts as an alternative to the official analysis method ISO 14088 – IUC 32, so that a correlation between the two methods is established.

From the point of view of the chemical composition, tannins are classified into two major groups:

i) condensed tannins, also called flavanols or catechins, and ii) hydrolysable tannins, also called pyrogallic tannins.

Today, the most widely used conventional extracts are quebracho, mimosa, chestnut, and tara. Quebracho and mimosa are condensed tannins, whereas chestnut and tara are hydrolysable tannins.

The following extracts were used in this study: tara powder, commercial mimosa and quebracho extracts and extracts derived from grape seed, containing both condensed and hydrolysable extracts.

The development of this new method will allow a faster and less expensive estimate of the amount of tannins present in a tannin extract.

Introduction

This study proposes the implementation of an alternative method to the standard method for the determination of tanning agents in vegetable tanning products (ISO 14088 - IUC 32 quantitative analysis of tanning agents by filter method). The new method proposes the determination of the composition of tannin extracts in aqueous solution and subsequent determination of the compounds present by a separation process that uses reverse phase high-performance liquid chromatography (HPLC).[1-3]

This method does not intend to replace the standardized method, but to develop a new method that has an acceptable correlation with the official method and allows for a faster and less expensive estimate.[4]

At the present stage of research, the test method used is based on the correlation between the sum of all peak areas obtained in the chromatograms of the samples of grape seed extract analyzed and the percentage in tannins obtained by official test method. The correlation between these two methods is studied.

On the other hand, the relationship between the total of peak areas obtained in each chromatogram with respect to its percentage ratio for each of the peaks identified by the Grape Tannins spectra library was also investigated. The Grape Tannins library, a library integrating the different peak values obtained in chromatography was created using different samples of tara tannins and extracts from mimosa and quebracho. All samples were analyzed using the same chromatography method. Retention time and spectral similarity between the compounds were considered to identify them from the calculations using a spectral comparison algorithm.

Retention times and spectra of different analysis patterns of catechin type and hydrolysable type polyphenols were also incorporated into the library (gallotannins and ellagitannins). In this study, spectra and retention times of the following compounds are incorporated into the library:

Procyanidin A2, Procyanidin B1, Procyanidin B2 (catechin dimers), (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Gallocatechin, mixed dimers such as (-)-Epigallocatechin gallate, (-)-Epicatechin gallate, (-)-Catechin gallate, (-)-Gallocatechin gallate. The pattern of gallic acid and a gallotannin (penta-O-galloyl-B-D-glucose) are also analyzed as representative units of hydrolysable tannins.

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Materials and Methods

Introduction

The production of grape pomace as a wine by-product is around 750,000 Tn/year. This by-product contains the stems, pulp and seeds of the fruit in variable proportions (25%, 55% and 20% on average, respectively). 100 Kg of treated grape pomace yield around 16-20 Kg of dry grape seeds. Grape seed is intended for companies producing oil for consumption. The by-product is subject to a process of grinding and oil extraction. This product is currently valued as biofuel after the use of grape seed oil, with a production of 60,000 Ton/year[3] in Spain.[5] There are several studies focused on polyphenolic compounds obtained from defatted grape seeds.[6-10] Frequently, methanol, ethanol, acetone and ethyl acetate are used as extractants. Also, supercritical fluid extraction of grape seed oil has been studied.[11,12]

Obtaining grape seed extract

To obtain grape seed extracts with tanning capacity, a process of aqueous extraction by pressure and temperature is applied over a given period of time, with the addition of a solubilizer.[13] The raw material used in this process is grinded grape seeds from a Spanish winery. The grape seed tannin extract obtained by extraction has an average tannin percentage of 3% as determined by filter method (ISO 14088 - IUC 32).

Next, the tannin extract is evaporated for concentration and application to vegetable tanning processes. Using the Emeco's evaporator CONCENTRA RV150. The extracts obtained have a 22% tannin concentration according to the standardized test. As of today, a Spanish tannery is using the new grape seed extract on a semi-industrial scale. The results obtained are satisfactory with respect to the tanning degree. The beige color of the leathers and the physical and chemical properties and fastness comply with the recommended values for the manufacture of vegetable tanned leather goods.

HPLC Method for Determination of Tannins

This study proposes the implementation of an alternative method to the standard method for the determination of tanning agents by filter method (ISO 14088 - IUC 32) to assess the percentage of tannins in the tannin grape seed extracts generated over the course of this study.

Table I

Summary of patterns analyzed by reverse phase HPLC-DAD. (1) *W.C. (without correlation).

