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# Structural Analysis of Flavonoids and Related Compounds – A Review of Spectroscopic Applications

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## 1. Introduction

In 1936 St. Ruzsnyák and A. Szent-Györgyi described, in a paper in *Nature* (Ruzsnyák & Szent-Györgyi, 1936), the relief of certain pathological conditions, characterized by an increased permeability or fragility of the capillary wall, by extracts of Hungarian red pepper containing flavonols, a type of flavonoids, which were then named “vitamin P”. The following five decades saw a slow but steady rise in the interest in the group of flavonoids, and their benefits in the treatment of a vast number of diseases and conditions, including pregnancy toxemia, rheumatic fever, diabetes and cancer. In the late 1980s and throughout the 1990s flavonoids were intensely studied concerning their actions as mutagenic agents and as antioxidants and pro-oxidants as their likely roles in biological systems (for example, Aviram & Fuhrman, 1998; Lambert & Elias, 2010). In the early 90s the antioxidant activity of flavonoids was extensively studied *in vitro*, and it was assumed that such activity would be at the basis of the health promoting benefits of these compounds. However, in the late 90s and early 00s the metabolism of flavonoids was deeply scrutinized, and the results indicated that their antioxidant activity *in vivo* could not account for the overall actions attributed to them (Fraga et al., 2010). The paradigm for flavonoid action changed towards the establishment of flavonoids as inflammation modulators, and more recently their role in neuroprotection, memory and cognition has been under scrutiny (Gomes et al., 2008; Spencer et al., 2009; Spencer, 2010). However, exact mechanisms for many of the actions attributed to flavonoids have not yet been established, but the relationship between their activity and the presence of specific functional groups in the molecules is undeniable. Moreover, each role attributed to flavonoids has been linked to different structural features – for example, while antioxidant activity depends essentially on the number and location of OH groups in the molecules, their antagonist effect towards adenosine receptors depends more on the overall planarity than on the hydroxyl groups; in fact, the latter even appear to be counter-productive (González et al., 2007).

The work developed in this area strongly depends on powerful analytical techniques for quantification and structural identification, as circulating forms are usually found in the low micromolar range, and intracellular levels are even lower. This chapter will briefly review the analytical techniques employed to determine the flavonoids structure from *in vitro* and *in vivo* studies. Albeit it will be focused on the more common classes of flavonoids (flavones,

flavonols, flavanones, catechins, isoflavones and anthocyanidins), it will also address recent developments in minor flavonoid classes. Two fundamental works must be distinguished here. The first is the 1982 book by Markham entitled *Techniques of Flavonoid Identification*, which addresses the state-of-the-art of flavonoid structural identification at the time, and focused largely on UV spectroscopy (Markham, 1982). More recently, in 2005, Markham and Andersen have edited *Flavonoids: Chemistry, Biochemistry and Applications*, which is a reference book for those studying either structure or activity (or both) of flavonoids, containing large tables of collected NMR data and addressing many of the topics under research at that time (Fossen & Andersen, 2005). Hence, this chapter will be mainly focused on developments in the field from 2005 onwards.

## 2. Overview of flavonoid structural classification

Flavonoids are polyphenols of plant origin that are among the most important compounds in human diet due to their widespread distribution in foods and beverages. They can occur both in the free form (aglycones) and as glycosides, and differ in their substituents (type, number and position) and in their insaturation. As mentioned, the most common classes are the flavones, flavonols, flavanones, catechins, isoflavones and anthocyanidins, which account for around 80 % of flavonoids. Figure 1 shows the basic structure of the different flavonoid classes addressed in this chapter.

All flavonoids share a basic C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> phenyl-benzopyran backbone. The position of the phenyl ring relative to the benzopyran moiety allows a broad separation of these compounds into flavonoids (2-phenyl-benzopyrans), isoflavonoids (3-phenyl-benzopyrans) and neoflavonoids (4-phenyl-benzopyrans) (Figure 1). Division into further groups is made

