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Treball Final de Grau

Estimation of total polyphenols content in food supplements and nutraceuticals: Antioxidant assays *versus* high performance liquid chromatography.
Estimació del contingut total de polifenols en suplements dietètics i nutracèutics: Assaig de poder antioxidant *versus* cromatografia de líquids d'alta resolució.

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Report

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1. SUMMARY

Polyphenols are molecules present in nature and synthesized mostly in plants as a product of their secondary metabolism. Depending on their structure they can be classified in phenolic acids, flavonoids, stilbenes, lignans and tannins. One of the most important effect of polyphenols is to eliminate toxic products that can harm our organism through an oxidative reaction (antioxidant capacity).

In this research, antioxidant capacity is measured as the total content of polyphenols in food supplements and nutraceuticals by different methods. High-performance liquid chromatography (HPLC) and two antioxidant assays, ferric reducing antioxidant power (FRAP) and Folin-Ciocalteau (FC), were used for this purpose. On the one hand, HPLC fingerprints were obtained at different wavelengths and with those results a principal component analysis (PCA) model was built to evaluate sample distribution according to their typology. On the other hand, chromatographic fingerprints and the results obtained by the antioxidant assays were analyzed by partial least square (PLS) for correlation purposes.

PCA confirms that HPLC fingerprints are acceptable sample chemical descriptors to classify the analyzed samples according to their nature and origin. Moreover, PLS shows that a model with a good prediction capacity can be obtained to predict the analytical response of the antioxidant assay in function of the HPLC chromatographic fingerprints. Determination coefficients (R^2) in the model for FRAP assay and FC assay were 0.903 and 0.939, respectively. To conclude, HPLC result a better methodology than the antioxidant assays to predict natural extracts antioxidant capacity because of the simplicity of use and the presence of fewer interferences with this methodology.

Keywords: Polyphenols, antioxidant capacity, natural extracts, HPLC, antioxidant assays, PCA and PLS.

2. RESUM

Els polifenols són unes molècules presents a la natura sintetitzades sobretot en les plantes com a un producte secundari del seu metabolisme. Segons la seva estructura poden ser classificats en àcids fenòlics, flavonoides, stilbenes, lignans i tanins. Entre els diversos efectes positius dels polifenols en el nostre organisme, destaca la seva capacitat per eliminar productes tòxics mitjançant reaccions d'oxidació (capacitat antioxidant).

En aquest treball es vol determinar la capacitat antioxidant expressada com la quantitat total de polifenols en suplements dietètics i nutracèutics per diferents mètodes. La cromatografia de líquids d'alta resolució (HPLC) i dos assajos antioxidants, poder antioxidant reductor del ferro (FRAP per les seves sigles en anglès) i Folin-Ciocalteau (FC) han estat utilitzats per a aquest propòsit. Per una banda, es van obtenir els cromatogrames de totes les mostres analitzades a diferents longituds d'ona mitjançant HPLC, amb els quals es va fer una anàlisi de components principal (PCA per les seves sigles en anglès) per avaluar la distribució de les mostres en funció de la seva tipologia. Per l'altra banda, es van analitzar les empremtes cromatogràfiques i els resultats obtinguts dels assajos de capacitat antioxidant mitjançant mínims quadrats parcials (PLS per les seves sigles en anglès) per avaluar les possibles correlacions.

El PCA realitzat va confirmar que les empremtes cromatogràfiques obtingudes són uns acceptables descriptors químics per a classificar les mostres analitzades en relació a la seva natura i origen. A més, el PLS va mostrar que es pot obtenir un bon model per a predir la resposta analítica dels assajos de capacitat antioxidant en funció de les empremtes cromatogràfiques obtingudes per HPLC. Els coeficients de regressió (R^2) en el model del FRAP i del FC van ser 0.903 i 0.939, respectivament. Finalment, es va concloure que l'HPLC és un millor mètode per predir la capacitat antioxidant d'extractes naturals perquè, a més de ser molt menys laboriós, presenta menys interferències de les que poden aparèixer en els assajos antioxidants degudes al seu treball experimental.

Paraules clau: Polifenols, extractes naturals, capacitat antioxidant, HPLC, assajos antioxidants, PCA i PLS.

3. INTRODUCTION

3.1. POLYPHENOLS

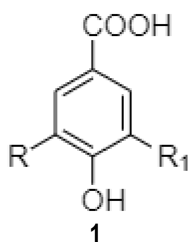
A lot of compounds with one or more phenolic rings are present in nature and they are called polyphenols. The most important effect of polyphenols is their capacity to eliminate toxic products that can harm our organism through an oxidative reaction (antioxidant capacity). This type of molecule is synthesized mostly in plants like a product of their secondary metabolism; consequently, it can be also found in fruits and vegetables. Several studies have proved how plants have a protective effect against cancer or cardiovascular diseases and these studies are investigating the probable role of the polyphenols. Experimental research has proven that polyphenols, in addition to the prevention of the disease, can also impact in the propagation and even in the healing of the disease.

Thousands of polyphenols can be found in plant-based products and most of them can be classified depending on their structure on phenolic acids, flavonoids, stilbenes, lignans and tannins (1–3).

3.1.1. PHENOLIC ACIDS

Phenolic acids occupy the third part of the total polyphenols that are consumed, and they are classified in two groups: hydroxybenzoic acids and hydroxycinnamic acids, with their corresponding derivates *(Figure 1)*. The main sources of phenolic acids are fruits, vegetables (e.g., spinach and broccoli), berry fruits, tea, coffee, wine, and beer. On the one hand, they are found as esters which are soluble and accumulate in the vacuoles, and on the other hand, these esters can be insoluble and become a cell-wall component (4).

Hydroxybenzoic acids



Hydroxycinnamic acids

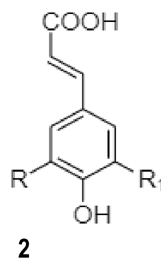


Figure 1: Chemical structures of hydroxybenzoic (1) and hydroxycinnamic (2) acids.

3.1.1.1. Hydroxybenzoic acids

The most frequent hydroxybenzoic acids are gallic acid (GA) and ellagic acid (*Figure* 2). Gallic acid is being studied in prostate cancer research while ellagic acid has a lot of properties that are beneficial to human health such as being anti-inflammatory, anti-carcinogenic or antioxidant. The anti-carcinogenic properties are due, among others, to the selective cytotoxicity of almost all the polyphenols. This characteristic function allows polyphenols to be toxic for cancer cells and have no toxicity for the healthy cells. Because of their beneficial effects against many diseases this type of polyphenols have a promising future in medicine (3,5).

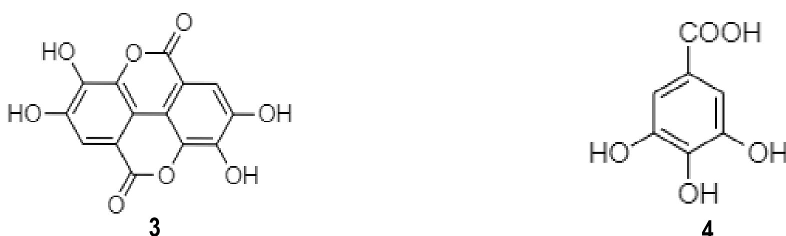


Figure 2: ellagic acid (3) and gallic acid (4) structure.

