CHAPTER 2

ISOLATION AND IDENTIFICATION OF FLAVONOIDS

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1. INTRODUCTION

Flavonoids and their conjugates form a very large group of natural products. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. The chemical structures of this class of compounds are based on a C_6-C_3-C_6 skeleton. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns (Figure 2.1). The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups (often prenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core. The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton. Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. The multiplicity of possible modifications of flavonoids result in more than 6,000 different compounds from this class were known in the end of the last century and this number continues to increase (Harborne and Williams, 2000). Condensed tannins create a special group of flavonoid compounds formed by polymeric compounds built of flavan-3-ol units, and their molecular weights often exceeding 1,000 Da.

In the plant kingdom, different plant families have characteristic patterns of flavonoids and their conjugates. All these compounds play important biochemical and physiological roles in the various cell types or organs (seed, root,

green part, fruit) where they accumulate. Different classes of flavonoids and their conjugates have numerous functions during the interactions of plant with the environment, both in biotic and abiotic stress conditions (Dixon and Paiva, 1995; Shirley, 1996). Additionally, flavonoid conjugates, because of their common presence in plants, are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Beck et al., 2003; Le March, 2002; Boue et al., 2003; Fritz et al., 2003; Nestel, 2003). For the mentioned reasons, methods for the efficient and reproducible analysis of flavonoids play a crucial role in research conducted in different fields of the biological and medical sciences.

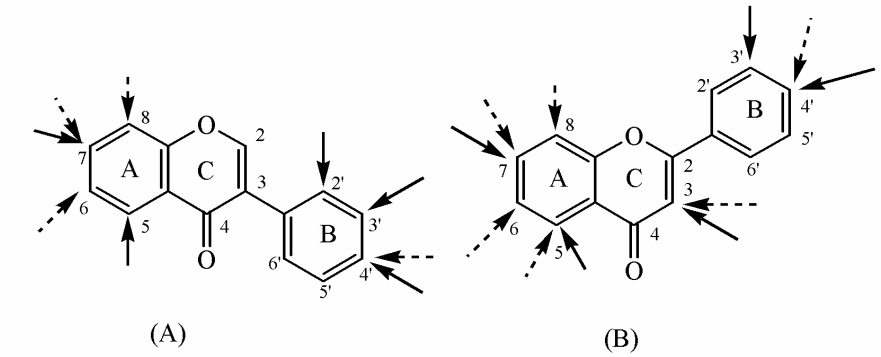

Figure 2.1 *Flavonoid structures, ring labeling, and carbon atom numbering. (A) Isoflavones. (B) Flavones and flavonols. Full arrows indicate most frequent hydroxylation sites and dashed arrows indicate most frequent C- and/or O- glycosylation sites.*

The identification and structural characterization of flavonoids and their conjugates isolated from plant material, as single compounds or as part of mixtures of structurally similar natural products, create some problems due to the presence of isomeric forms of flavonoid aglycones and their patterns of glycosylation. A number of analytical methods are used for the characterization of flavonoids. In many cases, nuclear magnetic resonance (NMR) analyses (^{1}H and ^{13}C) are necessary for the unambiguous identification of unknown compounds; other instrumental methods (mass spectrometry, UV and IR spectrophotometry) applied for the identification of organic compounds fail to provide the information necessary to answer all the structural questions. Utilization of standards during analyses and comparison of retention times as well as spectral properties, especially when compounds are present in a mixture, is critical. An important area of research on flavonoids is the identification of their metabolites in animal tissues and body fluids (urine, blood, spinal fluid). For this, investigators have to deal with different modifications of the flavonoid moieties, modifications often not found in plant tissues (Blaut et al., 2003). The metabolism of flavonoids in human and animal organisms, among others, is based on glucuronidation, sulfation, or methylation (Sfakianos et al., 1997;

Yasuda et al., 1994). The first two above-mentioned types of flavonoid aglycone modifications, occurring after their consumption by humans or animals, are less often found in the samples of plant origin.

One of the goals in functional genomics and systems biology studies is metabolite profiling. Qualitative and quantitative monitoring of flavonoid derivatives, together with information about the level of transcription and protein expression, enables the elucidation of gene functions (Fiehn, 2001; Hall et al., 2002; Sumner et al., 2003; Fernie et al., 2004). Another challenge in the field is to establish the flavonoid conjugate profiles in genetically modified plant lines, e.g. engineered for higher resistance against environmental conditions (pathogenic microorganisms, insects, and physical stress factors such as temperature, drought, or UV light). The plant performance in different environmental conditions and the resulting effect on crop yield may be accompanied by increased synthesis of desired and/or undesired natural products in particular plant tissues, among the biologically active flavonoids or isoflavonoids (Bovy et al., 2002; Dixon and Steel, 1999; Fisher et al., 2004; Trethewey, 2004; Verpoorte and Memelink, 2002).

2. ISOLATION OF FLAVONOIDS AND THEIR CONJUGATES FROM BIOLOGICAL MATERIALS

The analysis of flavonoids and their conjugates is one of the most important areas in the field of instrumental analytical methods, helping to solve problems in biological and medical sciences. Different methods of isolation of the natural products may be applied, and the utilization of various strategies is dependent on the origin of the biological material from which the target natural products are to be extracted (plant or animal tissue or body fluids). In the case of polyphenolic compounds, it often is important to initially determine whether the researchers are interested in the identification of individual components present in a mixture of target compounds or whether they would like to estimate the total amount of phenolic compounds in the biological material investigated. This second approach most often takes place during the nutritional studies on different foods or fodders, mainly of plant origin.

The presence of carbohydrates and/or lipophylic substances may influence the profile of the qualitative and quantitative composition of flavonoids and their derivatives in the obtained extracts. One has to consider the above-mentioned selection of the methods for sample preparation and extraction, and in many cases additional cleaning based on solid-phase extraction (SPE) of the extracted samples is required.

