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Metabolism and Bioavailability of Flavonoids in Chemoprevention: Current Analytical Strategies and Future Prospectus

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Abstract: Flavonoids are structurally diverse and among the most ubiquitous groups of dietary polyphenols distributed in various fruits and vegetables. Many have been proposed to be bioactive compounds in the diet that are responsible for lowering the risk of cancer and have been used in chemoprevention studies using animal models of this disease. As for any xenobiotic, to evaluate the potential risks and benefits of bioflavonoids to human health, an understanding of the physiological behavior of these compounds following oral ingestion is needed as well as their absorption, distribution, metabolism, and excretion (ADME). The study on metabolism and bioavailability is very important in defining the pharmacological and toxicological profile of these compounds. Due to great structural diversity among flavonoids, these profiles differ greatly from one compound to another, so that the most abundant polyphenols in our diet are not necessarily the ones that reach target tissues. Therefore, careful analysis of flavonoids and their metabolites in biological systems is critical. Mass spectrometry in various combinations with chromatographic methods has been a mainstay in applications that involve profiling and quantification of metabolites in complex biological samples. Because of its speed, sensitivity and specificity, liquid chromatography−tandem mass spectrometry (LC−MS/MS) has become the technology of choice for sample analysis. This review describes the chemistry of polyphenols and flavonoids, their ADME, and the various mass spectrometry-based strategies used in the analysis of flavonoids, including future trends in this field.

Keywords: Flavonoids; chemoprevention; bioavailability; LC−MS/MS

1. Introduction

Over the past 20 years, there has been an increasing interest in plant-derived polyphenols, in particular, the bioflavonoids, with respect to human health.[1–5] Many of these compounds are found in plant foods that have been in common use in different societies around the world for many

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centuries. Since by definition a food is not toxic at the levels normally consumed, it has been assumed that the polyphenols in the food are also nontoxic. This has encouraged the use of such foods and the polyphenols therein for their chemoprevention properties and/or as adjuvants in chemotherapy of cancer.[6–12] In reality, these compounds are all xenobiotics and are handled by the body in the same way as any synthetic, nonphysiologic agent. Their apparent "safe" nature may more reflect their lower dose levels in the foods than their intrinsic safety.

The purpose of this review is to present to the nutritionist, clinician, oncologist, botanist, and pharmacologist a unifying view of how polyphenols are made in the plant, are altered in the production of dietary supplements and foods, and are absorbed from the intestinal tract, distributed, metabolized, and excreted (ADME). In particular, we define bioavailability and discuss the reasons for its person-to-person variability and its importance in preclinical models. Finally, we describe the use of LC−mass spectrometry methods that allow investigators to follow the polyphenols as they proceed down this path. We encourage readers of this paper to refer to our previous reviews of the methods used for analysis of polyphenols,[13–16] in particular applications of mass spectrometry methods. This current paper emphasizes research reported between 2003 and 2007. With regard to bioavailability of polyphenols, there are excellent reviews by Manach et al.[17] Williamson and Manach[18] and Scalbert et al.[19] Russo[20] recently reviewed dietary phytochemical bioavailability in the context of chemical and dietary chemoprevention. Nielson and Williamson[21] have reviewed the factors governing isoflavone bioavailability in humans, and Silberberg et al.[22]]reported on the metabolism of flavanones in normal and tumor-bearing rats.

2. Chemistry of Polyphenols and Bioflavonoids in Plants

Modern DNA sequencing has suggested that the genomes of plants are more complex than those of the readers of these words.[23] Plants, literally with their roots firmly in the ground and unable to run away, have to employ chemical wizardry in order to attract those that can assist with their reproduction (bees) and repel those (bacteria and other microorganisms, beasts, birds and insects) who would otherwise attack them. To achieve this requires genes that can accomplish far more complex chemistry than their walking, crawling, and flying protagonists. In addition, plants have to defy gravity, stand in the hot sun and cold breezes, move water vertically without the benefit of a heart, and convert the CO_2 produced by beasts and their so-called civilized world into life-giving oxygen. A limited group, the legumes, form an alliance with soil bacteria to accomplish the most important chemical reaction for the creation and sustaining of life, the conversion of inert nitrogen gas into its fixed forms, leading to proteins, enzymes and nucleic acids.

Accordingly, plants contain rich sources of chemically diverse compounds. In a world where biologically beneficial compounds are highly prized to improve human health, it is worth noting that all the compounds in plants have been selected by evolution to have biological importance. There would be no advantage to a plant to casually make a particular compound. This gives plants a distinct advantage over those constructing random chemical combinatorial libraries. Chemical space, like the universe we live in, is immense, but only has very few useful locales biologically (cf. star formations). Thus, searching the plant chemical

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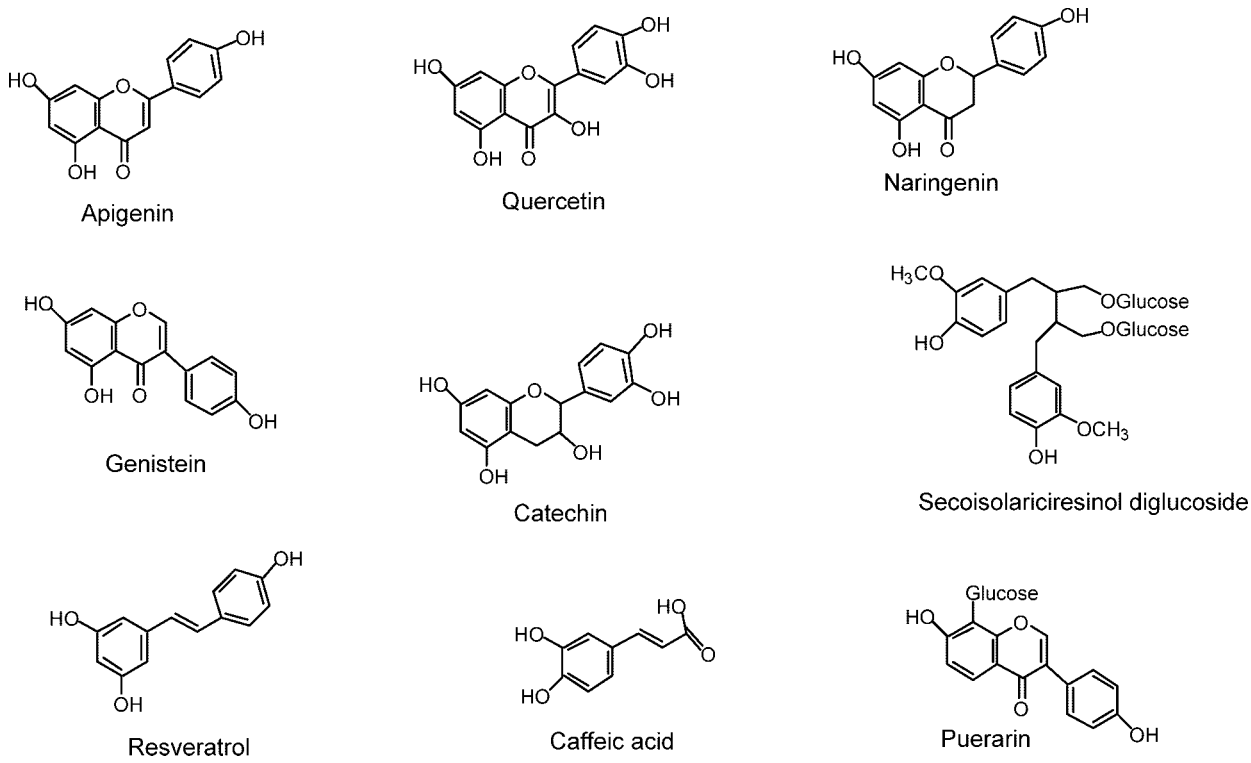

Figure 1. Chemical structures of some common dietary flavonoids and polyphenols.

libraries has had a history of being more productive in discovery of biologically useful compounds than scouring random chemical space. Plants also generate novel compounds with extreme stereospecificity, far better than even the most sophisticated organic chemist.

It is not therefore surprising that 50% of the anticancer therapeutic agents used in modern medicine are derived from plants.[24] Compounds such Taxol[25] and the vinca alkaloids[26] act on destabilizing the microtubules, essential for proper replication in dividing cells and particularly affecting the rapidly proliferating cancer cells. However, these agents also affect the proliferation of normal cells and therefore have an unavoidable toxicity.[27]

Over the past 30 years, there has been a new interest in discovering compounds that prevent cancer, so-called chemoprevention or chemopreventive agents. Since such agents are used *before* the appearance of the cancer, it is an essential requirement that they do no other harm. Because of this, the most likely plant category for the discovery of chemopreventive agents is edible plants. However, humans and many animals have long realized that only a limited number of plants can be eaten. Before humans turned the use of fire into cuisine, the list was quite short. The advent of modern humans (since the last ice age) has been the selection of plants (crops) with specific nutritional value and growth characteristics for the different parts of the world.