3.1.1.2. Hydroxycinnamic acids

Those of interest are caffeic acid coming from apple, grape, or tomato and ferulic acid being found, for instance, in wheat (*Figure 3*). Besides having anticancer properties, caffeic acid has demonstrated his antibacterial and antioxidant capacity in vitro. Moreover, through the combination of caffeic acid and quinic acid, chlorogenic acid (*Figure 3*) is obtained being the most abundant polyphenol in coffee. In addition, a dimer of ferulic acid linked by methylene is called curcumin (*Figure 3*) which is the major yellow pigment in turmeric and mustard (4).

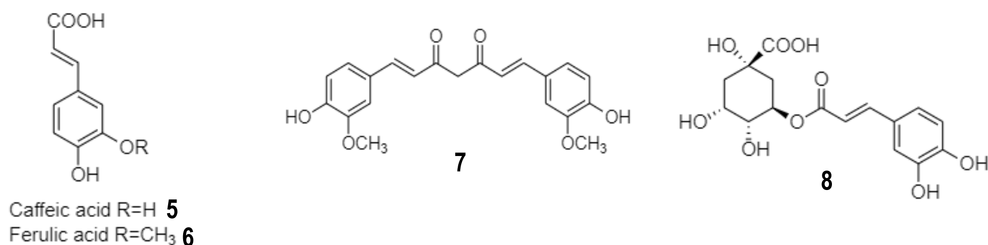


Figure 3: caffeic acid (5), ferulic acid (6), curcumin (7) and chlorogenic (8) acid structure.

3.1.2. FLAVONOIDS

Flavonoids chemical molecules have the general structure of a 15-carbon skeleton containing two aromatic rings (A and B) and a heterocyclic ring (C, which contains an oxygen) (*Figure 4*). This type of polyphenols is the most common in our diet being 60% of the total polyphenols we ingest. Furthermore, almost all flavonoids have a bound sugar in their structure in the form of β-glycosides such as glucose, galactose, rhamnose, among others. Depending on which carbon of the C ring is linked the B ring and the degree of unsaturation and oxidation of the C ring there are different subgroups: flavones, flavonols, flavanones, catechins, isoflavones, chalcones, and anthocyanidins (3,6–8).

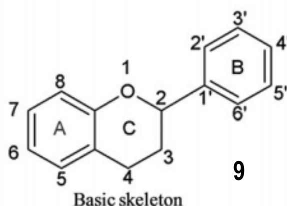


Figure 4: basic skeleton of a flavonoid (9).

(Figure extract from A. N. Panche et al. J. Nutr. Sci. (2016), vol. 5, e47, page 1 of 15)

3.1.2.1. Flavones

The major sources of flavones are red peppers, mint, chamomile, celery and others. They have a double bond in the position 2 and 3 and a ketone in the position 4 in the C ring. In addition, this class usually have a hydroxyl group in positions 5 and 7 of ring A and in the 4' of ring B *(Figure 4)*. Apigenin and luteolin are the most common flavones (*Figure 5*). The first one is being studied for its possible chemopreventive effect due to its advantages against others flavonoids (anti-mutagenic, anti-inflammatory, anti-viral and less toxic than others flavonoids) (3,6,7,9).

Figure 5: structures of apigenin (10) and luteolin (11).

3.1.2.2. Flavonols

Although flavonols are an extended group of flavonoids composed of 380 flavonols glycosides they are consumed in low quantity in our diet. They also have a double bond in the position 2 and 3 and a ketone in position 4 in the C ring *(Figure 4)*. Additionally, they have a hydroxyl group in the position 3 in the C ring (*Figure 4)*. The most important flavonols are kaempferol and quercitin (*Figure 6*) and can be obtained by ingesting fruits, vegetables and some medicinal plants. Quercitin is the flavonol more ingested in our diet and it has a high antioxidant power (7,8).

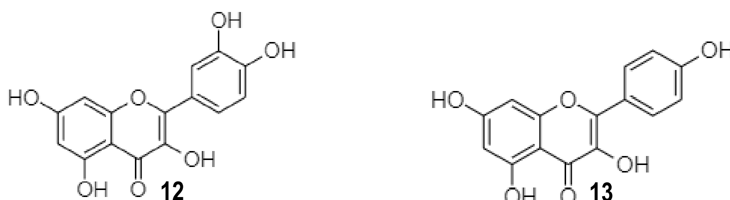


Figure 6: structures of quercitin (12) and kaempferol (13).

3.1.2.3. Flavanones

Flavanones are a small group that mainly come from citrus fruits such as orange, lemon and grapes. Unlike flavonols and flavones, flavanones have the C ring saturated and those of interest are hesperetin and naringenin (*Figure 7*). Although flavanones do not have the hydroxyl group in position 3 of the C ring and it decrease its reactivity, they have a high free radical scavenging capacity (7,8).

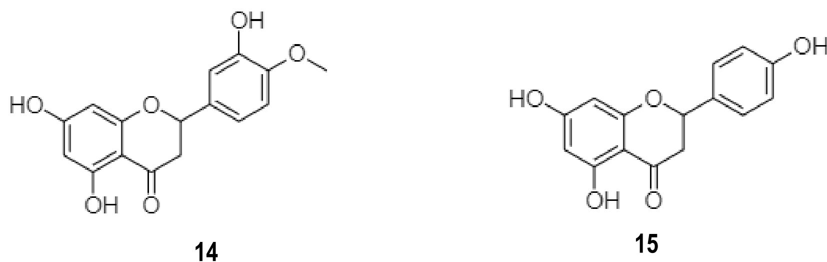


Figure 7: hesperetin (14) and naringenin structures (15).

3.1.2.4. Catechins

Catechins or flavan-3-ols, unlike the previous classes, neither have the ketone in position 4 nor the double bond in position 2 and 3 while a hydroxyl group is linked to the position 3 in the C ring. The most significant catechins are (+)-catechin, (+)-gallocatechin and (-)-epicatechin *(Figure 8)* which can be found in fruits, berries, chocolate, nuts and tea. Furthermore, they have potential medicinal properties such as the inhibition of carcinogenesis, tumorigenesis, or mutagenesis and antibacterial or antiviral protections (3,7,10).

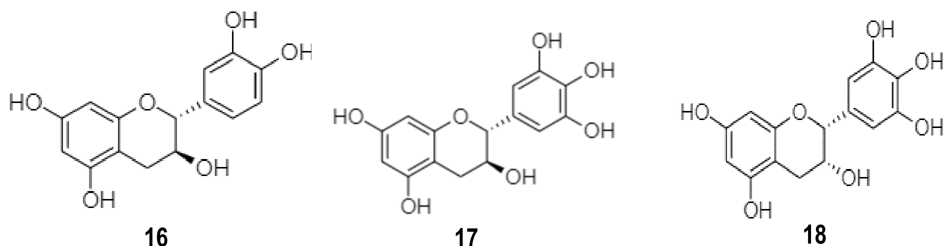


Figure 8: (+)-catechin (16), (+)-gallocatechin (17) and (-)-epicatechin (18) structures.

3.1.2.5. Isoflavones

This class has an extensive variety of molecules, and they are called phytoestrogens. The word phytoestrogen is formed by "phyto" which means plant and "estrogen" which is the female sex hormone. Taking this into count, isoflavones act as natural estrogens and the structure of the molecule is different compared with the other flavonoids. B ring is linked to the C ring in position 3 and they have a ketone in position 4 of the C ring. The principal source of isoflavones are legumes plants and the most studied are daidzein and genistein (*Figure 9*) (3,7).

