



ارائه شده توسط:

سایت ترجمه فا

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از نشریات معتبر

Assessment of phenolic compounds in biological samples

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Summary. A short review of extraction, purification and cleanup procedures for the analysis of phenolic compounds (anthocyanins, flavonoids, catechins, phenolic acids, etc.) in animal and human biological fluids and organs is presented. The rising interest about the antioxidant capacity of these widespread constituents of fruit and vegetables enables these procedures an invaluable tool prior of the separation and identification of these compounds and their metabolites occurring in the organism. Finally, the usual chromatographic and spectrophotometric analytical techniques for their separation and identification are briefly summarized.

Key words: phenolic compounds, flavonoids, analysis, extraction, sample cleanup, biomatrices.

Riassunto (*Valutazione dei composti fenolici in campioni biologici*). È presentata una sommaria rassegna delle tecniche di estrazione e di purificazione dei composti fenolici (antociani, flavonoidi, catechine, acidi fenolici, ecc.) da fluidi biologici o da organi animali o umani. L'interesse crescente sulle proprietà antiossidanti di questi composti assunti con la dieta di origine vegetale rende queste tecniche un valido ausilio per lo studio delle biotrasformazioni che essi subiscono nell'organismo. Sono poi brevemente illustrate le tecniche analitiche cromatografiche e spettrofotometriche idonee alla loro separazione e identificazione.

Parole chiave: composti fenolici, flavonoidi, analisi, estrazione, purificazione, biomatrici.

INTRODUCTION

Phenolic compounds include a large class of phytochemicals that are endowed with interesting biological properties. Among the most important are anthocyanins, flavonoids, catechins, phenolic acids, secoiridoids, stilbenes, coumarins and isoflavones which are widespread in vegetable crops such as fruits, vegetables, herbs, grains and seeds and derived foods such as juices, wines, oils, etc. [1, 2].

In recent years there have been many reports in the literature on the role of these natural compounds in counteracting the negative effects of oxygen and nitrogen reactive species (ROS/RNS), maintaining the redox homeostasis of biological fluids and preventing human disease such as cardiovascular diseases, atherosclerosis, and other degenerative pathologies such as cancer, diabetes, Alzheimer's and Parkinson's diseases [3, 4].

Biochemists have used several strategies to evaluate the antioxidative power of phenolic compounds and to determine the amounts of individual species in foods and beverages.

Many methods have been used to measure the *in vitro* total phenolics, the individual phenolic contents and the total capacity of scavenging ROS/RNS and other free radicals. The information contained in these studies has contributed to understanding the significant role that phenol-rich foods play in maintaining

a healthy lifestyle [5]. Various public institutions have published appropriate guidelines to encourage the consumption of food and beverages of vegetable origin.

The *in vitro* analysis, however, does not measure the efficiency of these compounds in improving the defense of the human body against free radicals. Absorption, bioavailability, pharmacokinetics and metabolism of these compounds may greatly affect the efficacy of their action *in vivo*.

There are literature reports on the actual amounts of these compounds or their metabolites in animal and human serum, plasma, blood, urine and body organs and the concentration levels of phenolic compounds in the fluids and tissues over time that are necessary to effectively protect human health [5].

The purpose of this paper is to present the specific procedures for the extraction, isolation and purification suitable for the analysis of phenolic compounds in animal and human biomatrices.

DISTRIBUTION OF PHENOLIC COMPOUNDS IN ORGANS, TISSUES AND FLUIDS

Absorption

The phenolic compounds may have simple to very large structures endowed of one or more hydroxyl

groups (polyphenols) and in most cases they are glycosylated with simple or complex sugar moieties. The type and number of glycosylation are the main factors that affect the absorption of phenolic compounds. They are primarily absorbed in the stomach and in the gastrointestinal (GI) tract.

Bioavailability

Bioavailability is affected by the fat and protein content, by the type of glucoside and by the aglycones occurring in the phenolic compound [6]. The maximum concentration in the plasma is generally reached within 15-30 minutes after consumption [7]. The half time disappearance for the same phenolic aglycones proceeds in the order galactose > glucose > arabinose, whether in the case of the same sugar moiety, the order followed is trihydroxy > dihydroxy > methoxy > dimethoxy > trimethoxy phenol [8, 9].

Generally the bioavailability is very low, less than 1% of the quantity consumed.

Metabolism

The biological fate of flavonoids is highly complex, although they are typically metabolized in the liver [10]. They undergo by the liver enzymes and are transformed into glucuronates. A lot is metabolized to sulphonated derivatives or methoxylated. These are the forms in which they are excreted in the urine. This complicates very much the situation because from a single phenol several derivatives may be obtained [11].