Plant-based chemoprevention agents include the vast array of polyphenols (bioflavonoids, stilbenoids, etc.) (Figure 1)[28–37] and nonpolyphenols (isothiocyanates, terpenoids, etc., as well as the more familiar vitamins A, C, D, and E).[38–44] Bioflavonoids are synthesized by first reacting phenylalanine

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and malonyl CoA to form 4-coumaroyl CoA, followed by condensation with further malonyl CoAs by chalcone synthase to yield naringenin.[45] This flavonoid is the precursor to a host of flavonoids, flavanoids, isoflavonoids, anthocyanins, proanthocyanins, and coumestanes.[46,47] Stilbenoids are formed in a similar way from 4-coumaroyl CoA and malonyl CoA and stilbene synthase.[45,48]

Many of these compounds in the plant are in a pro-form, i.e., they are chemically modified to limit their biological activity or to cause them to be sequestered within the plant in concentrated forms. This allows them to be made available quickly to the plant in times of stress or when it is under attack. Bioflavonoids and terpenoids are usually found as *O*-glycosides,[49,50] whereas isothiocyanates are complexed to alkyl or aryl moieties.[51] For the former, specific enzymes (glycosidases) are utilized to release the bioactive phytochemical.[52] The isothiocyanates precursors are hydrolyzed by myrosinase in the plant to release isothiocyanate.[53,54] Similarly, alicin is formed when fresh garlic is crushed and thereby releases aliinase.[55] In contrast, the bioflavonoid and terpene glycosides are degraded by enzymes originating in the eater.[56,57]

Because of the chemical forms of polyphenols in a plant, there are important considerations regarding how they are chemically changed by the processes involved in their preparation (prior to purchase) and cooking (prior to eating). These changes may have strong influence on how well the polyphenols are absorbed from the intestines and carried via the bloodstream to the organs of the body. The isoflavones in soybeans are 7-*O*-β-glucosides and are esterified in the 6-position of the glucose with malonic acid.[49,58,59] This substantially alters the ADME of isoflavones.

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2.1. Consequences of Glycoside Chemistry to Bioavailability. 2.1.1. *C*- vs *O*-Glycosides: Malonyl Esters. For the biosynthesis of many plant natural products, including polyphenols and terpenes, glycosylation is the final step necessary for storage (and therefore accumulation) of the metabolite in the plant. Therefore, most polyphenols exist in plants in glycosidic forms. Glycoside conjugates of polyphenols (*O*- and *C*-glycosides) are the major naturally occurring forms in leguminous plants, including soy (*Glycine max merrill*)[48,58–60] and kudzu (*Pueraria lobata*).[61] We recently identified several isoflavone *C*-glycosides in *Pueraria lobata* root cell cultures by tandem mass spectrometry.[62] The sugars of *C*-glycosylisoflavones are linked to the isoflavonoid by a carbon−carbon bond which is resistant to acid hydrolysis; in contrast, the sugars of isoflavone *O*-glycosides are *O*-linked at phenolic hydroxy groups, forming a carbon−oxygen−carbon bond, and are acid-labile. Well known examples of mono-*C*-glycosylisoflavones include puerarin (daidzein 8-*C*-glucoside), vitexin (apigenin 8-*C*-glucoside), and isovitexin (apigenin 6-*C*-glucoside).

Most of the polyphenol *O*-glucosides undergo intestinal hydrolysis to release the respective aglycons by intestinal glucosidases/hydrolases, both from the host[56] and intestinal bacteria.[57] Because the aglycons and their metabolites are more hydrophobic, they are more efficiently transported across the wall of the gastrointestinal tract than their respective glucosides. They are converted both in the gut wall and the liver, as well as at peripheral tissue sites, into phase I and phase II metabolites. In many animals, there are also extensive bacterial metabolites. Indeed, except for humans, the principal form of the soy isoflavone daidzein in the blood and urine of animals used in chemoprevention experiments is (*S*)-equol, a bacterial metabolite.[63]

There is a complex interplay between the chemical structure of glycosides and their rate of intestinal transport.[64] Our preliminary studies on puerarin's metabolism and pharmacokinetics have indicated that puerarin is rapidly absorbed intact, reaching a maximum concentration and then declining within 1 h after oral administration.[65] Unlike bioflavonoid *O*-glycosides, unconjugated puerarin is the major component in the blood and urine after its oral administration, indicating that phase II metabolism is not the major metabolic pathway for puerarin excretion.[66]

3. Metabolism in the Intestinal Wall: Glucuronides/Sulfates

Evolution has engineered the intestinal wall to provide a first line of defense against the toxins in plants and other foods. The needed materials for energy and construction of cells (carbohydrates and amino acids, respectively) have specific transporters that actively carry them across the intestinal membrane. Those compounds lacking transporters rely on passive diffusion−their transport rate is associated with the degree of hydrophobicity (i.e., is higher for the methoxylated bioflavonoids than their nonmethoxylated counterparts). However, increased hydrophobicity is also a cause of insolubility in the aqueous phase in the intestinal lumen. In that case, hydrophobic compounds, once released from the matrix of the dietary supplement/chemopreventive agent, are solubilized by forming mixed micelles with bile acids and monoglycerides. The micelles maintain a moderate concentration of the hydrophobic compounds in the region of the unstirred water layer on the surface of the enterocytes by acting as carriers from the bulk phase.[67]

Once in the enterocyte, a second level of defense occurs in the form of phase I and phase II metabolism. The principal reaction is the formation of β-glucuronides, but methylation and sulfonation also occur. A simplified general scheme for flavonoid metabolism in the small intestine and liver is shown in Figure 2. Using a Caco-2 cell monolayer system, it is also apparent that the β-glucosides could be transported back into the lumen from the serosal side by the multidrug resistance

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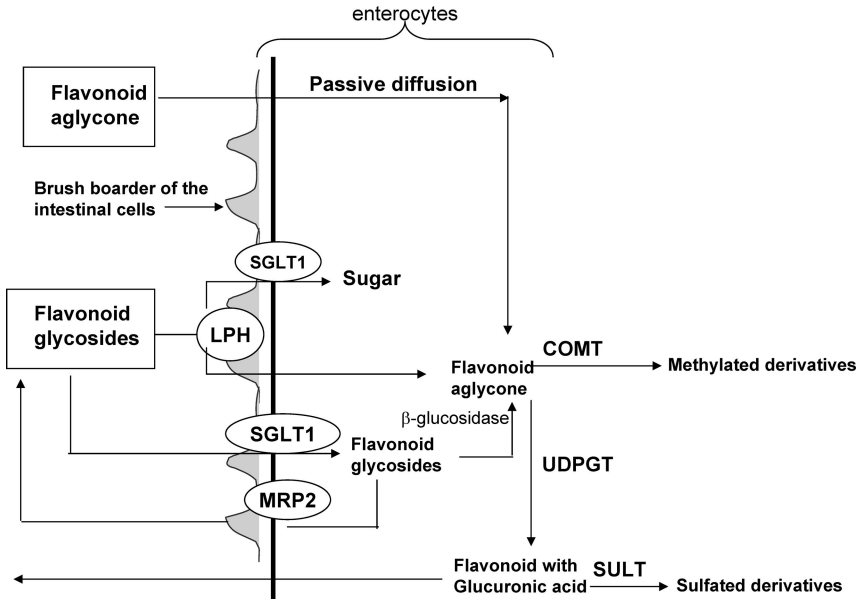

Figure 2. Schematic outline of flavonoid transport and metabolism. Key: SGLT1 = sodium dependent glucose transporter 1, LPH = lactase phlorizin hydrolase, UDPGT = uridine diphosphate glucuronosyl transferase, MRP2 = multidrug resistance protein2, SULT = sulfotransferase.

protein MRP2.[68] However, this restricted to the Caco-2 cell model as demonstrated by results obtained using the rat intestinal perfusion model.[69]

4. Role of Gut Microflora

As indicated previously, the gut bacteria contain glycosidases that can break down not only the simple β-glycosides such as daidzin (7-*O*-β-glucosyldaidzein) and genistin (7-*O*-β-glucosylgenistein) but also the 6″-malonyl-7-*O*-β-glucosyl and 6″-acetyl-7-*O*-β-glucosyl isoflavones.[57] The latter hydrolysis only occurs in the colon, thus accounting for the 6.5 h peak of blood isoflavone concentrations when volunteers consumed soy protein isolate that is enriched in 6″-malonyl-7-*O*-β-glucosyl isoflavones.[70,71]

Once the bioflavonoid aglycons are released in the colonic lumen, they undergo further reactions in the prevailing anerobic conditions. These consist of reduction and ring fission of the heterocyclic B-ring. In this way, daidzein is converted to dihydrodaidzein (7,4′-dihydroxyisoflavanone), equol (7,4′-dihydroxyisoflavan), and *O*-desmethylangolensin.[72–75] These compounds are absorbed and enter the enterohepatic circulation—they are hydroxylated, methylated, and conjugated (glucuronidation and sulfonation).[76–79] Of great importance in carrying out experiments to determine the biological activity of these metabolites is that the majority of them contain a chiral center at the C_2 (flavanoids) or C_3