2.1. Preparation of Plant or Animal Tissue and Foodstuffs for Flavonoid Analysis

The utilization of dried plant material for extraction may cause a substantial decrease in the yield of flavonoid conjugates. Acylated flavonoid glycosides are especially labile at elevated temperatures and are frequently thermally degraded during the process of drying plant tissues. This is important during the profiling of

this class of natural products in research directed toward the investigation of their physiological and biochemical roles in plants under the influence of environmental factors, or in studies of genetically modified plants for the elucidation of changes in metabolic pathways.

Free flavonoid aglycones exuded by plant tissues (leaf or root) may be washed from the surface with nonpolar solvents, such as methylene chloride, ethyl ether, or ethyl acetate. However, more polar glycosidic conjugates dissolve in polar solvents (methanol and ethanol), and these organic solvents are applied for extraction procedures in Soxhlet apparatus. Mixtures of alcohol and water in different ratios are applied for the extraction of flavonoids and their conjugates from solid biological material (plant or animal tissues and different food products). The extraction efficiency may be enhanced by the application of ultrasonication (Rostagno et al., 2003; Herrera et al., 2004) or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60°C to 200°C (Rostagno et al., 2004). Supercritical fluid extraction with carbon dioxide also may be used (Kaiser et al., 2004). However, the temperature conditions during the extraction procedures have to be carefully adjusted because of the possibility of thermal degradation of the flavonoid derivatives. In many cases, further purification and/or preconcentration of the target compound fraction is necessary. In these cases, liquid–liquid extraction (LLE) or SPE are most commonly used. For estimation of the extraction yield it is necessary to spike biological materials with proper internal standards. Most suitable are compounds structurally similar to the studied analytes but not present in the sample. Compounds labeled with stable isotopes (^{2}H or ^{13}C) are useful when mass spectrometric detection is applied. In the case of the extraction of flavonoids from biological materials, different classes of phenolic compounds are often added. On the other hand, quantitative analysis of consecutive components of the analyzed flavonoid mixture needs reference standard compounds necessary for preparation of calibration curves essential for a precise quantification.

The choice of the extraction procedure for obtaining flavonoid conjugates from biological material is very important and depends on the goals of the conducted research. The evaluation of the spatial distribution of target compounds on the organ, tissue, cellular, or even subcellular level is of special interest in some projects. In these situations, the amount of biological material for the isolation of natural products may be extremely small, and the application of microextraction techniques is necessary (reviewed in Vas and Veckey, 2004). In many cases, it is necessary to avoid the chemical and/or enzymatic degradation of the metabolites. This is of special importance in the profiling of flavonoid glycosides in research directed toward plant functional genomics or during physiological and biochemical studies that need information about all classes of flavonoid conjugates present, even the thermally labile acylated derivatives. On the other hand, in the phytochemical analysis of plant species or phytopharmaceutical studies of plant material, the repeatable isolation of all biologically active flavonoid aglycones with a good yield is more important. In these cases, more drastic extraction conditions are acceptable. Excellent reviews have been published on isolation strategies for the determination of active phenols in plants tissue or food and foodstuff (Naczk and Shahidi, 2004; Robards, 2003).

Robust multistep chromatographic methods are necessary for the isolation of individual components from plant extracts containing new uncharacterized compounds. Various stationary phases are used in column chromatography, including polyamide, Sephadex LH-20, and different types of silica gels (normal and reversed phase with chemically bonded functional groups). The proper choice of solvent systems is necessary, often requiring the application of gradients of more polar (normal phases) or more hydrophobic solvents (reverse phases), together with the above-mentioned chromatographic supports in different chromatography systems. The sequence and kind of separation methods used depends on the composition of the sample and the experience of the researcher. However, minor flavonoid components are difficult to obtain as pure compounds. In cases of analysis of samples containing a number of compounds present in small amounts, the application of an analytical chromatographic systems enhanced by proper detectors (UV, NMR, and/or MS) gives spectrometric information sufficient for establishing the structure of minor target components. When liquid chromatography is used for separation of compounds, multiple detector systems are available (UV diode array detector, mass spectrometers, and nuclear magnetic resonance spectrometer). It is possible to achieve complete structural information about isomeric flavonoids and their conjugates in this way.

2.2. Preparation of Body Fluids

For the isolation of flavonoids and their derivatives from liquid samples like beverages (wine or fruit juice) and physiological fluids (blood or urine), two different approaches are usually applied. The first one is based on liquid–liquid extraction and the second one on solid-phase extraction of target natural products mainly on RP C-18 silica gel cartridges. In the case of body fluids, special procedures have to be considered to avoid degradation of target compounds due to the activity of different enzymes present (Martinez-Ortega et al., 2004). However, in some cases, flavonoid conjugates can be enzymatically hydrolyzed with external glucuronidases and sulfatases prior to the isolation and analysis of products.

3. STRUCTURAL CHARACTERIZATION AND/OR IDENTIFICATION OF FLAVONOID AND THEIR CONJUGATES

All physicochemical methods applied in the field of organic chemistry are useful for structural characterization or identification of individual flavonoids and their conjugates. The separation approaches mentioned above may be considered in different ways. The first one is directed toward the analysis of single compounds obtained after exhaustive isolation and purification procedures. The method of choice in this approach is NMR of ^{1}H hydrogen and/or ^{13}C carbon isotopes, dependent on the intensity of the interactions between different atoms within a molecule placed in a high-intensity magnetic field. Different NMR experiments have been developed to achieve information concerning chemical structure of the studied molecule on this basis. Particularly useful are methods enabling recording

of two-dimensional spectra showing homonuclear interactions [**co**rrelation **s**pectroscop**y** (COSY) and **n**uclear **O**verhauser **e**ffect **s**pectroscop**y** (NOESY)] as well as heteronuclear [**h**eteronuclear **s**ingle **q**uantum **c**orrelation (HSQC) and **h**eteronuclear **m**ultiple **b**ond **c**orrelation (HMBC)] to facilitate the acquisition of all the structural information about an aglycone and the corresponding sugar substitution. In the case of diglycosides, information on the placement of the interglycosidic bonds and the possible acyl group substitutions on the sugar rings, and the position of anomeric proton(s) also can be obtained. The limitation of NMR methods is the lower sensitivity in comparison with other instrumental methods. For obtaining good quality spectra containing all the necessary structural information, relatively high amounts of purified compound (more than 1 mg) are necessary, especially when magnets of medium frequency (300 MHz) are used in the NMR spectrometer. The NMR spectrometers may be connected on line to liquid chromatographs (LC-NMR), giving a powerful tool to study mixtures of natural compounds present in complex samples. Important structural data also can be obtained from mass spectra registered on different types of mass spectrometers (MS). The application of ultraviolet and infrared spectrophotometers may give valuable information about specific compounds.