Figure 9: genistein (19) and daidzein (20) structures.

3.1.2.6. Chalcones

Chalcones is another subclass of flavonoids that is characterized by the absence of the C ring in the basic skeleton (*Figure 4*). This fact makes necessary the existence of another link between the two aromatic rings which is a three-carbon α,β-unsaturated carbonyl system. The most abundant chalcones, among others, are naringenin chalcone and xanthohumol (*Figure 10*) and can be found in hops (beer) or in fruits and vegetables (3,7). Several studies demonstrated that xanthohumol has the capacity to modulate different enzymes, favoring the scavenge of hydroxyl and peroxyl radicals and decreasing the inflammation (11).

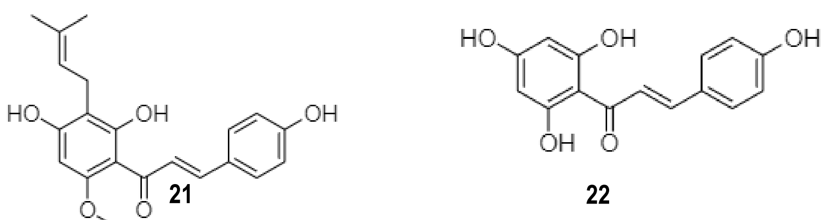


Figure 10: xanthohumols (21) and naringenin chalcone (22) structures.

3.1.2.7. Anthocyanidins

Anthocyanidins, the aglycones of the anthocyanins, are related to the color of many fruits and vegetables and it depends on the pH. Hence, they are called the universal plant colorant. A study demonstrated that anthocyanins are not a cancer cell proliferation inhibitor while anthocyanidins inhibit cancer cell growth in a high percent. For instance, cyanidin, delphinidin and petunidin (*Figure 11)* inhibited the cancer cells growth by 47, 66 and 53%, respectively (12). The principal sources of anthocyanidins are fruits, vegetables, nuts and dried fruits (3,7).

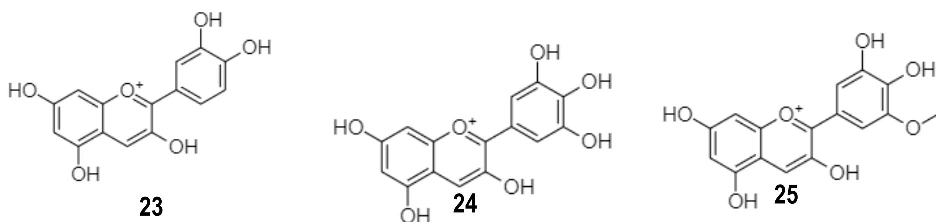


Figure 11: cyanidin (23), delphinidin (24) and petunidin (25) structures.

3.1.3. STILBENES

This family of polyphenols includes the hydroxy and methoxy derivatives of the simple stilbene and the principal sources are nuts, berries and, especially, grapes and grape products. So that, one of the main suppliers in our diet is red wine having 0.27 mg/100 mL (9). The most abundant stilbenes are resveratrol, pterostilbene and piceatannol *(Figure 12)*.

Resveratrol is an antioxidant, a free radical scavenger and it has a cardioprotective effect in humans. In 1992 a term called "French paradox" appeared and it started lot of research. This term refers to the low impact of coronary heart diseases (CHD) in France compared with other European countries and EEUU despite the high consumption of saturated fats. Several studies demonstrated that low CHD in France was probably related to their higher consumption of wine. For this reason, the interest on the main polyphenol in wine, resveratrol, increased (13,14).

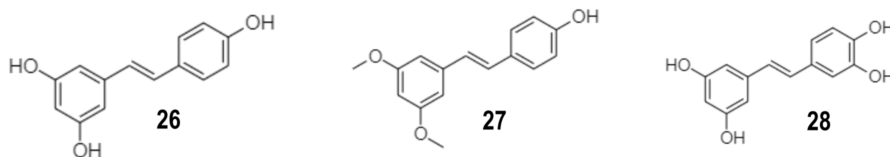


Figure 12: resveratrol (26), pterostilbene (27) and piceatannol (28) structures.

3.1.4. LIGNANS

Lignans have a polyphenolic structure formed by two rings of phenylpropane. They can be found in a high variety of sources such as soybeans, fruit, vegetables and extra virgin olive oil; it has to be said that lignans represent the higher number of phenolic compounds in olive oil. As well as isoflavones, lignans are the second principal group of phytoestrogens (3).Moreover, studies suggest that this type of polyphenols have an inhibitory effect on prostate cancer and breast cancer. 7-Hydroxymatairesinol is the most studied lignan in this field and his structure is shown in *Figure 13* (15).

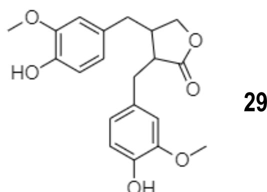


Figure 13: 7-Hydroxymatairesinol structure (29).

3.1.5. TANNINS

Tannins represent the last main group of polyphenols and they can be found mainly in grapes and wood. Unlike the previous classes, tannins are compounds of intermediate to high molecular weight being classified in condensed and hydrolysable tannins.

Non-hydrolysable oligomeric (of catechins) and polymeric proanthocyanins were classified as condensed tannins.

Hydrolysable tannins are formed by a glucose (or other carbohydrate) esterified with gallic acid (called gallotannins) or with ellagic acid (called ellagitannins) (3,16).

3.2. METHODOLOGIES FOR THE DETERMINATION OF POLYPHENOLS

Due to the high number of polyphenolic molecules and their presence in a wide variety of food products which are complex matrices, the separation and determination of polyphenols becomes a difficult task. Analytical separation methodologies such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) coupled with several detection systems, including UV-Vis, photodiode array (PDA) and mass spectrometry (MS) detection have been described. The last one is nowadays among the most widely employed for the identification and characterization of polyphenols.

While HPLC (17) is the most common technique for separating and quantifying individual polyphenols, there are many spectrophotometric assays to determine the total polyphenol content and consequently, the antioxidant capacity. These spectrophotometric assays are based on chemical reactions involving hydrogen atom transfer (HAT) or a single electron transfer (SET). For instance, oxygen radical absorbance capacity (ORAC) is a HAT assay, Folin-Ciocalteau (FC) and ferric reducing antioxidant power (FRAP) (18) are SET assays, and there are some of them using a combination of the two types such as trolox equivalent antioxidant capacity (TEAC); 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic) acid (ABTS) (19) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (20) will be used to quantify TEAC indexes. Moreover, the antioxidant capacity could also be measured by electrochemical techniques especially by differential pulse voltammetry (DPV). Due to the mechanisms involved in the reactions, the different standards used and considering that depending on each polyphenol the sensitivity could be different, it becomes difficult to find equivalences between these assays. All these methods will be explained in the experimental section.

3.3. DATA PROCESING: PRINCIPAL COMPONENTS ANALISYS (PCA)

Some of the results of this study will be based on the principal components analysis (PCA) of the obtained data. PCA, in statistics, is a multivariate analysis technique that allows us to reduce the number of variables eliminating the correlated ones and creating a new group of variables called principal components (PCs) which are orthogonal to each other. The original variables can then be expressed in function of the PCs and vice versa (21).