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Table 1. In Vitro and in Vivo Metabolites of Some Common Dietary Flavonoids

dietary source	identified metabolite	ref
soy	dihydrogenistein	73
	tetrahydrogenistein	73
	hydroxyphenyl-2-propionic acid	74
	6′-hydroxy-*O*-desmethylangolensin	73
	4-ethylphenol	143
	dihydrodaidzein	75, 70, 73
	tetrahydrodaidzein	73
	equol	70
	cis-4-OH-equol	145
	3′,7-dihydroxyisoflavan	139
	3′-hydroxydaidzein	142
	6-hydroxydaidzein	142
	O-DMA	73
	6-hydroxy-ODMA	142
onion	isorhamnetin	112, 124, 128
	4-Dihydroxyphenylacetic acid	141
	m-Hydroxyphenylacetic acid	141
	4-Hydroxy-3-methoxyphenyl acetic acid	141
green tea or tea phenolics	catechin or epicatechin	134
	3′-methylcatechin/epicatechin	127
	EGCG, ECG, GC, CG, GCG	134
	3′-*O*- and 4′-*O*-methylEGCG	134, 135
	3″-*O*- and 4″-*O*-methylEGCG	137
	4′,4′-di-*O*-methylEGCG	137
	(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	136
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	136
	(−)-5-(3′,5′-dihydroxyphenyl)-γ-valerolactone	136
	3′-*O*-methylepicatechin gallate	144
	4′-*O*-methylepicatechin gallate	144)
	4″-*O*-methylepicatechin gallate	144
	3′,4″-di-*O*-methylepicatechin gallate	144
	GSH conjugate of gallic acid	115
	GSH conjugate of EGCG	115
orange juice	hesperidin	138
	narirutin	138
	naringenin	124, 141
	p-hydroxyphenylpropionic acid	140
grape wine or grape seed extract	catechin or epicatechin	104
	3′-methylcatechin or epicatechin	104

(isoflavonoids) carbon atom.[80] However, chemically synthesized metabolites, unless specifically purified, are diastereoisomers. Setchell et al.[63] have shown that the biological isomer of equol is its *S*-isomer. (*S*)-Equol is a far better ligand than (*R*)-equol for estrogen receptor α (6.4 nM versus 27 nM) and estrogen receptor β (0.73 nM versus 15 nM).

The *C*-glycosides of bioflavonoids are quite resistant to hydrolysis, even in the colon. Following administration of puerarin to rats, daidzein, dihydrodaidzein, and equol only appeared (in small amounts) on the third day of treatment.[66] Various flavonoid metabolites identified *in vitro* and *in vivo* studies are listed in Table 1.

5. Definition of Bioavailability

A discrepancy often exists in the minds of nutritionists and pharmacologists over the term *bioavailability*. In many papers in the nutrition literature, bioavailability is assessed by how much of the phytochemical that was orally consumed appears in urine.[81] The logic is that the compound has to enter the blood compartment before it can be transported to the kidney to undergo urinary excretion. By reverse logic, it is assumed that a phytochemical appearing in the urine must have had access to the blood compartment and therefore was distributed all over the tissues of the body. The greater the proportion of the dose found in the urine, the more

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bioavailable it must be. This logic has a fallacy in that rapid excretion of the compound in the urine also means that it did not stay long in the body. A compound that is retained in tissues is excreted more slowly in the urine. Another issue concerns how well a compound is reabsorbed in the proximal tubules of the kidney after initial filtration by the renal glomeruli or is excreted by the organic anion transporter. Bile acids are substantially reabsorbed in the kidney, so very little of them appear in the urine.[82] They would appear to have poor bioavailability by the nutritionists' definition, even though 95–98% of an oral dose is taken up from the intestines.[83]

Another, and perhaps more relevant to chemoprevention, definition of bioavailability comes from the pharmacology community. The time-dependent change in the blood concentration of an oral dose of the compound is compared to the changes observed when the same dose is administered systemically.[84] This definition takes into account how a compound is distributed to tissues when it is directly introduced into the blood stream. The areas under the concentration−time curves for each route of administration are compared to give an estimate of oral bioavailability. This method also assesses how much of the active form of the compound is available, an important issue for a chemoprevention agent. For instance, over 70% of orally administered genistein is taken up from the small intestine in the rat and is excreted into bile;[85] however, only 5% or less of the genistein is present in the blood in an unmetabolized form. The latter value is similar to the *oral bioavailability*. Nonetheless, this may underestimate true bioavailability since the circulating bioflavonoid β-glucuronides and sulfates can be utilized in a site-specific manner if there is a local tissue invasion of neutrophils, eosinophils, and macrophages. These cells, when activated, secrete enzymes (β-glucuronidases, sulfatases) that may release the aglycons that would then be rapidly taken up into the local tissues.[86]

The pharmacokinetic approach to studying disappearance of compounds from the blood can generate other useful information that is not provided by studying only urinary excretion. Extrapolation of the plasma−time disappearance curve to zero time allows for estimation of the *volume of distribution*. This is a very useful parameter since it reveals the extent to which a phytochemical is taken up by tissues. A polyphenol that has a volume of distribution equivalent to the plasma volume is confined to the blood compartment, whereas one that has a much larger volume of distribution must have significantly entered the tissue space.

5.1. Factors Governing Bioavailability. The matrix in which the bioflavonoid/polyphenol is contained will have an important role in determining when and to what extent it enters the aqueous phase. Plant-derived bioflavonoids typically associate with the protein fraction and therefore are not available until the protein matrix is digested.[87] Therefore, the host's capacity to digest food materials is a variable−and is reduced in pancreatic insufficiency due to pancreatitis, cystic fibrosis and aging. Some plant materials have protease inhibitors that interfere with this process−most beans, including soybeans, are soaked to remove these soluble protease inhibitors prior to initiation of cooking. An advantage of a compound being dispersed within a food matrix is that it has a very high surface area of contact with the aqueous phase. In contrast, an extracted bioflavonoid compressed into a pill form has to be actively dispersed, a phenomenon well-known to the pharmaceutical industry. Many compounds in the pill form are mixed with sodium bicarbonate to ensure such a dispersal.

Other important variables in the bioavailability of bioflavonoids concern the variations in gene expression and protein abundance of the important components that control ADME, i.e., specific transporters and metabolic enzymes in the intestine, hepatocytes, and kidney. In addition, single nucleotide polymorphisms of these genes and their promoters regions can affect copy number at the mRNA level and the functional properties of the translated proteins and their turnover. A mostly unexplored area is the variability caused by the organisms that populate the intestinal tract. A glimpse of the importance of this is the variable production of the daidzein metabolite equol.[88–90] This isoflavan has strong estrogenic properties and is produced in significant amounts (blood concentrations >20 nM) in 20–30% of the human population (it should be noted that in most animals it is the

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dominant daidzein metabolite). Subjects who produce equol do so consistently, suggesting that equol is formed by particular (and rare) microorganisms that are established in their gastrointestinal tract. Whether these organisms also have single nucleotide polymorphism (SNP)-based variation is unknown at this time.

6. Mass Spectrometry in a Bioavailability/ Chemoprevention Experiment

Modern chemoprevention experiments are placing a much greater emphasis on the need for precise knowledge of the composition of the agent that is being tested particularly at the time the experiment is initiated, the diet is mixed, and the diet is administered. At our own institution, we've encountered problems with the Animal Resource Program staff not wanting to go to the refrigerator at weekends to get the diet to be administered to the animals. In that scenario, there is the risk that a polyphenol such as epigallocatechin 3-gallate may decompose when left at room temperature. The National Center for Complementary and Alternative Medicine has instituted a procedure for grant applications that are determined by NIH Study Sections to be in the funding range. Each principal investigator is sent a series of questions by the Product Quality Working Group about the composition of the product to be studied, how it will be analyzed, what the quality control measures will be in place to determine if another batch is equivalent to the first batch used, and how the composition varies in the diet over the period of the batch of diet is used and over the entire experiment? Answers to these questions require serious consideration by the investigator since the grant will not be activated until the Product Quality Working Group issues a *Satisfactory* statement. In our own experience, it took two 6-page letters, three rounds of response, and 4 months to satisfy the Working Group with regard to the use of curcumin.

In addition to characterizing what is administered to the animal (in the diet or by gavage), it is also essential to know what is circulating in the blood or tissues and what's excreted in the urine. For instance, in almost all the animal models used in cancer chemoprevention, the isoflavone daidzein is mostly converted to (*S*)-equol (equol is 6 times higher than daidzein in the blood of rats,[91] whereas in humans only one-third make any equol and in those that do daidzein still exceeds equol).[89]

Mass spectrometry is an invaluable analytical tool to establish the composition of the chemoprevention agent and the quality control procedures needed to conduct an experiment. It is also well suited to discover the nature of the metabolites and detemine the concentrations of the starting compound and the metabolites. In the following sections, the principal mass spectrometry techniques to carry out these assays are described and examples given.

7. Mass Spectrometry as an Analytical Platform

As is well appreciated by the pharmaceutical industry, the gold standard for the analysis of bioactive agents, either synthetic or derived from plants, both in the pharmaceutical product and in body fluids and tissues, is the use of highly specific and quantitative, hyphenated chromatography–mass spectrometry techniques.[92] It is essential to correctly identify the starting compound(s) and every metabolite derived from them−analyses based on LC methods followed by UV absorption, fluorescence, or electrochemical detection are not adequate. Although gas chromatography–mass spectrometry analysis of polyphenols was the first hyphenated chromatography–mass spectrometry technique to be implemented, the introduction of suitable ionization interfaces has led to the majority of analysis of polyphenols in 2007 being carried out using LC−mass spectrometry.