MS applied for the analysis of organic compounds utilize different ionization methods and may be equipped with different types of analyzers. In addition, these instruments may be combined with GC/LC or capillary electrophoresis (CE) apparatus. However, simple chemistry based on single reactions such as silylation, methylation, and acetylation blocking polar functional groups has to be done on the studied samples prior to GC-MS analyses. Derivatization of polar groups improves structural information obtained from MS spectra and ameliorates the volatility of analytes, decreasing the thermal degradation of compounds within the GC capillary column. The variety of MS techniques being available in laboratories is a reason that this technique has a wide range of scientific or practical applications in biological and medical disciplines. The general information about the ionization methods and types of analyzers in MS used in phytochemical analysis is presented in Table 2.1.

Analysis of natural products is possible with different types of MS available on the market. The instruments are equipped with various sample introduction systems and ionization methods, as well as diverse physical phenomena are used for separation of the created ions in MS analyzers. Positive and negative ions are analyzed in MS; the choice of the ionization mode (negative or positive) is sometimes a very important feature. The ionization methods may be divided into two groups differing with respect to the amount of energy transferred to the molecule during the ionization process. Electron ionization (EI) belongs to the first group. The transfer of energy occurs during the interaction of electrons with the molecule in the vapor state; it may cause the cleavage of chemical bonds and fragmentation of the molecule, which is characteristic for the analyzed compound. Other ionization methods deliver lower energy to the studied molecules during the protonation (positive ion mode) or deprotonation (negative ion mode) processes. In both cases, the absorbed energy is too low to cause intense fragmentation. In this situation, techniques of collision-induced dissociation with tandem MS (CID MS/MS) have to be applied for the structural characterization of compounds.

Different designs of tandem analyzers in MS may be used (McLafferty, 1983; Jennings, 1996). An example of such instruments enabling multistage tandem MS (MS^n) are instruments with ion trap analyzers. In these analyzers, the fragment ions created with CID may be further studied using additional MS^n stages (March, 1997).

Table 2.1 Ionization methods and types of analyzers for phytochemical and clinical analysis of flavonoids and their conjugates using mass spectrometry

Sample introduction system	Ionization method	Analyzer type	Comments
Direct insertion or infusion of single compound	Electron ionization – EI Chemical ionization – CI Liquid secondary ion mass spectrometry – LSIMS[b] Fast atom bombardment – FAB[b] Atmospheric pressure chemical ionization – APCI[b] Electrospray ionization – ESI[b] Matrix assisted laser desorption – MALDI[b]	Electromagnetic – B/E or E/B[a] Quadrupole – Q Ion trap – IT Time of flight – ToF[a] Fourier transform ion cyclotron resonance – FT ICR[a]	When soft ionization methods are applied, MS/MS techniques Increase the amount of structural information.
Gas chromatography – GC[c]	Electron ionization – EI Chemical ionization – CI	Electromagnetic – B/E or E/B[a] Quadrupole – Q Ion trap – IT Time of flight – ToF[a]	Need of derivatization, only analysis of free aglycones is possible.
Liquid chromatography – LC or capillary electrophoresis – CE	Continuous flow fast atom bombardment – CF FAB[b] Atmospheric pressure chemical ionization – APCI[b] Electrospray ionization – ESI[b]	Quadrupole – Q Ion trap – IT Time of flight – ToF[a] Fourier transform ion cyclotron resonance – FT ICR[a]	Only soft ionization methods are applied, MS/MS techniques increase the amount of structural information

[a] High resolution analyzer, ions with mass difference of 0.1 Da may be resolved.
[b] Soft ionization method.
[c] Possible thermal degradation of analyte on GC column.

However, the fragmentation mechanisms are different during high- and low-energy collisions used in electromagnetic or quadrupole and ion trap analyzers. It has been shown that low-energy CID MS/MS spectra of *C*-glycosidic flavonoids differ from those obtained when high-energy measurements are performed. In tandem electromagnetic analyzers, high-energy collisions are performed, while other types of tandem MS require low-energy collisions. Mass spectra registered with these two approaches may give slightly different fragmentation pathways (Waridel et al., 2001). During the analysis of flavonoid glycosides in negative ion CID

MS/MS experiments, the cleavage of aglycone fragment ions and intensities of the product ions in registered mass spectra are dependent on the ions chosen for the fragmentation. March and coworkers (2004) registered CID MS/MS product ion mass spectra of the fragment ions at 268 m/z $(Y_0 - H)^{\bullet -}$ and at 269 m/z (Y_0^-), created after cleavage of the glycosidic bond of genistein 7-*O*-glucoside and demonstrated that both spectra differed substantially.

In the last few years some review articles have been published describing methods of MS analysis of flavonoids and their conjugates including information on fragmentation pathways of flavonoid aglycones and their glycosidic conjugates (Justesen et al., 1998; Stobiecki, 2000, 2004; Careri et al., 2002; Cuyckens and Claeys, 2004; Prasain et al., 2004).

3.1. Structural Characterization of Individual Flavonoids and Their Conjugates

3.1.1. Nuclear Magnetic Resonance

NMR is a well-established and the most commonly used method for natural product structure analysis. The studies of flavonoid structures using ^{1}H-NMR were initiated in 1960s (Markham and Mabry, 1975) and along with ^{13}C-NMR have became the method of choice for the structure elucidation of these compounds. The chemical shifts and multiplicity of signals corresponding to particular atoms and their coupling with other atoms within the molecule allow for easy identification of the aglycone structure, the pattern of glycosylation, and the identity of the sugar moieties present. The literature of this topic is abundant and rapidly growing (Markham and Geiger, 1994; Albach et al., 2003; Kazuma et al., 2003; Francis et al., 2004).