Furthermore, the samples can be represented in the PC space having the scores plot and the original variables can be exposed in the PC space having the loading plot. In the scores plot it could be seen if samples form clusters (being close together) or if they are scattered in the diagram. The loading plot shows the correlation between the variables. Finally, the PCA model is useful to recognize patterns or trends and it could be express by the matrix in *Figure 14*:

$$X = T \times P^T + E$$

Figure 14: the matrix that represents PCA model. T corresponds to the score matrix, P^t to the loading matrix and E is the error matrix.

3.4. PARTIAL LEAST SQUARES (PLS)

Partial least squares (PLS) is a common chemometric technique used to relate two matrices X and Y using a linear multivariate method. A combinate algorism is applied to X and Y obtaining the principals components of the two matrices. The hybrids PCs obtained are called latent variables (LV) and are combinations of the old ones. So, the PLS method makes a model that allow us to predict the Y in function of X. For instance, in this work, PLS is going to be applied to predict the response in the antioxidants assays (Y) in function of the chromatographic data obtained (X).

4. OBJECTIVES

This work focuses on the determination of the total polyphenol content (antioxidant capacity) in agri-food products, food supplements and nutraceutical products by HPLC and some antioxidant assays, with the aim of studying the correlation between HPLC and antioxidant assays Briefly, the work can be separated in the following objectives:

- To analyze several agri-food products, food supplements and nutraceutical products by HPLC and calculate their antioxidant capacity expressed as milligrams of gallic acid per kilogram/liter of sample.
- To study the analyzed sample distribution by PCA using the obtained HPLC fingerprints.
- To determine the antioxidant capacity of the analyzed samples by using different spectrophotometric antioxidant assays.
- To study the correlation by PLS between antioxidant capacity calculated with HPLC and spectrophotometric antioxidant assays.

5. EXPERIMENTAL SECTION

5.1. REAGENTS AND SOLUTIONS

The polyphenols needed for this work are gallic acid (97.5%) purchased from Sigma Aldrich (St Louis, MO, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) purchased from Carbosynth (Berkshire, UK). Solvents that are used for the preparation of the stock and working solutions are: dimethylsulfoxide (DMSO) and ethanol from Merck (Darmstadt, Germany) and methanol from Panreac (Barcelona,Spain). Milli-Q water purified using a Elix 3 coupled to a Milli-Q system was employed. Stock solutions of the standards were prepared at 1000 mg/L (stored in an amber vial at 4ºC) and the solutions for the calibrations were prepared by dilution with ethanol:water (1:1, v:v) to obtain concentrations in the range of 0.2-5 mg/L.

Furthermore, to carry out the antioxidant indexes the following reagents were needed: formic acid from Sigma Aldrich, hydrochloric acid (37% w:v), Fe (III) chloride and sodium carbonate from Merck, FC reagent from Panreac and 2,4,6-tripyridyl-S-triazine (TPTZ) from Alfa Aesar (Kandel, Germany).

All the reagents used in this work were of analytical grade.

5.2. INSTRUMENTS AND METHODS

5.2.1. HPLC

Chromatographic data was obtained with an Agilent Series 1100 chromatograph (Agilent technologies, Palo Alto, California, USA) equipped with a quaternary pump (G1311A), a degasser (G1322A), a diode array detector (G1315B), a fluorescence detector (G1321A) and an automatic injector system. The chromatographic column used was a core-shell Kinetex C18 reversed phase column (150 mm x 4.6 mm, 2.6 µm partially porous particle size) (Torrance, California, USA). A 0.1% formic acid solution (A) from Sigma Aldrich and acetonitrile (B) from Honeywell/Riedel-de Haën (Seelze, Germany) were used as mobile phase components under gradient elution (gradient program is summarized in Table *1)*. The injection volume was 5 µL and the mobile phase flow rate was 1 mL/min. UV-acquisition was carried out at 210, 230, 250, 280 and 320 nm (22).

Table 1: Gradient elution.

TIME(MIN)	%B
0-12	20-40
12-22	40-60
22-22.1	80-20
22.1-27	20

5.2.2. ANTIOXIDANT INDEXES

The spectrophotometric measurements were performed in a Perkin Elmer UV/VIS/NIR Lambda 19 spectrophotometer (Waltham, MA, USA). The QS quartz cuvette used were from Hellma Analytics (Jena, Germany) and it has a 10 mm of optical path.

5.2.2.1. Folin-Ciocalteau.

The FC index started waiting 8 minutes after mixing 250 µL of FC reagent and 1 mL of water in an amber vial. Then, 113 µL of a sodium carbonate aqueous solution 7.5% (w:v) and the appropriate volume of sample/standard to be in the concentration range 0.2-5 mg/l were added. To conclude, water was added up to 5 mL and after 2h the absorbance was measured at 795 nm using the reagent blank as the reference.

5.2.2.2. FRAP assay.

FRAP reagent was prepared mixing 20 mmol/L $FeCl_3$, 10 mmol/L TPTZ (with 50 mmol/L HCl) and 50 mmol/L formic acid aqueous solution in the ratio of 1:2:10 (v:v:v). The assay was performed by mixing 300 µL of FRAP reagent with the appropriate volume of sample/standard to be into de calibration range (0.2-5 mg/L). Finally, water is added to obtain a final volume of 2.5 mL and, after 5 minutes, the absorbance is measured at 595 nm using the reagent blanc as the reference.

5.3. SAMPLES AND SAMPLE TREATMENT

Several sample matrices were studied in this research and they are summarized in *table 2:*

Table 2: studied samples in this work.

Sample	Amount and identification code	Abundant polyphenols	Observations	References
Cranberry extracts	EC1, EC2, EC3, C02, C04, C05	Proanthocyanins (PACs), flavonols and flavan-3-ols.	Antibacterial activities	(23)
Raspberry	C17, C18, C19	Phenolic acids and flavonoids.	Slimming activities	(8)
Grape	C20, C21	Anthocyanidins, flavonols and catechins.	Antioxidant and antiaging	(8)
Grape and pomegranate	C22	Anthocyanidins, flavonols and catechins.	Antioxidant and antiaging	(8)
Vine	C23	Anthocyanidins, flavonols and catechins.	Protective of arteries, veins and capillaries	(8)
Artichoke	C25, C26	Hydroxycinnamic acids.	Hepatoprotective and hypolipidemic.	(8)
Turmeric and curry	T1, T2, T3, T4, T5, CY1, CY2, CY3	The most important is curcumin.	Coloring, seasoning and anti-inflammatory.	(24)
Coffee	CO1, CO2, CO3, CO4, CO5, CO6	Hydroxycinnamic acids.	Different coffees varieties.	(8,24)
Pepper	P1, P2, P3	Flavonoids.	Coloring and seasoning.	(8)
Tea	Te1, Te2, Te3	Flavonols and epicatechin.	Stimulant, antioxidant and sliming.	(8,24)
Juice	J1 (peach) J2 (pineapple) J3 (tomato) J4 (apple) J5 (pear)	Epicatechin (J1, J4) Flavonols (J4, J3) Hydroxycinnamic acid (J4, J5)	-	(8)
Wine	W1 (white wine), W2 (red wine)	Flavonols, epicatechin, anthocyanidins and resveratrol.	Stimulant and cardioprotective.	(8,24)

