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-OH What? A Review of Mass Spectrometry Techniques for the Analysis of Phenolic Compounds

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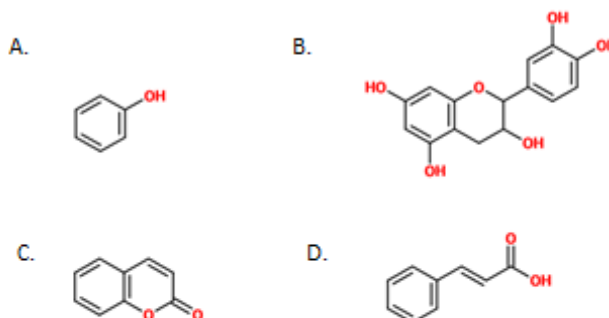
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ABSTRACT

Phenolic compounds, also called phenolics, are found in a variety of plant sources and can provide pharmacological and nutritive effects as well as taxonomic information. A single source can contain hundreds of phenolic compounds making characterization of these compounds time consuming and expensive. The recent leaps in mass spectrometry (MS) development including coupling to chromatographic columns, UV-vis, and tandem MS MS/MS) has renewed the study of phenolic compounds in both the agricultural and medical fields through its capability for fast, accurate and high throughput analyses. This paper will summarize recent discoveries, methodologies, and applications of MS in studies of phenolic compounds.

INTRODUCTION

Phenolic compounds are one of the most abundant phytochemicals. Phenolics are secondary plant metabolites, many of which are generated through the pentose phosphate pathway, which can exist in free, reversibly bound, or irreversibly bound forms^[1]. The classification of a molecule as a phenolic compound differs between researchers due to the lack of an accepted definition and categorization system. For the purpose of this article, phenolic compounds will be defined in a broad manner as: a compound that contains a hydroxyl group bound to an aromatic hydrocarbon, **Figure 1** shows some examples of phenolic compounds^[1,2]. This definition will allow the inclusion of as many phenolic compounds as possible, including: phenols, polyphenols, cinnamic acids, tannins, coumarins, and flavonoids^[1,3,4].



Four examples of phenolic compounds, a: phenol, b: catechin, a polyphenol, c: cinnamic acid and d: coumarin

Figure 1. Structures of phenolic compounds.

Phenolic compounds have attracted attention due to their potential medicinal abilities and functionality as food stabilizers or enhancers. (de la Luz Cádiz-Guerrea et al.,) Of particular interest is their antioxidant ability which contributes to both aforementioned applications [3]. Phenolics can act as free-radical scavengers, which is associated with anti-inflammatory, anti-cancer, anti-viral, and anti-bacterial functions [3,5-7]. In plants, this antioxidant ability is studied using a variety of methods such as FRAP (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity), DPPH, and TEAC (Trolox equivalent antioxidant capacity) [3,5-7]. Whereas the vanillin assay and Folin-Ciocalteu assay are used to determine total phenolic compounds present [3,6]. (de la Luz Cádiz-Guerrea et al.,) The benefit of mass spectrometry is its ability to generate structural information to identify compounds and their post-translational modifications, which former methods, e.g., UV-vis, are unable to generate as quickly and accurately [3,8].

Phenolic compounds are a part of the human diet through consumption of plants, plant extracts, and other foods such as honey. Flavanols, a polyphenol, are commonly consumed and both the compounds themselves and their metabolic products may be beneficial [4,9]. The heavy metabolism of these phenolics into multiple compounds make analysis of serum concentrations of phenolics a complex process that is simplified by mass spectrometry, particularly ultra-high performance liquid chromatography tandem mass spectrometry, UHPLC-MS/MS, and high performance liquid chromatography tandem mass spectroscopy, HPLC-MS/MS [4]. Mass spectrometry causes molecules to be ionized into a gas state wherein fragmentation results in peaks (m/z, mass to charge, ratio) that are used to characterize molecules, such as phenolic compounds, and may be coupled to chromatography columns and UV-vis analyzers to improve resolution and increase the accuracy of compound identification. Furthermore, the phenolic compound of a plant varies according to species, variety, season, environmental situation, and vasculature harvested (leaves, roots, stems, etc.) which requires analysis of a large amount of samples in order to determine optimal harvest time, location, and species, which is neither cost nor time efficient if other chromatography-spectroscopy or spectrometry techniques are used instead of MS [6,7,9].