3.1.2. Mass Spectrometry

MS is a very sensitive analytical method used to identify flavonoid conjugates or to perform partial structural characterization using microgram amounts of sample (Cuyckens and Claeys, 2004). Indeed, significant structural data can be obtained from less than 1 mg of the analyzed compound when different MS techniques are used in combination with chemical derivatization of the characterized compounds (Franski et al., 1999, 2002, 2003). A strategy for the combined application of different MS techniques and chemical derivatization are presented in Figure 2.2.

Flavonoid glycosides are thermally labile compounds and the evaporation without decomposition of the analyte is impossible, even in the ion source of a MS, where high vacuum exists (about 3×10^{-5} torr). In this situation, soft ionization methods need to be applied for the analysis of this group of compounds, and the analyte molecules are ionized without evaporation in high vacuum (FAB or LSIMS, MALDI) or under atmospheric pressure (ESI, APCI). From normal mass spectra, information can be obtained about the molecular weight of the whole conjugate, the size of the sugar moieties attached to the aglycone, and the molecular weight of the aglycone (Stobiecki, 2000; Cuyckens and Claeys, 2004).

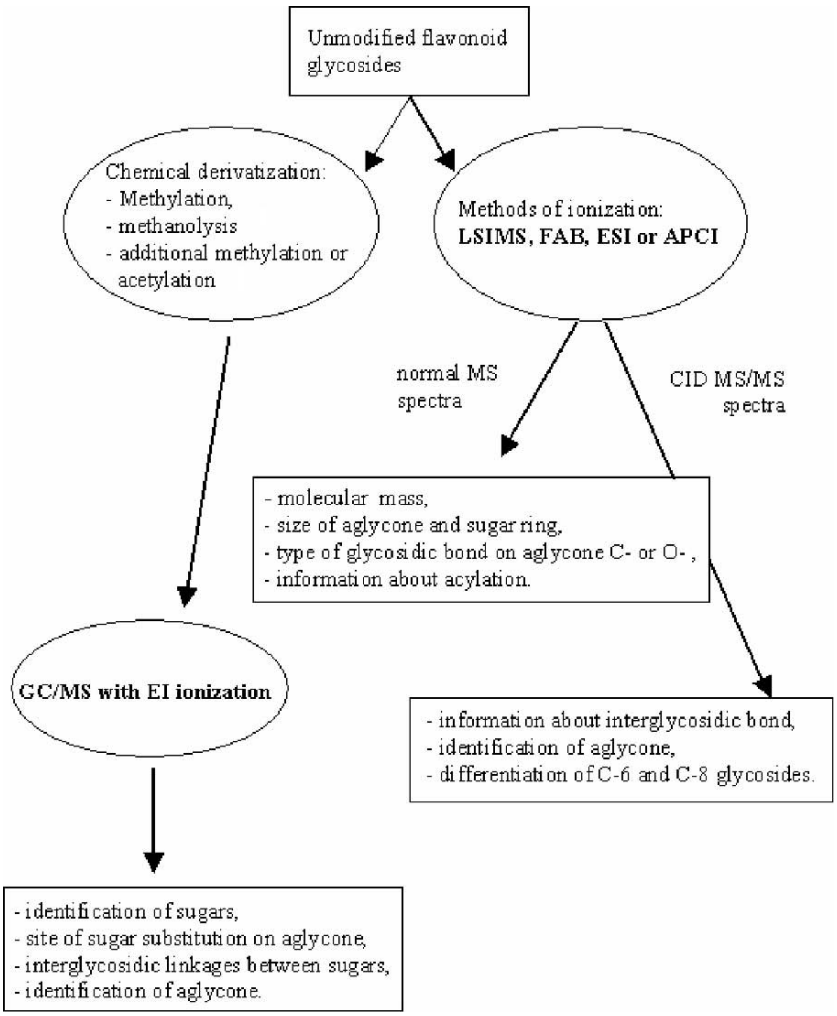

Figure 2.2 *Structural information obtainable with different mass spectrometric methods.*

With FAB or LSIMS ionization, the desorption of the analyte molecule ions from the liquid matrix may be improved when the interactions of the polar groups of the analyte with the matrix decrease. Improved efficiency of ion desorption may be further achieved after the methylation of the analyzed compounds. In addition, the methylation of a flavonoid glycoside may help to elucidate the glycosylation pattern of the aglycone hydroxyl groups (Stobiecki et al., 1988).

The *O*-glycosides of flavonoids give positive ion mass spectra containing intense $[M+H]^+$ ions as well as fragment ions created after the cleavage of glycosidic bonds

between sugar moieties or sugar and aglycone, in this case Y_n^+ type ions (for the ion nomenclature, see Domon and Costello, 1988; Claeys et al., 1996). A slightly different situation is observed in the negative ion mass spectra mode, where much lower fragmentation of the deprotonated molecule ions $[M-H]^-$ occurs. In the case of flavonoid *O*-glycosyl-glycosides, the rearrangement of sugars may take place during the fragmentation process, and the sequence of sugar losses does not correspond to the sequence of the sugar moieties in the intact molecule (Cuyckens et al., 2001, 2002). This situation was observed for the first time in flavonoid rutinosides α(1-6) and neohesperidosides α(1-2), in which rhamnose occurs on the nonreducing end of the diglucoside moiety and glucose is bound to the aglycone (Ma et al., 2001). A different fragmentation pathway may be observed in the flavonoid *C*-glycosides, in which rupture of the sugar ring takes place and strong $[M+H-120]^+$ ions are created. The nomenclature of the fragment ions in mass spectra of *C*-glycosides was introduced by Becchi and Fraisse (1989). The cleavage of the sugar moiety in all types of flavonoid *C*-glycosides is observed in both positive and negative mode mass spectra. The possible fragmentation patterns of flavonoid *O*- and *C*-glycosides are presented on Figure 2.3.