Beer	B1, B2, B3	Tannins.	-	(8)
Sparkling wine	S1, S2, S3, S4, S5	Flavonoids and hydroxycinnamic acids.	-	(25)
Chocolates	CH1, CH2, CH3	Catechins	Stimulant and antioxidant.	(8)

Regarding the sample treatment three different procedures were followed, depending on the samples:

- Liquid samples (juice, wine, beer and sparkling wine) were just filtered with a syringe filter nylon of 0.2 µm from Agilent Technologies (Waldbronn, Germany) and data were expressed in milligrams of gallic acid per liter of sample.
- Chocolates were defatted before doing the extraction. For that purpose, 10 mL of cyclohexane (Thermo Fisher Chemical, Massachusetts, USA) were added to 2 grams of sample and sonicated for 15 minutes. The solution was centrifuged for 5 mins at 4500 rpm and the liquid was filtered with a syringe filter nylon of 0,2 µm (Agilent Technologies). Data were expressed in milligrams of gallic acid per kilogram of sample.
- The rest of the samples were processed by adding 10 mL of methanol/water/hydrochloric acid (70:29:1, v:v:v) to 0.2 grams of sample weighted with an analytic balance (ALT261, Mettler Toledo). Then, the solution was sonicated (Branson 5510 sonicator bath, Marshall Scientific, Hampton, USA) for 30 minutes. Finally, centrifugation (Rotanta 460 RS) for 15 minutes at 3500 rpm was done and the liquid was removed and filtrated with a syringe filter nylon of 0.2 µm (Agilent Technologies). Triplicates were done for each sample. Data were expressed in milligrams of gallic acid per kilogram of sample.

Moreover, a quality control (QC) was prepared by mixing 25 µL of each sample extract. This QC solution was analyzed every 10 sample injections with the proposed HPLC method.

6. RESULTS AND DISCUSSION

6.1. HPLC DETERMINATIONS AND PCA ANALYSIS

The first objective of this work is to obtain the chromatographic data of all the samples shown in *table 2.* The HPLC method explained in the experimental section was applied and the obtained chromatograms were acquired. To obtain useful information for the correlations that will be evaluated in this work, a standard solution of 15 mg/L of gallic acid was also analyzed to evaluate the global polyphenolic content expressed as gallic acid equivalents (GAE) using the overall chromatographic area as the analytical response. Then, the total area of the sample chromatograms was transformed into concentration using the average area corresponding to 3 gallic acid replicates as a reference to quantify total chromatographic detected bioactive substances. Finally, considering the volume of the sample solutions (10 mL) and the weighted grams of each sample, concentrations in the extracts were transformed in a value of mg gallic acid/kg of sample. If the sample was liquid, the result was expressed in mg gallic acid/L of sample. For instance, *figure 15* shows the chromatograms (registered at 280 nm) of samples EC2.1 (cranberry), CY1.1 (curry) and C25.1 (artichoke), and the quantitation of GAE is given in *table 3 in Appendix 1*.

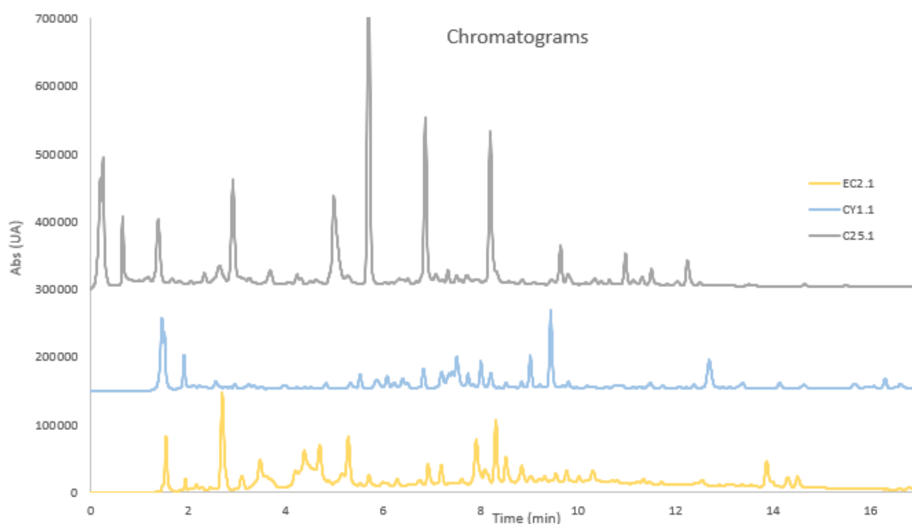


Figure 15: HPLC-UV chromatographic fingerprint of some samples at 280 nm.

As can be seen after HPLC determinations, samples have significant differences in their fingerprints *(Figure 15)*. These were the expected results because a wide variety of samples was analyzed. To confirm this fact, a matrix was built using the HPLC fingerprints as chemical descriptors and PCA was applied with Stand Alone Chemometrics Software (SOLO, Eigenvector Research, Manson, WA, USA).

Regarding the data preprocessing, normalization (1-norm, area=1) and autoscaling were used to equalize the contributions of majors and minor components to the model. The obtained PCA score plot (PC1 vs. PC2) is depicted in *figure 16*. The model shows that PC1 and PC2 retained 48,59% of variance from the data. As can be seen in the figure, QC replicates are grouped, showing the good repeatability and robustness of the employed HPLC method, and of the obtained chemometric results. Regarding the analyzed samples, they tend to be grouped according to their typology (classes) as described in *Table 2*. This confirms that HPLC fingerprints are acceptable sample chemical descriptors to classify the analyzed samples according to their nature and origin.

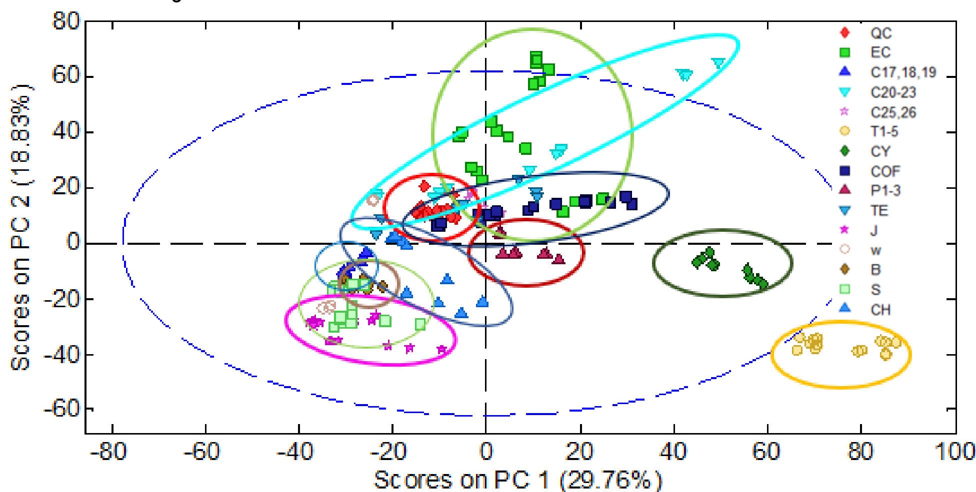


Figure 16: PCA score plot of PC1 vs PC2 when using HPLC fingerprints (registered at 280 nm) as sample chemical descriptors.

