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"Polyphenols for improving food quality and nutraceuticals"

*Review*

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# An Overview of Plant Phenolics Measurement

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## ABSTRACT

Plant phenolics are widely distributed in nature and are well known for their remarkable antioxidative properties in biological systems such as plants and non-biological systems such as foods. Substantial work has been done to measure plant phenolics in different matrices; the four common steps involved in a measurement process are sampling, sample preservation, sample preparation, and analysis. This review addresses how sampling and sample preservation affect phenolic compounds levels and stability in different plant materials. Other important measurement factors including various sample extraction techniques and analysis methods are also reviewed in this paper.

**KEYWORDS:** Plant phenolics; Phenolic extraction; Phenolic analysis.

## INTRODUCTION OF PHENOLIC COMPOUNDS

Phenolic compounds are widely distributed in nature as secondary metabolites produced through different pathways in plants.[1] More than 8,000 phenolic compounds with diverse structures and functionalities have been found and reported from various plant sources.[1] Figure 1 shows the three major categories of phenolic compounds: simple phenols, also known as phenolic acids, polyphenols, and a miscellaneous group.[2]

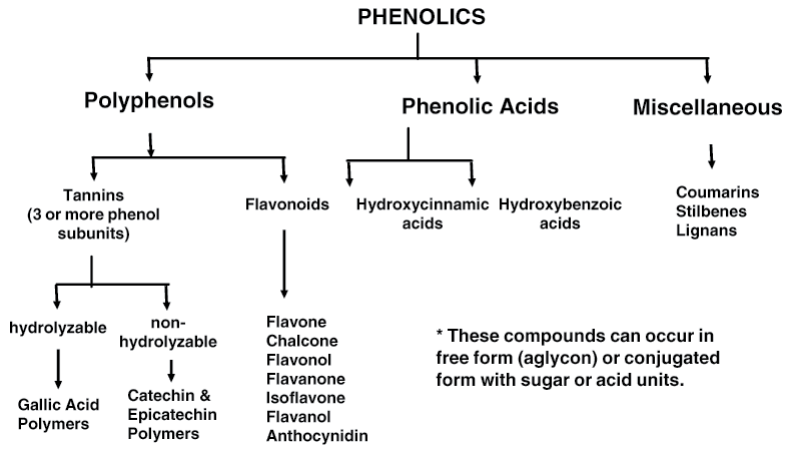


**Figure 1:** Classification of phenolic compounds.[2]

Studies of plant phenolic compounds have dramatically increased during the past few decades due to the unique and remarkable properties of the compounds in biological systems such as plants and human beings as well as in non-biological systems such as foods. The basic structural building block of all phenolic compounds is a phenyl ring bearing a hydroxyl group; this unique structure allows them to be either hydrogen-donors or acceptors.[3] Thus, phenolic compounds exhibit antioxidant properties due to their ability to scavenge free radicals generated from lipids, proteins, and oligonucleic acids. The free radical scavenging ability of phenolic compounds has been widely studied and associated with multiple biological effects including health benefits for human beings.[4-6] With the potential to reduce the risk of certain chronic

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diseases,[7,8] plant extracts that are rich in phenolics have attracted increasing interest in academia Figure 2 and the food industry.

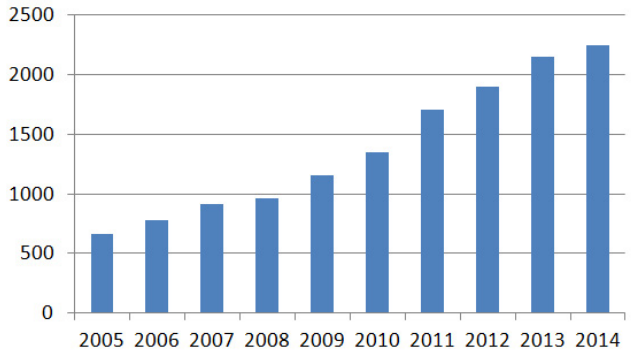


**Figure 2:** Number of publications containing "phenolics" from 2005 to 2014. Source: SciFinder Scholar.

Plant extracts usually contain a combination of various phenolics rather than a single phenolic compound. For example, epicatechin, epigallocatechin, epicatechin gallate, caffeic acid, and quinic acid were identified by direct infusion Electrospray Ionization Mass Spectrometry (ESI-MS) in a study in which the authors were looking at different solvents for the extraction of phenolics from green tea.[9] As shown in Figure 1, the complexity of analyzing phenolic compounds is that they have different properties polarity, solubility, etc. due to different structural moieties. Therefore, it is of critical importance to develop rapid, reliable and accurate analytical methods in order to establish relationships between phenolic compounds and their corresponding bio-physiochemical properties.