Mass spectrometry is an analytical technique capable of producing and separating ions according to their mass-to-charge ratio (*m*/*z*). In mass spectrometers, electric or magnetic fields are generated inside the instrument to separate ions. Therefore, a mass spectrum of a compound consists of a plot of ion abundance versus its *m*/*z* ratio. A molecular ion is usually $[M + H]^+$ or $[M - H]^-$ thereby knowing its *m*/*z*, the molecular weight of the compound can be deduced. Over the past two decades, mass spectrometry has been one of the most effective and sensitive techniques with a wide range of applications in biomedical research. Its high sensitivity and easy hyphenation with liquid chromatography (LC−MS) has enabled it to become the method of choice.

There are various types of ionization sources that can be used as the interface between LC and mass spectrometer. Electrospray ionization (ESI−MS) and atmospheric pressure chemical ionization (APCI−MS) are now the most common ionization sources for small molecule metabolites such as bioflavonoids, offering excellent mass range and sensitivity.[14,92,93] Owing to the soft ionization of ESI and APCI, intact conjugated metabolites can be detected as protonated or deprotonated molecules together with diagnostic fragment ions. Recently, a new ionization method for LC/MS, atmospheric pressure photoionization (APPI), has been introduced which is based on charge transfer to the analytes from dopant molecules (e.g., toluene) that have been ionized using 10 eV photons produced by a vacuum-ultraviolet lamp. APPI has become an important ionization source because it generates ions directly from solutes with

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relatively low background and is capable of analyzing relatively nonpolar compounds.[94]

Matrix-assisted laser desorption ionization (MALDI) is another soft ionization technique, first introduced by Karas et al. for analysis of nonvolatile compounds.[95] Although MALDI-TOF-MS is well-known as powerful tool for analysis of a wide range of biomolecules, such as peptides and proteins, its potential in food analysis has only been explored recently.[96]

7.1. Sample Preparation. When studying polyphenols in *in vivo* models, typically blood and urine specimens are collected. However, particular physiological samples may be pertinent to certain projects. For instance, to investigate the enterohepatic circulation, bile and intestinal fluids may be collected. In a study of soy isoflavones in men at risk for prostate cancer, Hedlund et al.[97] examined expressed prostatic fluid. For those interested in the body distribution of polyphenols, solid tissues (e.g., adrenals, brain, kidney, liver, mammary gland, ovary, testis, and uterus) are examined. It is worth noting that in such experiments the blood contamination of the tissue must be avoided. This can be accomplished in rodent models by flushing 50–100 mL ofice-cold isotonic saline through the heart and the rest of the body prior to the collection of a tissue.

Sample preparation is a crucial step both for qualitative and quantitative analyses. In complex biological matrices, salt, small organic and inorganic components, and other protein/nonprotein macromolecules contribute to the overall matrix effect that interferes with quantitative analysis of target compound(s). The performance of the analytical process heavily depends on the sample preparation method and the quality of the preparation. It is not unusual to spend more time preparing samples for analysis than in analyzing them. Therefore, sample preparation is a bottleneck in bioanalytical science.

Since most of the flavonoid metabolites exist as phase II metabolites such as glucuronides and sulfonate conjugates in biological fluids, the first step of their analysis is to hydrolyze them 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 at 37 °C for few hours to overnight with shaking.[68,84] In order to assess the efficiency of hydrolysis, 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 flavonoids conjugates have been prepared and are available.[98–100] The hydrolyzed sample is cleaned up and extracted using liquid–liquid extraction (LLE) or solid-phase extraction (SPE) [[101]]. SPE is relatively simple, easy to automate, and widely used to cleanup samples by removing interferences that would otherwise compromise analysis. It generates extracts that are sometimes with less recovery than those obtained by LLE.

Solid-phase extraction using C_8 or C_{18}-E SPE cartridges has been used for sample preparation by several investigators.[101,102] In this method, the cartridge was conditioned with methanol (1 mL) followed by 5% methanol (1 mL) before extraction of the samples. After loading the sample, the cartridge was then washed with 5% methanol (800 μL) before the aglycons were eluted in 1:1 ethyl acetate/acetonitrile (400 μL). In the case of liquid–liquid extraction, common solvents used for extraction of flavonoid aglycons are ethyl acetate, diethyl ether, and methylene chloride. Solvent extraction is preferred for extraction of less-polar flavonoids that are soluble in water-immiscible organic solvents and where the extraction fraction is large (>80%).

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.[99,100] However, the availability of

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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.[103] 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.[104] Dihydroflavone was used as the IS to study the pharmacokinetics of daidzein and genistein in serum samples of premenopausal women.[99] The benzophenones, 2,4,4′-trihydroxybenzophenone and 4-hydroxybenzophenone, have been used as IS to analyze isoflavones in urine and plasma, respectively,[105,106] and curcumin in plasma.[107]

Protein precipitation is an attractive, generic sample pretreatment strategy that is high-throughput. It cannot be considered as a real sample preparation technique because it only removes plasma proteins. Various protein precipitation techniques are available, including organic solvent, acid, salts, and metal ions. 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 methanol without further extraction.[65] This approach is particularly helpful if it is intended to measure both the conjugated and unconjugated forms of the flavonoids. Romanova et al. 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.[108]

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 rats consuming soy can be analyzed directly by LC−MS.[109] The only workup needed is centrifugation or filtration of the urine to remove particles that would otherwise clog up the HPLC column. Direct injection of urine samples or diluted urine samples has been reported for LC−MS/MS analysis.[109,110] The real advantage of direct injection is that it is rapid and no metabolites are lost during the sample preparation. Figure 3 shows the MRM chromatograms of puerarin in urine samples after SPE and using "dilute-and-shoot" method. Compared to SPE, these results indicate no significant interference of urinary matrix components when samples are extensively diluted. However, the high-salt content in urine can cause ion suppression and adduct formation in the electrospray process and can also cause a rapid deterioration of instrument performance due to contamination by non-volatile residues.[111] In this type of analysis, separation is carried out using gradient elution with acetonitrile or methanol after first trapping the bioflavonoid metabolites in a low concentration of the organic solvent. This elutes the electrolyte and other hydrophilic components of urine that would interfere with detection of the flavonoids before the gradient is begun.

For metabolite profiling, especially urinary metabolites, analysis without enzymatic hydrolysis is preferred. For example, Mullen et al. identified 23 quercetin metabolites when unhydrolyzed urinary extract was analyzed by LC−MS/MS.[112] A similar approach "dilute and shoot" can be applied for bile samples. The concentration of flavonoids is typically so high that the bile has to be diluted with the starting HPLC solvent.[85] Again, preanalysis filtration or centrifugation is used to remove any particulate matter. In contrast, 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. Moreover, direct injection of protein-rich serum or plasma samples can cause poor chromatographic metabolite separation and a rapid increase of column backpressure, resulting in degradation of column performance.[113] An alternative approach is to use SPE to recover

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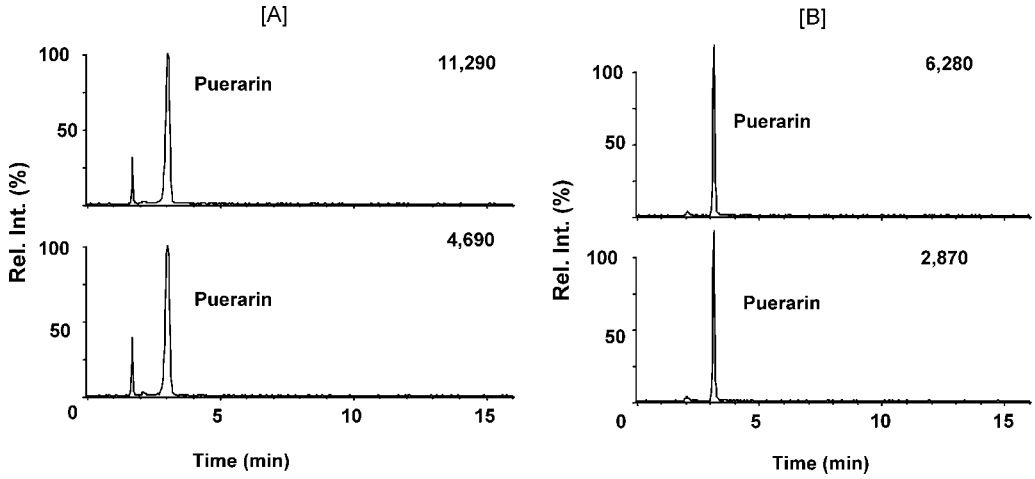

Figure 3. Comparison of MRM chromatograms of puerarin in a urine sample after SPE (A) and using a dilute-and-shoot method (B). The sample prepared by SPE (A) was 2-fold more concentrated than the sample prepared by the dilute-and-shoot method (B). The numbers on the top right corner of each chromatogram represent the fullscale value of the ion abundance.

the conjugated polyphenols from serum/plasma or urine. Because of the higher hydrophilic nature of the conjugates, their percentage recovered from the SPE method is low. To increase the hydrophobicity of the conjugates (and hence their absorption onto the SPE phase), a hydrophobic counterion (triethylammonium sulfate) should be added to the sample.[70]

7.2. MS Acquisition Strategies. The application of MS for ADME of bioflavonoids has increased tremendously with the advent of API technology.[14,92,93] In particular, since LC−MS/MS is the single most important method for qualitative and quantitative analysis of metabolites in biological samples. Commonly used tandem mass spectrometers include triple quadrupoles (tandem mass spectrometry in space), three-dimensional ion traps, and quadrupole time-of-flight (Q-TOF) instruments. The ability of ion traps to perform multiple stage MS (MS^n) and the powerful implementation of data-dependent operation in the instrument control software are particularly advantageous in structural elucidation. The use of high resolution and accurate mass MS has further enabled the detection of large numbers of parent ions present in a single extract and can provide accurate information on the chemical composition and thus the putative identity of many unknown metabolites.

