Free Radical Biology & Medicine, Vol. 37, No. 9, pp. 1324–1350, 2004

0891-5849/$-see front matter

ELSEVIER

doi:10.1016/j.freeradbiomed.2004.07.026

Serial Review: Flavonoids and Isoflavones (Photoestrogens): Absorption, Metabolism, and Bioactivity
Serial Review Editor: Catherine Rice-Evans

MASS SPECTROMETRIC METHODS FOR THE DETERMINATION OF FLAVONOIDS IN BIOLOGICAL SAMPLES

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(*Received* 5 *April* 2004; *Revised* 15 *July* 2004; *Accepted* 22 *July* 2004)
Available online 12 August 2004

Abstract— There is an ever-increasing interest in the biological effects of the bioflavonoids, members of the large group of plant polyphenols. Because of the aromatic character of these compounds, they have been analyzed by several chromatographic methods. In the case of high-performance liquid chromatography, they are readily detected by their ultraviolet absorbance or electrochemical properties. More evidence that the bioflavonoids undergo extensive metabolism during uptake from the gut and distribution around the body and in specific tissues is accumulating. In addition, free radical products at sites of inflammatory processes react with bioflavonoids and their metabolites, generating important new compounds of as yet unknown properties. For these reasons, careful examination of the chemical nature of bioflavonoids and their products in biological systems is absolutely required. Combination of mass spectrometry with the various chromatographic methods has proved to be highly successful in this regard. This review of the literature on the bioflavonoids is focused on the methods that are currently available for their qualitative and quantitative analysis by mass spectrometry and covers the period 2001–2003. Emphasis is placed on the description and value of existing methods, followed by an examination of emerging technologies.

Keywords—Flavonoids, Mass spectronomic analysis, Free radicals

Contents

This article is part of a series of reviews on "Flavonoids and Isoflavones (Phytoestrogens): Absorption, Metabolism, and Bioactivity." The full list of papers may be found on the homepage of the journal.

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INTRODUCTION

Flavonoids are among the most ubiquitous groups of plant secondary metabolites distributed in various foods and medicinal plants. During the past decade, an increasing number of publications on the health beneficial effects of flavonoids, such those as in cancer and coronary heart diseases, have appeared [1–5]. Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of substitution: hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavone, flavonol, flavanone, and flavanol types depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 with an OH group at C-3, respectively (Fig. 1).

Although they are sometimes found as their aglycones, flavonoids most commonly occur in plant materials as flavonoid *O*-glycosides, in which one or more hydroxyl groups of the aglycones are bound to a sugar, forming an acid-labile glycosidic O-C bond. There are certain hydroxyl groups in flavonoids that are usually glycosylated. These are the 7-hydroxyl group in flavones, flavanones, and isoflavones and the 3- and 7-hydroxyl groups in flavonols and flavanols. 5-*O*-Glycosides are rare for compounds with a carbonyl group at C-4, since the 5-hydroxyl group participates in hydrogen bonding with the adjacent carbonyl at C-4. Isoflavonoids are flavonoids with ring B attached to the C-3 position of ring C. This precludes the existence of a hydrogen-bonded hydroxyl group at the 3 position, diminishing the probability of significant contribution by such a group to the antioxidant activity of an isoflavone [6]. All are structurally related to the parent compound, flavone (2-phenyl benzopyrone).

Isoflavones such as genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone) are commonly regarded to be phytoestrogens because of their estrogenic activity in certain animal models. A major dietary source of isoflavonoids is soy products. There are at least 12 known isoflavone compounds in soybeans (3 aglycones, 3 glucosides, 3 acetyl-ester glucosides, and 3 malonyl-ester glucosides). Daidzein and genistein occur in four different chemical forms, namely, the aglycones (daidzein and genistein), the 7-*O*-β-glucosides (daidzin and genistin), the 6″-*O*-acetylglucosides (6″-*O*-acetyldaidzin and 6″-*O*-acetylgenistin), and the 6″-*O*-malonylglucosides (6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin). Similarly, glycitein (6-methoxydaidzein) has been found in four different forms, namely, the aglycone (glycitein) and the 7-*O*-β-glucosides (glycitin), 6″-*O*-β-acetylglycitin, and 6″-*O*-malonylglycitin. In addition, 4′-methylated derivatives of daidzein and genistein, biochanin A and formononetin, respectively, are present in red clover (*Trifolium pratense* L) [7]. Significant amounts of the isoflavone genistein as its glucosyl glucoside have also been reported in the tubers of the American groundnut (*Apios americana*) [8]. Mazur et al. [9] estimated the isoflavone concentrations

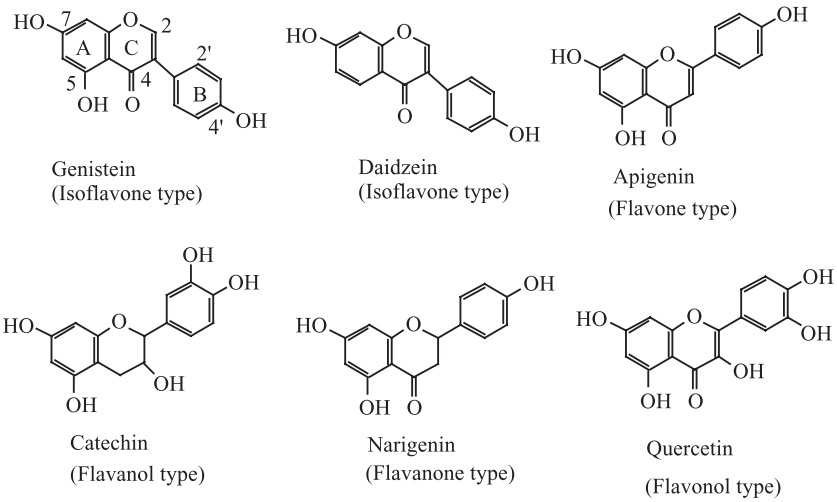


Fig. 1. Structures of flavonoids commonly found in dietary sources.

in 68 cultivars of 19 common leguminous food species and four forage legumes. The highest total isoflavone concentration was found in kudzu root (*Pueraria lobata*) (>2 mg/g dry weight). Puerarin (daidzein 8-*C*-glucoside) has been reported to be the major isoflavonoid in kudzu dietary supplements [10].

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most abundant flavonol-type flavonoids found in fruits and vegetables, such as apples and onions, and is a strong antioxidant [11]. The prominent flavonoids in tea are the flavanols catechin, epicatechin, epicatechin gallate, epigallocatechin (EGC), and epigallocatechin gallate (EGCG) and their fermentation products, derived tannins—theaflavins and thearubigins. A dietary supplement, marketed for natural breast enhancement [12], contains several prenylated flavonoid derivatives such as 8-prenylnaringenin that have been also isolated and identified from hops [13]. Propolis (a resinous hive product collected by honey bees from different parts of plants) is a rich source of flavonoids [14]. There are nearly 900 naturally occurring isoflavone aglycones which can be divided into nine major classes on the basis of difference in their carbon skeletons [15]. These polyphenols show biological properties through their free-radical-scavenging antioxidant activities and metal-ion-chelating abilities. They are also known for their ability to inhibit enzymes such as protein kinase C, several protein-tyrosine kinases, or cyclin-dependent kinases [16–18].

Although there are many reports of in vitro studies designed to elucidate the biological properties of flavonoids, these studies may not be easily extrapolated to in vivo systems. Therefore, an understanding of the metabolism and bioavailability of these compounds is important. With regard to the chemistries involved in metabolism of flavonoids, our understanding of the absorption of conjugated and unconjugated flavonoids and their biotransformation in humans or in experimental animals is incomplete. In the case of unfermented foods, flavonoids largely occur in conjugated forms as their glycosides, malonylglycosides, and acetylglycosides. In contrast, in fermented samples such as the soy products miso, tempeh, and fermented bean curd, unconjugated aglycones predominate due to the hydrolytic enzyme activity of the microorganisms used in their manufacture [19,20].

Although some investigators have suggested that flavonoid glucosides may utilize the sodium-dependent glucose transporter for uptake by the gut, it has been shown that the β-glycosides genistin and daidzin, and by implication other flavonoid glucosides, are hydrolyzed in the gut wall by lactose phlorizin hydrolase, an enzyme in the apical membrane of the villi of the small intestine [21], and by intestinal microflora that convert them into aglycone forms [22]. The flavonoid algycones that are produced by hydrolysis are then absorbed into the intestinal cells by passive mechanisms. This is followed by a reconjugation step in the intestinal cell with glucuronic acid by the phase II enzyme UDP-glucuronosyltransferase. Those aglycones that escape this initial metabolism pass into the circulation and are converted to glucuronidated, methylated, and sulfated phase II metabolites by enzymes in the liver and other organs [23,24].

The flavonoid phase II metabolites are taken up from the blood by the liver and are excreted in bile, thus transporting them back into the intestines. Intestinal β-glucuronidases and sulfatases then release the aglucones—these can be reabsorbed or enter the bacterial-rich large bowel for further metabolism. For example, reduction (daidzein to equol) [25], ring opening (daidzein to *O*-desmethylangolensin) [26], and ring cleavage [daidzein to *p*-ethylphenol and/or 2-(4-hydroxyphenyl)-propionic acid] of the heterocyclic ring of the isoflavonoids can occur [27]. Flavonoids are converted to several other phenolic acids. Some of these metabolites have shown higher anti-oxidative and estrogenic activities (measured in vitro) than their parent compounds, for instance equol compared with daidzein [28].

Most interest has been focused on the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. To establish the role of flavonoids as antioxidants in vivo, it is critical to understand the chemical nature of the absorbed forms in the circulation in vivo. The antioxidant efficacy of flavonoids in vivo is poorly documented, presumably because of the limited knowledge on their uptake and distribution in humans. Most ingested flavonoids are extensively degraded to various phenolic acids, some of which still possess a radical-scavenging ability. Biochemical events such as deglycosylation before absorption and conjugation in the small intestine leading to phase II metabolites may influence their localization and biological activities in vivo [29].

Furthermore, the realization that flavonoids form novel compounds following their reaction with free radicals and other oxidant species produced at sites of inflammation has further increased the range of compounds that have to be studied to define the roles of flavonoids in health and disease [30–32]. Therefore, a number of analytical techniques have been utilized to evaluate the metabolism and bioavailability of flavonoids in vitro and in vivo [33,34]. These methods include gas chromatography (GC), reverse-phase high-pressure liquid chromatography (HPLC), and capillary electrophoresis (CE) in combination with UV absorbance, fluorescence, electrochemical detection, and mass spectrometry (MS); nonchromatographic techniques such as matrix-assisted laser desorption time-of-flight mass

spectrometry (MALDI-TOF-MS) and immunoassay procedures are also used. We have recently reviewed the relative merits of these different approaches [35]. This present review surveys the mass spectrometric techniques (and accompanying sample preparation methods) being used for the analysis of flavonoids in plant products and biological samples and covers reports published in the last 3 years.

MASS SPECTROMETRIC METHODS

Over the past 2 decades, mass spectrometry has proved to be one of the most effective techniques in biomedical research, particularly for the analysis of complex mixtures in biological samples. Its high sensitivity, specificity, and easy combination with chromatographic techniques have placed it as the method of choice of many analysts. We herein describe briefly the most common mass spectrometric methods used for flavonoid analyses.

Gas chromatography–mass spectrometry (GC-MS)

Many volatile compounds are directly amenable to analysis by gas chromatography–mass spectrometry, a technique that combines the unsurpassed separation properties of GC with the sensitivity and selectivity of electron impact ionization (EI) mass spectrometry. The latter mostly depends on the ion source and the ionization mode. In an EI interface, an electron beam interacts with the molecules of the analyzed compound, giving them increased internal energy and producing a complex mixture of ions. These ions are either molecular ions or fragment ions; their relative abundance can also be used for compound identification. Often due to excessive fragmentation, the molecular ions may be absent in the mass spectrum. Thus, this ionization mode is less applicable for analysis of a complex mixture. However, by the application of the selected ion monitoring (SIM) method, a simplified ion chromatogram for the ions of interest can be generated [34]. In a typical mass spectrometric analysis, the mass spectrometer is set to scan a specific mass range. This can be over a wide range (as in the full scan analysis) or a SIM scan. In SIM, the mass spectrometer is set to scan over a very small mass range (often 1 amu or less) so that only compounds with the selected mass are detected and plotted. The SIM plot is a more specific plot than the full-scan total-ion current plot and considerably more sensitive. Another method, reconstructed ion chromatogram or extracted ion current profile, uses the data for a selected ion from a full-scan analysis. The selection is carried out after the data have been acquired and as a result is much less sensitive than SIM.