As an example, from the simple pelargonidin 3-glucoside three monoglucuronides are obtained, one sulfoconjugate and the pelargonidin itself [12].

Recoveries

The highest recoveries of almost intact phenolics or their glucuronates/sulphates occur in the urine and in the bile, ranging from 5 to 50% of the quantity assumed with the food or drink. The maximum recovery peak is reached in 3-6 hours, and all compounds or their metabolites are completely recovered within 24 hours [13-15]. In other tissues and in the plasma the distribution is very different according to the phenolic compound involved. Generally liver and kidneys are the organs in which the phenolic content occurs in the highest amount.

Methoxylated phenolics or acylated phenol glycosides are highly absorbed, and their presence in the urines will be very low [13].

EVALUATION OF THE TOTAL PHENOLIC CONTENT

The measurement of the Total Phenolics as GAE equivalent with the FCR

Among the several chemical methods to evaluate the total antioxidant power *in vitro* or *in vivo* [16, 17] based on the capacity to exchange protons (HAT methods: ORAC, TRAP, crocin bleaching) or electrons (ET methods: TEAC, FRAP, TAP, DPPH, Cu⁺⁺

ions reducing methods), belonging the latter category, there is the Folin Ciocalteu Reagent (FCR) method, mainly the most simple and widely used method of evaluation of the antioxidant potential.

This method is based on the Folin-Ciocalteu Reagent (or simply Folin or phosphomolybdate assay). It is a slightly yellowish golden mixture of hexavalent salts of Mo and W in acidic media that are reduced to blue colored complexes, mainly by the phenolic hydroxyls. The color develops completely after addition of an alkaline salt such as sodium carbonate.

This colorimetric test was developed for the first time as a protein reagent due to the fact that the ubiquitous tyrosine occurring in proteins, give positive response to this test. Today FCR is used to measure the total polyphenolic content in foods and beverages. As standard reference compound is typically used gallic acid, a compound cheaply available in very pure form, endowed of an average reactivity. Therefore the total phenolic content is conventionally expressed as Gallic Acid Equivalent (mg/L GAE) [18]. Other than phenolics, some other reducing compounds occurring in foods may react with FCR. They are ascorbic acid, glutathione, and few others. In foods rich in phenolics this is generally low relevance interference. On the other hand these compounds participate to the antioxidant potential of the food. The blue color produced by the FCR with the phenolics is measured by colorimetry at 700-765 nm. Due to its simplicity it can be easily automated for routine analyses.

The test does not entail any special sample preparation other than a homogenization of the sample, followed by a centrifugation when necessary.

Generally this test gives the first information on the overall phenolic content occurring in a sample, an important data in view of further analyses and in order to operate dilutions/concentrations when needed.

EXTRACTION AND ISOLATION METHODS

The variety of classes of phenolic compounds occurring in nature, their different properties, and the necessity of isolate these compounds from the other constituents presents in the complex matrices that are the biological fluids or organs, particularly rich in proteins, has conducted to the pinpoint of specialized procedures for their extraction, isolation and subsequent determination.

PPT (protein precipitation)

Biological fluids are characterized by the presence of proteins, which may affect further steps of processing such as isolation, purification and analysis. The polarity of the compounds to be extracted limits for a while the use of solvents. Therefore a first step is the elimination of interference due to the proteins by precipitation and subsequent centrifugation. It is the usual simplest way to eliminate them. Deproteinization is usually done by addition of 5

volumes of acetonitrile to the sample. This procedure gives better results and high recovery [19].

Otherwise, classical precipitation with trichloacetic acid or other acidic compounds may affect phenolic compounds when conjugated as glucuronates or sulphates.

SPE (solid phase extraction)

The SPE is done in factory-made cartridges of different volumes and supports. Several types of stationary phases and dimensions are available according to the compounds to be isolated.

This is the most frequently used procedure [20]. When employed on the serum it reduces the background very well, particularly as in the case of subsequent mass spectrometry (MS).

The advantages of this isolation technique are:

- high selectivity;
- speed of extraction;
- possibility of automation;
- low quantity of solvents involved.

The SPE in some cases requires acidification or alkalization of the sample before extraction. In the case of urine or serum it is possible to fully automatize the process with evident advantages in term of safety.