7.2.1. Full Scan (Q1 Scanning). Soft ionization techniques such as ESI allow profiling of parent ions of polyphenol metabolites (usually 200–1000 Da). The starting point for metabolite profiling is to perform a Q1 scan using both positive and negative ion modes. Care should be taken in adjusting orifice voltage especially for conjugated metabolites since glucuronides and sulfonates of bioflavonoids are cleaved in the orifice (in-source fragmentation) with high voltages. The molecular ions are accelerated in this region as they enter the mass spectrometer and collide with residual gas molecules in the interface.

The nonselective nature of LC−MS Q1 scanning has the advantage of ensuring that most ionizable metabolites will generate a mass spectrometric response. It is important to recognize whether the ions extracted from total ion current (TIC) of LC−MS ion chromatograms correspond to protonated, deprotonated, or adduct ions or to fragment ions. In the positive-ion mode, flavonoids bearing a carbonyl functional group usually produce ions with ammonium or metal ion adducts (e.g., sodium or potassium). Selected ion monitoring (SIM) by Q1 is widely used for a full scan over a narrow mass window. The advantage of SIM over full scan spectral acquisition is the increase in sensitivity. In a quadrupole mass spectrometer, this sensitivity enhancement is due to the increased collection time of selected ions. SIM is also helpful in reducing the complexity when there ions are clustered in a narrow range. For example, Figure 4 shows SIM chromatograms detecting only β-glucosides and acetyl-β-glucosides of isoflavones in a soy product.

An enhanced mass scan mode (EMS), which is the full-scan mode where all ions in the specified m/z range are trapped in Q3 prior to detection, is used as the survey scan in QTRAP instruments. While full scan analysis provides useful information on total profiling of metabolites with respect to control samples, detection of minor metabolites is compromised due to the low sensitivity of Q1 scans.

7.2.2. Precursor Ion and Constant Neutral Loss Scanning. The precursor ion scan and constant neutral-loss scan are commonly used to profile all metabolites that result in that particular product ion and spectrum of precursor ions that undergo a particular neutral loss, respectively. In precursor ion scan, Q3 is held to measure the occurrence of a particular fragment ion and Q1 is scanned; in a neutral loss scan, Q1 is scanned as in the precursor ion scan but this time Q3 is also scanned to produce a spectrum of precursor ions that undergo a particular neutral loss. Neutral loss scanning is widely used for detection of phase II conjugates such as glucuronides (loss of 176) and sulfates (loss of 80),[114] as well as for detection of glutathione (GSH) adducts (loss of 129).[115] The loss of a caffeic acid unit was also observed with the loss of 162 mass units in the triple

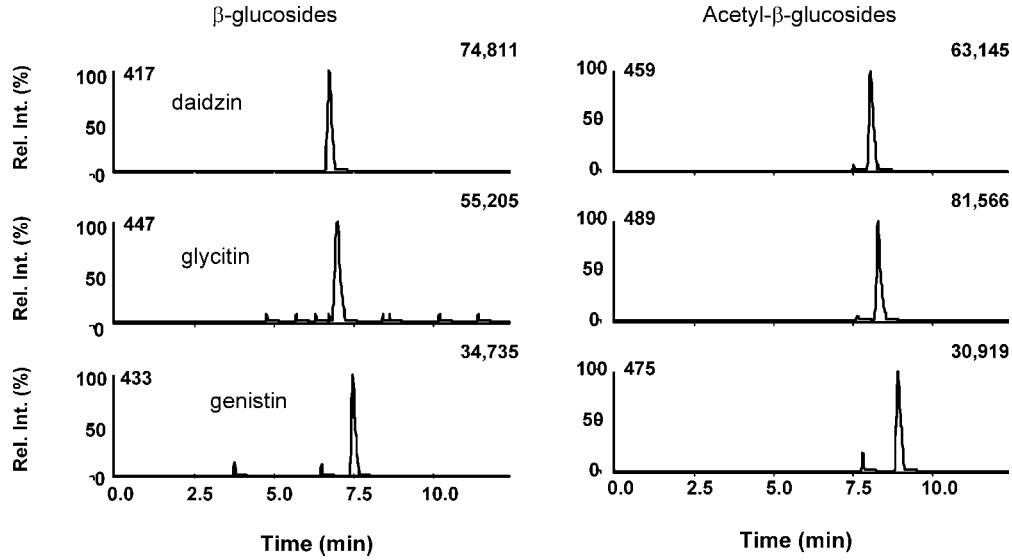

Figure 4. SIM chromatograms in positive ion mode corresponding to isoflavone glucosides daidzin (*m*/*z* 417), glycitin (*m*/*z* 447), genistin (*m*/*z* 433), and their acetyl analogues *m*/*z* 459, 489, and 475, respectively, in a soy product.

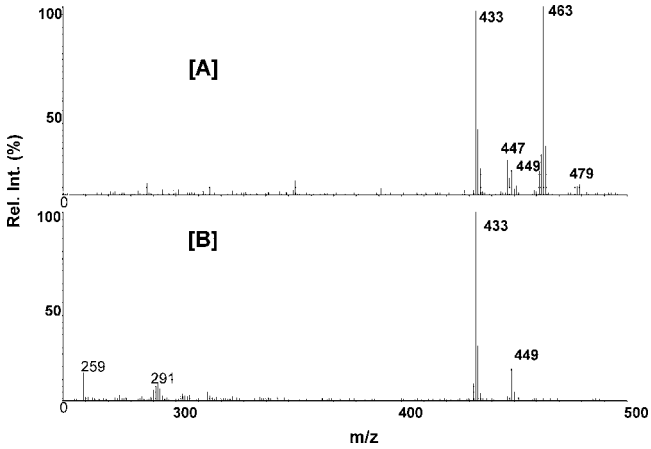

Figure 5. ESI−MS/MS neutral loss scan to identify flavonoid glycosides in a cranberry extract; spectra of flavonoids with neutral loss of 162 (A) corresponding to hexosides and 132 (B) to pentosides of flavonoid.

quadrupole mass spectrometer.[116] We used neutral loss of 162 and 132 Da to profile flavonoid glycosides in the cranberry juice concentrate (Figure 5). The results showed that the ions *m*/*z* 433, 447, 449, 463, and 479 were flavonoid bearing hexoside sugar, and *m*/*z* 433 and 449 corresponded to flavonoid pentosides. This method provides a fast method for the "dereplication" of natural products.

Similarly, the neutral loss of 56 Da (2 × CO), which was a common feature of all isoflavones in API(+), can be used to identify isoflavone structures.[117] Precursor-ion analysis specifically can be used to detect the molecular cations of each category of anthocyanins by scanning the precursors of anthocyanidins (cyanidin, peonidin, and pelargonidin).[118] Furthermore, since metabolites usually share a common structural feature with the parent compound, product ions produced by the fragmentation of a metabolite ion bear resemblance with those of the parent compound. Thus, a precursor ion scan can be used to search for metabolites structurally related to the parent compound. These scan types are possible in the classical triple quadrupole instrument. However, the sensitivity of triple quadrupole instruments may not always suffice when operated in full-scan mode, and for this reason, the use of ion trap mass spectrometers has increased. Although no definitive structure assignment can be carried out in the absence of standards, neutral loss and precursor ion scans help in identifying conjugated derivatives of parent flavonoids.

Ion-trap mass spectrometers provide high full-scan sensitivity, and in addition, their MS^n (multistage mass spectral scans) capability is highly efficient in the structural analysis of metabolites. One limitation of conventional ion trap MS,

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(118) Tian, Q.; Giusti, M. M.; Stoner, G. D.; Schwartz, S. J. Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring. *J. Chromatogr. A* **2005**, *1091*, 72–82.