As we have noted previously [35], a particular disadvantage of GC-MS for bioflavonoids is the need for derivatization of these involatile compounds (Table 1). This is an even greater problem for thermally labile biologic or chemical metabolites of flavonoids.

Fast-atom bombardment (FAB) and liquid secondary ion mass spectrometry (LSIMS)

In both cases, these mass spectrometry methods do not require derivatization of the flavonoids. In FAB, the impact of an energetic particle initiates both the sample vaporization and the ionization processes, so that separate thermal volatilization is not required. In the case of LSIMS, a liquid matrix is used and a primary beam of cesium ions instead of fast atoms causes evaporation and ionization. The matrices most often used (each of which is a high-boiling-temperature solvent) are glycerol, nitro-

Table 1. Comparison of Mass Spectrometry Ionization Techniques for Flavonoid Analysis

Ionization technique	Major application	Advantages	Disadvantages
EI	Mainly aglycone analysis (qualitative and quantitative)	Easy combination with GC Highly sensitive Identification of unknown possible	Derivatization needed, labor intensive Limited mass range possible thermal decomposition High fragmentation
FAB	Flavonoid glycosides (plant samples)	Extended mass range up to 7000 Da Soft ionization technique	Often results in no observable molecular ion Low sensitivity Requirement of solubility of sample in matrix High background matrix peaks
MALDI-TOF	Flavnoid glycosides, proanthocyanidins, and condensed tannins	High mass limit Tolerant of mM concentration of salts High-throughput analysis	Low resolution high matrix background signals little use for small molecules May be not good for laser-sensitive compounds
APCI	Flavonoid aglycones	Practical mass range up to 2000 Da Highly sensitive (femtomole) HPLC/MS capable	Sensitivity may be variable with compound type Possibility of thermal decomposition
ESI	Wide range of flavonoids (qualitative and quantitative)	High mass range HPLC/MS capable Multiple charge resolution ~2000 Sensitivity femtomole to picomole	Relatively low salt tolerance Multiple charge states can be confused in mixtures Analysis may be difficult for non-ionizable compounds No or less tolerance for heterogenous mixtures

benzyl alcohol, dithiothreitol/dithioerythritol, 5:1 w/w (so-called magic bullet), and thioglycerol. The choice of matrix has a great impact on the signal-to-noise ratio. Both methods cause a relatively mild ionization process, so that fragment ions are generally of low abundance. The strength of these techniques is the ability to analyze a wide range of thermolabile and ionic compounds. There are several examples on the application of FAB and LSIMS for the analysis of polyphenols, particularly, flavonoid glycosides. A review on the application of FAB-MS for identification of flavonoid glycosides has been published [36]. FAB is a useful soft ionization MS technique for molecular weight determination. However, the low *m/z* region is crowded with signals resulting from the matrix. These matrix signals are not very reproducible. Therefore, spectra correction and interpretation are not easily accomplished.

Electrospray ionization (ESI)-MS and atmospheric pressure chemical ionization (APCI)-MS

ESI is a method of generating highly charged droplets from which ions are ejected by an ion evaporation process. An electric field is generated at the tip of a sprayer by applying a high voltage, with a close proximity of a counter electrode. Ions of one polarity are preferentially drawn into the drops by the electric field as they are separated from the bulk liquid (Fig. 2). This technique is typically performed either in the infusion mode or in combination with HPLC or capillary electrophoresis. In the infusion mode, the sample is introduced into a continuous liquid stream via an injection valve. ESI interfaces (also referred to as ion-spray interfaces for certain commercial variations) are most often used with quadrupole mass spectrometers. Quadrupole instruments have limited mass-to-charge (*m/z*) ranges (typically up to *m/z* 2000 or 4000). They are also relatively simple, robust instruments. The electrospray sources are even simpler than the mass spectrometers and, depending on the design, equally robust. Several ESI interface designs exist, but all depend on the ability to form a microfine spray from a needle maintained at a high voltage potential.

The term APCI denotes those atmospheric pressure ionization processes that involve ion–molecule reactions to create ions in the gas phase (using corona or Ni^{63} discharge). With the advent of atmospheric pressure ionization (API) technology (both ESI and APCI), a wide range of flavonoids and other polyphenols can now be analyzed directly without derivatization. The sensitivity of ESI is at least two orders of magnitude higher than those of FAB or LSIMS. Another advantage of ESI is a better signal-to-noise ratio, due to the reduced number of ions in the spectral range of <300 amu originating from the matrix and spraying solvent, a very important region for flavonoids. These two interfaces (ESI and APCI) are highly sensitive, show greater ionization stability, and have become the methods of choice for flavonoid analysis. In a recent development, the efficiency of ionization in these interfaces has been enhanced by use of a photoionization technique [37]. It should be noted that ionization in both processes is quenched by the use of common HPLC mobile-phase modifiers such as trifluoroacetic acid and sodium or potassium phosphate, thereby requiring modification of existing chromatographic methods. Alternative modifiers include formic acid in place of trifluoroacetic acid and ammonium acetate or ammonium formate for the phosphate buffers. Both techniques can be used equally well in positive- and in negative-ion modes, although most flavonoids are measured in the negative-ion mode (for details see below).

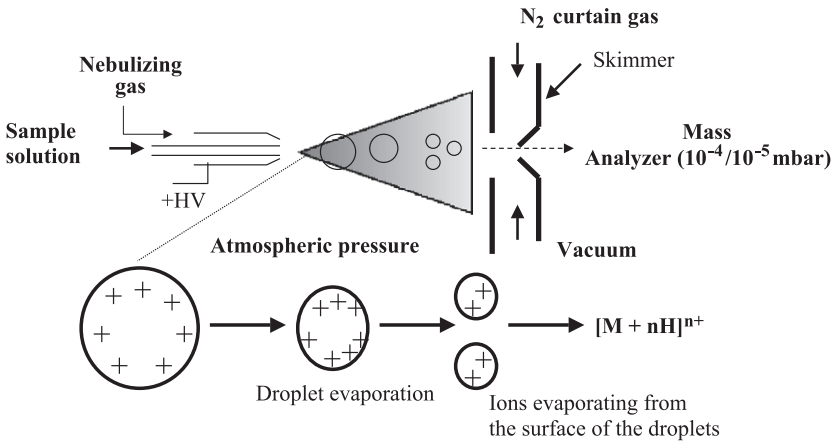


Fig. 2. Major components of the ESI source. The sample solution is passed through an electrically charged needle and the liquid takes the shape of a Taylor cone as it comes under the influence of the flow and the electrostatic field (the force on the ions drags the liquid along while surface tension tries to pull it into a sphere). There is rapid evaporation of the droplet and the capillary into which the droplets fly is heated to aid solvent evaporation. In the case of the IonSpray interface, the sample is dispersed by a nebulizing gas. Illustrated here is operation in the positive-ion mode. Negative ions can also be selected.

The application of tandem mass spectrometry (MS-MS) is useful in the identification and quantification of a compound. A tandem mass spectrometer is a mass spectrometer that has more than one analyzer, in practice usually two. The two analyzers are separated by a collision cell into which an inert gas (e.g., argon, xenon) is admitted to cause collision with the selected sample ions and to bring about their fragmentation. Using the entire mass spectrum of the fragmented ions, this information can then be pieced together to generate structural information on the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected or quantified in complex mixtures due to their specific and characteristic fragmentation patterns. Commonly used MS-MS techniques involve product ion, precursor ion, and neutral loss scanning. A product ion mass spectrum contains the fragment ions generated by collision of their parent (usually molecular) ion. A precursor ion mass spectrum is generated by limiting the fragment ion to a single ion of interest. The parent ions are scanned to determine which of them gives rise to the fragment ion. In neutral loss mass spectra, fragment ions that have a particular set loss of mass are allowed to pass to the detector. For quantitative analysis, multiple-reaction ion monitoring (MRM) in which a combination of precursor ion and one of its product ions is used to characterize a particular compound in complex mixtures is widely used. By comparing the ratios of peak area of analytes to a constant internal standard, the amount of an analyte in the mixture can be determined.

Capillary electrophoresis–mass spectrometry (CE-MS)

Capillary electrophoresis, first described by Jorgenson and Lukacs in 1981 [38,39], is a relatively new separation technique compared to other chromatographic methods such as GC and HPLC. Unlike other chromatographic methods which are based on interaction between the sample compounds and the stationary phase, separation by CE is a result of differences in electrophoretic mobilities in solution of charged species in an electric field in small-diameter capillaries. The use of capillaries, with 50–100 μm i.d. and 150–360 μm o.d., offers the advantages of rapid, high-resolution separation (up to 10^6 theoretical plates) with minimal sample volume required (in the nanoliter range), resulting in excellent mass detection limits (femto- to attomole of components). Based on the format of the buffers used in the capillary, the CE technique has been developed into several modes, such as capillary zone electrophoresis (CZE), capillary gel electrophoresis, or micellar electrokinetic chromatography (MEKC). The theory of CE has been discussed in detail in many references [40–42]. These techniques also have been applied to analyses of various classes of samples, including macromolecules such as proteins or small molecules such as drug metabolites. Applications of CE in the analysis of natural products, including tea components and resveratrol in wine, have been reviewed [43–45].

CZE is the basic mode of CE techniques. Charged species are separated from each other in the capillary, whereas all neutral species migrate at the same speed. Since most of the flavonoids are weak acids, alkaline buffers are used to ensure that the phenolic moiety is charged for electrophoretic separation. Borate buffer, which forms a charged complex with the *cis*-diol moiety of the sugar ring, is also useful for analyses of β-glycosides of flavonoids. The influence of structure and buffer composition on electrophoretic behavior of flavonoids has been discussed in several papers [46–49]. MEKC, a modified CE technique, is performed by adding surfactants, such as sodium dodecyl sulfate (SDS), at levels above their critical micellar concentration in the running buffer. The surfactants form charged micelles and migrate in the CE capillary under the electrical field, similar to all charged species. The analytes, both neutral and ionic species, partition between the micelle and the running buffer, which contributes additional selectivity to the separation. Therefore, the micelle is referred to as a pseudo-stationary phase, similar to the stationary phase in LC separation. MEKC has been extensively applied to separate various compounds including neutral and hydrophobic species [50,51]. Chiral separation of diastereomeric flavonoids can also be performed by CE with cyclodextrins added to the running buffer. Gel-Moreto et al. [52] have reported separation of the diastereomers of six major flavanone-7-*O*-glycosides (naringin, prunin, narirutin, hesperidin, neohesperidin, and eriocitrin) for the first time by chiral CE using 0.2 M borate buffer at pH 10.0 and with 5 mM β-cyclodextrin as chiral selector.

The instrumentation format of CE is similar to HPLC—therefore most detection methods used in HPLC, such as UV, fluorescence, and electrochemical detections, can be adapted to monitor CE separation. Mass spectrometry has been shown to be an excellent detector for CE separation with features of sensitivity, universal detection, and selectivity with the capability of providing structural information. The relatively low flow rates of CE (<1 μL/min) compared to those of conventional HPLC (1 mL/min) make it much better suited to interface with MS. The CE effluent can be introduced into the mass spectrometer through an ESI interface without splitting. However, caution must be taken to maintain the CE separation efficiency and resolution while maintaining the electrical continuity for CE separation and ESI interface. The first CE-MS interface, using silver metal deposition onto the capillary terminus as the point for electrical contact, was reported by Smith and coworkers in 1987 [53]. Development of other CE-MS interfaces and their applications have been described in many reviews [54,55].

Aramendia et al. [56,57] explored the use of on-line CE-MS for separation and characterization of selected isoflavones. A triaxial electrospray probe was used to interface CE with a single-quadrupole MS operating in the negative-ion mode. Fast separation of genistein, daidzein, biochanin A, and isoliquirtigenin was achieved with baseline resolution, while the comigrating pseudobatigenin, formononetin, and biochanin A were resolved by MS [56]. The sensitivity of this system relied on many factors including the buffer composition and the operation mode of the MS. Under optimum conditions and in selected-ion recording mode, a limit of detection of about 100 mol, equivalent to 6–7 nM in solution, was reported for almost all the isoflavones [56]. Although over 180 reports using CE for analysis of flavonoids have been published, no further application of CE-MS on analysis of flavonoids has occured in the past few years (based on a Medline search). This could be due to the difficulty of interfacing CE with MS and little need for high sensitivity in analysis of flavonoids from plants, given their high concentrations. The importance of CE-MS techniques may assume greater significance for the analysis of flavonoids and their metabolites from physiological samples such as tissue extracts, interstitial fluids, blood, and urine.