6.2. DETERMIATION OF DIFFERENT ANTIOXIDANT ASSAYS

Once the calculations and the explorations were done with HPLC, the determination of the sample antioxidant capacity by employing several antioxidant assays was carried out. It should be mentioned that the concentration found using the HPLC data (as GAE) allowed to have an approximation of which volume of sample was needed to each one of the spectrophotometric assays employed. The antioxidant capacity of each sample was then calculated by FC and FRAP assays following the description in 5.2.2. Theoretically, the results obtained with antioxidant assays and the ones obtained with HPLC may have a linear correlation. To evaluate this, the obtained HPLC concentrations were represented versus the concentrations obtained with FC and FRAP assays, separately. In *figure 17* the representation of HPLC vs FC assay is shown, and in *figure 18* the representation of HPLC vs FRAP assay is shown. As can be observed, in both representations, there are differences between HPLC results and antioxidant indexes. This fact could be attributed to interferences on the antioxidant assays from other non-phenolic reducing substances and interferences on the chromatographic data from other absorbing (non-polyphenolic) components. However, a correlation trend was observed between HPLC measurements and antioxidant assays, with determination coefficients (R^2) of 0.6938 and 0.7745 for FC and FRAP indexes, respectively.

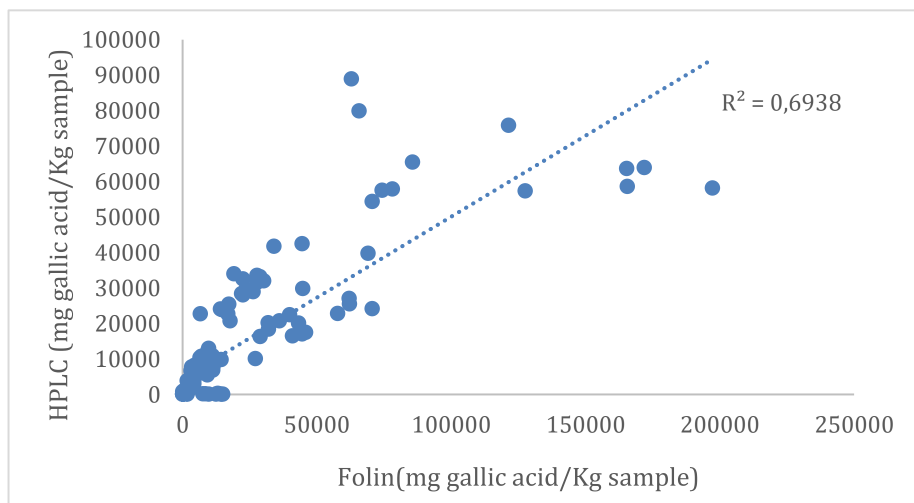


Figure 17: Correlation between concentrations obtained by HPLC vs FC assay.

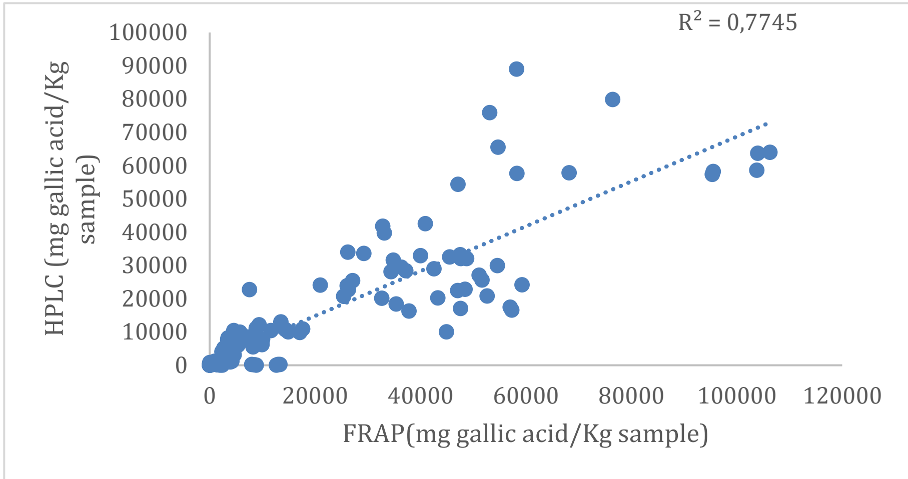


Figure 18: Correlation between concentrations obtained by HPLC vs FRAP assay.

Moreover, the results obtained with FC represented versus the FRAP results displayed a quite linear correlation with a R^2 of 0.8369 as shown in *figure 19*.

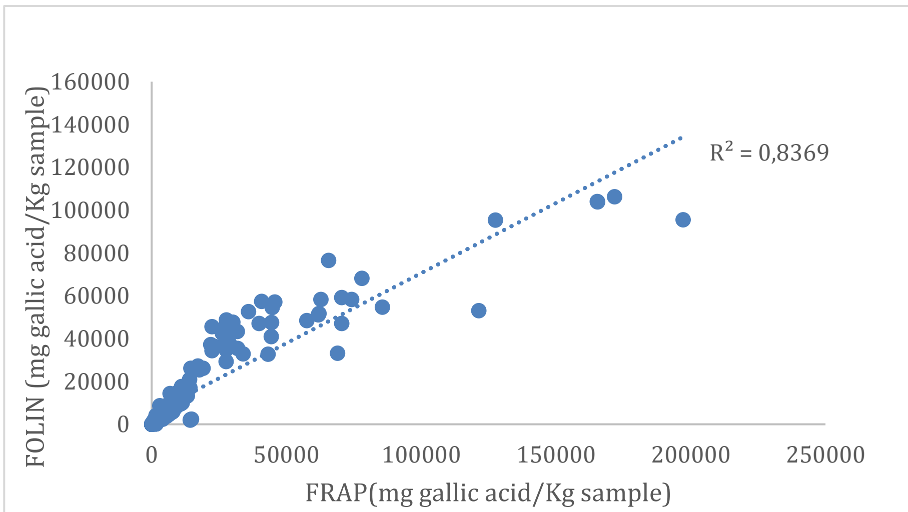


Figure 19: Correlation between concentrations obtained by FRAP assay vs FC assay.

6.3. PLS ANALYSIS

After studying the correlations between HPLC and antioxidant assays, a PLS study was carried out to, as explained in section 3.4, calculating a model that allows to predict the response of the antioxidant assay (matrix Y) in function of the chromatographic data obtained (matrix X).

First, a PLS study was done with the HPLC data in matrix X and the results of the FRAP assay in matrix Y. Before carrying out the PLS model, the chromatographic segments where no data is present were removed, as can be seen in *figure 20.* Hence, the first and last section the chromatograms as well as a small section in the middle were deleted from the analysis as no relevant information among samples was observed.