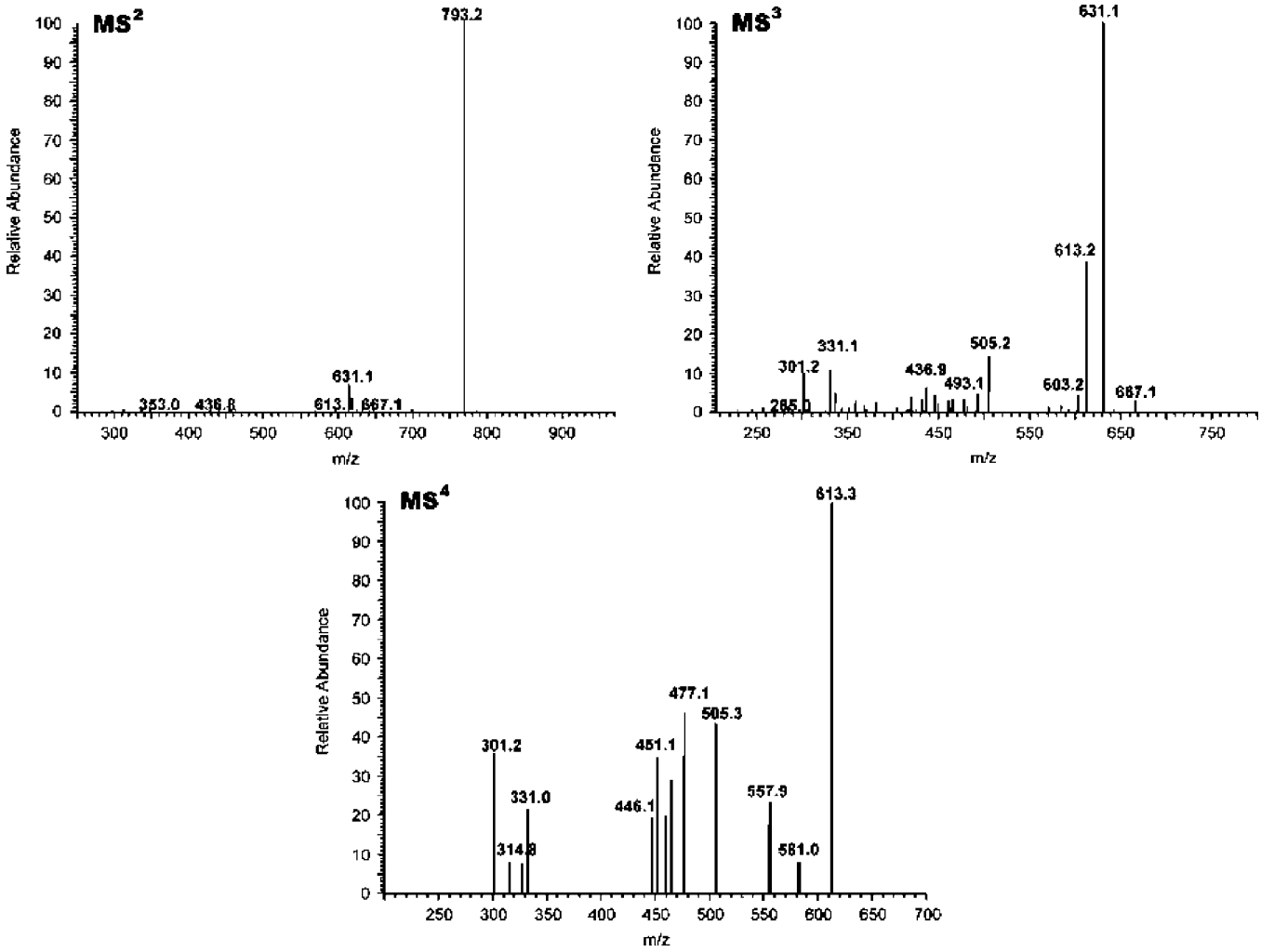

Figure 6. MS^2, MS^3, and MS^4 spectra of the molecular ion at *m*/*z* 955, composed of peonidin, malvidin, and two glucoses. MS^2 and MS^3 spectra were obtained in HPLC-DAD-ESI-MS^n analyses and the MS^4 spectrum in ESI-MS^n direct infusion analyses, which might explain the existence of fragment ions in the latter, which do not seem to belong to the fragmentation of ion at *m*/*z* 631. (Reprinted with permission from *J. Mass Spectrom.* Vol. 42. 2007 John Wiley & Sons, Ltd.)

however, is that neutral loss scan and precursor ion scan analyses cannot be performed.

In an attempt to hybridize quadrupole and ion trap capabilities, triple quadrupole-based linear ion traps, namely Q-TRAP, have been developed and proved to be extremely powerful for metabolite identification due to faster duty cycles and the availability of information-dependent acquisition (IDA) software.[119] This avoids the need for separate injections for acquisition of product ion spectra. Thus, the maximum dimension of data is collected with minimum number of sample injections. A detailed description of this topic is beyond the scope of this discussion and has been reviewed elsewhere.[120]

7.2.3. Product Ion (MS^2) and (MS^n) Scanning. The product ion spectrum of a compound provides a characteristic molecular fingerprint which can be obtained using product ion scans on a tandem mass spectrometer or occasionally in-source fragmentation. In metabolite identification, LC−MS/MS analysis is first performed to obtain a product ion spectrum of the parent compound as a reference and compare it to those of unknown metabolites. The structures of product ions are proposed to deduce partial structure of unknown metabolites. Product ion scan functions are available on various types of mass analyzers including triple quadrupole, ion trap, quadrupole ion trap, quadrupole time-of-flight (TOF), and FT-ICR MS, as well as magnetic sector instruments. A typical product ion scan experiment on a triple quadrupole mass spectrometer where Q1 is used to select the parent ion of interest to be fragmented in the collision

(119) Xia, Y. Q.; Miller, J. D.; Bakhtiar, R.; Franklin, R. B.; Liu, D. Q. Use of a quadrupole linear ion trap mass spectrometer in metabolite identification and bioanalysis. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1137–1145.

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cell (frequently referred to as Q2) and Q3 is set to mass-analyze the product ions from low mass to high mass. There is growing interest in constructing MS/MS phytochemical libraries that would enable rapid identification of various phytochemicals from biological samples. In this effort, Lee et al. constructed MS/MS spectra libraries of flavonoids using the results of LC−ESI−MS/MS with data-dependent acquisition on an ion-trap mass spectrometer.[121]

Continuous generation of product ion spectra from the fragment ion produced by the previous stage leads to MS^n data. This multistage MS^n analysis not only provides a convenient way for assigning fragmentation mechanisms but also proves to be a very useful means for structural elucidation of metabolites. Recently Alcalde-Eon et al. reported the use of MS^n fragmentation approach to identify the structures of dimeric anthocyanins.[122] As can be seen in Figure 6, the MS^2 yielded dimeric aglycone (loss of 2 × 162 amu) as the major ion. Other fragments in the MS^3 spectra were those originating from the losses of 180 (glucose residue and a water molecule) and 288 (glucose moiety and a phloroglucinol molecule).

Feng et al. used ion-trap MS to analyze the metabolites of baicalin in rats.[123] Detailed investigations on the identification of flavonoid metabolites after the consumption of onions were published by Mullen et al.[124] The analysis was performed by LC−ion trap mass spectrometry and 23 metabolites of quercetin were identified. As mentioned earlier, methylation of the aglycon and the formation of mono- and diglucuronides and sulfate conjugates are the major metabolic product of flavonoids. An increase in mass by 14, 16, 176, and 80 indicates the addition of methyl, hydroxyl, glucuronide, and sulfate group in the parent molecule (Table 2).

The choice of ionization source is also crucial in the identification of phase II metabolites. ESI has been reported to be the most suitable ionization method. Glucuronide and sulfate conjugates are ionized most efficiently with ESI. MS/MS experiments showed that the site of glucuronidation or sulfation could not be determined, since the primary cleavage was a loss of the conjugate group (glucuronic acid or SO_3),

Table 2. Mass Shift with Possible Metabolic Reactions and Their Mass Spectrometric Detection

metabolic reaction		neutral loss scan (amu)
glucuronidation	+176	176
hexose sugar	+162	162
pentose sugar	+132	132
sulfation	+80	80
glutathione	+129	129
methylation	+14	
hydroxylation	+16	
acetylation	+42	60
carboxylation	+44	44
decarboxylation	−44	44
demethylation	−14	

and no site-specific product ions were formed.[125] However, it may be possible to determine the site of methylation, since methylated products are more stable than glucuronides or sulfates. Cren-Olive demonstrated that differentiation of four isomeric *O*-monomethylated catechins (on phenolic positions) by the analysis of the fragmentation behavior of catechin.[126] Differences observed in the relative intensities of the major peaks detected in the spectra of methylated catechin isomers can be used for the identification of the site of methylation.

7.2.4. Multiple-Reaction Monitoring (MRM). One of the major advantages of LC−MS/MS is its high selectivity and sensitivity that allow the quantitative analysis of metabolites at very low concentrations in complex biological matrices. MRM is widely used for detection and quantification of metabolites in biological samples. It delivers a unique product ion that can be monitored and quantified in the midst of a very complicated matrix. Figure 7 shows that epigallocatechin gallate (EGCG) can be quantified with lower limit of quantification (5 nM) in a serum sample using MRM. MRM experiment is accomplished by specifying precursor ion/product ion mass transition of a compound. Knowing the mass and structure of the target analytes, it is possible to predict theoretical MRM transitions (the precursor *m*/*z* and a fragment *m*/*z*) for many unknown metabolites of flavonoids. Due to the high specificity of an MRM scan, very little chemical background is observed, allowing the detection of very low levels of the analyte. It also allows quantification of analytes over a wide dynamic range. Table 3 lists the MRM transitions of used for quantification of common

(121) Lee, J. S.; Kim, D. H.; Liu, K. H.; OH, T. K.; Lee, C. H. Identification of flavonoids using liquid chromatography with electrospray ionization and ion trap tandem mass spectrometry with an MS/MS library. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3539–3548.