Structural information obtainable from the mass spectra may be achieved when tandem mass spectrometric techniques are applied. It is possible to increase the degree of fragmentation by applying CID MS/MS technologies; however, in LC/MS instruments with atmospheric pressure ionization (APCI or ESI) the increase of potential between the entry slit to the analyzer and the skimmer also promotes the fragmentation of the molecule ions, especially in the positive ion mode. The analysis of metastable ions is also possible in MS with electromagnetic analyzers equipped with collision cells in the field-free regions (McLafferty, 1983; Stobiecki et al., 1988; Becchi and Fraisse, 1989). When a full CID MS/MS system is used, the identification of the isomeric aglycones, for example kaempferol and luteolin or apigenin and genistein, is also possible (Ma et al., 1997). An important advantage of some CID MS/MS systems is the possibility of discrimination between *C*-6 and *C*-8 flavonoid glycosides. There are several reports showing that the differentiation of both isomers is possible on the basis of the relative intensities of the fragment ions obtained after CID fragmentation of the $[M+H]^+$ ions (Becchi and Fraisse, 1989; Li et al., 1992; Waridel et al., 2001; Bylka et al., 2002; Cuyckens and Claeys, 2004). The analysis of the CID mass spectra of flavonoid *O*-diglucosides permits one to distinguish between α(1-2)- and α(1-6)-linked sugars in the investigated molecules (Cuyckens et al., 2000; Ma et al., 2001; Franski et al., 2002).

Mass spectrometers with laser desorption ionization combined with the time of flight analyzer (MALDI ToF) also were used for the analysis of isoflavone glycosides in soy products (Wang and Sporns, 2000a), and $[M+H]^+$ and Y_n^+ type ions were observed in the mass spectra. The same authors tried to apply this MS technique for the analysis of flavonol glycosides in food (Wang and Sporns, 2000b).

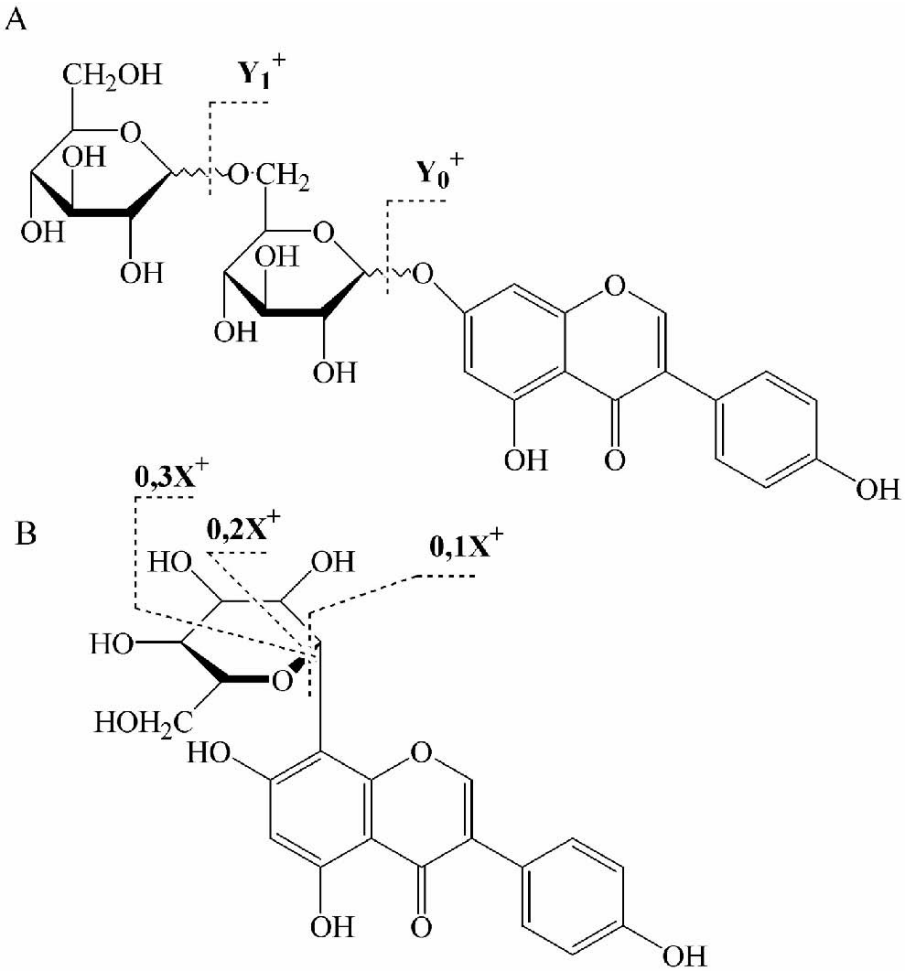

Figure 2.3 *Fragmentation pattern and daughter ion nomenclature of different glycosides of isoflavonoids. (A) Genistein-7-O-glucosyl-glucoside. (B) Genistein-8-C-glucoside.*

Studies on the application of the MALDI ToF technique for the quantitative analysis of flavonoid glycosides also were performed in the same laboratory; in this case the proper choice of the matrix and the deposition of samples in target wells was very important (Frison and Sporns, 2002). MALDI ToF also has been used for the analysis of condensed tannins isolated from plant material. In the first paper on this subject (Ohnishi-Kameyama et al., 1997), the MALDI and FAB ionization for the identification of flavan-3-ol oligomers in apples was compared. The presence of up to pentadecamer polymers composed of catechins was reported. However, only tetramers were identified in the same plant source when FAB ionization was used in earlier experiments (Self et al., 1986). This comparison of

results obtainable with both ionization methods shows the advantages of the MALDI technique in studies of polymers and other compounds of high molecular weight. The efficiency of the ion desorption from the ion source depends on the compound used for the matrix cocrystalized with the analyte. Different compounds, mainly organic acids such as trans-3-indoleacrylic acid or dihydroxybenzoic acid, are used. However, the signal-to-noise ratio may vary substantially depending on the matrix utilized. Generally, MALDI instruments are used for analysis of compounds with molecular weights above 500 Da. MALDI ToF MS studies of polygalloyl polyflavan-3-ols in grape seeds and wine were performed by several groups (Wang and Sporns, 1999; Yang and Chien, 2000; Krueger et al., 2000; Perret et al., 2003). Reed and coworkers (2003) identified procyanidines in sorghum. The isolation of target compounds from plant extracts with different solvents permitted one to distinguish two groups of compounds: oligomers of flavan-3-ols and a series of their 5-*O*-glucosylated derivatives. It was demonstrated that the fragmentation obtainable in ToF analyzers due to the postsource decay (PSD) technique permits the achievement of fragment-protonated molecules of flavan-3-ol trimers and tetramers together with sequence information (Behrens et al., 2003).