Another limitation of current methodologies is that only a subset of the compounds present in an extract are identified, characterized, and quantified, mass spectrometry provides the ability to quickly characterize and quantify more compounds present in the extract. This leads to the ability to study the interactions between compounds, such as phenolics, that endow plants with medicinal abilities, whose presence was not even detected using other techniques [10]. An important application of phenolic compound MS is to assess the amount of beneficial compounds, phenolics, which are present and may be extracted from wasted parts of plants, e.g., seeds and stems of grapes [10]. Extraction and MS protocols are currently being established and optimized for the various sources of phenolics, however establishing a standardized protocol is challenging due to the variety of contaminants, phenolic compounds, and sources (dried beans, fresh leaves, etc.), a summary of MS techniques is available in **Figure 2** [7]. The high throughput and sensitivity of MS encourage researchers to use this method for phenolic profiling, despite the challenges [11].

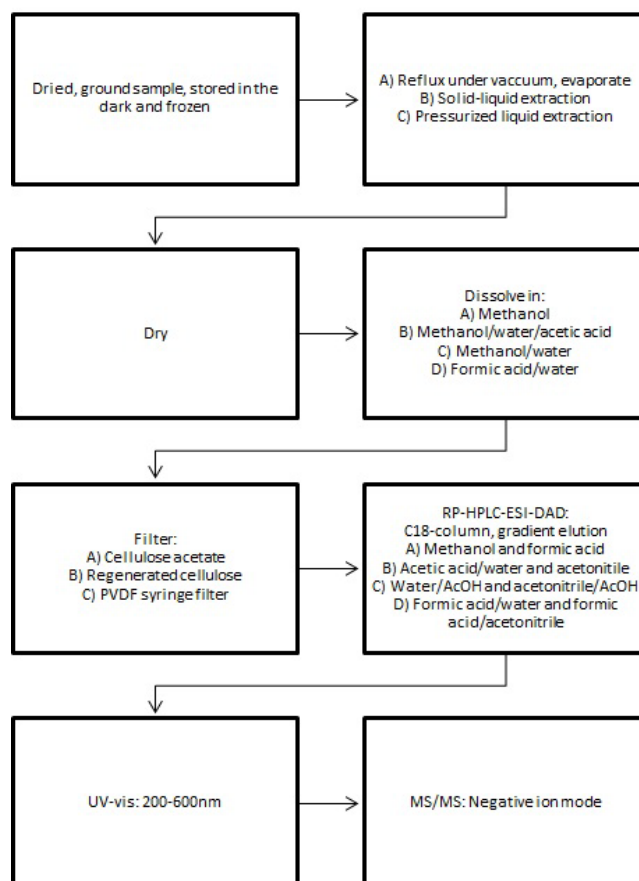


Figure 2. Summary of applications.

The rest of this article will discuss the applications of MS in phenolic-based research. First the studies assessing phenolic sources for antioxidant and medicinal applications will be discussed, subsequently, phylogenetic and metabolomic research, new protocols, and other applications of MS for phenolic profiling, a summary is found in **Figure 3**.

Figure 3. General procedure for detection of phenolic compounds.

Purpose	Mass Spectrometry Technique	Examples of Identifiable Compounds	Studies
Phenolic Profiling – Medicinal Plants	HPLC-QTOF tandem MS with UV-vis (220-600nm) With C18 column and m/z 100-1000	Hydroxycinnamic acid derivative, Hydroxybenzoic acid derivative, hydroquinone, flavanoids, coumarins, megastigmane glycosides	Zhang, Z. et al. 2014
	HPLC-ESI tandem MS with UV-vis	Flavanoids, glycosides, glucosides, chlorogenic acids, stilbenes, phenolic acids, anthocyanidins	Benayad et al. 2014, Chen, F., et al. 2014, Garcá-Salas et al. 2014, Jiménez et al. 2014, Ma et al. 2014, Mirali et al. 2014, Regueiro et al. 2014
Metabolomic Studies	LC-QTOF-MS	Cinnamic acid, flavanols, proanthocyanidins	Cedahia et al. 2014
	GC-MS	Catechin, coumaric acid, cinnamic acids, benzoate derivatives, cinnamate derivatives	Cedahia et al. 2014, Chang et al. 2014
	ESI-QTOF-MS	Diosmetin, apigenin, luteolin	Barrajon-Catalán et al. 2013
Food Safety	HPLC-ESI-QTOF MS	Cinnamides, procyanidin	Rodriguez-Pérez et al. 2013
Organic Waste Composition	HPLC-ESI-TOF MS	Lignin, flavones, ellenoic acids, phenols	Deási et al. 2014, Liang and McDonald, 2014, Lozano-Sánchez et al. 2014
	HPLC-ESI MS with UV-vis	gallic acid, catechin hydrate, epicatechin gallate	Ramirez-Lopez and DeWitt, 2014