The efficiency of SPE depends on several factors such as: type of solvent employed, sample volume and pH, solvent and modifier content if present. Availability of different types of cartridges (C_{18} , C_8 , C_2 , SAX, etc.) may consent selectivity for a particular type of phenolic compounds. The most recent SPE cartridge very advantageous for phenolic compounds is the Oasis Hlb distributed by Waters [21].

This is a wetttable polystyrene divinylbenzene copolymer cartridge which polar characteristics consent 5 to 10 times loadings higher than usual hydrophobic silica based cartridges. Furthermore it permits fast elution time, high recovery and constant reproducibility for all classes of phenolic compounds.

LLE (liquid liquid extraction)

This technique is ideal for lipophylic compounds. In the case of phenolics selection of an appropriate solvent is necessary. Flavonoids are extracted with ethyl acetate, possibly after acidification.

Ethyl ether can be used as an alternative for low molecular weight phenolics. By acidifying the media, the phenol-phenate equilibrium completely shifts towards the less polar phenyl form, thus facilitating extraction with non polar solvents. High molecular weight phenolics can be extracted with butyl or isoamyl alcohols. This is very efficient for less polar compounds such as flavonoid aglycones, or procyanidins.

In urine, due to the high concentration of phenolic compounds, extraction can be avoided and usually a simple dilution is sufficient. When suppression of the matrix interference is important, as in the case of liquid chromatography-mass spectrometry (LC-MS), LLE is insufficient and needs to be combined with SPE or, in some cases PPT is combined with both SPE and LLE.

Some authors used an internal standard added to the sample in order to estimate losses during the extraction/purification steps. A useful compound is trihydroxycinnamic acid, a phenolic compound not naturally occurring in foods but endowed with the same general properties of other food constituents [22].

IN VIVO ANALYSIS OF PHENOLIC COMPOUNDS

The *in vitro* analysis presents several limitations because when the data are transferred to *in vivo* experimentation some mechanism may affect the actual antioxidant concentration in the blood and organs:

- 1) bioavailability appears to be, generally, relatively low. For example when healthy volunteers assume 4.5 g of green tea dissolved in 500 mL of water, peak plasma concentrations of individual catechins are lower than 2 micromoles/L;
- 2) subject variability is another factor that may affect antioxidant activity. A single dose of pure catechins gave an average peak plasma concentration of 5.0, 3.1 and 1.3 to three volunteers [23];
- 3) metabolism may affect the efficiency in antioxidant power. Some metabolites may have a different antioxidant capacity than their precursors. Usually phenolics undergo biotransformation in the GI tract, in the liver and in the kidneys [24]. They are usually glucuronidated, sulphonated or methylated. Conjugates concentrations are generally 4 to 6 times higher than unconjugates;
- 4) in addition, the intestinal microflora may metabolize phenolic compounds. In the case of catechins, a ring fusion produced by the bacteria, form new compounds with different antioxidant power than in the precursors;
- 5) another factor is the composition of the other food constituents introduced with the phenolics. It may affect bioavailability as in the case of fats. Although not completely clear, several studies have demonstrated that consumption of proteins together phenolic rich foods (e.g., milk with tea phenolics), may reduce their antioxidant power or annul them completely [25].

EXTRACTION FROM BIOLOGICAL FLUIDS

From blood/serum/plasma [26, 27]

Usually blood is collected by venipuncture in the brachial vein. A procedure consists of collecting about 20 mL of blood in vacutainer tubes. It is then left to clot at room temperature for 30 minutes. Later it is centrifuged ($1000 \times g$) for 15 min at $5^\circ C$ to recovery the serum. It may be stored at $-80^\circ C$ until analysis. In some case proteins may interfere, therefore precipitation can be attempted with acetonitrile, followed by SPE on alumina as for catechins [28]. Blood samples were collected before and at 0.5, 1, 2, 3, 4 and 6 hours after consumption of the phenolic-containing food.

The EDTA blood samples were centrifuged at $3000 \times g$ for 10 min at $4^\circ C$ and the plasma was re-

moved quickly and stored at $-80\text{ }^{\circ}\text{C}$ until high-pressure liquid chromatography (HPLC). A $500\text{ }\mu\text{L}$ portion of plasma was adjusted to pH 2.5 with $150\text{ }\mu\text{L}$ of 1 mol potassium dihydrogen phosphate solution and $15\text{ }\mu\text{L}$ phosphoric acid. Each sample was vortexed mixed with 2.5 mL acetonitrile for 1 min and centrifuged at $3500 \times g$ for 10 min at $5\text{ }^{\circ}\text{C}$.

The supernatant liquor was evaporated to dryness at $35\text{ }^{\circ}\text{C}$ in a speedvac.