3.2 Identification of Flavonoid Conjugates in Mixtures

During the past few decades, GC has been utilized for the separation of free flavonoids present in mixtures. This method offers a good efficiency of separation, but the necessity of derivatization of the compounds prior to the analysis decreases its applicability. Moreover, the derivatization procedures (e.g., methylation and trimethylsilylation) have their own limitations. When GC-MS is used, the mass spectra of silylated compounds are dominated by fragmentation products derived from the elimination of silyl groups; thus, less structural information on the desired compound is obtained and the utilization of standards becomes necessary. On the other hand, some rearrangements may take place during the methylation of flavonoid –OH groups, and additional compounds become present in the reaction mixture after the derivatization. This is especially the case for flavanones, which rearrange to chalcones. However, mass spectra of methylated flavonols, flavones, or isoflavones give good structural information about the corresponding isomers. The comparison of mass spectra of silylated and methylated isoflavones from extracts obtained from lupine roots was previously demonstrated (Stobiecki and Wojtaszek, 1990). It is possible to obtain additional information when CD_3I is used for the methylation of flavonoids, where the placement of native methyl group on the aglycone can be established (Bednarek et al., 2001). The chromatographic separation of the trimethylsilyl derivatives of components of a given mixture was better than that of the methylated compounds, but the mass spectra of methylated isoflavones provided more structural information.

The two other chromatographic techniques, LC and CE, mentioned before, allow one to work without the chemical derivatization of analytes and permit the simultaneous separation of flavonoid aglycones and glycosidic conjugates. The combination of LC or CE with MS permits the use of different soft ionization

methods. The most important are atmospheric pressure ionization, ESI and APCI, used in negative or positive ion mode. Other ionization methods, such as thermospray (TSP) and continuous flow fast atom bombardment (CF FAB), are no longer in common use due to their technical complications. However, significant descriptions of the use of LC/TSP/MS or LC/CF FAB/MS for the identification (profiling) of secondary metabolites were published in the last decade of the twentieth century (Stobiecki, 2000, 2001). Various analyzers are widely used in the MS, both high resolution (time of flight) and low resolution (quadrupole and ion trap). All the mentioned analyzers may be utilized in CID MS/MS mode. For the detection of flavonoid conjugates after their separation with LC or CE systems, other instruments besides MS are also commonly applied as detectors, including UV detectors [single wavelength or diode array (UV DAD)], electrochemical, laser-induced fluorescence (LIF), and NMR (Sumner et al., 2003). The sensitivity of LC-MS or CE-MS systems depends on the detector applied. The highest sensitivity achievable is estimated to approach 10^{-21} mole in the case of CE combined with a LIF detector. The detection limit of LC-MS or LC-UV instruments is in the range of 10^{-12} to 10^{-15} mole, whereas the LC-NMR sensitivity is much lower and the minimal amount of compound that can be detected is around 10^{-5} mole.

Many reports describe the combination of different detectors coupled in line. The most promising system, permitting in many cases an unambiguous structural characterization of components in extract from unknown source, consists of UV DAD detector, NMR, and mass spectrometer in line. The analysis of plant extracts using this system was demonstrated for the first time almost 10 years ago (Wolfender et al., 1998).

The structural elucidation of compounds using NMR experiments and their separation by HPLC are well-established analytical methods. This is why the connection of LC and NMR spectroscopy for analysis of mixtures of unknown compounds was originally devised. The first such experiment was performed in 1978 (Watanabe and Niki, 1978). However, until the mid-1990s, not much progress was achieved in the development of this technique. Several reviews discussed serious obstacles, which had to be overcome to develop LC-NMR as a useful method (Albert, 1999; Wolfender et al., 2003). The major problems concerned a low sensitivity of NMR, the presence of protonated solvents used in the reversed phase HPLC covering some signals of protons of analytes, and the possibility of running only ^{1}H-NMR experiments.

Most of these problems have been solved partially within the last decade. The low sensitivity of the NMR techniques has been addressed in several ways. Using high-field magnets (at least 500 and up to 750–900 MHz) in spectrometers connected to HPLC gives satisfactory results (Wolfender et al., 2001). Another method for increasing the sensitivity is a “stop-flow” mode of the LC-NMR system operation, allowing for a prolonged time of acquisition of NMR spectra, of up to several days. In this method, the software controlling the whole system switches off the HPLC pump in the moment when a chromatographic peak, registered by another detector (most frequently UV), reaches the NMR probe (Holt et al., 1998). Different NMR experiments then may be performed, including two-dimensional ^{1}H-^{13}C

HSQC and HMBC spectra that could be obtained using even 10 μg of sample (Wolfender et al., 2001). Preconcentration of the sample, using higher-field strength of the NMR instrument and optimization of the column parameters, further improve the detection limit by an order of magnitude (Hansen et al., 1999). Signals of solvents most frequently used in the reversed-phase chromatographic systems (CH_3CN-D_2O and CH_3OD-D_2O) may be suppressed using the WET (water suppression-enhanced through t_1 effects) technique (Smallcombe et al., 1995).

The LC-NMR technique has been applied successfully in several laboratories for the studies of flavonoid compounds from different plant species (Wolfender et al., 1997; Hansen et al., 1999; Lommen et al., 2000; Vilegas et al., 2000; Andrade et al., 2002; Queiroz et al., 2002; Le Gall et al., 2003; de Rijke et al., 2004a; Waridel et al., 2004). In most cases, the LC-NMR data are supported by results obtained with different types of LC-MS experiments. However, even though the direct connection of an HPLC with both spectrometers in one system is possible, it is not a frequent arrangement. The reason for this is the need of use of deuterated water as a solvent for NMR. In these conditions, some protons in the flavonoid molecules (especially hydroxyl groups of the aglycone as well as those of the glycoside moieties) are exchanged easily for deuterium atoms, causing the increase of the apparent molecular weights of analytes and the eventual differences in the MS spectra. In these cases, running additional LC-MS with H_2O instead of D_2O in the mobile phase is recommended (Hansen et al., 1999).