Phenolic Profiling – the Hunt for Super foods

A phenolic profile is the quantitative description of all phenolic compounds present in a specific source. Phenolic profiling is conducted in order to determine potential candidates for drug development, sources of medicinal abilities, interacting compounds, and the location of original source acquisition (see section 2: Proteomic and Metabolomic studies). Phenolics are known for their antioxidant capabilities, which contribute to the interest in determination of their sources, for use as medicine, and improving food product shelf life ^[1].

The use of plants for medicine predates modern medicine; this makes traditional remedies a good starting point for drug development. *Ficus pandurata* H. is used in Chinese medicine for indigestion and was investigated using HPLC-QTOF-MSn to identify new compounds that contribute to the plant's medicinal properties. The roots of *F. Pandurata* were powdered, extracted (n-butanol) and filtered, then injected into the HPLC-MS (C18 column at 30 °C) and both mass spectra (m/z 100-1000) and UV-vis spectra (220-600 nm) were obtained ^[12]. Retention times when compared to standards were used to identify compounds known to be present in the extract. Whereas UV-vis spectra, fragmentation patterns (MS/MS), and molecular weight (MS) were used to identify other compounds. In all, 37 phenolic compounds, hydroxycinnamic and hydroxybenzoic acid derivatives and hydroquinone, flavanoid, coumarin, and megastigmane glycosides, were identified. The combination of HPLC-ESI-MSn and UV-vis was used by many researchers to characterize the phenolic profiles of fenugreek, peanut skins, soursop pulp, eggplant, lentil seed coat, Jerusalem artichoke leaves, walnuts, and *Cyclopia genistoides* L. When researching fenugreek, *Trigonella foenum-graecum*, the positions of glycosylation, linkage type (O- or C-), and isomers were distinguished using the MS/MS fragmentation, however, this method is unable to distinguish stereoisomers ^[13-18].

Other methods such as HPLC-ESI-MS (without UV-vis accompaniment), direct injection ESI-MS, UHPLC-MS, GC-MS (gas chromatography), and LC-ESI MS (liquid chromatography) were also used for the analysis of phenolic compounds. Studies of *Moringa oleifera*, honey, and *Polygonum minus* used principal component analysis (PCA) in order to discriminate between samples i.e., different locations of acquisition, or organs ^[8,9,12,19-24]. It is clear, however, that HPLC-ESI-MSn is the most popular method for the analysis of phenolic compounds ^[19-21].

Metabolomic studies – Phytochemical Detectives

Metabolomics is the analysis of all metabolites in an organism. Due to the high variability in metabolite characteristics, such as polarity and volatility, metabolite profiling is a very challenging ^[25]. Furthermore, metabolite profiles vary between crops grown in different locations, or that have differing ancestors, even amongst one species ^[25]. Consequently, a high throughput, robust technique is required for metabolomic profiling. Mass spectrometry's customizable instrument and experimental design allows it to meet these requirements.