The residue was reconstituted in $100\text{ }\mu\text{L}$ methanol and an aliquot injected in HPLC.

From urine

Phenolic compounds in urine can be extracted by an appropriate solvent, such as ethyl acetate [29]. The urine samples are generally acidified (fundamental in the case of the anthocyanins) with the addition of $20\text{ }\mu\text{L}$ of concentrated hydrochloric acid per mL of sample.

Urine samples are stored at $-80\text{ }^{\circ}\text{C}$ until analyses.

EXTRACTION PROCEDURES FROM TISSUES [24, 30, 31, 32]

Animal and human tissues (liver, brain, etc.) containing polyphenols are homogenized (by an Ultraturrax or similar apparatus) together with the extraction solvent, usually ethyl acetate, which gives excellent results, is not water miscible, and is easily removable. Sonication may increase extraction efficiency.

Foam formation can be avoided by addition of an antifoam agent such as silicone oil or octyl alcohol.

For anthocyanins or other glycosylated flavonoids the use of methyl alcohol is an excellent procedure. Methanol must be acidified, possibly avoiding inorganic acids, because they may hydrolyze glycosides. Good results are given by formic, acetic or trifluoroacetic acid.

Animals (rats, guinea pigs, etc.) were anesthetized in the morning after overnight fasting, by inhalation of isoflurane, using NO-O_2 (1:1, v.v.) as a carrier.

They were fully bled via the abdominal aorta. Blood was collected in EDTA-tubes and plasma was subsequent obtained by centrifugation. The further procedure follows as previously described for these fluids.

After blood collection, the tissues were dissected, weighed and immediately frozen in liquid nitrogen. In distribution studies GI tract, being the most directly exposed to polyphenol intake, is usually not collected [24].

Extraction procedure from organs

Prior to extraction all tissues were lyophilized before further processing. Then, the organ was chopped in small pieces in liquid nitrogen and milled in an appropriate mill. Small organs, like of the rats may be ground and homogenized in a mortar with a pestle.

Some organs like liver, kidney, lung, muscle and brain require additional homogenization in a mill. Samples are stored in airtight containers at $-20\text{ }^{\circ}\text{C}$.

Enzymatic hydrolysis [8, 24, 33, 34]

When a phenolic compound is present in body fluids it generally occurs in one or several conjugated forms. In order to simplify its identification, it is therefore hydrolyzed to liberate the aglycone in free form. Chemical hydrolysis must be avoided due to the degradation of the structure of the compound. Usually, the aglycone is obtained from their conjugates (glucuronates or sulfates) by an appropriate enzymatic treatment.

To $200\text{ }\mu\text{L}$ of plasma an adequate amount of an antioxidant and an anti clotting agent are added. Thus a mixture of EDTA and ascorbic acid are usually added. The pH is adjusted to 7 by means of appropriate amounts of H_3PO_4 and KH_2PO_4 . Subsequently 250 units of beta glucuronidase (from *Helix pomatia* or from *Ampullaria*) and 1 unit of sulphatase were added. Generally these enzymes are sold in combination by the manufacturer such as Sigma.

Under nitrogen the enzymatic process is conducted at $37\text{ }^{\circ}\text{C}$ for 45 to 60 minutes.

The enzymatic reaction is stopped by immersion in an ice bath. The enzymatic reaction product is then submitted to SPE for recovery of aglycones to be analyzed.

SEPARATION, DETECTION AND IDENTIFICATION TECHNIQUES

A very large number of phenolic compounds occur in nature; therefore no single procedure may be valid for their separation. Each class of phenolic compound, in relation to their chemical structure, spectral characteristics, polarity or glycosidic linkages, needs an appropriate analytical separation and identification technique.

Due to these facts there are very extensive reviews on the analytical procedures used for separating and identifying the phenolic compounds which is not the scope of this paper.

Analytical separation techniques for phenolic compounds have passed from classic paper, thin-layer chromatography (TLC) and gas chromatography (GC), to HPLC coupled with spectrometry to HPLC coupled with MS and nuclear magnetic resonance spectrometry (NMR) according to advances in technology in the recent years [35].

Usually the separations are performed by chromatography, mainly HPLC or in some cases GC. GC is currently a valuable tool for the separation of low molecular weight compounds such as phenols, phenolic and benzoic acids. TLC is used mainly for preparative purposes.

DETECTION OF PHENOLIC COMPOUNDS

Spectral properties are generally utilized for the identification of the phenolics.