The application of LC-NMR provides the most valuable structural information in studies of mixtures of flavonoids of natural origin and allows for a proper identification of compounds indistinguishable using other methods. Hansen et al. (1999) have shown in *Hypericum perforatum* that two different glycosides of quercitin exist, which coelute from the RP C-18 column and have the same MS and MS/MS spectra. However, according to the differences in the NMR spectra, they could be identified as quercitin 3-β-D-glucoside and 3-β-D-galactoside. The same compounds were found and similarly identified in the apple peel by Lommen et al. (2000). However, in this material, in addition there were three quercitin pentosides present, which could be identified only using LC-NMR as 3-α-L-arabinofuranoside, 3-β-D-xyloside, and most probably 3-β-L-arbinopyranoside. Similarly to the above examples, two different kempferol glycosides, obtained from transgenic tomato fruits, eluted from the column very close to each other and had almost identical MS spectra (LeGall et al., 2003). These compounds could be differentiated only on the basis of the spectra acquired in the LC-NMR system. The isoflavones formononetin and biochanin A along with their 7-*O*-β-D-glucosides and malonyl-glucosides were found in clover (*Trifolium pratense*) leaves (de Rijke et al., 2004b). Isomeric forms of malonyl-glucosides of both aglycones were present, and according to the LC-NMR analysis were identified to differ in the 4″ or 6″ esterification of the glucose ring with malonic acid.

Successful identification and quantification of components in analyzed samples is not only dependent on the applied detectors. The influence of the composition of the liquid phase on results obtained with mass spectrometry detectors was studied for different classes of compounds (Rauha et al., 2001; Zhao et al., 2002; Huck

et al., 2002). In addition, different interfaces applied for the coupling of LC with MS were described (Gelpi, 2002; Niessen, 1999). The type of LC columns used also influences the quality of data collected. The application of columns with SiO_2 particles of 3 μm diameter as well as the application of RP C-30 stationary phase (Vilegas et al., 2000) may improve the resolution and further reduce the time of analysis.

Most of the described LC separations of flavonoid mixtures obtained from different biological materials were performed on reversed-phase C-18 columns with acidified mobile phases, in most cases with acetic or formic acids. A strong suppression of cationized molecules ($[M+Na]^+$ and $[M+K]^+$ ions) was observed in mass spectra due to the acid addition to the phase (Stobiecki et al., 1997). An increase in sensitivity may be achieved this way owing to the presence of single $[M+H]^+$ ions. Comparable effects may be achieved after the addition of ammonium acetate to the mobile phase; in this case strong ammonium adduct ions $[M+NH_4]^+$ are observed (de Rijke et al., 2003). The flow rate of the mobile phase is an important parameter when API methods are applied. In LC-MS systems the column flow varies between 0.2 ml/min to 1 ml/min, for column diameters of 2 mm and 4 mm, respectively. The decrease of the column diameter may improve the sensitivity due to a lower dilution of the analyte in the liquid phase. The accessible sensitivity during the mass spectrometric analysis of flavonoids is in the low nanogram level of a single compound injected onto the column. However, in the case of extracts from biological origin, the sensitivity may decrease due to the coelution of different natural products and the suppression of the ionization efficiency of the target compounds. Extracts from 200 mg of fresh weight of plant tissue material (root or green parts) give reasonable amounts of natural products to obtain good quality single-ion chromatograms and mass spectra. The additional purification and/or preconcentration of target flavonoid compounds is not necessary; however, solid-phase extraction often was used to improve the quality of obtained results. On the other hand, many efforts have been undertaken to improve the sample preparation procedures and instrumental identification methods in order to achieve the sensitivity sufficient for secondary metabolites analysis in single cells. At present the utilization of micro- and capillary LC or CE allows one to approach this limit of sensitivity.

During LC/MS system utilization, increased structural information may be obtained when tandem mass spectrometric approaches are utilized. Different types of analyzers can be applied including triple quadrupole, quadrupole and time of flight, or ion traps. The last system makes it is possible to perform multiple MS/MS experiments (March, 1997). From the MS^n product ion mass spectra one can achieve data about structure of an aglycone (Sanchez-Rabaneda et al., 2003; de Rijke et al., 2003) and differentiation of *C*-6 and *C*-8 substituted glycosides (Sikorska et al., 2003; Waridel et al., 2001) and can draw conclusions about patterns of *O*-glycosylation (Cuyckens and Claeys, 2004). During MS^n experiments it is important to establish proper conditions of mass spectrometric analysis. These include the voltage between the entrance slit and skimmer or choice of parent ions. Both characteristic fragmentation pathways of parent ions and their relative intensities

bring information about the structure of flavonoid glycosides present in the mixture. Comparison of positive ion mass spectra registered during LC/MS and LC/MS/MS analyses with ESI ionization is presented on Figures 2.4 and 2.5. In normal mass spectra, protonated molecules $[M+H]^+$ ions and Y_n^+ fragment ions are present, created after the cleavage of glycosidic bonds. Tandem experiments after further fragmentation of Y_0^+ ions allow the identification of the structure of an aglycone in *O*-glycosides. This approach creates possibilities for establishing mass spectral data bases of flavonoid glycosides or free aglycones (Bristow et al., 2004). However, during analyses of crude extracts, the coelution of different secondary metabolites is possible and in this situation simultaneous ionization of compounds in the column eluate occurs; sometimes ratios of ion intensities of target compound with these, originating from other substances, are very low. In this situation, the application of MS/MS systems is useful, enabling the identification of natural product in extracts of complex composition.