Compound	CAS Nº	Category	Molecular weight	Retention time (min)	(R^2)
(+)- Catechin	154-23-4	Flavan-3-ol	290.3	11.8	0.9994
(-)- Epicatechin	490-46-0	Flavan-3-ol	290.3	16.1	0.9991
(-)- Epigallocatechin	970-74-1	Flavan-3-ol	306.3	10.3	0.9997
(-)- Gallocatechin	3371-27-5	Flavan-3-ol	306.3	5.0	0.9999
Procyanidin B1	20315-25-7	Procyanidin dimer	578.5	10.9	0.9982
Procyanidin B2	29106-49-8	Procyanidin dimer	578.5	15.4	0.9807
Procyanidin A2	2031-25-7	Dimeric catechin	576.5	23.7	0.9728
Gallic acid	149-91-7	Phenolic acid	188.14	3.0	0.9993
Penta-O-galloyl-B-D-glucose	14937-32-7	Gallotannin	940.7	25.1	S.C.
(-)- Epicatechin gallate	1257-08-5	Flavan-3-ol	442.4	22.7	0.9650
(-)- Catechin gallate	130405-40-2	Flavan-3-ol	442.4	22.9	W.C.*
(-)- Epigallocatechin gallate	989-51-5	Flavan-3-ol	458.4	17.3	W.C.
(-)- Gallocatechin gallate	4233-96-9	Flavan-3-ol	458.4	18.1	1.0000

The equipment used in this study is Water's Alliance HPLC System. A Water's XBridge Phenyl column is used (particle size: 3.5µm; length:15 cm; pore diameter: 130 Å) with a PDA detector between 200 and 400 nm. XBridge Phenyl columns are applicable in separations requiring an alternative selectivity, particularly when the desired analytes have an aromatic ring. Compared to straight-chain alkyl columns, XBridge Phenyl columns provide great flexibility in difficult separation resolutions.[3,14] Detection wavelength is set at λ=271.1nm. All chromatograms are detected at such wavelength since the baseline is much more stable, without the drifts that typically occur below 230nm.[15,16]

The mobile phase consists of two eluents: eluent A (acidified ultrapure water with 0.1% of formic acid) and eluent B (acidified acetonitrile with 0.1% of formic acid). The procedure in reverse phase is made up of an eluent gradient according to the following program: minutes 0-6, constant at 95%A-5%B; minutes 6-30, linear increase at 74%A-26%B; minutes 30-34, linear increase at 0%A-100%B. Mobile phase flow rate is set to 1 ml/min and the temperature to 35ºC.[17]

All the reagents used meet the quality standards required for HPLC tests. Millpore's ultrapure water is used for the preparation of solutions. Prior to the HPLC-DAD analysis, all solutions are filtered using 0.45µm nylon filter membranes.

Results and Discussion

Analysis of Patterns

Different polyphenol patterns of the following types of flavan-3-ols are analyzed: monomeric units such as (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Gallocatechin; catechin dimers (procyanidin A2, procyanidin B1, procyanidin B2); mixed dimers such as (-)-Epigallocatechin gallate, (-)-Epicatechin gallate, (-)-Catechin gallate, (-)-Gallocatechin gallate. The pattern of gallic acid and a gallotannin (penta-o-galloyl-B-D-glucose) are also analyzed as representative units of the hydrolysable tannins. All patterns analyzed are purchased from Extrasynthése (France).

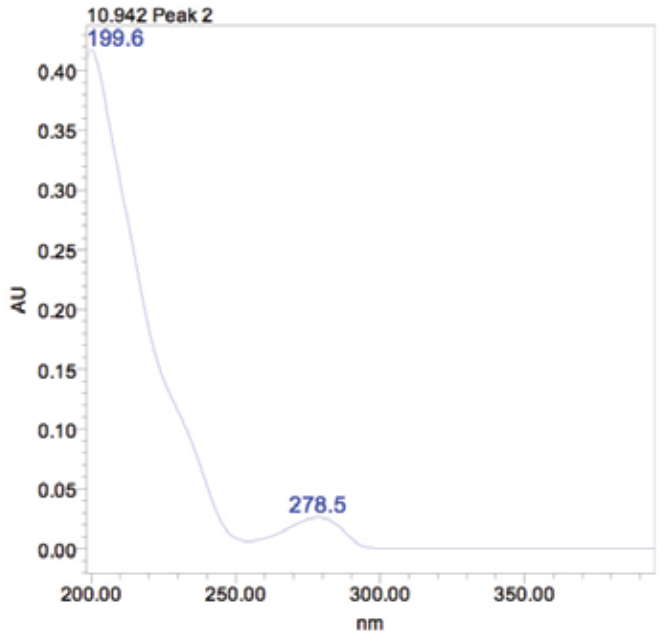

Figure 1. Typical UV-Vis spectrum of catechin compounds.