The four common steps involved in a measurement process are sampling, sample preservation, sample preparation, and analysis including separation and detection of components. as shown in Figure 3.[10]

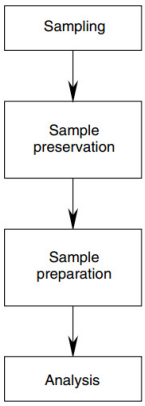


**Figure 3:** Steps in a measurement process.[10]

## SAMPLING

Sampling is the first step in any analysis, where a sample of interest is collected for further tests. It should be kept in mind that how and where the sample is collected have an important effect on the final analytical results. For example, when collecting samples to determine anthocyanins in grapes, their levels can vary greatly depending upon the cultivar of the grapes, the environment that the grapes were grown in, and what part of the grapes seed, skin, etc. is used for analysis. Researchers are usually aware of this and the details of sample information are well documented in recently published literature. Table 1 is a representative example that shows the total phenolic distribution measured by the Folin-Ciocalteu method of peel, juice, and seed in four different pomegranate cultivars.[11] The highest total phenolic content of 3547 µg/g was found in the peel of "Lefan", which is 20 times higher than that of the seed in the same cultivar. Other examples include influence of berry size on sugar content in berries collected from the same grape cluster bunch but different locations top, middle, and bottom of the bunch.[12] Different catechin contents in three tea cultivars grown in 10 locations which showed how environment especially climate exerts a significant effect on biosynthesis of tea catechins,[13] and varied flavonoids levels in different parts of blueberry leaves, buds, and berries.[14]

| Cultivar | Peel | Juice | Seed | Total |
|---|---|---|---|---|
| Lefan | 3547.8 | 1551.5 | 125.3 | 5224.6 |
| Katirbasi | 3127.0 | 1229.5 | 121.2 | 4477.8 |
| Cekirdeksiz-IV | 2537.1 | 784.4 | 117.0 | 3438.5 |
| Asinar | 1775.4 | 1307.3 | 177.4 | 3260.1 |
| Mean | 2746.8 | 1218.2 | 135.2 | 4100.3 |

**Table 1:** Total phenolic unit: µg gallic acid equivalent GAE./g. distribution of peel, juice, and seed in four pomegranate cultivars.[11]

## SAMPLE PRESERVATION

Since there is usually a delay between sampling and sample preparation and analysis, it is important to preserve the samples appropriately so any possible quality changes can be minimized. For example, in a study to evaluate green tea catechins in commercial tea leaves in the dry state during storage for six months, a progressive decrease in the total catechin content was observed, especially of the most abundant phenolics, −.-epigallocatechin 3-gallate EGCG. and −.-epicatechin 3-gallate ECG.[15] Murcia et al evaluated the antioxidant activity of 25 vegetables artichoke, asparagus, beetroot, broad bean, broccoli, Brussels sprout, carrot, cauli flower, celery, chicory, cucumber, eggplant, endive, garlic, green bean, leek, lettuce, maize, onion, pea, pepper, radish, spinach, Swiss chard, and zucchini. under different storage/preservation conditions, and found that refrigerated storage 7 days, frozen 1st day, frozen storage 8 months, canned 1st day, and canned storage 18 months resulted in different antioxidant activity loss across all 25 vegetable species.[16]

Therefore, it is suggested that when samples are stored/preserved for subsequent preparation and analysis, it is very important to inactivate possible enzymatic, metabolic, and chemi-

cal reactions in order to maintain sample integrity.[2]

## SAMPLE PREPARATION

Sample preparation is usually a multi-step process. Such processes may consist of sample size reduction milling and grinding and homogenization, followed by extraction, then concentration, purification, and fractionation, and sometimes other steps, such as derivatization and hydrolysis. Normally, the steps chosen are dependent upon the chemical and physical properties of the sample and the analysis objectives. Due to their diverse structures and properties eg. polarity, solubility, and stability, etc., there is no universal method available to extract all phenolic compounds from any given set of samples. Procedures used to extract compounds from plant materials consist of distillation, solvent extraction, and cold pressing. These methods have been widely adopted in academia and industry for many years, and the effects of parameters such as sample size, extraction time, temperature, solvent type, and solvent-to-sample ratio on extraction efficiency have been widely discussed in published literature. Thus, this section will focus on reviewing several modern non-conventional extraction techniques.