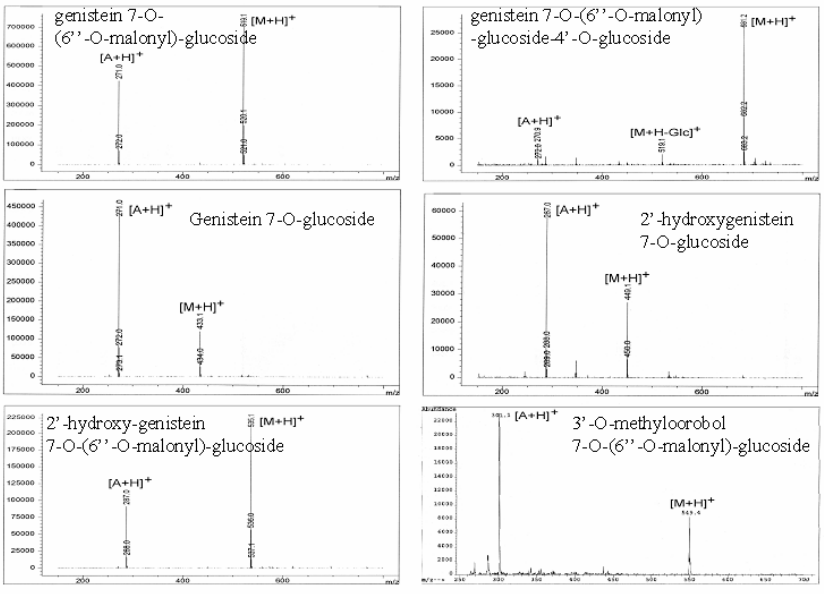

Figure 2.4 *Mass spectra of different isoflavonoid glycosides obtained using ESI HPLC-MS system.*

Metabolite profiling of flavonoids and isoflavonoids and their conjugates gained a wide interest in last few years. Many papers presenting applications of LC-UV or LC-MS systems for the analysis of the above-mentioned natural products in tissues of different plant species have been presented. Roles of isoflavones under the influence of abiotic stress or as phytoalexins or phytoanticipins during interaction of plants with pathogenic microorganisms were studied (Graham, 1991a, 1991b; Lozovaya et al., 2004; Bednarek et al., 2001, 2003). The same group of

compounds was analyzed in different plant tissues (Gu and Gu, 2001; de Rijke et al., 2001, 2004a, 2004b; Klejdus et al., 2001; Lin et al., 2000; Sumner et al., 1996). Numerous conjugates of flavones and flavonols were identified in extracts obtained from herbs, vegetables, fruits, and berries or juices. In the last few years, numerous papers related to the analysis of anthocyanins in wine and grape or catechin derivatives in green tea also were published (reviewed in Justesen et al., 1998; Stobiecki, 2000, 2001; Careri et al., 1999, 2002; Prasain et al., 2004).

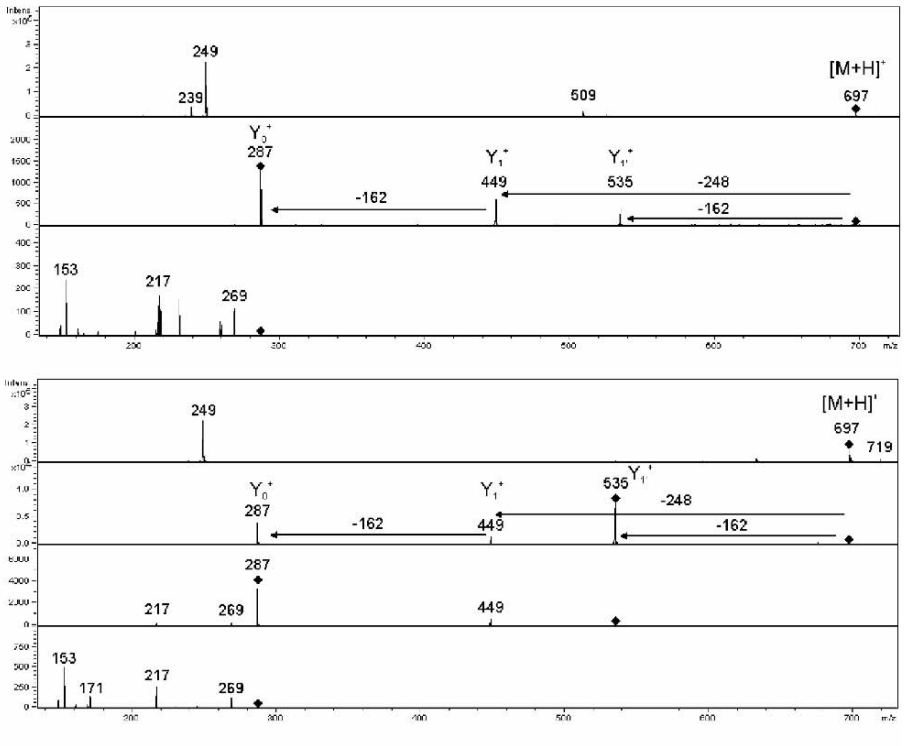

Figure 2.5 *MS^n spectra of different malonyl-diglucosides of 2'-hydroxy-genistein obtained using ESI HPLC/MS ion trap system.*

Different methods of CE also can be also applied for the analysis of flavonoids and their conjugates in various biological materials (Mellenthin and Gallensa, 1999; Urbanek et al., 2002). The comparison of LC and CE chromatographic systems for flavonoids analysis also has been published (Tomas-Barberan and Garcia-Viguera, 1997; Vanhoenacker et al., 2001; Smyth and Brooks, 2004; Wang and Huang, 2004).

4. CONCLUSIONS

Due to a wide range of biological activities of flavonoids consumed by humans and animals, there is a high interest in the metabolism of these compounds. The most important groups of this class of natural products are the phytoestrogenes (isoflavones: genistein derivatives) and antioxidants (anthocyanins: flavonols and flavones); their interactions with proteins (tannins) and their metabolites are monitored in physiological fluids (urine, blood, milk) and tissues (Prasain et al., 2004). These kinds of studies will help to elucidate the influence of the flavonoids on human and animal health and permit the evaluation of their role in different kinds of epidemiological studies (see Chapter 8 for a detailed description). MS techniques are the methods of choice in these research areas, especially the combination of chromatographic systems (GC, LC, or CE) with powerful detectors which enable the identification of single compounds in complex mixtures.

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