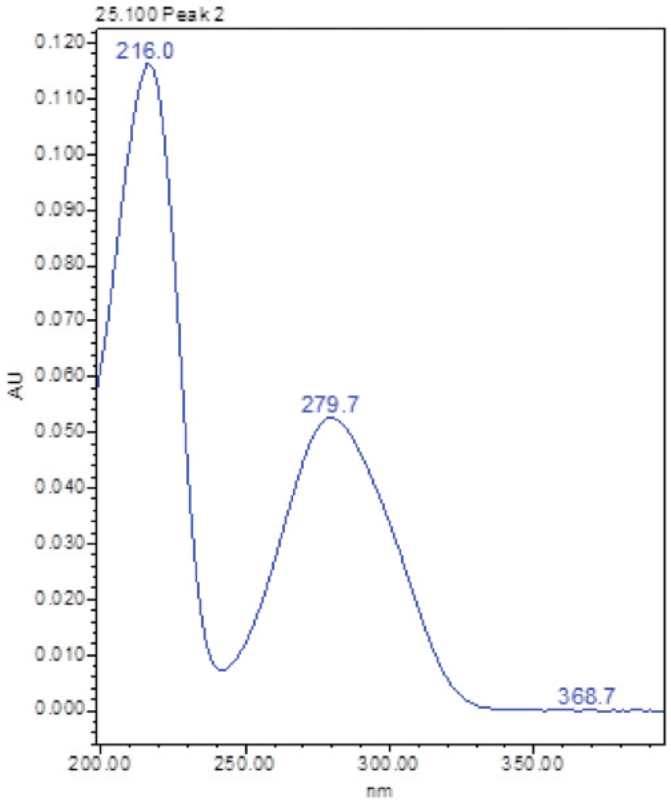

Figure 2. Typical UV-Vis spectrum of gallotannins.

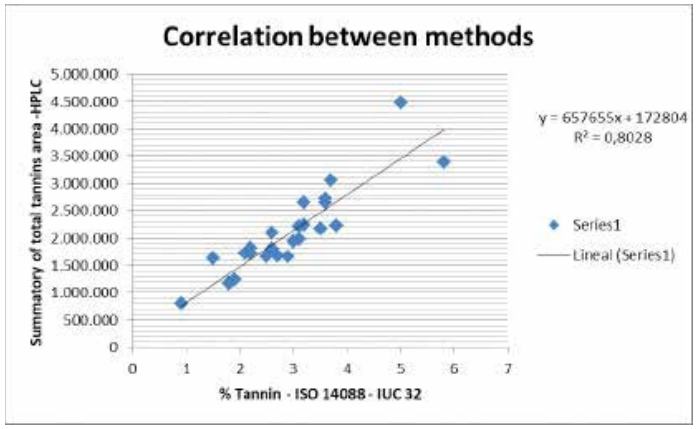

Figure 3. Correlation between standard filter method ISO 14088 – IUC 32 and total of peak areas by HPLC.

Four patterns of each compound are prepared in a concentration range of 5 and 100 mg/l. The compounds are dissolved in Millpore's ultrapure water and acidified with formic acid. The same test method in reverse phase is applied to the four analyzed samples. Chromatogram detection is set to 271.1 nm. The results obtained are shown in Table I.

It can be seen how not all patterns have a strong correlation in the calibrations due to the instability of the solutions of some of the polyphenols analyzed. Figures 1 and 2 correspond to the

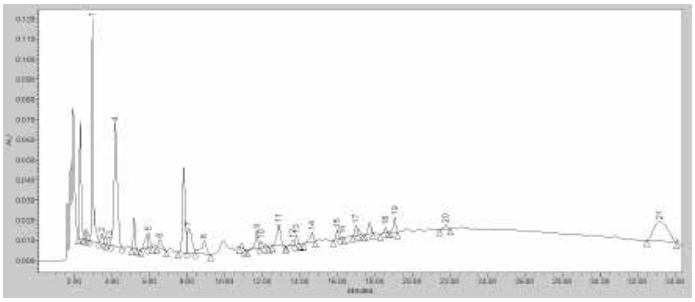

Figure 4. Grapetan chromatogram, PP2 sample.

Table II

Compounds identified on the Grapetan PP2 sample chromatogram.