### Supercritical Fluid Extraction (SFE)

When temperature and pressure are above their critical points, a supercritical fluid SCF forms having density and dissolving capability comparable to that of liquids and penetration power intermediate between that of gas and liquids.[17] This unique feature of SCF can be favorably exploited to extract phenolic compounds from plants. The most commonly used SCFs and their critical properties are listed in Table 2.[18] Among these solvents, carbon dioxide ($CO_2$) is the most popular for SFE due to its non-toxicity, low flammability, and low cost; it also allows SFE operation at relatively low pressure and temperature.[19] Overall, SFE reduces the use of solvents and avoids degradation reactions of the extracted compounds in the absence of air and light; this makes SFE an environmentally friendly and promising extraction technique. However, the high equipment cost may limit its application compared to conventional extraction methods.

| Solvent | Pc bar | Tc °C | Density g/mL |
|---|---|---|---|
| Methane | 46.41 | -82.4 | 0.16 |
| Carbon dioxide | 73.87 | 31.2 | 0.47 |
| Ethane | 48.84 | 32.5 | 0.20 |
| Propane | 42.46 | 97.3 | 0.22 |
| Ammonia | 113.99 | 132.6 | 0.24 |
| Ethanol | 63.83 | 243.6 | 0.28 |
| Benzene | 48.94 | 289.1 | 0.30 |
| Water | 221.19 | 274.3 | 0.32 |

**Table 2:** Critical properties of commonly used SCFs.[18]

### Subcritical Water Extraction (SWE)

When water is heated under high pressure in order to maintain its liquid state Figure 4, its polarity measured by dielectric constant is reduced due to the breakdown of intermolecular hydrogen bonds between water molecules.[20,21] By adjusting temperature and pressure, different polarities of subcritical water can be achieved[21] and used to extract a wide range of compounds with different polarities from fruits, vegetables, and herbs, examples include pomegranate,[22] red grape skin,[23] potato peel,[24] and rosemary.[25]

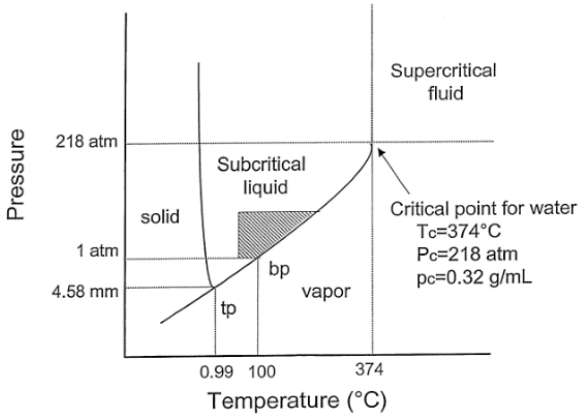


**Figure 4:** Phase diagram of water as a function of temperature and pressure.[26]

### Pressurized Fluid Extraction (PFE)

PFE, also known as accelerated solvent extraction (ASE), pressurized solvent extraction (PSE), pressurized liquid extraction (PLE), and enhanced solvent extraction (ESE), has attracted attention in recent years since this technique allows for relatively fast extraction and consumes less solvent than other standard extraction methods. A typical PFE system setup is shown in Figure 5.[27] The solvent is pumped into the extraction cells, which are then heated to a desired temperature to perform extraction. The pressure is then released and the extract is collected. The extraction process usually takes less than 30 minutes.[28] Under elevated pressure up to 150 bars and temperature around 100-200 °C, the solvent viscosity decreases which helps to disrupt the solute-matrix interaction and solubilization of the compounds being extracted increases which favors efficient and fast extraction.[27,29]