UV-Vis (ultraviolet visible spectrophotometry)

For chromatographic analyses, usually the simplest way is the use of a UV-Vis detector. Almost all phe-

phenolic compounds absorb with a maximum in the UV region at 270-280 nm. Additional spectra/shifts occur in the 220-230 nm band e.g., for catechins, hydroxytyrosol, or in the 330 nm region as in the case of hydroxycinnamic acids and flavonoids. Anthocyanins alone absorb in the visible region showing a maximum at 520-550 nm.

DAD (diode array detection)

Many flavonoids possess typical absorption spectra that are useful in their identification.

Today DAD detectors give much more information about the full spectra of these compounds, which may be a great help in their identification. Usually the spectra, collected in the range 200-700 nm, are compared with the spectra of reference compounds by evaluating the degree of overlapping. This is generally sufficient for routine analyses or when phenolics or their metabolites are well known.

FL (fluorimetry)

Several classes of phenolics such coumarins, hydroxycinnamic acids, and catechins, are strongly fluorescent. This detection technique has been implemented for the analysis of phenolics in biomatrices [28, 36, 37]. Excitation and emission spectra, usually falls in the UV range. As an example hydroxytyrosol has an excitation wavelength of 281 nm and an emission wavelength of 316 [37].

This property is very advantageous to the analyst because fluorescence increases sensitivity and minimizes background interference, thus simplifying sample preparation.

CAD (coulometric array detection)

Recently, electrochemical detection of phenolics, based on their redox properties has gained diffusion. These techniques are very sensitive and more selective than those previously mentioned. Thus HPLC separation coupled with CAD, or amperometric or voltammetric detection of phenolic compounds has been extensively applied in the analysis of polyphenols containing biomatrices [38-40, 26]. Nevertheless, due to the utilization of buffers and delicate sensors, these instruments need further improvement.

Other techniques such as capillary zone electrophoresis (CZE) were found utilized for the separation of phenolics [41].

Due to unavailability of the majority of standard reference compounds, identification is made using chemical or enzymatic hydrolyses in order to disassemble their molecular structure or by collecting their mass spectra. This is particularly important in the case of the identification of phenolic metabolites. Additionally, NMR may be used to define its final structure [42].

MS (mass spectrometry)

Further information, in some cases largely definite, can be obtained by collecting the MS spectra of the

compound. It is particularly evident in the case of research projects, when the phenolic compounds involved may be numerous and unknown or partly unknown they and their metabolites [43, 44, 34, 20].

Better performance in identification can be obtained by MS-ESI (electrospray ionization) [30], by MS-MS [19] or, as in the case of high molecular weight compounds, like complex structures of anthocyanins, by fast atom bombardment-mass spectrometry (FAB-MS) [45].

Generally this approach gives the desired results, but the above mentioned techniques are not currently available in all laboratories.

Many analytical procedures have been published for the separation of single classes of phenolic compounds, each devoted to give better performance within the compounds belonging to that class and researchers may refer to this specialized literature.

Thus, we can indicate papers or reviews devoted to pinpointing chromatographic analysis of phenolic acids [46, 47, 48], hydroxytyrosol [49, 37], resveratrol [50, 51], coumarins [52], anthocyanins [45, 20, 53, 54, 15] flavonoids [36, 55, 56], isoflavonoids [21, 41, 57], secoiridoids [42, 37], catechins and procyanidins [58, 26, 28, 33] in foods and/or in biological matrices.

CONCLUSIONS

Analyzing phenolic compounds present in biological fluids and in animal and human organs is a delicate task with very important implications for our health. At the present time of the knowledge and of the technical development the analytical procedures available for the classes of phenolic compounds occurring in foods are highly experimental.

They consists mainly of the HPLC separation techniques coupled with the common spectrophotometric detection instruments (UV-Vis, DAD, FL) or they are based on other chemical-physical properties of the phenolic compounds (CAD, amperometric, voltammetric, etc.) or on the more sophisticated mass spectrometers, which are less available due to their cost. They will be enhanced in sensitivity and precision when further coupled with the MS-MS detectors or by ESI detectors. In relation to these premises, the extraction, isolation, and purification of the biological sample are an important task for the success of all the procedures of analysis and the collection of the data, from which our health depends on. Thus, several procedures to extract, isolate and purify phenolic compounds have been illustrated in this paper. Their particular characteristics have not only been taken into account, but above all of the distance they have gone inside the organism to reach the target organs. The biotransformation they have undergone has also been taken into account.

Submitted on invitation.

Accepted on 18 October 2007.

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