(122) Alcalde-Eon, C.; Escribano-Bailón, M. T.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Identification of dimeric anthocyanins and new oligomeric pigments in red wine by means of HPLC-DAD-ESI/MSn. *J. Mass Spectrom.* **2007**, *42*, 735–48.

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(126) Cren-Olivé, C.; Déprez, S.; Lebrun, S.; Coddeville, B.; Rolando, C. Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14* (23), 2312–9.

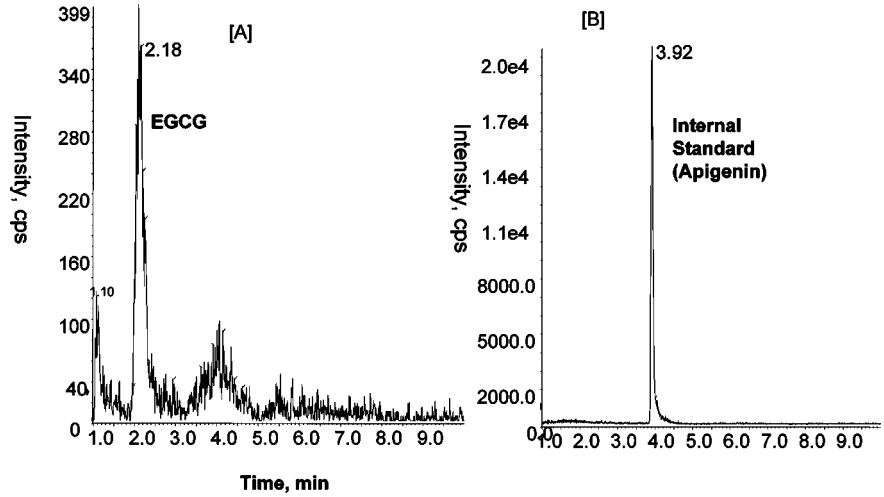

Figure 7. LC-MS-MRM chromatograms of rat serum spiked with (A) EGCG (5 nM) and (B) the internal standard apigenin (1μM). The mass transitions were *m*/*z* 457/169 and *m*/*z* 269/117 for EGCG and apigenin, respectively.

Table 3. MRM Transitions Used in Analysis of Flavonoids and Their Metabolites

compd	Q1	Q3	LLQ/LOD[a]	ref
genistein	269	133	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
daidzein	253	223	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
puerarin	415	267	2 ng/mL (LLQ)	65
genistein sulfates	349	269	1.51 ng/mL (LOD)	147, 98
			5.03 ng/mL (LLQ)	
genistein glucuronide	445	269	1.85 ng/mL (LOD)	127, 98
			6.17 ng/mL (LOD)	
daidzein sulfates	333	253		98
daidzein glucuronide	429	253		98
dihydrodaidzein	255	149	12.8 ng/mL	147
equol	241	121	100 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
O-DMA	257	109	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
glycitein	283	268	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
enterolactone	297	253	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
enterodiol	301	253	10 pg/mL (LOD)	100
			0.1 ng/mL (LLQ)	
quercetin	301	151	1.3 pg in column (LLQ)	146
isorhamnetin	315	151	0.4 pg in column (LLQ)	146
kaempferol	285	187	1.3 pg in column (LLQ)	146
epicatechin	289	245	13.3 ng/mL (LLQ)	151
			4 ng/mL (LOD)	
EGCG	457	169	5 ng/mL (LLQ)	149
naringenin	273	153	5 ng/mL (LLQ)	129, 150
naringin	581	273	5 ng/mL (LLQ)	150
hesperidin	611	303	5 ng/mL (LLQ)	150

[a] LLQ = lower limit of detection, LOD = limit of detection.

flavonoids and their metabolites. It appears that using MRM the lower limit of quantification of flavonoids, i.e., the concentration level where reproducible results are obtained, may be as low as 1–2 pg injected on column. The use of LC−MS/MS for quantification of flavonoids has been demonstrated in numerous studies.[70,127–130] A reliable LC−MS/MS method operating in MRM mode was developed in our laboratory that allows for the characterization and simultaneous quantification of 10 isoflavones metabolites and chrysin (internal standard) with mass transitions *m*/*z* 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108 (*O*-desmethylangolesin), 269/133 (genistein), 283/184 (glycitein), 267/191 (formononetin), 272/65 (6-hydroxy-*O*-desmethylangolesin), 289/109 (biochanin A), 253/143 (chrysin), and 267/91 (coumestrol) (Figure 8). This method permits a lower limit of quantification of these isoflavones (LLQ, 5 nM, S/N = 10).

8. Recent Sample Introduction Techniques

A number of sample introduction strategies have been developed to offer higher sensitivity, resolution, ability to work with minute sample sizes and shorter run time, and better compatibility with MS. Here are some strategies currently being used.

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(128) Wang, L.; Morris, M. E. Liquid chromatography-tandem mass spectroscopy assay for quercetin and conjugated quercetin metabolites in human plasma and urine. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2005**, *821*, 194–201.

(129) Ma, Y.; Li, P.; Chen, D.; Fang, T.; Li, H.; Su, W. LC/MS/MS quantitation assay for pharmacokinetics of naringenin and double peaks phenomenon in rat plasma. *Int. J. Pharm.* **2006**, *307*, 292–299.

(130) Lee, J. I.; Narayan, M.; Barrett, J. S. Analysis and comparison of active constituents in commercial standardized silymarin extracts by liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2007**, *845*, 95–103.

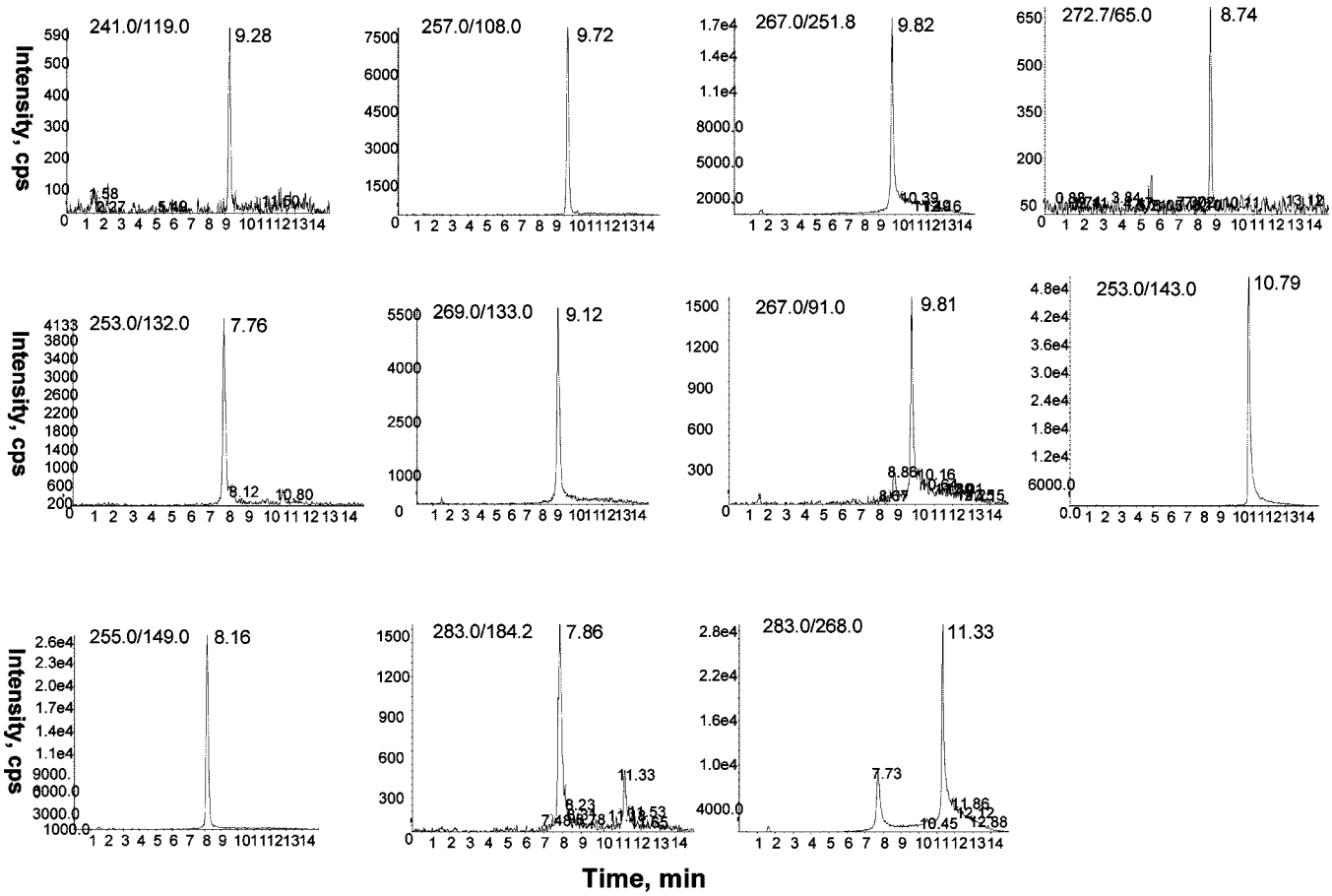

Figure 8. MRM chromatograms of phytoestrogens with mass transitions *m*/*z* 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108 (*O*-desmethylangolensin), 269/133 (genistein), 283/184 (glycitein), 267/191 (formononetin), 272/65 (6-hydroxy-*O*-desmethylangolesin), 289/109 (biochanin A), 267/91 (coumestrol), and 253/143 (chrysin, internal standard).