Matrix-assisted laser desorption ionization time-of-flight-mass spectrometry (MALDI-TOF-MS)

MALDI was first introduced by Karas et al. [58] to convert samples from the solid form into the ionized form in gas phase for MS analysis. In this technique, samples are cocrystallized with a matrix, usually an aromatic organic acid, which absorbs energy from laser pulses and allows a soft desorption ionization of the sample. The sample ions are then analyzed by a time-of-flight mass analyzer (Fig. 3). Increased mass resolution is obtained by bouncing the ions off an electronic mirror (a reflectron). This increases the focus of the ions.

Post source decay (PSD) has been used to obtain a fragmentation spectrum of compounds. In this technique, a precursor molecular ion is first isolated in a distinct mass window by timed ion selection following the initial laser pulse. The precursor ions passing the mass window spontaneously fragment on their way to the detector. By varying the voltages applied to the reflectron ion mirror (see Fig. 3), the fragment ions can be brought into focus. By mathematically stitching together 6–12 of these spectra, PSD spectrum can be obtained. This method has been superseded by use of the TOF-TOF instrument (see Combined techniques below).

MALDI-TOF-MS has advantages over other methods, including high speed of analysis, good sensitivity, and good tolerance toward contaminants [59]. Additionally, MALDI-TOF-MS produces mainly singly charged ions, unlike ESI-MS. These attributes allow for the simultaneous determination of masses in complex samples of low- and high-molecular-weight compounds. Although MALDI-TOF-MS is well known as a powerful tool for analysis of a wide range of biomolecules, such as peptides and proteins, its potential in flavonoids analysis

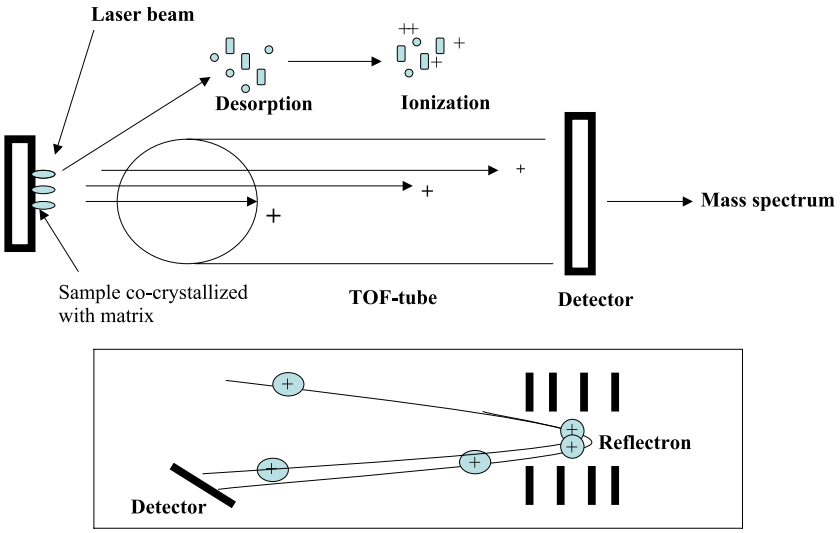


Fig. 3. Schematic of MALDI-TOF-MS analysis. Samples that are analyzed by MALDI are first mixed with crystalline matrix and spotted on a stainless steel target. Upon drying, it is inserted into the ion source of mass spectrometer which is under a high vaccum. A laser (a pulsed nitrogen laser at 337 nm) is fired onto the sample, resulting in a desorption event. The ions are repelled from the target surface and drift through the flight tube and their arrival at the detector is carefully timed, smaller ions fly faster than larger ions. Thus ions are separated according to their mass-depending velocities. In some analyses, the ions are "bounced" off of a reflectron to obtain a higher-resolution mass spectrum.

has been explored only recently by Ohnishi-Kameyama et al. [60] in the identification of catechin oligomers in apples, by Sporns's group [61–64], and by Sugui et al. [65] on analyses of flavonols in red wine and foods.

Combined techniques and Fourier transform–ion cyclotron resonance–mass spectrometry (FT-ICR-MS)

As noted earlier, there are many other techniques based on mass spectrometry that can be applied to the analysis of flavonoids. For instance, in LC-ESI-MS, the quadrupole detector can be replaced by an ion trap or a TOF detector. The ion trap has the advantage that it can carry out sequential fragmentation first of the parent molecular ion and second of the daughter ions. This would be of particular advantage in the analysis of glycosides of isomeric flavonoids. For example, the glycosides of genistein (an isoflavone) and apigenin (a flavone) have the same molecular weight (*m/z* 431 for their [M-H]- molecular ions) and chromatographically have very similar mobilities. Fragmentation of these glucosides in a triple-quadrupole instrument leads to an aglycone daughter ion (*m/z* 269), thereby not distinguishing them. However, as can be seen in Fig. 4, fragmentation of the *m/z* 269 ion leads to unique daughter ions (*m/z* 133 for genistein and *m/z* 149 for apigenin), allowing for their independent measurement [35].

Hybrid instruments take advantage of easily creating and isolating molecular ions of flavonoids. The quadrupole orthogonal time-of-flight (Q-TOF) mass spectrometer is related to triple-quadrupole instruments. Ions are generated with ESI or MALDI, selected in the first quadrupole, and fragmented by collision with argon gas; the fragments are accelerated orthogonally and injected into a TOF analyzer. The advantage of the TOF detector is its higher sensitivity and better mass accuracy (at least 20 ppm) than the quadrupole detector in a triple-quadrupole instrument. A related instrument is the quadrupole ion trap. In this case the TOF detector is replaced by an ion trap detector.

Those in the field of proteomics are excited about the hybrid TOF-TOF instrument. In this case, ions separated in the first TOF analyzer are selected using a timed ion gate. These ions are collided with argon gas and then reaccelerated in a second TOF analyzer. This method is particularly rapid and many hundreds of samples could be analyzed per hour by this tandem mass spectrometry approach [66]. However, the low *m/z* values of bioflavonoids and their metabolites are a complication as noted earlier because of the difficulty of separating them from the ions derived from the crystal matrix. Whether a TOF-TOF instrument will be as valuable in flavonoid research remains to be seen.

Fourier transform–ion cyclotron resonance mass spectrometers can be used with both ESI and MALDI interfaces. Their particular advantages are their sensitivity, extreme mass resolution, and mass accuracy. The latter allows for determination of the empirical formulae of compounds under 1000 Da. In FT-ICR-MS, the ions are inserted into a cylindrical trap at the center of the magnetic field of a superconducting magnet. Mass resolution is a function of the magnetic field. The ions precess around the center of axis of the magnetic field in tight orbits. Application of ion cyclotron radiation increases the energy of the ions which leads to their

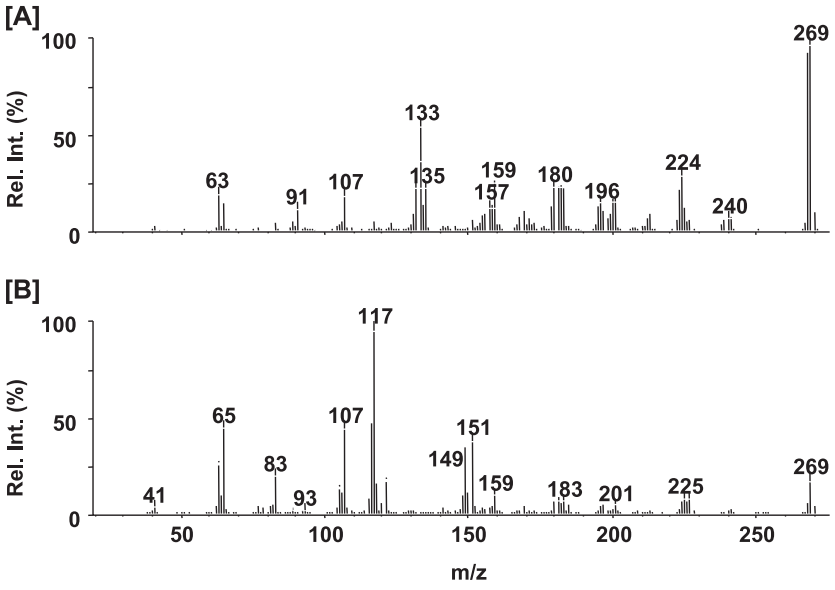


Fig. 4. ESI-MS-MS spectra of deprotonated genistein [A] and apigenin [B] at *m/z* 269.

having larger orbits. When the radiation is turned off, the excited ions decay back to their ground states. As they do so, they interact with solenoids placed around the ICR cell and create a free induction decay (FID) signal (analogous to that in an NMR experiment). The FID signal (in the time domain) is processed by a fast Fourier transform procedure into the frequency domain and hence can be converted into a mass spectrum. Since the observed mass resolution is a function of the number of data points collected at any one moment and the total time of collection, there are limitations in its application to chromatography techniques. Currently, mass resolution of more than 100,000 can be achieved using a 1-s transient in a 4-s analysis cycle in a 9.4-Tesla magnet that is compatible with most LC analyses. Each cycle involves the introduction of charged ions into an ion-selecting quadrupole, fragmentation, and collection of daughter ions in a hexapole or capture of ions in a 2D- or 3D-ion trap, passage to the ICR cell, excitation, and recording of the FID signal, and finally export of the data to a computer. In the most recent instrument developments, 75% of the cycle period can be devoted to recording the FID. This enhances the observed mass resolution since for most cases mass resolution increases linearly with the time of recording of the FID signal. Therefore, a 9.4-Tesla magnet using a 1-s recording is over two times less effective than a 7-Tesla magnet using a 3-s recording. The longer timescale of FT-ICR-MS necessitates the use of MALDI matrices such as dihydroxybenzoic acid rather than α-cyano-4-hydroxycinnamic acid or sinapinic acid because of the metastability of ions generated by the latter [67]. Additionally, the MALDI ions have to be collisionally cooled to be suitable for FT-ICR-MS analysis.

The much higher vacuum needed for the ICR cell presents a problem for traditional collisional dissociation of molecular ions. The gas that is introduced to initiate ion fragmentation has to be pumped out before the analysis can begin. There are two solutions to this problem. In the first solution, ions in the ICR cell are excited and hence dissociated by the application of infrared multiphoton dissociation (IRMPD) [68], or by electron capture dissociation (ECD) [69], or by a combination of these two processes [70]. Both IRMPD and ECD cause fragmentation without altering the vacuum. In the second solution, fragmentation is carried out outside of the ICR cell while the previous sample is undergoing analysis. In a rapidly developing method, the fragment ions are generated in an external ion trap. This allows for collection of a constant number of ions for the measurement in the ICR cell, leading to a much more consistent mass accuracy by avoiding variable space–charge effects. This method has shown great promise in the analysis of peptides produced in proteomics analysis [71].

Although FT-ICR-MS has not yet been applied to flavonoids, its ability to determine mass with an accuracy approaching or better than 1 part per million may ensure the correct identification of flavonoid metabolites recovered from tissue and cell preparations.

SAMPLE PREPARATION

The isolation of flavonoids and their metabolites from biological samples is of immense importance for both qualitative and quantitative analyses. Certain obstacles have to be overcome to achieve this. First, because of the complexity of the biological matrix, many compounds interfere in the analysis of the target analytes. Second, some of the flavonoids and their metabolites are present at very low concentrations, requiring a high level of sensitivity. Sample preparation is a crucial step in the analysis of biological samples. We therefore surveyed the recent literature for original papers on flavonoids and isoflavonoids that involved the use of techniques for sample preparation of biological fluids and tissues.

The first step in the analysis of flavonoids is a decision whether to first carry out an extraction step. For physiological fluids (bile, plasma, serum, or urine), an initial extraction may not be necessary. For these fluids, flavonoids may be first hydrolyzed with β-glucuronidase, sulfatase, or a mixture containing both enzymes. In the latter method, sodium acetate buffer (0.14 M, pH 5), internal standards, and β-glucuronidase/aryl sulfatase from a crude solution of *H. pomatia* are added to a biological sample and the hydrolysis of conjugates is allowed to proceed overnight at 37°C, with shaking. To ensure that these enzymes are active in the incubates, known amounts of synthetic substrates (such as phenolphthalein glucuronide and 4-methylumbelliferone sulfate) are usually added to the mixture. In certain cases, ^{13}C-labeled flavonoid conjugates have been prepared and are available [72]. The hydrolyzed sample is cleaned up and extracted using liquid–liquid extraction or solid-phase extraction (SPE) [73]. Grace et al. [74] used solid-phase extraction with a Strata C18-E SPE cartridge (Phenomenex, Macclesfield, UK). The cartridge was conditioned with methanol (1 mL) followed by 5% methanol (1 mL) before extraction of the samples. The cartridge was then washed with 5% methanol (800 μL) before the aglycones were eluted in 1:1 ethyl acetate:acetonitrile (400 μL). In the case of liquid–liquid extraction, common solvents used for extraction of flavonoid aglycones are ethyl acetate, diethyl ether, and methylene chloride. Solvent extraction is preferred for extraction of less-polar flavonoids that are soluble in water-immisible organic solvents and the scale of extraction is large.