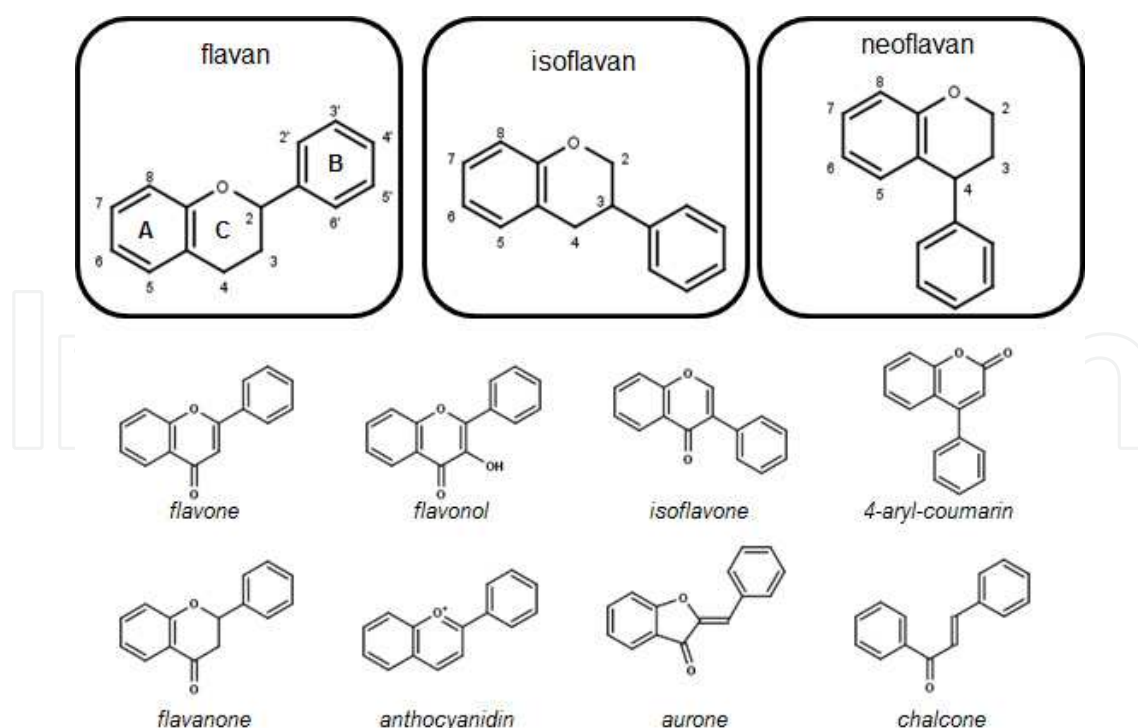


Fig. 1. Structure of the structural backbones of the main flavonoid groups (flavan, isoflavan and neoflavan) and of relevant flavonoid classes. Atom numbering and ring nomenclature are also included.

on the basis of the central ring oxidation and on the presence of specific hydroxyl groups. Most common flavonoids are flavones (with a C2-C3 double bond and a C4-oxo function), flavonols (flavones with a 3-OH group) and flavanones (flavone analogues but with a C2-C3 single bond), and abundant isoflavonoids include isoflavones (the analogue of flavones). 4-arylcoumarin (a neoflavonoid with a C3-C4 double bond) and its reduced form, 3,4-dihydro-4-arylcoumarin, are the major neoflavonoids. Other natural compounds, such as chalcones and aurones also possess the C6-C3-C6 backbone, and are henceforth included in the general group of flavonoids.

### 3. Mass Spectrometry

Mass Spectrometry (MS) has proved to be one of the most effective techniques in biomedical research, in special when complex matrixes of biological samples must be analysed. The main advantages of MS are its high sensitivity, which allows analysis of compounds present in the  $\mu\text{g}$  scale, and high specificity, as it is able to separate molecules of the same molecular weight but different atom composition, and sometimes even to differentiate stereoisomeric compounds. Its easy coupling to separation techniques such as liquid and gas chromatography is also an excellent advantage. A review of separation methods, applied to flavonols, isoflavones and anthocyanidins, has been recently published (Valls et al., 2009). Sample preparation may also be critical, but that varies from sample to sample; nevertheless, some general guidelines have been reviewed (Prasain et al., 2004). For more detail on mass spectrometry equipment and experiments, an excellent book has been published in 2007 (de Hoffmann & Stroobant, 2007). Specifically for electrospray and MALDI mass spectrometry applications in the biology area, the work edited by Cole (Cole, 2010) is highly adequate.

Gas Chromatography (GC) is one of the key techniques for the separation of organics and, coupled to MS, one of the most common techniques of structural identification. However, flavonoids are largely nonvolatile, and need be derivatized; also, they are usually thermally unstable. Both these characteristics have led to the establishment of Liquid Chromatography, in particular High Performance (HPLC), as the fundamental separation technique for flavonoids. Consequently, LC-MS coupling is routinely used for the overall structure elucidation of flavonoids (Fossen & Andersen, 2005).