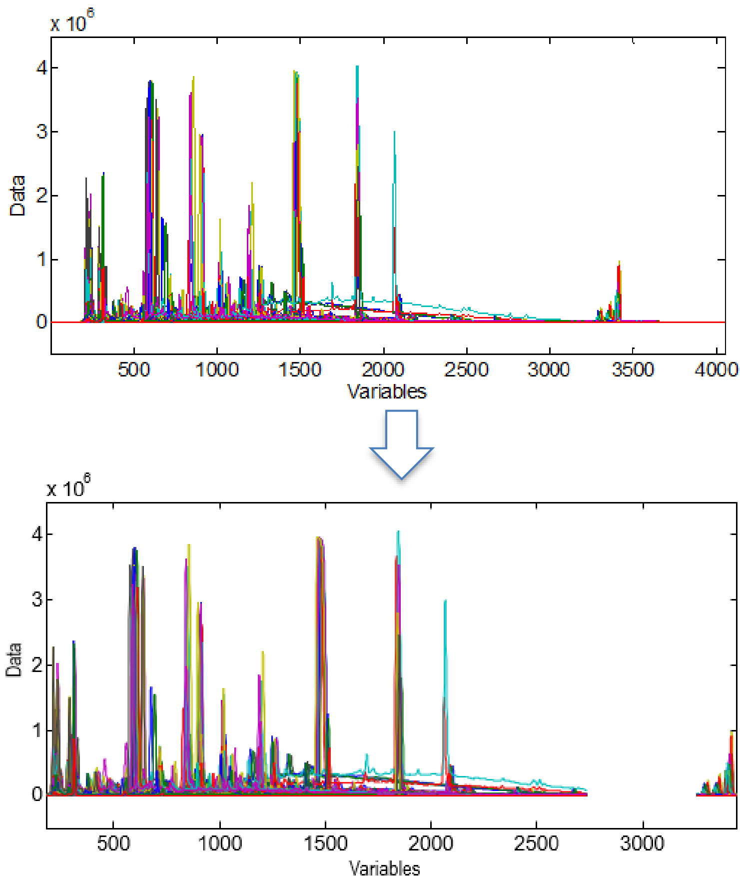


Figure 20: Raw and selected HPLC sections to carry out the PLS study.

Then, a PLS model was calculated with all the samples analyzed in this work. As can be seen in *figure 21*, the graphic of Q residuals reduced vs Hotelling T^2 reduced shows that the sample C21.3 is an outlier and consequently it must be eliminated.

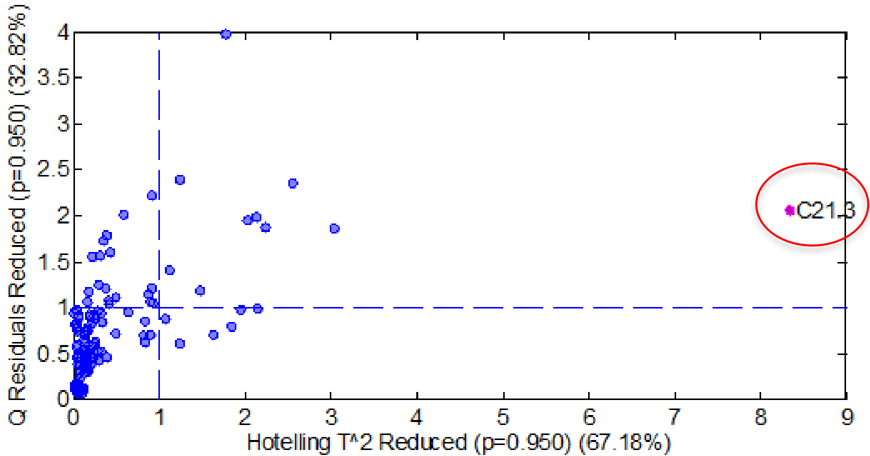


Figure 21: Q residuals reduced vs Hotelling T^2 reduced.

On the one hand, a PLS model using FRAP results was built. Three LV were found to be optimal to carry out the calibrations as it was deduced by cross validation (CV) under a Venetian blind approach. Hence, the variance explained was 56.58% for the X-block and 94.98% for the Y-block. In *figure 22,* the Y measured vs the Y CV predicted is represented. The R^2 is 0.903 for the CV which indicates a good prediction with the model.

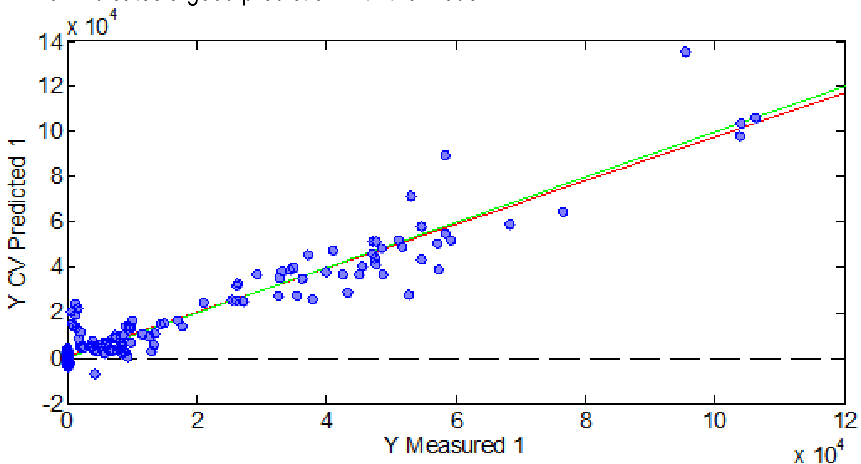


Figure 22: Y measured vs Y CV predicted for FRAP assay.

On the other hand, for the FC results 3 LV were chosen explaining the 57,31% of the variance for the X-block and 96,55% of the Y-block. As can be seen in *figure 23,* the R^2 of the cross-validation is 0.939, so the model has also a good prediction capacity.

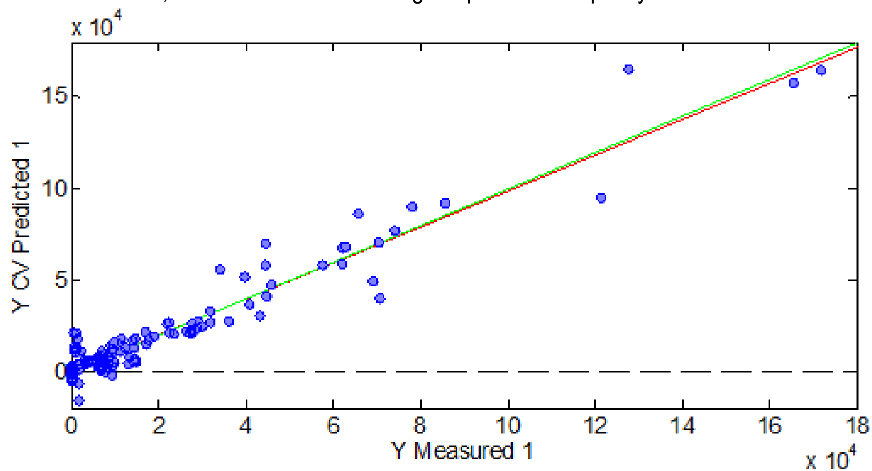


Figure 23: Y measured vs Y CV predicted for FC assay.