To carry out quantitative measurements of flavonoids using chromatographic methods, it has proved necessary to include internal standards (IS) to correct for unknown losses during the procedure used. Usually, deuterated (^{2}H) or carbon-13 (^{13}C)-labeled stable isotope forms of the flavonoids of interest have been used as IS [75,76]. However, the availability of labeled compounds is limited; so alternatively, compounds with similar chemical structures and properties that are not naturally present in the sample to be studied can be used as IS. For example, apigenin has been used as an IS in the analysis of isoflavonoids [77,78]. Taxifolin has been used as the IS for the quantification of catechin in human plasma by GC-MS because it is flavonoid and not available in wine samples [79]. Dihydroflavone was used as the IS to study the pharmacokinetics of daidzein and genistein in serum samples of premenopausal women [72]. The benzophenones, 2,4,4′-trihydroxybenzophenone and 4-hydroxybenzophenone, have been used as IS to analyze isoflavones in urine and plasma, respectively [80].

In some cases, protein precipitation has been used in the first step of the extraction process. We recently analyzed puerarin and its metabolites in serum and urine samples after protein precipitation with acetonitrile without further extraction [78]. This approach is particularly helpful if it is intended to measure both the conjugated and the unconjugated forms of the flavonoids. Romanova et al. [81] also used a similar method for the determination of apigenin in plasma by HPLC in which samples were directly analyzed after protein precipitation with methanol.

In the case of GC-MS analysis, extraction is followed by liquid chromatographic purification and conversion of the resulting flavonoid fractions into their trimethylsilyl ether (TMS) derivatives. Setchell et al. [72], in a study of pharmacokinetics of daidzein and genistein in healthy humans, reacted the phenolic fraction with *tert*-butyldimethylsilyl (tBDMS) and the ether derivatives were analyzed by GC-MS.

The sample preparation of plant or food samples starts with grinding, before extraction with aqueous ethanol or methanol. Several investigators have followed the method by Coward et al. [82]. A freeze-dried food sample (0.5 g) and 5 μg of deuterated daidzein (internal standard) were dispersed by sonication and extracted with 80% aqueous methanol (5 mL) with stirring for 1 h at 60°C. The mixture was cooled and centrifuged at 8000*g* for 5 min, the solvent extract was aspirated off, and the residues were resuspended in aqueous ethanol (2× 2.5 mL). The combined extract was concentrated and lipids were removed by partitioning into hexane. After drying the aqueous alcoholic phase under nitrogen, the residue was redissolved in 50% aqueous methanol (10 mL) prior to LC analysis. Recently, highly glycosylated acylated flavonoids were characterized from cauliflower [83]. In this method, freeze-dried cauliflower (70 g) was extracted by boiling with 3 L of distilled water for 60 min. The extract was further mixed with Amberlite XAD-2 particles and stirred to retain the phenolic compounds on the surface of the nonionic Amberlite particles. The Amberlite particles were packed into the chromatography column, washed with distilled water (5 L), and eluted with methanol. The methanolic extract was dried and redissolved in 50% aqueous methanol for further analysis.

It should be noted that extraction of flavonoid glycosides in heated solvents may lead to changes in composition. This is a particular issue for the malonyl esters of the flavonoid glycosides. These undergo extensive hydrolysis of the malonyl group to the simple glycoside even at room temperature. This can also occur in an autosampler while awaiting analysis (Michelle Johnson, Stephen Barnes, unpublished observations). Malonyl esters also undergo heat-induced decarboxylation (to form acetylglycosides) in the dry state. For several foods containing flavonoids, this may have occurred during processing prior to their use in an experiment [82].

To overcome these problems, newer sample preparation techniques such as countercurrent supercritical fluid extraction and pressurized liquid extraction have been used in analysis of plant flavonoids [84,85]. In the countercurrent technique, a liquid sample is introduced in the middle of the packed column, located over the inlet of the CO_2, creating a countercurrent between the flow of sample (downward) and the flow of CO_2 (upward). Supercritical CO_2 has been widely used in conventional citrus processing applications [86]. This method is suitable for extraction of volatile components.

A particular advantage of liquid chromatography-mass spectrometry (LC-MS) is its capability to determine both free and conjugated forms of flavonoids. Unlike for GC-MS, when using LC-MS it is often unnecessary to use any extraction. For example, urine samples from human subjects consuming soy can be analyzed directly [76]. The only work up needed is centrifugation or filtration of the urine to remove particles that would otherwise clog up the HPLC column. In this type of analysis, separation is carried out using gradient elution with acetonitrile or methanol. The electrolyte and other hydrophilic components of urine that would interfere with detection of the flavonoids elute before the gradient has begun. For bile samples, the concentration of flavonoids is so high that the bile has to be diluted with the starting HPLC solvent. Again, filtration or centrifugation is used to remove any particulate matter. In the case of serum, the concentrations of flavonoids and their

metabolites are much lower and, except when using very high doses, it is usually necessary to extract and thereby concentrate the samples first.

A summary of recently reported sample preparation methods for flavonoid analysis is given in Table 2. Hydrolysis followed by solvent extraction is recommended when GC-MS is used for analysis because it is easier to form volatile di- or trimethylsilyl derivatives from the aglycones than from the glycosides. The majority of GC-MS analyses have focused on determining the total flavonoid aglycone concentration in biological samples. Solvent extraction methods are equally suitable for LC-MS analysis for quantification of total aglycones. Sample preparation using reversed-phase SPE is also widely used for flavonoid analysis in biological samples. Biological fluids such as urine and serum can be processed using SPE in a 96-well format for high-throughput quantification of compounds of interest. The analytes are preferentially adsorbed by the solid phase and can be eluted using a small volume of an appropriate organic solvent, resulting in a preconcentration prior to analysis.

The study of flavonoids' action requires a full identification of their metabolites to develop a through understanding of the metabolic pathways of flavonoids

Table 2. Methods for the Determination of Flavonoids and Their Metabolites in Biological Samples

Analytes	Sample size	Sample preparation	Recovery[a] (%)	Detection	References
Puerarin in urine and serum	0.2 mL	IS (apigenin) addition Acetonitrile protein precipitation Centrifugation Direct analysis of supernatant	Not quoted	LC-MS/MS (MRM)	[78]
Daidzein and genistein in serum	0.25–0.50 mL	IS (dihydroflavone) addition Diluted with triethylamine sulfate Heated 64°C, SPE C_{18}-cartridge Enzymatic hydrolysis, SPE and Sephadex LH-20 fractionation. Derivatization with tBDMS	Not quoted	GC-MS (SIM)	[72]
Daidzein and genistein in urine	0.5 mL	IS (dihydroflavone) addition Enzymatic hydrolysis SPE on C_{18}-cartridge	Not quoted	LC-MS	[72]
Isoflavones and lignans in serum	0.2 mL	^{13}C standards addition Enzymatic hydrolysis SPE on C_{18}-cartridge	92.3–100.7	LC-MS-MS (SRM)	[74]
Isoflavones and their conjugates in urine	5 mL	SPE with LC-18 cartridge Concentrated and freeze drying Dissolved in 75% aqueous MeOH	Not quoted	LC-MS-MS	[158]
Lignans and isoflavones in human feces	0.3–0.6 g	Deuterated IS addition Solvent extraction (ethanol:acetone 9:1 v/v) Fats and protein precipitation Cleaned up with SPE C_{18} Ion exchange chromatography	97	GC-MS (SIM)	[73]
Lignans and isoflavones in plasma and prostatic fluid	plasma (1ml) EPS (0.05–0.2 mL)	IS addition Enzymatic hydrolysis Ion exchange chromatography Derivatization with BSTFA	Not quoted	GC-MS-SIM	[110]
Flavonoid glycoside naringin in human urine	1 mL	IS (hesperidin) addition Sep-Pak extraction Enzymatic hydrolysis	Not quoted	LC-MS	[145]
Procyanidin B1 in human serum	0.5 mL	Enzymatic hydrolysis Extracted with methanol/formic acid	Not quoted	LC-MS	[141]
Genistein, daidzein, and conjugates in rat blood	0.075 μL	Acetonitrile protein precipitation Enzymatic hydrolysis IS addition, ethyl acetate extraction	85	LC-MS	[75]
Catechins in urine	0.3 mL	Treated with dichloromethane Centrifuged, filtered the aqueous layer Analyzed the filtrate	Not quoted	LC-MS	[134]
Polyphenols in urine	0.035 mL	IS (syringin) added Enzymatic hydrolysis Ethyl acetate extraction Centrifuged, dried Reconstituted with aqueous methanol	Not quoted	LC-MS-MS (MRM)	[139]

EPS, expressed prostatic secretion; BSTFA-*N*,*O*-bis(trimethylsilyl)trifluroacetamide; tBDMS, *tert*-butyldimethylsilyl.

[a] At higher concentration of standards.

after consumption. Conjugated metabolites such as β-glucuronides and sulfate conjugates are polar and are not amenable to solvent extraction/partition. However, solvent-induced protein precipitation methods are gaining popularity as they do not involve extraction and there is a low chance of workup losses during sample preparation.

MASS SPECTROMETRIC ANALYSIS OF FLAVONOIDS

Plant samples

As alluded to earlier, flavonoids are structurally diverse and are generally part of a complex mixture isolated from a plant extract. Medicinal plants containing flavonoids are currently being advocated for preventing and treating many diseases, provided that they are of adequate quality and properly used. So, the quality control of these natural products existing in a complex matrix is an essential part of research. However, the rapid and systematic analysis of natural products is a serious challenge for analytical chemists because of their inherent structural diversity.

Although GC-MS has been the mainstay for analysis of small molecules for the past 20 years, nowadays it is not widely used in flavonoid analysis due to the limited volatility of flavonoid glycosides which are found widely in fruits and vegetables. With the advent of API sources, LC-MS has become the most widely used method for the analysis of flavonoids in a complex mixture. Since LC-MS provides the molecular weight of each component with its retention time, it is used for identification of known compounds. Further characterization of known or unknown components can be done by LC-tandem mass spectrometry. It is employed for quantitative analysis also.

The ionization efficiency of different API sources, i.e., ESI and APCI, has been previously reviewed by Rauha et al [87]. ESI-MS in the negative- ion mode with an acidic ammonium acetate buffer as the mobile phase provides the best sensitivity. De Rijke et al. [88] reported a comparative study on the analytical performance of the APCI and ESI techniques, in both the positive- and the negative-ion modes. MS responses were best in the negative-ion modes, with APCI generally better than ESI. The results obtained with negative APCI and ESI were similar for all flavonoid aglycones.

The Claeys group has published a series of papers on the use of mass spectrometry for structure assignment of flavonoid glycosides from plants [89–92]. Recently, they reported the application of LC-ESI-MS and collision-induced dissociation (C/D) in the structural characterization of acylated flavonol *O*-glycosides from the seeds of *Carrichtera annua*[90]. The acyl groups produced characteristic product ions in the $[M + H]^+$ and $[M + Na]^+$ CID spectra and radicalar-acid-related product ions at high-energy collisional activation. Tandem mass spectrometric methods have been used for the characterization of the type and the differentiation of the interglycosidic linkage of isomeric flavonoid *O*-diglycosides. Based on the occurrence of internal monosaccharide residue loss and the relative abundances of Y-type ions formed by fragmentation at glycosidic bonds, isomeric flavonoid *O*-diglycosides can be unambiguously differentiated. Cleavage at the glycosidic O linkage with a concomitant H arrangement leads to the elimination of dehydrated monosaccharide residues, i.e., the loss of 162 u (hexose), 146 u (deoxyhexose), and 132 u (pentose). Methylated flavonoids are characterized by the loss of 15 u, resulting in a $[M\text{-}H\text{-}CH_3]^{-\cdot}$ [93]. ESI-MS/MS is powerful enough to distinguish several methylated flavonoid isomers based on their product ion spectra. For example, glycetin and biochanin A are isomers (protonated molecular ion at *m/z* 285), and their product ion profiles are different (Fig. 5).