Peak	Ret.Time (min.)	Identification	% Area / Total area
1	2.97	Gallic acid	16.1
2	3.46	Catechin tannin (chromophore present in Quebracho -1-)	1.2
3	3.87	Catechin tannin (chromophore present in Mimosa -1-)	1.4
4	4.17	Catechin tannin (Same chromophore as Procyanidin B2)	20.5
5	5.93	Catechin tannin (Same chromophore as (+)- Catechin)	1.7
6	6.54	Catechin tannin (chromophore present in Mimosa -2-)	1.5
7	8.07	Catechin tannin (chromophore present in Mimosa -3-)	5.3
8	8.91	Catechin tannin (Same chromophore as Procyanidin B2)	3.4
9	11.71	Catechin tannin (Same chromophore as (-) -Epicatechin)	2.5
10	11.96	Hydrolysable-catechin tannin (Same chromophore as (-) -Epigallocatechin gallate)	0.9
11	12.88	Hydrolysable-catechin tannin (Same chromophore as (-) -Epicatechin gallate)	3.8
12	13.63	Catechin tannin (chromophore present in Mimosa -4-)	0.7
13	13.83	Gallic tannin (chromophore present in Tara -1-)	1.1
14	14.67	Hydrolysable-catechin tannin (Same chromophore as (-) - Epigallocatechin gallate)	1.6
15	15.99	(-)- Epicatechin	1.1
16	16.25	Catechin tannin (Same chromophore as (-)- Epicatechin)	0.2
17	17.01	Tannin derived from gallic acid	1.3
18	18.57	Hydrolysable-catechin tannin (Same chromophore as (-)-Epicatechin gallate)	0.6
19	19.07	Hydrolysable-catechin tannin (Same chromophore as (-)-Epigallocatechin gallate)	1.7
20	21.78	(-)-Epicatechin gallate	0.6
21	33.17	Catechin tannin (chromophore present in Quebracho -2-)	13.7

Table III
Percentages of tannins, non-tannins and unknown compounds.

Tannins	**% Area / Total area**
Catechin tannins	52.1
Hydrolysable tannins	2.3
Hydrolysable-Catechin tannins	8.6
Total	**63.0**
Non-Tannins	**% Area / Total area**
Gallic acid	16.1
Ellagic acid	0
Catechins	1.7
Total	**17.8**
Unknown compounds	**% Area / Total area**
Total	**19.2**

spectra of Procyanidin B1 (catechin dimer) and Penta-o-galloyl-B-D-glucose (gallotannin) as examples of the UV-Vis spectra of a catechin compound and a gallotannin, respectively:

Analysis of Tannin Extracts
25 grape seed tannin extracts were obtained in pilot plant through the patented process (P201630673).[13] Each extract is analyzed by the standard filter method (ISO 14088 - IUC 32) to determine tannin content. Next, extracts are analyzed by reverse phase high-performance liquid chromatography. Detection wavelength of chromatograms is 271.1nm.

Once the samples are readied for testing, each of the samples is diluted in ultrapure water (1:25). The solution is filtered with 0.45µm filter and the chromatogram of the sample is obtained. A volume of 25µl of the test solution is injected to all the tested samples of grape seed extract. A correlation is established between the total sum of peak areas and the results obtained in percentage of tannins by the standard filter method. The correlation used is shown in Figure 3.

Figure 4 shows the chromatogram obtained from the analysis of a vegetable extract resulting from the above process mentioned in section 2.2. The peaks on the chromatogram identified by the library are numbered; peaks corresponding to non-tannins and unknown compounds are not numbered. Table II shows the peaks identified by spectral similarity to the substances present in the library of spectra. It includes a semi-quantitative estimate of the content of each component calculated from each peak area in relation to total area of peaks.

The integration process applied is *ApexTrack*. The peaks identified on the chromatogram belong to entries in the Grape Tannins library corresponding to the polyphenol patterns mentioned in section 3.1, samples of commercial tara tannin and mimosa, quebracho and chestnut extracts.

Once the total peak area of the chromatogram is obtained, the peak areas of the identified tannins are added together from. The group corresponding to non-tannins and unknown compounds is identified (see Table III).

Conclusions

A new way of identifying tannin composition is explored by reverse phase high-performance liquid chromatography. A new method of determination of tannin content by HPLC is generated by correlating the total peak area of the compounds identified as tannins and the results obtained by the standard filter test (ISO 14088) from a population of 25 tested samples.

On the other hand, the study involved the detection and semi-quantitative estimate of the compounds separated on the chromatograms from the data incorporated into the Grape Tannins library relative to the tested patterns and compounds present in the commercial samples of Tara tannins and Mimosa, Quebracho and Chestnut extracts. The peak areas of each peak are summed and each peak area is expressed as a percentage of the total. They are then grouped according to types (catechin tannins, hydrolysable tannins and catechin and hydrolysable tannins). Non-tannins and unknown compounds are also indicated. The results obtained show that the sample of tannins of grape seed contains, in comparison, a higher amount of catechin tannins (52.1%). Future investigations will aim to identify a greater number of compounds to try and reduce the number of unknown tannins.

Acknowledgements

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