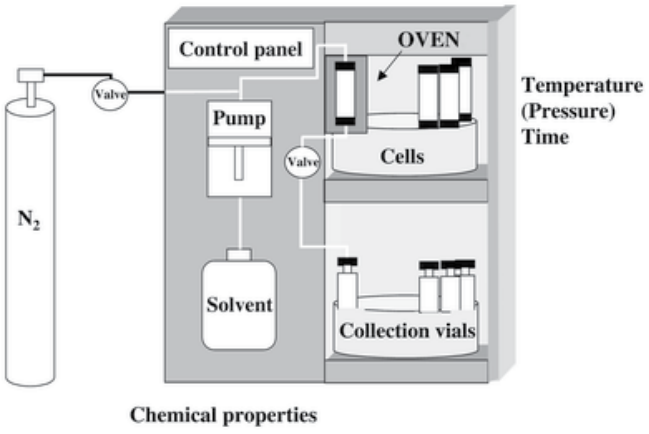


**Figure 5:** Principle of a PFE system and influencing parameters.[27]

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### Ultrasound-assisted Extraction (UAE)

Ultrasound radiation with frequencies higher than 20 kHz can create cavitation bubbles near the sample and collapse of these bubbles facilitates extraction of compounds from the solid matrices.[30] There are two UAE modes that can be used to extract phenolics from plants: static and dynamic. Static mode refers to a closed vessel system without continuous transfer of solvent, while dynamic mode refers to a flowing system in which fresh solvent is supplied continuously.[31] Static systems are easier to set up than dynamic systems; however the latter reduces extraction time and solvent consumption, and also helps avoid thermal degradation of extracted compounds caused by heat generated during sonication.[31,32] The UAE techniques do not require complex equipment. They are inexpensive and easy to use not only for small scale laboratory experiments but also for large scale industrial purposes.[33]

### Microwave-assisted Extraction (MAE)

Microwaves frequencies between 300 MHz and 300 GHz can generate heat by inducing molecular motions in molecules both the samples being extracted and solvents used for extraction that have dipoles.[27] Heat penetrates into the extracted material which helps to extract active compounds. In the case of phenolic compounds, the hydroxyl groups enable the compounds to absorb microwave energy therefore MAE can be used to extract phenolics from plant material.[27] Solvents play an important role in MAE, because many solvent parameters affect the extraction efficiency. These parameters include their boiling points, dielectric constant, and dissipation factor.[34] These parameters for the most commonly used MAE solvents are listed in Table 3.[35] Advantages of applying MAE to extract phenolics from plant material include reduced use of organic solvents and decreased extraction time with higher extraction yields compared to conventional solvent extraction methods; however, MAE is not suitable for the extraction of thermally labile compounds such as anthocyanins or compounds with a higher number of hydroxyl-type substituent's such as tannins.[34,36]

## SAMPLE ANALYSIS

Historically, polyphenol content of plant material had been performed using paper chromatography,[37,38] thin layer chromatography,[39] low-pressure column chromatography,[38,40] and gas chromatography,[41] the last of which improved the quantitative aspects of the analyses. Since the mid-1970's, the polyphenolic content of plant material, plant extracts, and biological material has been performed by High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection.[42,43] In more recent times, various HPLC conditions have been used with the majority being reversed-phase employing C18 column technology with acidic mobile phases and UV or photodiode array (PDA) detection.[44-58] In the recent literature, there can also be found some instances of the use of the newer fused-core column technology which allows for shorter run times. This is due to the fact that these columns run at much lower back pressure than do traditional porous particle columns and they can thus be run at faster flow rates and produce very similar chromatographic separations in shorter times.[59,60] The C18 column dimensions used, both porous and fused-core, generally range from 125 to 300 mm in length and 2 to 4.6 mm internal diameter with particle sizes ranging from 3 to 5 µm.

Additionally, there are an increasing number of instances of the use of Ultra-high Performance Liquid Chromatography (UPLC) analysis in the more recent literature[61-68] again using C18 columns and acidic mobile phases. The columns used in UPLC analyses are 50 to 100 mm in length, 2.1 mm inner diameter, with a particle size of 1.7 µm. The use of UPLC technology has the potential to significantly decrease analysis time and increase sensitivity of detection due to the decreased particle size of the column packing which results in narrower, sharper chromatographic peaks than those obtained with traditional HPLC columns. UPLC technology also results in decreased solvent consumption due to lower mobile phase flow rates and shorter run times.