Cuyckens and Claeys [89] have studied optimization of a liquid chromatography method based on simultaneous ESI-MS and ultraviolet photodiode array detection (DAD) for analysis of flavonoid glycosides. In the positive-ion mode, ESI-MS analysis in methanol containing 1% acetic acid was by far the most sensitive, whereas an acetonitrile/water mobile phase containing 0.5% formic acid gave the best sensitivity in LC/ESI-MS/UV-DAD analysis. In the negative-ion mode, the highest sensitivity was obtained with a mobile phase containing 0.1% formic acid, while addition of bases decreased the sensitivity. We recommend the negative-ion mode for flavonoid analysis in biological samples because of its better sensitivity and limited fragmentation. Trifluoroacetic acid decreases the sensitivity due to its strong ion-pairing effect, making the analyte ions appear as neutrals [94]. The opposite effect was observed for acetic acid. However, acetic acid in the mobile phase decreases retention times on the LC column.

Hvattum and Ekeberg [95] demonstrated that flavonoid glycosides undergo both collision-induced homolytic and heterolytic cleavage of the *O*-glycosidic bond, producing deprotonated radical aglycone and aglycone product ions. According to the report, quercetrin (quercetin-3-*O*-rhamnoside) is capable of producing a stable radical aglycone anion after CID of the $[M\text{-}H]^-$ precursor. It has been reported that such radical anions are also produced while acting as antioxidants. The ability of quercetin glycosides to provide radical fragments is apparently similar to the electron-or hydrogen-donating antioxidant property of the flavonoids. Figure 6 shows the product ion spectra of deprotonated quercitrin (*m/z* 447) at different collision energies. Hvattum [96] also reported the identification in rose hip extract of an anthocyanin, i.e., cyanidin-3-*O*-glucoside, several glycosides of quercetin, and glycosides of taxifolin and

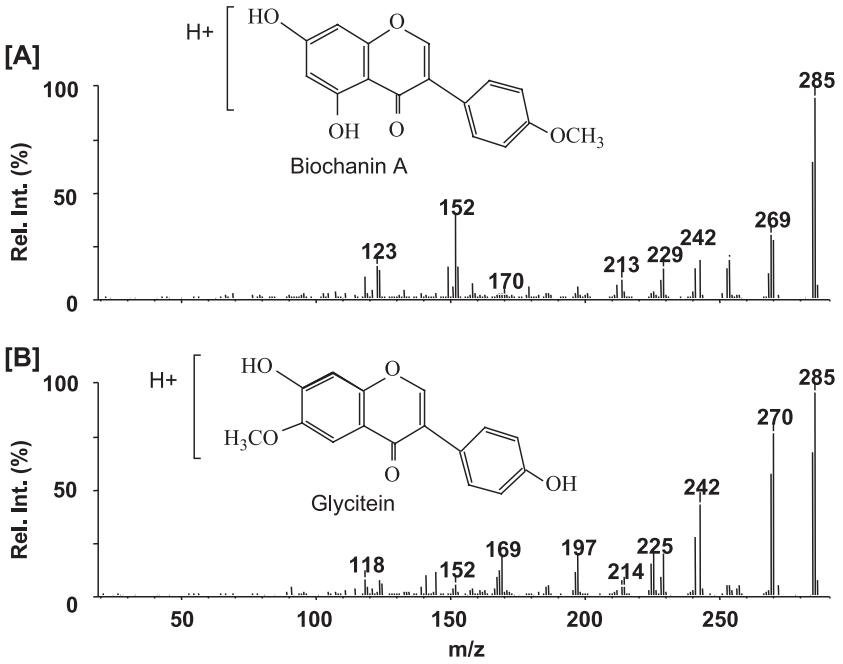


Fig. 5. ESI-MS-MS spectra of protonated biochanin A [A] and glycitein [B] at *m/z* 285.

eriodictyol. Phloridzin was identified, and several conjugates of methyl gallate were also found, one of which was tentatively identified as methyl gallate-rutinoside. Furthermore, the stereochemical assignment of hexose and pentose residues in acetylated flavonoids has been reported using a tandem mass spectrometric method [97]. In addition to *O*-glycosides, flavonoid *C*-glycosides are also found in many medicinal plants and vegetables [98,99].

In the case of *C*-glycosides, the sugar is directly linked to the flavonoid moiety via an acid-resistant C-C bond. MS-MS experiments with CID allow the characterization of *C*-glycosides both in positive- and in negative- ion modes. Recently, we examined isoflavonoid *C*-glucosides such as puerarin (daidzein-8-*C*-glucoside) from kudzu dietary supplements using ESI-tandem mass spectrometric methods [10]. The MS-MS spectrum of the protonated ion of puerarin showed characteristic product ions of the *C*-glycoside unit itself, whereas daidzin (the *O*-glucoside isomer) generated an abundant $Y(0)^{(+)}$ aglycon ion in its product ion spectrum (Fig. 7). A base peak due to the loss of 120 Da $[M + H - 120]^{(+)}$ is the diagnostic ion for *C*-glycosides [100]. Fabre et al. [101] applied a MS^n technique in an ion trap to determination of flavone, flavonol, and flavanone aglycones. They observed losses of CO, CO_2, and C_3O_2 in the negative-ion mode.

Wang and Sporns [63,64] were the first to use MALDI-TOF mass analysis of isoflavones in soy products. Isoflavones were predominately ionized in a protonated form with a very small amount of sodium and potassium adduct ions. The loss of the glycosidic residue was observed in the analysis. Interestingly, daidzin showed more than twice the response of genistin using MALDI-TOF MS. MALDI-TOF-MS has also been used to analyze grape tannins [102].

Although the MALDI-based approach has not been recognized as a quantitative technique, Sporns et al. [103,104] further reported their effort on quantitative analysis of flavonol glycoside composition of almond seedcoats using MALDI-TOF-MS. In these studies, 2′,4′,6′-trihydroxyacetophenone monohydrate was used as MALDI matrix and preformed on the target plate as a lawn of small homogeneous crystals by dissolving the matrix in acetone. The flavonoids extracted from almond seedcoats were dissolved in 70% methanol solution and spotted on top of the precoated matrix bed. The matrix was redissolved by the 70% methanol and recrystallized with the flavonoids in a similar small crystal form that showed good spot-to-spot repeatability. Rutin, quercitin-3-rutinoside, was used as internal standard in this MALDI-TOF-MS approach to quantitatively determine four flavonol glucosides, isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside [102]. Individual peak ratios were reported to be very consistent across triplicate analyses of 16 almond seedcoat samples; the average standard deviation was 9%. Isorhamnetin rutinoside was the most abundant flavonol glycoside, and the total content ranged from 75 to 250 μg/g [104].

Analysis of condensed tannins by MALDI-TOF-MS was first described by Ohnishi-Kameyama et al. [60]. Polymerized catechins up to pentadecamers in apple

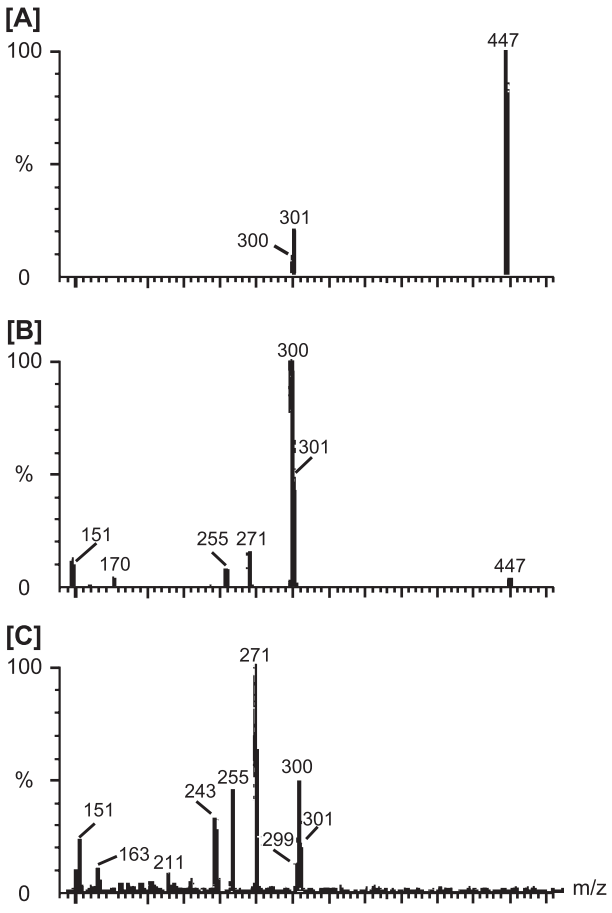


Fig. 6. Negative-ion CID mass spectra of the $[M-H]^-$ precursor ion of standard quercitrin at *m/z* 447. Collision energies (A) 20 eV; (B) 40 eV; (C) 60 eV. The product ion spectra presented are representative of three analyses. (Reprinted from *J. Mass Spectrom*. Vol. 38, 2003, Hvattum et al., "Study of the Collison-Induced Radical Cleavage of Flavonoid Glycosides Using Negative Electrospray Ionization Tandem Quadrupole Mass Spectroscopy," copyright 2004 with permission from John Wiley & Sons Ltd.)

were observed using trans-3-indoleacrylic acid (IAA) as matrix in the presence of silver ions. Yang and Chien [105] demonstrated the detection of oligomers composed of (+)-catechin, (-) epicatechin, and their galloylated derivatives in the grape seeds using MALDI-TOF MS. In this study, Yang and Chien claimed that dihydroxybenzoic acid (DHB) was a better matrix for studying grape procyanidins than IAA and other matrices, due to the broader mass range of the procyanidins and the lower background noise from DHB.

Krueger et al. [106] also reported characterization of polygalloyl polyflavan-3-ols (PGPF) in grape seed extracts using MALDI-TOF MS [106]. PGPF in grape seed extracts were isolated by ytterbium (Yb^{3+}) precipitation followed by cation exchange. Masses of PGPF up to undecamers were observed in the positive-ion linear mode, while masses corresponding to a series of PGFG units up to nonamers were observed in the positive-ion reflectron mode. Reed's group [107] further demonstrated the use of MALDI-TOF MS to determine the structural diversity of polyflavans in plants, Ruby Red sorghum. In that study, the crude extract of the plants was fractionated using a Sephadex LH-20 column with different elution solvents before analysis by MALDI-TOF MS. Polyflavans that eluted with methanol/acetone showed a series of masses corresponding to heteropolyflavan-3-ols differing in degree of hydroxylation and nature of the interflavan bond (A type and B type), while those that eluted with ethanol/methanol revealed a series of masses corresponding to heteropolyflavan-5-*O*-β-glucosides that varied in the extent of hydroxylation and contained a flavanone as the terminal unit. By combining chromatographic separation with MALDI-TOF MS for characterization of polyflavans in plants, they demonstrated that the structural heterogeneity is much greater than previously reported.

Behrens et al. [108] used MALDI-TOF MS and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. The PSD product ion spectra of condensed tannin tetramer and trimer from lime (*Tilia cordata*) are shown in Figs. 8A and 8B, respectively. The fragmentation of the tetramers (*m/z* 1177) produced signals at *m/z* 889,887 and *m/z* 601,599 (Fig. 8A). The peaks at *m/z* 889 and 601 corresponded to the loss of one and two catechin/epicatechin units, respectively.

Other biological samples

As noted earlier, flavonoids are absorbed and metabolized following ingestion. Urine and blood (plasma or serum) are the two main biological fluids that have been analyzed for metabolism studies [26,72,74]. Two additional biological fluids that have been used in the analysis of metabolites are breast milk and prostatic fluid [109,110]. Tissues from various organs such as liver, kidney, and brain have also been examined using mass spectrometric analysis [111].