#### 3.1 Ionization techniques

Various ionization techniques are available, and each has their own specificities which make them more or less useful depending on the molecules under study and on the aim of such study. The ones most applied to flavonoid research are hereafter presented.

##### 3.1.1 FAB-MS and LSIMS

Fast-Atom Bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS) are ionization techniques used in Secondary Mass Ion Spectrometry (SIMS) in which secondary ions, emitted by sample irradiation with a beam of energetic (primary) ions, are analyzed. Typically, these techniques are able to produce ions from polar compounds with molecular weights up to 10 kDa, but they require the analyte to be dissolved in a matrix, which may lead to the formation of more complex spectra. Also, being soft techniques, ion abundance is

low, and it has been used essentially to identify flavonoid glycosides and for molecular weight determination (Stobiecki, 2000; Prasain et al., 2004).

### 3.1.2 ESI and APCI

Electrospray Ionization (ESI) is a technique in which ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe, or coupled to separation techniques such as LC or capillary electrophoresis. In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period of time. ESI interfaces are mostly coupled to quadrupole mass spectrometers; both are simple and robust equipments, able to produce either positive or negative ions, and their main limitation is the relatively limited  $m/z$  range, usually below 2 kDa. In Atmospheric Pressure Chemical Ionization (APCI) sources ionization occurs via a corona discharge on a heated solvent spray, which produces solvent-derived primary ions that will, in turn, ionize the solute (Prasain et al., 2004; de Hoffmann & Stroobant, 2007). Both ESI and APCI use atmospheric pressure and high collision frequency, and thus generate large amounts of ions; as they involve solvent evaporation, the decomposition of the analytes is reduced, and full scans show limited fragmentation. The main disadvantage of both these techniques is that some HPLC solvents interfere with the ionization process, and thus chromatographic separations need to be specifically designed (Prasain et al., 2004).

### 3.1.3 MALDI

Matrix-Assisted Laser Desorption/Ionization (MALDI) is a soft ionization technique in which the analytes are co-crystallized with a matrix; this mixture is deposited on a plate upon which a laser beam is aimed. The laser discharge ultimately leads to analyte ionization and projection from the matrix and onto the analyser. Typical matrixes are derivatives of 4-hydroxycinnamic acid, and also 2,5-dihydroxybenzoic acid (2,5DHB) (de Hoffmann & Stroobant, 2007). Due to the structural similarity between these matrixes and the flavonoids, only recently has MALDI been applied to flavonoid structural elucidation, using a FT-ICR spectrometer, and a significant, although still informative, number of flavonoid-matrix clusters are observable (Madeira & Florêncio, 2009).

## 3.2 Mass analysis

Mass analysis is the second step in a MS experiment. Following ion generation, mass analysers measure the mass-to-charge ratio,  $m/z$ , of the ions, by using a combination of electromagnetic fields. There are many types of mass analysers, as there are of ion sources and detectors. The most common are of the ion type and of the quadrupole type, which analyse  $m/z$  ratios by the resonance frequency and by the trajectory stability, respectively, and time-of-flight (TOF) analysers, which measure ion velocity (or flight time). More recent resonance frequency analysers, namely Fourier Transform (FT) ion cyclotron resonance (FT-ICR) and FT orbitraps, are now starting to be applied to flavonoids (de Hoffmann & Stroobant, 2007). A recent work has compared the performance of different mass analysers coupled to the same ion source and it was concluded that fragmentation patterns are transferable among different mass analysers, only the relative abundances are changed; although applied to cyano dyes (Volná et al., 2007),

the conclusions also apply to other classes of compounds, such as flavonoids, where different mass analyzers lead to similar fragmentation patterns.

### 3.3 Tandem mass spectrometry

Tandem mass spectrometry, usually abbreviated MS/MS, or MS<sup>n</sup> for *n*<sup>th</sup> order fragmentation, is any method that involves at least two stages of mass analysis, in conjunction with a fragmentation process, either dissociation or reaction, which causes a change in the *m/z* ratio on an ion. Most commonly, a mass analyser is used to isolate a precursor ion, which is then fragmented to yield product ions (and, eventually, neutral fragments) that will be detected in the second mass analysis – a typical MS<sup>2</sup> experiment. This can, at least conceptually, be expanded with further successive modification and detection steps, giving rise to MS<sup>3</sup>,... , MS<sup>n</sup>. However, as only a very small fraction of ions detected in one analyser makes it to the following analysers, MS<sup>3</sup> is usually the highest order achieved. This spatial arrangement of equipment, analyser-modified-analyser, corresponds to tandem MS in space, where ions are treated in different regions of space. Alternatively, tandem MS can be performed in time, with analysers such as ion traps, orbitraps or FT-ICR, where the same analyser performs different tasks successively (de Hoffmann & Stroobant, 2007).