The most common detection methods have been UV

| Solvent | Dielectric constant[a], ε′ | Dipole moment[b] | Dissipation factor, tan δ ×10$^{-4}$ | Boiling point[c] °C | Closed-vessel temperature[d] °C |
|---|---|---|---|---|---|
| Acetone | 20.7 | | | 56 | 164 |
| Acetonitrile | 37.5 | | | 82 | 194 |
| Ethanol | 24.3 | 1.96 | 2500 | 78 | 164 |
| Hexane | 1.89 | | | 69 | –[e] |
| Methanol | 32.6 | 2.87 | 6400 | 65 | 151 |
| 2-Propanol | 19.9 | 1.66 | 6700 | 82 | 145 |
| Water | 78.3 | 2.3 | 1570 | 100 | |
| Hexane–acetone 1:1. | | | | 52 | 156 |

[a]determined at 20 °C
[b]determined at 25 °C
[c]determined at 101.4 kPa
[d]determined at 1207 kPa
[e]indicates no microwave heating

**Table 3**: Physical constants and dissipation factors for some solvents commonly used in MAE.[35]

and PDA for both HPLC and UPLC analyses but the advent of more affordable mass spectrometers in the last 15 years or so has resulted in an increased number of methods using MS and MS/MS detection in order to more fully characterize the polyphenols present in various samples. Most of the methods use either single quadrupole or triple quadrupole mass spectrometers but there are some examples of the use of time of flight (TOF) and Orbitrap™ mass spectrometers as well. Single quadrupole mass spectrometers provide molecular weight information of the species detected and can be used for quantitation[69-71] while triple quadrupole mass spectrometers add the possibility of MS/MS experiments to aid in structure elucidation and confirmation.[72-91] Triple quadrupole mass spectrometers can also be used in the multiple reaction monitoring (MRM) mode, also called selected reaction monitoring (SRM), for very selective detection of low level species in order to confirm their presence and for trace level quantitation.[92-94] TOF and Orbitrap™ technology provide the possibility of accurate mass determination which is a major step toward identification of unknowns especially when coupled with MS/MS fragmentation data.[95-103]

In addition to UV, PDA, and MS, other detection methods for liquid chromatographic separations have been successfully applied over the years, including chemiluminescence reaction detectors, fluorescence detectors, and electrochemical detectors, especially for quantitation.[104-111]

Gas chromatography/mass spectrometry (GC/MS) has been employed for the analysis of phenolic microbial fermentation products in biological samples in order to study the metabolic impact of flavonoid consumption[112-114] but has also been used for the direct analysis of the polyphenolic content of plant material.[115-118] The major drawback to GC analysis of polyphenols is that derivatization is generally required while with liquid chromatographic analyses derivatization is not required. Derivatization adds a time consuming step to the sample preparation procedure.

Less commonly used methods of analysis found in the literature include high-performance Capillary Electrophoresis (CE) or Capillary Zone Electrophoresis (CZE)[119-122] for the separation of mixtures and for the quantitation of the polyphenols present in them.

When unknown polyphenols have been detected and isolated from plant materials, structure elucidation and identification has been accomplished with spectroscopic techniques, including 1D and 2D, $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy (NMR)[123,124] and infrared spectroscopy (IR) in conjunction with high resolution and MS/MS mass spectral data.[125]

Given the availability of lower cost, simpler to operate, and lower maintenance mass spectrometers, it can be expected that future analyses will very likely employ mass spectrometric detection to a much greater extent than has been seen in the past. The power of UPLC to shorten analysis times while maintaining, or even increasing chromatographic performance, will likely be exploited by analysts working in the field in order to detect and characterize new polyphenols that have not been previously detected. UPLC, when coupled to mass spectrometry, especially high resolution accurate mass spectrometry (HRAMS) instruments such as TOF and Orbitrap™ mass spectrometers, will dramatically improve attempts at characterization of new structures due to the availability of chemical formula prediction from the accurate mass data. One can envision work flows where UPLC is coupled to an HRAMS instrument as well as to an NMR resulting in full structure elucidation of new polyphenols in a single analysis.

**CONFLICTS OF INTEREST:** None.

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