In vitro. Although flavonoids are metabolized largely in organs such as the liver, intestine and kidney, an understanding of flavonoid metabolism at the cellular level is important to explain their pleotropic activities in different cell lines. The biological activities of compounds may be related to their metabolic behavior. For example, genistein and biochanin A are 10- and 6-fold more potent inhibitors, respectively, of EGF-stimulated growth of normal human mammary epithelial (HME) cells than of MCF-7-transformed human breast cancer cells. Based on these observations, Peterson et al. [112] demonstrated that human breast cancer MCF-7 cells extensively metabolized both isoflavones, producing two genistein metabolites with molecular weights of 350 and 380 Da and three biochanin A metabolites with

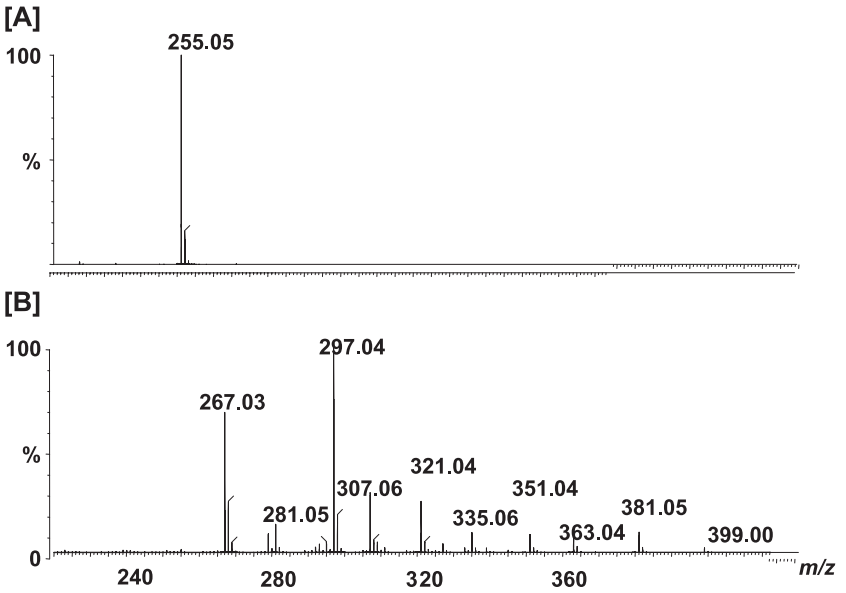


Fig. 7. Comparison of the products ions obtained in ESI-MS-MS experiments of protonated daidzin [A] and puerarin [B].

molecular weights of 270, 350, and 380 Da. In contrast, significant genistein or biochanin A metabolism was not observed in HME cells.

Using mass spectrometry and nuclear magnetic resonance analysis, metabolite 350 from genistein and biochanin A experiments was identified as genistein 7-sulfate. The product ion spectrum at *m/z* 349 showed a prominent ion at *m/z* 269 due to the loss of 80 Da (-SO_3). The genistein metabolite with molecular weight 380 Da was not unequivocally identified but appeared to be a hydroxylated and methylated form of genistein sulfate. In MCF-7 cells, genistein 7-sulfate and metabolite 380 Da were detected primarily in the cell media fraction, suggesting that, once formed, these polar metabolites were exported from the cells, perhaps as substrates of the multidrug resistance (MDR) transporter. Intestinal CaCo-2 cells expressing MDR-2 also export flavonoid β-glucuronides in the basolateral-to-serosal direction [113]. The biochanin A metabolite 270 Da was identified as genistein, formed by demethylation, and the biochanin metabolites 350 and 380 Da were the same as those observed for genistein.

In a similar vein, Spencer et al. [114] investigated intracellular metabolism and bioactivity of quercetin and its in vivo metabolites 3′-*O*-β-methyl quercetin, 4′-*O*-methyl quercetin, and quercetin 7-*O*-β-D-glucuronide on dermal fibroblasts. Uptake experiments indicated that exposure to quercetin led to the generation of two novel cellular metabolites, one identified as a 2′-gluthathionyl quercetin conjugate and another product, putatively a quinone/quinone methide. These compounds were analyzed by ESI-MS-MS. A similar product was identified in cells exposed to 3'-*O*-methyl quercetin but not in the lysates of those exposed to its 4'-*O*-methyl counterpart, suggesting that its formation is related to oxidative metabolism. There was no uptake or metabolism of quercetin 7-*O*-β-D-glucuronide by fibroblasts. Formation of oxidative metabolites may explain the observed concentration-dependent toxicity of quercetin and 3'-*O*-methyl quercetin, whereas the formation of a 2'-glutathionyl quercetin conjugate is interpreted as a detoxification step.

The Williamson group published a series of papers on metabolism of quercetin [114–118]. They presented a very interesting paper showing that quercetin is an effective inhibitor of H_2O_2-induced lens opacification. Using a LC-MS method, it was confirmed that the intact rat lens is capable of converting quercetin aglycone to 3′-*O*-methyl quercetin (isorhamnetin; *m/z* 317 in the positive-ion mode ESI) [115]. Interestingly, quercetin and 3′-*O*-methyl quercetin both inhibited H_2O_2-induced sodium and calcium influx and lens opacification. These observations indicate that dietary quercetin and its metabolites are active in inhibiting oxidative damage in the lens and thus could play a role in prevention of cataract formation.

Generally, glucuronidation of drugs is regarded as an inactivation step for excretion. Quercetin-3- and quercetin-7-glucuronides are major products of small intestinal epithelial cell metabolism. O'Leary et al. [118] demonstrated that both compounds can be further metabolized following the pathways of methylation of the catechol functional group and hydrolysis of the glucuronide by endogenous β-glucuronidase followed by sulfation to quercetin-3′-sulfate using the HepG2

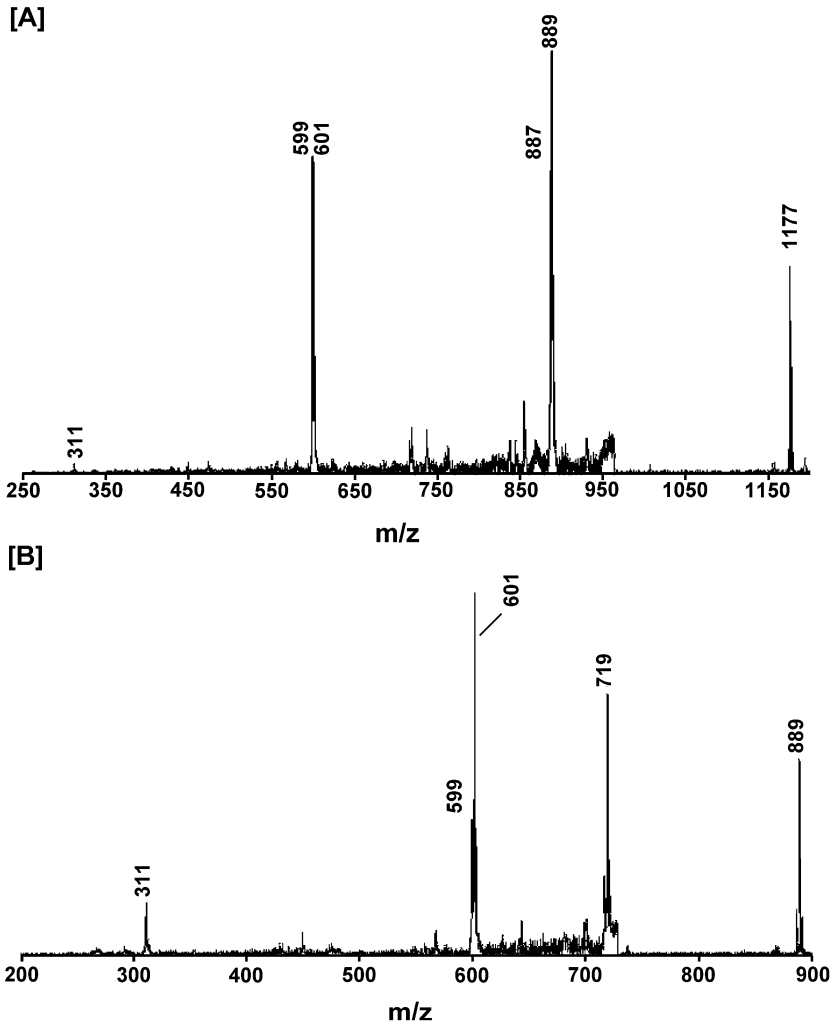


Fig. 8. PSD spectra of condensed tannin tetramer (A) and trimer (B) from lime (*Tilia cordata*). (Reprinted from *Phytochemistry*, Vol. 62, 2003, pp. 1159–1170, Behrens et al., copyright 2004, with permission from Elsevier.)

cell model. Identities of these metabolites were confirmed by LC-ESI-MS. Selected-ion monitoring was conducted for *m/z* 303 (quercetin), 317 (3′-*O*-methyl quercetin), 383 (quercetin-3′-sulfate), 397 (methylquercetin sulfate), 479 (quercetin-7-glucuronide), and 493 (3′-methylquercetin-7-glucuronide or 4′-methylquercetin-7-glucuronide).

Kulling et al. published a series of papers on oxidative metabolism of soy isoflavonoids [119–121]. They identified a variety of catechol metabolites using both GC-MS and LC-MS methods. Although the microsomal metabolism of formononetin and biochanin A is dominated by demethylation to daidzein and genistein, respectively, catechols of the parent isoflavones and of the demethylation products are also formed. Thus, oxidative metabolism appears to be common among isoflavones and may have implications for their biological activities. Oxidative metabolism of isoflavonoids in vitro is outlined in Fig. 9. To understand the ability of recombinant cytochrome P450 to metabolize dietary isoflavonoids, Breinholt et al. [122] recently reported the metabolism of genistein and tangeretin (a flavonoid from citrus food) by human and murine cytochrome P450s. Analysis of the metabolic profile from incubations with genistein and human liver microsomes revealed the production of five different metabolites. One of these metabolites was identified as orobol, the 3’-hydroxylated metabolite of genistein. The remaining two metabolites were also hydroxylated metabolites as evidenced by LC-MS. Orobol was the only metabolite formed after incubation with CYP1A2. The two major product peaks after incubation of tangeretin with human microsomes were identical with 4’-hydroxy-5,6,7,8-tetramethoxyflavone and 5,6-dihydroxy-4’,7,8-trimethoxyflavone.

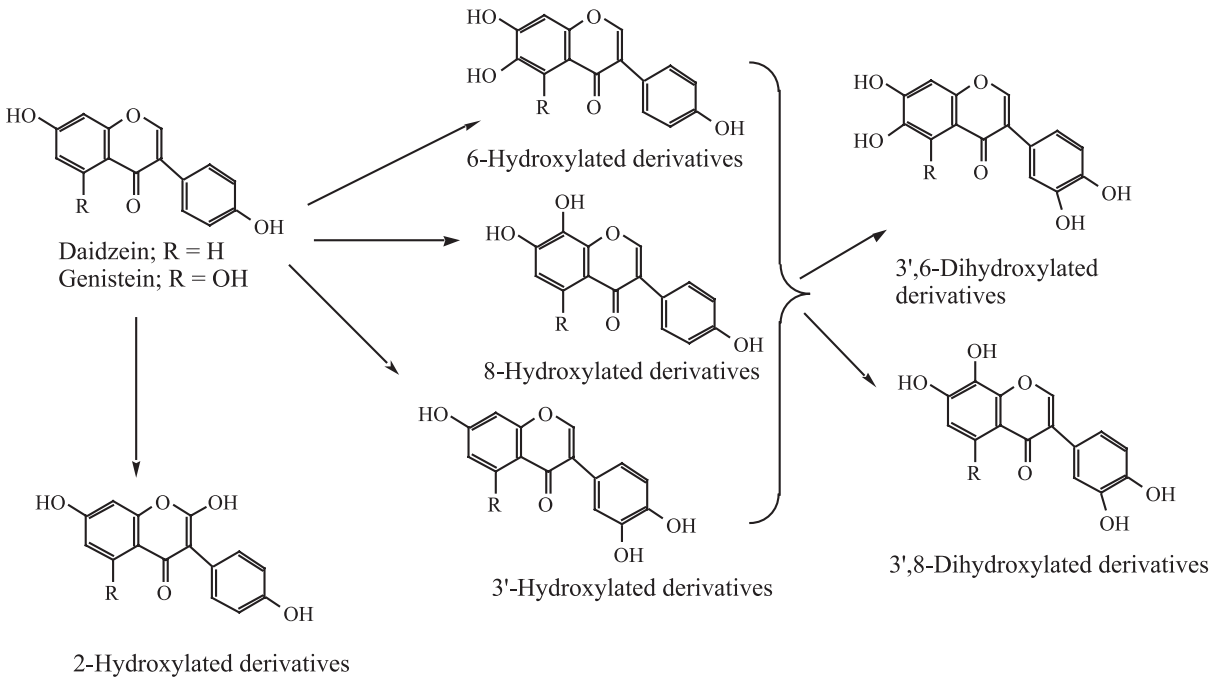


Fig. 9. Oxidative metabolites of genistein and daidzein in vitro.

It has been observed that dietary polyphenolics with phenol rings are metabolized by peroxidases to form prooxidant phenoxyl radicals which in some cases were sufficiently reactive to cooxidize GSH or NADH, accompanied by extensive oxygen uptake and reactive oxygen species formation.