7. CONCLUSIONS

The main objective of this work was to develop a method that allow to determine the antioxidant capacity of a wide range of natural extracts by HPLC and antioxidant assays. For that purpose, determinations with HPLC and antioxidant assays were performed to find correlations between the two types of methodologies. After comparing the results of the HPLC determinations and the antioxidant assays via chemometrics several conclusions can be made:

- First, a high number of natural extracts were analyzed based on HPLC fingerprints and spectrophotometric indexes of antioxidant capacity. All the results of the used methods are summarized in the *table 3* in *Appendix 1*.
- The PCA study confirms that HPLC fingerprints are acceptable sample chemical descriptors to classify the analyzed samples according to their typology.
- Despite the differences between the HPLC and the antioxidant assay measurements, a trend could be seen when plotting the calculated antioxidant capacities as GAE, showing acceptable correlations between HPLC fingerprints and both antioxidant assays under evaluation. Regarding the employed methodologies, it should be mentioned that the achievement of the experimental data using the HPLC method was the best in terms of overall advantages and convenience. The antioxidant assays are complex and time-consuming in their experimental work, and because of this, the possibility of interferences and errors increases.
- Finally, a PLS model built was able to predict antioxidant capacity data (equivalent to that obtained with the antioxidant assays under study) by employing the HPLC fingerprints, showing that the main objective of the present work was successfully accomplished.

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9. ACRONYMS

ABTS: 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic) acid

CE: capillary electrophoresis

CHD: coronary heart diseases

CV: cross-validation

DMSO: dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DPV: differential pulse voltammetry

FC: Folin-Ciocalteau

FRAP: ferric reducing antioxidant power

GA: gallic acid

GAE: gallic acid equivalents

HAT: hydrogen atom transfer

HPLC: high-performance liquid chromatography

LV: latent variable

MS: mass spectrometry

ORAC: oxygen radical absorbance capacity

PCA: principal components analysis

PCs: principal components

PDA: photodiode array

PLS: partial least square

QC: quality control

SET: single electron transfer

TEAC: Trolox equivalent antioxidant capacity

TPTZ: 2,4,6-tripyridyl-S-triazine

APPENDICES

Appendix 1: RESULTS WITH THE DIFERENT METHODS

Table 3: results with HPLC and the antioxidant assays in milligrams of GA/kg of sample or milligrams of GA/L of sample.

Sample	HPLC mg ga/kg	Folin mg ga/kg	FRAP mg ga/kg
EC1.1	27000	61900	51100
EC1.2	22800	57700	48500
EC1.3	25600	62100	51700
EC2.1	6880	9720	9070
EC2.2	7020	10980	9500
EC2.3	7840	11360	10100
EC3.1	58600	165500	103800
EC3.2	63700	165300	104000
EC3.3	64000	171800	106300
C02.1	20200	31900	43300
C02.2	16300	28900	37800
C02.3	18400	31900	35400
C04.1	3220	3640	4630
C04.2	3110	4210	4650
C04.3	2980	4040	4310
C05.1	5470	9150	8240
C05.2	6610	9680	9460
C05.3	6630	8930	9810
C17.1	41800	34000	32900
C17.2	39800	69100	33200
C17.3	42500	44400	40900
C18.1	57900	78100	68200
C18.2	57600	74200	58300
C18.3	54400	70500	47200
C19.1	65500	85600	54700
C19.2	75900	121400	53100
C19.3	79900	65700	76500
C20.1	24200	70600	59300

C20.2	16500	40900	57300
C20.3	17500	45700	57100
C21.1*	57300	0	0
C21.2	58200	127600	95400
C21.3	89000	197200	95600
C22.1	29900	62800	58300
C22.2	20800	44700	54600
C22.3	17100	36000	52700
C23.1	22500	44500	47600
C23.2	20100	39900	47100
C23.3	13000	43200	32700
C25.1	10100	9640	13500
C25.2	10400	9720	14900
C25.3	9850	9340	11700
C26.1	10900	14300	17100
C26.2	10700	11100	17700
C26.3	10400	6920	14400
T1.1	6890	6500	4600
T1.2	6450	5600	3800
T1.3	6890	6780	5410
T2.1	12200	11200	9990
T2.2	11100	9370	9390
T2.3	8780	9440	8840
T3.1	8640	7900	5930
T3.2	8920	8080	6480
T3.3	9980	8820	8240
T4.1	7520	7220	5780
T4.2	8650	7790	7100
T4.3	7640	7160	5910
T5.1	8380	7140	6550
T5.2	8770	7020	5840
T5.3	8180	7690	5760
CY1.1	5990	4480	3520

CY1.2	5950	4480	3900
CY1.3	6140	4530	5400
CY2.1	7640	6770	9950
CY2.2	6610	6840	6290
CY2.3	6440	6140	8270
CY3.1	7850	6960	8960
CY3.2	6650	6490	8540
CY3.3	10080	3110	8700
CO1.1	32100	27100	45000
CO1.2	33600	30200	47700
CO1.3	31600	27700	29300
CO2.1	32600	27700	34900
CO2.2	28400	22400	45600
CO2.3	28100	22000	37200
CO3.1	29500	22400	34500
CO3.2	29000	23500	36400
CO3.3	32100	26300	42600
CO4.1	33000	27800	48800
CO4.2	33200	27600	40000
CO4.3	34000	28700	47600
CO5.1	25400	19100	26200
CO5.2	23900	17200	27100
CO5.3	22700	14600	26100
CO6.1	24000	16800	26300
CO6.2	20700	14100	21000
CO6.3	22700	17700	25500
P1.1	7420	6560	7620
P1.2	8520	6650	7520
P1.3	7740	6250	7090
P2.1	3830	3390	3480
P2.2	4120	3370	3550
P2.3	3960	3340	3730
P3.1	5160	4150	2400

P3.2	4030	3940	2720
P3.3	3860	3890	2340
Te1.1	1180	1650	4050
te1.2	973	1750	4240
te1.3	1180	2160	3960
te2.1	613	585	973
te2.2	552	569	674
te2.3	473	563	831
te3.1	540	842	1660
te3.2	675	1020	1870
te3.3	766	1270	2030
J1.1	92	62	27
J1.2	87	63	29
J1.3	109	64	24
J2.1	196	253	41
J2.2	191	260	44
J2.3	191	250	40
J3.1	186	108	16
J3.2	194	136	26
J3.3	200	129	26
J4.1	65	65	45
J4.2	64	82	23
J4.3	66	61	34
J5.1	76	88	4
J5.2	73	99	11
J5.3	74	104	7
W1.1	155	179	153
W1.2	155	184	150
W1.3	150	175	152
W2.1	532	1210	1440
W2.2	467	1470	1370
W2.3	537	1220	1390
B1.1	293	252	219

B1.2	264	243	226
B1.3	258	265	232
B2.1	234	235	138
B2.2	239	219	156
B2.3	241	224	150
B3.1	390	386	397
B3.2	428	394	407
B3.3	436	387	428
S1.1	93	97	69
S1.2	94	104	67
S1.3	95	77	69
S2.1	81	87	62
S2.2	103	79	81
S2.3	73	74	67
S3.1	75	120	119
S3.2	101	143	123
S3.3	101	138	116
S4.1	103	181	160
S4.2	102	185	132
S4.3	103	178	95
S5.1	134	1640	153
S5.2	133	1630	156
S5.3	133	1610	153
CH1.1	48	14500	2300
CH1.2	55	14300	1980
CH1.3	73	14900	2440
CH2.1	205	9740	8890
CH2.2	171	7520	8020
CH2.3	44	8520	8560
CH3.1	249	12500	12700
CH3.2	255	13300	13300
CH3.3	72	12900	13000

*The sample C21.1 was lost in the experimental work.