The metabolomic profile of European beech leaves using the supernatant of chloroform then water:methanol extracted beech

leaves were directly subjected to LC-QTOF-MS or derivatised then analyzed by GC-MS, to find 56 phenolic compounds of which 38 were newly identified in this species. Caffeinated coffee has been observed to prevent Alzheimer's disease more effectively than decaffeinated coffee, so researchers used GC-MS to compare the metabolomic profiles of caffeinated and decaffeinated coffee samples to find the phenolic compounds that contribute to the difference in efficacy^[25]. They found 69 metabolites whose concentration varies between caffeinated and decaffeinated samples using orthogonal partial least squares discriminant analysis to process the results^[26]. ESI-QTOF-MS was used to identify the major metabolites produced by JIMF1 (human breast cancer) cells when exposed to olive leaf extract^[26]. Diosmetin, apigenin, and luteolin were identified in JIMF1 cytoplasm, however the pure compounds do not exhibit G1 cell cycle arrest, even though luteolin inhibits the MAPK (mitogen-activated protein kinase) pathway^[20]. This result emphasizes the importance of metabolomic profiling since natural extracts exhibit effects dependent on multiple interacting compounds, not individual active ingredients^[20].

The interpretation of MS and MS/MS spectra is particularly difficult for phenolic compounds due to the lack of understanding of the behaviour of phenolic compounds during mass spectroscopy. The behaviour of low molecular weight phenolics is especially mysterious because there have been fewer studies examining them^[27]. In order to accurately characterize low weight phenolics, a study using LC-ESI-MSn was conducted. They found that low molecular weight phenolics tend to fragment causing molecular ion peaks to have low abundance, which charged clusters of compounds are often misinterpreted as single phenolics, and they were able to establish a set of rules to predict fragmentation of low molecular weight phenolics^[27]. Accurate identification of phenolic compounds and metabolomics profiling are necessary tools when studying the medicinal effects of plant extracts.

New Methodologies – Chasing Simplicity

Mass spectrometry has been used for many years to determine the biochemical content of samples. It is difficult to establish a standard protocol for mass spectrometric analysis due to the need for adjustments for specific compound(s) of interest, desired resolution, and similarity between components within a sample. However, there are many papers that present a method that is successful and can be used as a starting point for future studies.

For the determination and quantification of phenolic compounds in virgin and extra virgin olive oil, liquid-liquid microextraction and UHPLC-ESI-MSn in dynamic reaction monitoring mode was tested. The proposed method is sensitive, fast, and uses a small amount of both sample and reagents thereby lowering the cost^[28]. The limitation of decreased or increased quantification of compounds due to matrix effects is the weak point of this methodology. The metabolites, found in blood plasma, produced by rats after eating grape seed flavanols were detected using a micro solid phase extraction (μ -SPE) followed by HPLC-ESI-MS/MS. This method uses low plasma volumes (250 μ L), and decreases interference from other compounds, but the plasma volume should be further lowered by future innovations to increase the ease of sample acquisition^[4].

The measurement of phenolic compounds in plant extracts has been accomplished using HPLC-ESI-MS as previously described (section 1), but may also be accomplished using CE-QTOF-MS (capillary electrophoresis quadrupole time-of-flight mass spectrometry) with sheath flow, deuterated and DPPH, interface. This method allows the identification, quantification, and free-radical scavenging activity of phenolic compounds to be tested^[29]. Maringer et al.'s method also increases the accuracy of structure assignments however, the sheath liquids dilute the samples. An interesting application of MS for the study of phenolics is using GC-C-IR-MS (gas chromatography combustion isotope ratio mass spectrometry) to study BFRs (phenolic brominated flame retardants) and BPA (bisphenol A) and their degradation products which are harmful to the environment. This method has no observed carbon isotope effect and can be applied to determine the source and predict future effects of these phenolics^[30]. The final methodology that will be mentioned can be used for food safety regulation by identifying and quantifying moniliformin, a mycotoxin, which may be found in grain plants. Lim et al.'s method uses PHREE, a protein exclusion purification column, and LC-LIT-MS and LC-QTOF-MS to measure moniliformin, and is easy, robust, and sensitive^[31].