Moridani et al. [123] utilized ESI-MS and UV-vis spectroscopy to show that the naturally occurring flavonoid catechin undergoes enzymatic oxidation by tyrosinase in the presence of GSH. In this study, tyrosinase (50 units/mL) was added to a mixture of catechin (1 mM) and GSH (4 mM) in water and preincubated for 5 min at room temperature prior to mass spectrometric analysis. Ions at *m/z* 291 and 307 corresponded to catechin and hydroxycatechin, respectively. In addition to these, other ions at *m/z* 596, 901, and 1207 $[M+H]^+$ were assigned to the mono-, bi-, and triglutathione conjugates of catechin, respectively. Higher molecular ions at *m/z* 884 and 1189 corresponded to mono- and biglutathione conjugates of catechin dimer, respectively.

EGCG is a potent antioxidant and is unstable under alkaline and even neutral conditions. Hong et al. [124] studied its stability, uptake and biotransformation, and efflux of [^{3}H]EGCG in HT-29 human colon adenocarcinoma cells. EGCG was not stable in cell culture systems, and its oxidation led to the formation of dimers and H_2O_2. Furthermore, it is transported into the cells by passive diffusion and is subsequently converted to methylated and glucuronide metabolites. These metabolites were identified by LC-MS-MS experiments.

We have recently proposed that dietary polyphenolics may react with reactive oxygen species and reactive nitrogen species produced at sites of inflammation [125–127]. This is potentially significant since preclinical studies in cell culture and animal experiments have revealed that diets enriched in polyphenolics may provide protection against cancer [128] and cardiovascular diseases [129]. It is possible that chlorinated and nitrated polyphenolics are not only end products of metabolism but also new pharmacophores, with their own biological activities.

An LC-MS-MS approach was applied to the mass spectrometry analysis of some representative chlorinated and nitrated isoflavones (Fig. 10), potential metabolites of isoflavones in inflammatory cells [126]. In MS-MS experiments on the deprotonated ions of these compounds, a number of structurally characteristic product ions were produced. The product ion analysis of 3'- and 8-chlorodaidzein in tandem mass spectra led to ready differentiation of these isomers. 3′-Nitro derivatives of both genistein and daidzein have product ions due to the losses of HNO_2 and two OH groups. The product ion spectra of 3′-nitrogenistein are shown in Fig. 11. Chlorinated derivatives of isoflavones were detected in cell-based experiments and their structures were proposed by comparing the tandem mass spectra of their product ions with those of standards.

In vivo. Since flavonoids are extensively metabolized in in vivo, most of the biological activities of flavonoids reported based on in vitro experiments cannot be simply

extrapolated to the in vivo systems. For example, cocoa flavonoids (proanthocyanidins—condensed catechins) showed preventive effects on low-density lipoprotein oxidation susceptibility in vitro [130]. However, proanthocyanidins are poorly absorbed across the gut barrier because of their high molecular weights although Holt et al. [131] have shown procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa.

To evaluate the influence of flavonoids in our diet and their health beneficial effects, it is important to monitor the concentration of the dietary flavonoids occurring in biological/physiological samples. Flavonoids are substrates for β-glucosidase, UDP-glucuronosyltransferase, or catechol-*O*-methyltransferase in the small intestine and for a number of phase I and II enzymes in the liver and other tissue sites. Furthermore, ingested flavonoids are subjected to hydrolysis and degradation in the colon due to microbial enzyme catalysis. Accordingly, several investigations on metabolism and bioavailability of flavonoids using mass spectrometric methods have been conducted.

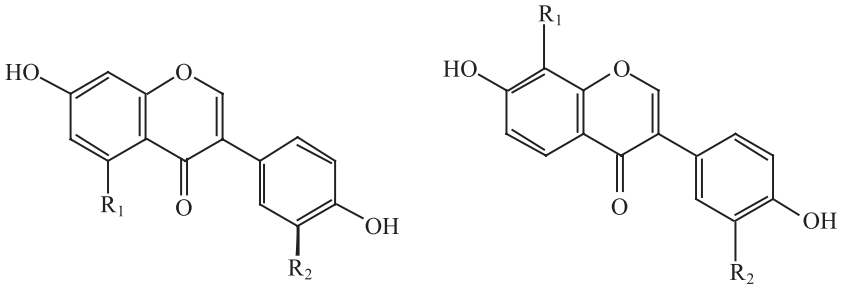
Catechin, a flavanol abundantly present in tea and grape seeds, has been studied extensively both in vitro and in vivo. Catechins (epicatechin and catechin) are *O*-methylated and glucuronidated in the small intestine. Plasma levels of catechin and its metabolites 3′-*O*-methylcatechin (3′MC) after consumption of red wine have been determined by GC-MS of the TMS derivatives [78]. Glucuronide and sulfate conjugates were determined after enzymatic hydrolysis. Before consumption, plasma levels of catechin, 3′MC, and all conjugates were <2 nM. After 1 h, their concentration increased to 91 ± 14 nM (red wine) and 81 ± 11 nM (dealcoholized red wine). Takino et al. [132] applied LC-MS with a turbulent flow chromatography method for the determination of five catechins in human plasma. In this method, after on-line extraction by its injection onto an extractor column at turbulent flow, five catechins were backwashed onto a reversed-phase column via on-line column switching and separated chromatographically at a laminar flow of 1 mL/min. Using this tandem LC-LC-MS system, the extraction, the separation, and the quantitation of five catechins in human plasma could be achieved with satisfactory selectivity and sensitivity. The limit of detection (S/N = 3) ranged from 0.6 to 2 ng/mL.

Cren-Olive et al. [133] used an LC/ESI-MS/MS method in the positive-ion mode for the structural characterization and differentiation of four isomeric *O*-monomethylated catechins by the analysis of the fragmentation behavior of catechin. To characterize the site of methylation, product ion spectra of methylated catechin $[M+H]^+$ ions were investigated. The maximum relative intensity of the product ions was achieved by collision energy scanning between 5 and 15 eV. The most obvious difference was the inversion of the base peak between 3′-*O*-methyl and 4′-*O*-methyl catechins: the ion at *m/z* 139 is the base peak for former, and the ion at *m/z* 137 is so for the later. Li et al [134] reported the use of LC/ESI-MS to determine urinary glucuronidated and sulfated tea catechins and their metabolites (including methylated and ring-fission metabolites) based on the detection of deprotonated molecular ions and aglycone fragment ions. The resolution of individual compounds was achieved both chromatographically and by mass spectrometry.

After green tea administration, the major conjugates appearing in human, mouse, and rat urine samples were identified as monoglucuronides and monosulfates of (-)-epigallocatechin and (-)-epicatechin. In addition to these, *O*-methyl-EGC-*O*-glucuronides and -*O*-sulfates and *O*-methyl-epicatechin-*O*-sulfates in human urine were detected. (-)-5-(3’,4’,5’-Trihydroxyphenyl)-γ-valerolactone and (-)-5-(3’,4’-dihydroxyphenyl)-γ-valerolactone, the ring-fission metabolites of EGC and (-)-epicatechin, respectively, were also predominantly found in their monoglucuronide and monosulfate forms in the urine. An LCQ ESI-ion trap mass mass spectrometer operating in the negative-ion mode was used for the analysis. The deprotonated aglycone ions for conjugated EGC, epicatechin, (-)-5-(3’,4’,5’-trihydroxyphenyl)-γ-valerolactone, (-)-5-(3’,4’-dihydroxyphenyl)-γ-valerolactone *O*-Me-EGC, and *O*-Me-epicatechin were at *m/z* 305, 289, 223, 207, 319, and 303, respectively.

Several investigators have contributed to the understanding of bioavailability and biotransformation of EGCG [135–137]. 4’,4’ ’-Di-*O*-methyl-EGCG (4’,4’ ’-diMeEGCG) was detected in human plasma and urine by LC-MS-MS following green tea ingestion [128]. (-)-5-(3’,4’,5’-Trihydroxyphenyl)-γ-valerolactone and (-)-5-(3’,4’-dihydroxyphenyl)-γ-valerolactone, along with another possible ring-fission metabolite, (-)-5-(3’,5’-dihydroxyphenyl)-γ-valerolactone, were detected in human urine after green tea ingestion. Structures of EGC, EGCG, and some of their metabolites are shown in Fig. 12.

Recently, the metabolic fate of (-)-epicatechin gallate, one of the major tea catechins, has been reported [138]. After intravenous administration of (-)-epicatechin gallate to Wistar male rats, its biliary metabolites were purified by HPLC after enzymatic hydrolysis. The metabolites were shown to be (-)-epicatechin gallate, 3’-*O*-methyl-(-)-epicatechin gallate, 4’-*O*-methyl-(-)-epicatechin gallate, 4’ ’-*O*-methyl-(-)-epicatechin gallate, and 3’,4’ ’-di-*O*-methyl-(-)-epicatechin gallate. These compounds were identified by FAB-MS and NMR studies. After oral administration, five major metabolites excreted in rat urine were purified in their deconjugated forms and their chemical structures identified. They were degradation products from (-)-epicatechin gallate, pyro-



3'-chlorogenistein; R_1=OH, R_2=Cl, $C_{15}H_9ClO_5$ mol. wt. 304

3'-chlorodaidzein; R_1=H, R_2= Cl, $C_{15}H_9ClO_4$ mol. wt. 288

3'-nitrogenistein; R_1=OH, R_2=NO_2, $C_{15}H_9NO_7$ mol. wt. 315

8-chlorodaidzein; R_1=Cl, R_2= H, $C_{15}H_9ClO_4$ mol. wt. 288

3'-nitrodaidzein; R_1=H, R_2=NO_2, $C_{15}H_9NO_6$ mol. wt. 299

3',8-dichlorodaidzein; R_1=Cl, R_2=Cl $C_{15}H_8Cl_2O_4$ mol. wt. 322

Fig. 10. Structures of chlorinated and nitrated derivatives of isoflavones.

gallol, 5-(3,4-dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid, 3-(3-hydroxyphenyl)propionic acid, and *m*-coumaric acid.

With regard to the metabolism of proanthocyanidins in humans, studies on the in vitro depolymerization of proanthocyanidins suggested that they might be degraded into catechins in the stomach or small intestine. However, it has been shown that proanthocyanidins are stable at stomach pH. On the other hand, several low-molecular-weight metabolites are formed from proanthocyanidins by the colonic bacteria. Rios et al. [139] recently analyzed several polyphenol-derived phenolic acids after chocolate intake in healthy humans using GC-MS and LC-MS-MS techniques. Detection by ESI was carried out at 45°C in the negative-ion mode—the data were collected in MRM mode in which precursor and product ions specific for each compound were monitored with a dwell time of 500 ms. Quantification was done using the following mass

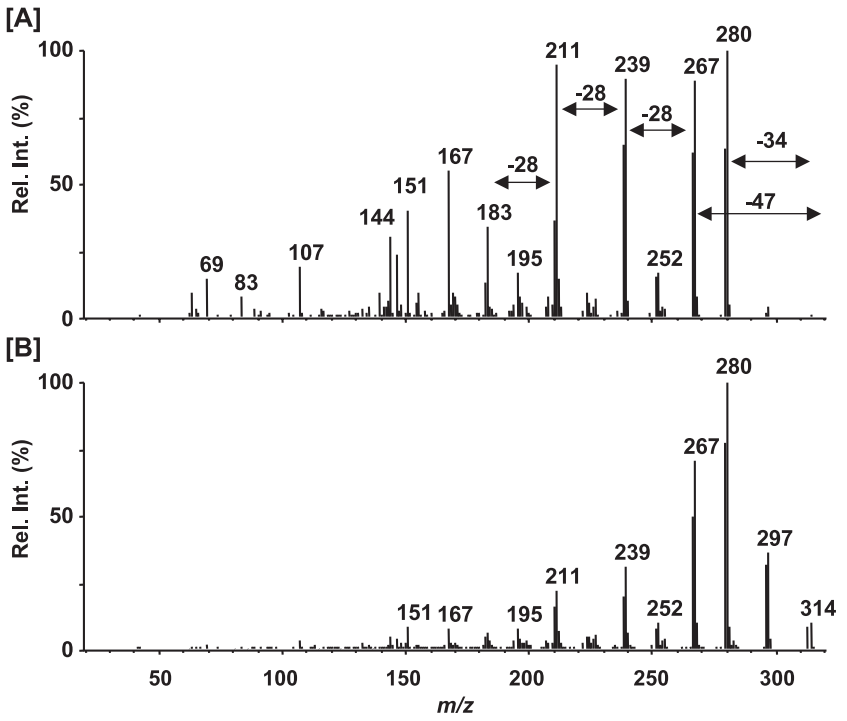
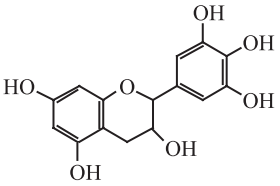
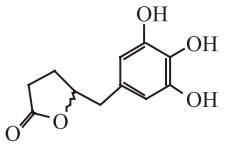


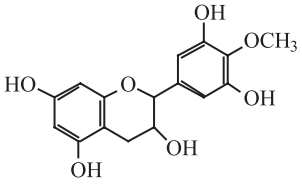
Fig. 11. Product ion spectra for the ion *m/z* 314 of 3′-nitrogenistein at collision energy [A] 35 eV; [B] 28 eV. (Reprinted from *J. Mass Spectrom*., Vol. 38, 2003, Prasain et al., "Mass Spectrometric Methods for the Analysis of Chlorinated and Nitrated Isoflavonoids," copyright 2004, with permission from John Wiley & Sons Ltd.)