8.1. Ultra-High-Performance Liquid Chromatography (UPLC). The use of smaller particles in packed-column LC is a well-known approach to shorten the diffusion path for a given analyte. As an approximation, the time required to achieve a given degree of resolution between two compounds decreases as the square of the particle diameter, assuming everything else is held constant.[131] UPLC shows shortened analysis time, sensitivity, and resolution compared to HPLC. The same separation on reversed-phase HPLC that takes over 20 min can be accomplished in under 3 min by reversed-phase UPLC (RP-UPLC).[132] Recent technology advances have made available reversed-phase chromatography media with sub-2 μm particle size along with liquid handling systems that can operate such columns at much higher pressures. Recently, Cooper et al. reported UPLC analysis of six major chocolate polyphenols using an Acquity UPLC BEH C_{18} column (1.7 μm, 50 $\times$ 2.1 mm) in 3 min (Figure

(131) Xu, R. N.; Fan, L.; Rieser, M. J.; El-Shourbagy, T. A. Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. *J. Pharm. Biomed. Anal.* **2007**, *44*, 342–355.

(132) Churchwell, M. I.; Twaddle, N. C.; Meeker, L. R.; Doerge, D. R. Improving LC-MS sensitivity through increases in chromatographic performance: comparisons of UPLC-ES/MS/MS to HPLC-ES/MS/MS. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2005**, *825*, 134–143.

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(135) Li, C.; Meng, X.; Winnik, B.; Lee, M. J.; Lu, H.; Sheng, S.; Buckley, B.; Yang, C. S. Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry. *Chem. Res. Toxicol.* **2001**, *14*, 702–707.

(136) Meng, X.; Sang, S.; Zhu, N.; Lu, H.; Sheng, S.; Lee, M. J.; Ho, C. T.; Yang, C. S. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem. Res. Toxicol.* **2002**, *15*, 1042–1050.

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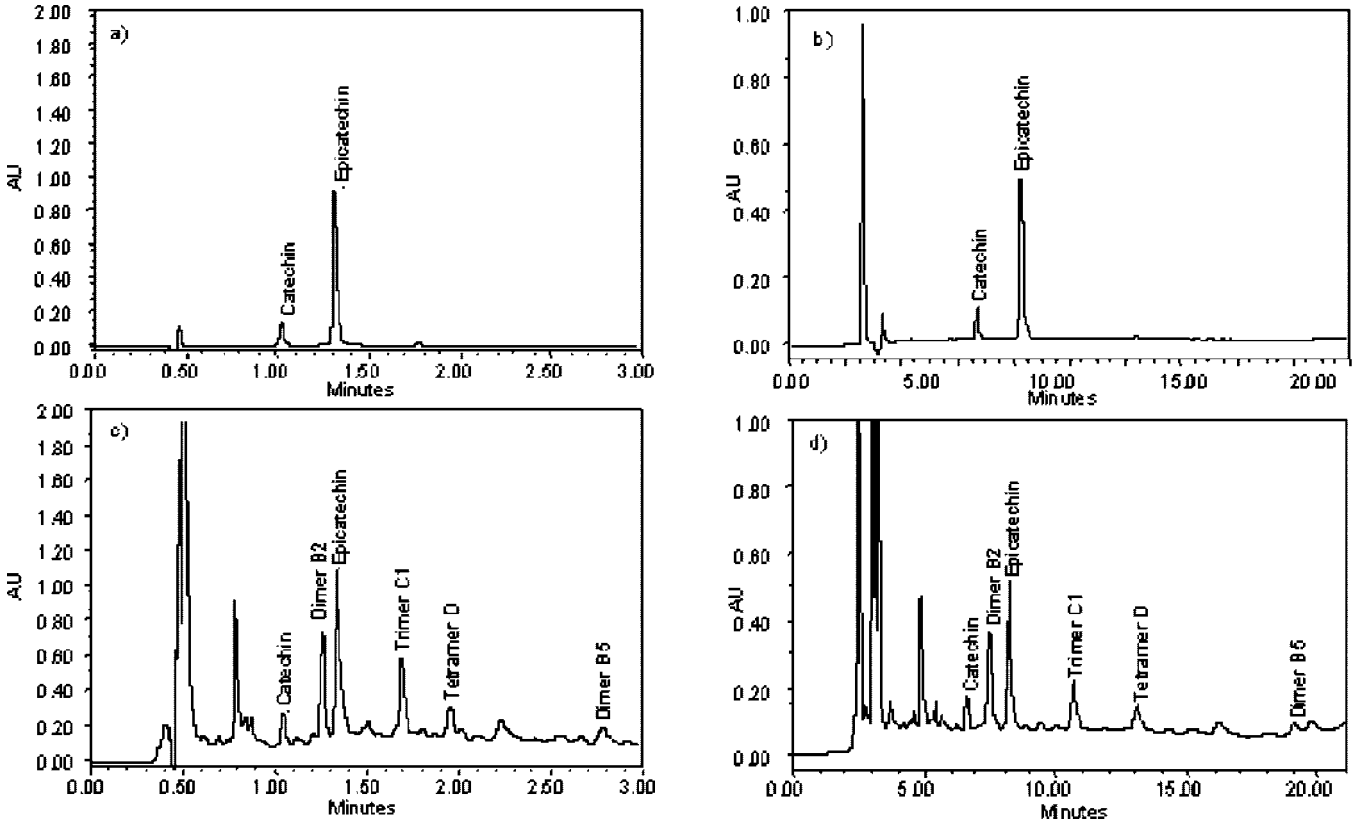

Figure 9. UPLC chromatograms (a, c) and HPLC chromatograms (b, d) of standard and SRM baking chocolate (the certified reference material), respectively. Reprinted from ref 133. Copyright 2007 American Chemical Society.

9).[133] It indicates that the same separation on reversed-phase HPLC that takes over 20 min can be accomplished in under 3 min by reversed-phase UPLC (RP-UPLC).

While UPLC offers significant theoretical advantages, when coupled with mass spectrometry it is important that the mass analyzer is capable of high-speed data acquisitions. ESI-TOF and ion trap instruments have an advantage over those with quadrupole analyzers. Also, 1.7 μm particles may be more sensitive than 2.5 μm and 3 μm particles to losses of efficiency associated with the effects of increased linear flow through the column.

8.2. Nano-Flow Approaches. Using much smaller column diameters (i.d., 50–300 μm) results in flow rates in the range from 100 nL/imin to 1–2 μL/min. As previously observed for peptides, this tremendously increases sensitivity. If a polyphenol MRM assay is performed in a 2.1 mm i.d. column, the limit of quantification (LOQ) is typically 50–100 fmol injected on column. If the assay is performed on a 75 μm i.d. column, then the increase in sensitivity is approximately the square of the ratio of diameter of the larger column divided by the diameter of the smaller column, i.e., $(2.1/0.075)^2 = 784$. Therefore, with this nanoLC arrangement, the LOQ will fall to 60–120 amol injected on column. Of course, there is a price with this—much longer run times due to dead volumes in the system. Nonetheless, with careful engineering, such as analysis on a chip, even this can be overcome.

9. Quantification and Validation

Quantification of biological active metabolites in physiological concentration is a real challenge. For this, sensitive and selective analytical methods for the quantitative analysis of metabolites is crucial. An LC−MS/MS method used in quantitative analysis should demonstrate that for certain analytes when analyzed in a given biological matrix, such as plasma, urine, or bile, it is reliable and reproducible. Following the development of an LC−MS/MS assay, it needs to be validated. The fundamental criteria of validation are precision, accuracy, linearity, reproducibility, selectivity, and stability of the method. With regard to selectivity, it refers the ability of an analytical method to differentiate and quantify the analyte in the environment of other endogenous matrix components. The relationship between instrument response and known concentrations of analytes represents

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calibration curves or standard curves. It is necessary to use a sufficient number of standards (five to eight, excluding blanks) to define adequately the relationship between concentration and response. The accuracy describes the closeness of mean calculated values obtained by the method to the known concentration (true concentration) of the analytes, while the precision of an analytical method is the closeness of individual measures of an analytes when analyzed in replicates. Other parameters such as stability, recovery, and lower limit of quantification and matrix effects need to be evaluated for any validated LC−MS/MS method.

10. Conclusion and Future Prospects

Identification and quantification of flavonoid and other polyphenols metabolites in physiological concentrations remain challenging tasks. While MS techniques based on ESI, APCI, and MALDI have greatly simplified this analysis, improvements in MS sensitivity and performance, for example, scan functions and scan speed, are critical for high-throughput analysis of biological samples. More improvements in current ionization techniques such as MALDI or ESI are expected in the near future as they still suffer from compound-dependent response and are prone to matrix effects, making quantitative analysis difficult. The use of MALDI-tandem time-of-flight (MALDI-TOF/TOF) instruments has revolutionized the visualization of the spatial distribution of small molecules in tissue levels. These techniques are expected to be used in assessing the health beneficial effects of flavonoids and other polyphenols.

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