Other Applications –Green Initiatives and Health Effects

Waste generated from the harvesting and processing of plants, e.g., leaves, stems, seeds, waste waters, may have a high content of phenolic compounds. Waste high in phenolic compounds are dangerous to the environment, so there are many initiatives to reuse waste and/or remove the phenolic compounds^[32]. If the phenolic compounds can be recovered, then they can be used for industrial reactions or drug production, or purified for use as standard reagents for research. There is a strong focus on olive oil production waste, waste waters, and filter cakes, as well as wine by-products such as grape pomace, since these crops have a lot of waste that can be salvaged for other uses. The investigation of olive oil waste waters and filter cakes used HPLC-ESI-TOF-MS to identify and quantify the phenolic compounds^[33]. In olive oil waste waters 27 phenolic compounds were identified, whereas in olive oil filter cakes 25 phenolic compounds were tentatively determined^[32,34]. Analysis of dried grape pomace used HPLC-ESI-MS and UV-vis to determine the phenolic content. The results showed that gallic acid, catechin hydrate, epicatechin gallate were the major phenolic compounds in dried grape pomace^[7]. Utilization of potato peel waste as biofuel was proposed after the analysis of the fermentation products revealed high lignin, a phenolic compound, and lipids using pyrolysis-GC-MS which could potentially be converted into biofuel^[33].

Phenolic compounds are well known for their antioxidant abilities however, their study has applications in other areas. The liberal definition of phenolic compounds as a compound with a phenol group (see introduction), enables the inclusion of estradiol

which contains a phenolic ring. The presence of hormones, such as estradiol, in wastewater threatens the ecosystems in which it is disposed [35]. (Fe³⁺-saturated montmorillonite aqueous system was shown to be effective in removing estradiol by transforming 17 β -estradiol (βE_2) into oligomers that are insoluble in water. Once the compounds have been converted into water insoluble forms they can easily be removed during regular water treatment and therefore harm to the environment, from increased estradiol concentrations, will be prevented. The conversion of phenolic compounds into phenolic lipids by lipase-catalyzed transesterification, naturally or artificially, may possess unique medicinal qualities. Virgin olive oil was used both to study lipid-phenolic interactions and to assess therapeutic ability [36]. LC-ESI-MSn was used to create the phenolic profile whereas therapeutic efficacy was determined by testing ACE (angiotensin converting enzyme) and amylase inhibition, as well as anti-tumorigenic activation in human colorectal cancer cells. Alu'datt et al., were able to elucidate the presence of lipid-phenolic interactions and to validate their potential to affect biological properties of phenolic compounds. A randomized controlled trial regarding changes in HDL (high density lipoprotein) phenolic composition before and after olive oil consumption was performed. The phenolic compounds were extracted by SPE and analyzed using UHPLC-ESI-MSn [37]. The analysis showed that the method, SPE and UHPLC-ESI-MSn, was able to measure the phenolic compounds present in the extract, and to determine that olive oil consumption imparts oxidation protective abilities to HDL.

Due to the high concentration of phenolic compounds in foods, the effect of long-term and short-term storage on phenolic compounds needs to be assessed to ensure food safety. The effect of gamma-irradiation, a process used commercially to increase shelf life, on phenolic compounds in cranberry syrup was assessed using HPLC-ESI-QTOF-MS to determine the shelf life of cranberry syrup by quantifying changes in phenolic compounds, e.g., degradation, change in concentration, in differing storage conditions [38]. They showed that most phenolic compounds are stable for 1 month. Overall, the applications of phenolic compound profiling are not limited to studies regarding antioxidant ability, but also have far-reaching implications for research, industrial process, and everyday life [38].

CONCLUSION

Phenolic compounds are present in a variety of sources. Their diversity in structure and properties, as well as the complex mixtures, in which they are found, makes phenolic profiling challenging. Fortunately improvements and innovations in the field of mass spectrometry have made phenolic profiling faster, more sensitive, and robust. HPLC-ESI-MS has gained popularity and may quickly become a routine step in studies of phenolic compounds. The antioxidant properties of phenolic compounds have been noted and the variety of sources can now be individually assessed to find the most likely candidates for therapeutics. Phenolic compound research also provides the opportunity to reuse what is currently waste and to drive the search for new drugs for therapeutic and preventative medicine. Metabonomic profiling can facilitate the identification of plant sources and mapping of the ancestry of crops to BFRs (phenolic brominated flame retardants) and BPA (bisphenol A) BFRs (phenolic brominated flame retardants) and BPA (bisphenol A) enable the selection of the most nutritious crops. Future research may attempt to reduce cost, optimize extraction and purification protocols, increase resolution of spectra, and develop standard procedures for mass spectrometry of phenolic compounds.

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