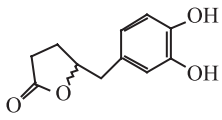
Epigallocatechin



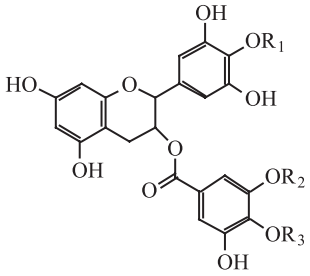
(-)-5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone



4'-*O*-Methylepigallocatechin



(-)-5-(3',4'-Dihydroxyphenyl)-γ-valerolactone



Epigallocatechinin gallate; R_1 = H, R_2 = H, R_3 = H
4"-Methylepigallocatechin gallate; R_1 = H, R_2 = H, R_3 = CH_3
4'4"-Dimethylepigallocatechin gallate; R_1 = CH_3, R_2 = H, R_3 = CH_3
4',3",4"-Trimethylepigallocatechin gallate; R_1 =CH_3, R_2 = CH_3, R_3 = CH_3

Fig. 12. Structures of EGC, EGCG, and their metabolites.

transitions: *m*- and *p*-hydroxybenzoic acid, *m/z* 137 and 93; *p*-hydroxyhippuric acid, *m/z* 194 and 100; vanillic acid, *m/z* 167 and 123; syringic acid, *m/z* 197 and 123; phenylacetic acid, *m/z* 135 and 91; *m*-hydroxyphenylacetic acid, *m/z* 151 and 107; dihydroxyphenylacetic acid, *m/z* 167 and 123; *m*-hydroxyphenylpropionic acid, *m/z* 165 and 121; dihydroxyphenylpropionic acid, *m/z* 181 and 59; and ferulic acid, *m/z* 193 and 134. It appears that the absorption of catechins and microbial phenolic acid metabolites may have collective roles in biological activities of chocolate. Gonthier et al. [140] also reported microbial aromatic acid metabolites formed in the gut of rats fed red wine polyphenols. Fourteen aromatic acid metabolites were assayed in urine collected for 24 h by an LC-MS-MS method. The 3 main metabolites formed from the catechin diet were 3-hydroxyphenylpropionic acid, 3-hydroxybenzoic acid, and 3-hydroxyhippuric acid.

Similar to Holt et al.'s [131] observation, Sano et al. [141] demonstrated that procyanidin B1 [epicatechin-(4β->8)-catechin] is detected in human serum after intake of proanthocyanidin-rich grape seed extract. Procyanidin B1 was detected in human serum with a concentration of 10.6 ± 2.5 nM using LC-MS methods.

Nielsen et al. [142] also reported a multistage APCI tandem mass spectrometric method for the determination and quantification of 12 dietary flavonoids (naringenin, naringin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, rutin, quercetin, kaempferol, isorhamnetin, tamarixetin, phloridzin, and phloretin) in human urine samples. They also investigated the biotransformation of tangeretin (5,6,7,8,4′-pentamethoxyflavone, which is concentrated in the peel of citrus fruits) and identified 10 new metabolites (demethylated or hydroxylated derivatives) with intact flavonoid structures by LC-MS and proton NMR methods [143].

Naringin and several other flavonoids possess a glycoside moiety at the 7 position of the flavonoid skeleton. The position and identity of the sugar moiety have been shown to have roles in activating polymorphonuclear leukocyte activation [144]. The observed activity of the polymorphonuclear activating property of naringin in vivo may be due to its intact glycosidic form after adsorption. Ishii et al. have published a series of papers on naringin metabolism [145–147]. They demonstrated that naringin can be absorbed from the human gastrointestinal tract as the glycoside using LC-MS-MS methods [145]. The positive-ion ESI-MS of authentic

naringin showed a prominent ion at *m/z* 598 $[M+NH_4]^+$. The same ion (*m/z* 598) was observed in urine sample obtained after a single oral administration of naringin. The MS-MS spectrum of the ion at *m/z* 598 yielded a product ion at *m/z* 273 (base peak) which corresponded to the protonated narigenin ion.

Recently, a detailed investigation on detection and identification of ^{14}C-labeled flavonol metabolites by HPLC-radiocounting and tandem mass spectrometry has been published by Mullen et al [148]. In this report, [2]quercetin-4′-*O*-β-glucoside was fed to rats and an extract from their gastrointestinal tracts was purified by partitioning and solid-phase extraction techniques. The analysis was performed by reverse-phase HPLC with on-line radioactivity detection and ion trap mass spectrometry. The presence of the ^{14}C-labeled metabolites was determined with the radioactivity monitor and the mass spectra produced were used to identify 16 of the 17 metabolites detected. These included methylation of the aglycone and the formation of mono- and diglucuronides and sulfate conjugates. In a similar vein, Day et al. [149] indicated that quercetin glucosides are not present in plasma of human subjects 1.5 h after consumption of onions. The major circulating compounds in the plasma after 1.5 h are identified as quercetin-3-glucuronide, 3'-methylquercetin-3-glucuronide, and quercetin-3'-sulfate. The existence of substitutions in the B and/or C ring of plasma quercetin metabolites suggests that these conjugates will each have very different biological activities. Other investigators have suggested that after consumption of quercetin glucosides, quercetin glucuronides are major metabolites in plasma [150,151]. Gross et al. [152] reported metabolism of quercetin in human urine.

There are several reports on metabolism of soy isoflavones [153–157]. Coldham et al. [157] determined systemic plasma pharmacokinetics of genistein in rats to evaluate the absolute oral bioavailability and made comparison with similar data in the literature derived from human subjects using LC-MS-MS methods. The absorption of total radioactivity from the gut (parent compound and metabolites) was 56 and 111% in male and female rats, respectively. In contrast, the absolute oral bioavailability of genistein in male and female rats was 7 and 15%, respectively. Selective plasma analysis by LC-MS-MS, without prior enzymatic hydrolysis, enabled ready discrimination between parent and conjugated metabolites and prevented gross overestimation of genistein bioavailability.

LC-MS-MS has been applied for the analysis of intact sulfate and glucuronide isoflavone conjugates in human urine using an isotope dilution LC-MS-MS method [76]. Similarly, Fang et al. [158] characterized isoflavones, metabolites, and their conjugates in female rat urine using a LC-MS-MS method following addition of soy protein isolate to the diet. Five isoflavones (daidzein, genistein, glycitein, dihydrodaidzein, and *O*-desmethylangolensin) were identified by comparison with authentic standards. Seventeen conjugates of isoflavones were characterized in the urine. Interestingly, they detected genistein 5-glucur-

Table 3. In Vivo Metabolites of Some Common Dietary Flavonoids

Parent compound	Metabolite	$[M-H]^-$	Reference
Genistein	Dihydrogenistein	271	[153]
	Tetrahydrogenistein	273	[153]
	4-Hydroxyphenyl-2-propionic acid	165	[156]
	6′-Hydroxy-*O*-desmethylangolensin	273	[153]
	4-Ethylphenol	121	[154]
Daidzein	Dihydrodaidzein	255	[153]
	Tetrahydrodaidzein	257	[153]
	Equol	241	[25,34]
	cis-4-OH-Equol	257	[34]
	3′,7-Dihydroxyisoflavane	241	[155]
	O-Desmethylangiolensin	257	[26,153]
Quercetin	3,4-Dihydroxyphenylacetic acid	167	[152]
	m-Hydroxyphenylacetic acid	151	[152]
	4-Hydroxy-3-methoxyphenyl acetic acid	181	[152]
EGCG	3′-*O*- and 4′-*O*-methylEGCG	471	[137]
	3″-*O*- and 4″-*O*-methylEGCG	471	[137]
	4′,4′-Di-*O*-methylEGCG	485	[137]
	(−)-5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone	223	[135]
	(−)-5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	207	[135]
	(−)-5-(3′.5′-Dihydroxyphenyl)-γ-valerolactone	207	[135]

onide and 4 glucuronide conjugates of reductive metabolites of daidzein by the application of LC-MS-MS. Some flavonoid metabolites identified in biological fluids are summarized in Table 3.

Although a large number of flavonoid metabolites have been now identified, there is a need to account for all of the ingested flavonoids. For this, more efficient and sensitive analytical methods are required to detect and identify minor metabolites. The use of multisite ^{14}C- and ^{13}C-labeled flavonoids should help elucidate their bioavailability.

RECENT ADVANCEMENTS IN HIGH SENSITIVITY OF MASS SPECTROMETRY

While the development of various API techniques has enabled the use of LC-MS for many varieties of chemical compounds, there are still many compounds such as hydrocarbons or polymethoxylated flavonoids that are difficult to ionize. In the latter case, the use of ammonium acetate-containing mobile phase and a lowered orifice potential may enable detection of $[M+NH_4]^+$ adducts. However, there are other complimentary ionization techniques that have been developed to assist ionization of molecules in the liquid phase. Of particular interest is electrochemistry/mass spectrometry (EC-MS). This technique greatly enhances MS sensitivity or even makes poorly ionizable compounds detectable. There are several reports on the use of EC-MS to mimic phase I oxidative metabolism [159–161].

Recently, Meyer et al. [162] reported liquid chromatography with on-line electrochemical derivatization and fluorescence detection for the determination of phenols. In this method, after liquid chromatographic separation of the analytes, an on-line electrochemical derivatization is carried out and the reaction products are detected fluorometrically. Phenols are oxidized in the electrochemical cell to form fluorescent dimers and higher oligomers, which were identified by on-line electrochemistry–mass spectrometry. Major advantages of the proposed method include enhanced selectivity and sensitivity. Without prior enrichment of the analytes, limits of detection down to 20 fmol may be reached for selected phenols, e.g., for 4-octylphenol, 4-ethylphenol, and 4-(1-indanyl)phenol. There is no report yet on the use of EC-MS for flavonoid analysis. However, we have recently studied the electrochemical reaction of puerarin in an electrochemical cell on-line with ESI-MS using various potentials. Puerarin underwent electrochemical oxidation, producing various deprotonated molecular ions. Some of the ions are assumed to be quinones (details will be published elsewhere).

CONCLUSIONS

Analysis of the bioflavonoids has taken giant steps forward as a result of the application of MS techniques. Much of the early literature on bioflavonoids and their metabolites was obtained in the period 1960–1985 and either was dependent on HPLC with UV or fluorescent detection or required extensive workup and derivatization prior to GC analysis. Modern MS techniques based on ESI, APCI and MALDI have greatly simplified this analysis, allowing investigators in the area of free radical biology to carefully examine the metabolism and fate of bioflavonoids in their in vitro and in vivo systems. Mass spectrometry will allow a substantial redefinition of what oxidative stress means in the presence of these compounds. It is probable that many new bioactive metabolites will be discovered at the tissue target sites.

Acknowledgments—Research on the mass spectrometry of bioflavonoids is supported by a grant (P50 AT00477) to the Purdue University-University of Alabama at Birmingham (UAB) Botanicals Center for Age-Related Disease (Connie Weaver, PI) from the National Center for Complementary and Alternative Medicine and in part by a grant (U54 CA100949) from the National Cancer Institute to the UAB Center for Nutrient-Gene Interaction (Stephen Barnes, PI). JP was supported by matching funds from the UAB School of Medicine Dean's office. Support for the purchase and installation of the mass spectrometers used at UAB came in the form of several grants from the National Center for Research Resources (S10 RR06487, S10 RR11329, S10 RR13795, S10 RR17261, S10 RR019231), the UAB Health Services Foundation-General Endowment Fund, and the UAB Office of the Provost. Ongoing support for the operation of the mass spectrometry facility has been provided by a grant (P30 CA13148) from the National Cancer Institute to the UAB Comprehensive Cancer Center (Al Lobuglio, PI).

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