*m/z* modification can be achieved by various techniques, but the most common is Collision Induced (or Activated) Dissociation (CID or CAD), where precursor ions undergo collisional activation with neutral atoms or molecules (such as inert gases) in the gas phase. CID is an example of a post-source fragmentation, in which energy is added to the already vibrationally excited ions. An alternative to CID is ECD (Electron Capture Dissociation), in which multiply charged positive ions are submitted to a beam of low energy electrons, producing radical cations. In opposition to post-source fragmentation, in in-source fragmentation, ions already possess sufficient internal energy and fragment spontaneously within the mass spectrometer. Although usually this is an undesired effect, because it leads to lower abundance of precursor ions, it may in some cases become useful (de Hoffmann & Stroobant, 2007; Abrankó et al., 2011).

#### 3.3.1 Scan modes

Four general types of tandem MS scans are possible, and all may generate valuable information. A *product ion scan* analyses all the fragment ions resulting from a single selected precursor ion (these are usually called *MS<sup>2</sup> spectra*). Conversely, a *precursor ion scan* will identify all the precursors of a selected product ion; a *neutral loss scan* is performed from a selected neutral fragment and will identify the fragmentations leading to the loss of that neutral fragment; these two techniques cannot be performed in time-based analysers (de Hoffmann & Stroobant, 2007). A particular application of neutral loss scans is in the identification of phase II conjugation metabolites, that can be identified by specific neutral losses (Table 1) (Prasain & Barnes, 2007). Neutral loss scans are widely used to detect phase II conjugation metabolites, such as glucuronides (loss of 176 Da) and sulfates (loss of 80 Da), as well as for the detection of glutathione adducts (loss of 129 Da). Scanning for neutral losses of 162 and 132 Da has also been used to separate flavonoids with hexose residues from those with pentose residues, respectively. More selective than these three techniques, *selected reaction monitoring* (SRM) will analyse if a specific product ion comes from the fragmentation of a specific precursor ion; although more sensitive, it is much more specific.

Metabolic Reaction	Mass change (Neutral loss)	Metabolic Reaction	Mass change (Neutral loss)
Glucuronidation	+176 (176)	Methylation	+14 (-)
Glycosylation, hexose	+162 (162)	Hydroxylation	+16 (-)
Glycosylation, deoxyhexose	+146 (146)	Acetylation	+42 (60)
Glycosylation, pentose	+132 (132)	Carboxylation	+44 (44)
Sulfation	+80 (80)	Decarboxylation	-44 (44)
Glutathionylation	+129 (129)	Demethylation	-14 (-)

Table 1. Mass shift associated with possible metabolic reactions of flavonoids and correspondent detection by neutral loss scanning. Adapted from Prasain & Barnes, 2007.

The following subsections will address the various applications of mass spectrometry to flavonoid structural elucidation, and are organized as a potential guide to explore the structure of novel compounds.

### 3.4 Flavonoid glycosides – differentiation and characterization

In plants, flavonoids are often found to be glycosylated; the glycoside residues can be attached to O and C atoms of the flavonoids, giving rise to *O*-glycosides, *C*-glycosides and *O*-*C*-glycosides. These can be differentiated by soft ionization techniques, with low fragmentation energy, usually by FAB-MS, in which glycoside loss from *O*-glycosides undergo heterolysis of their hemi-acetal O-C bonds, gives rise to  $Y_i^+$  ions; at low energy, *C*-glycosides only produce  $[M+H]^+$  ions, and, at higher energies, intraglycosidic cleavages give rise to  ${}_{ij}X$  fragments and water loss gives rise to characteristic ions (Cuyckens et al., 2000; Li & Claeys, 1994; Vukics & Guttman, 2010). Higher fragmentation energies lead to intraglycosidic cleavage in *O*-glycosides, to the generation of  $Y_i$  fragments in *C*-glycosides, and to both  ${}_{ij}X$  and  $Y_i$  fragments in *O*-*C*-glycosides, all of which correspond to complex, often misleading, mass spectra. (Li & Claeys, 1994). This nomenclature, proposed by Domon & Costello (1988) for the MS study of glycoconjugates, is presented in Figure 2.

The sugar type can be easily determined by the characteristic  $m/z$  values of the  $A_i$ ,  $B_i$  and  $C_i$  fragments arising from hexoses, deoxyhexoses and pentoses, which are not directly observable in the mass spectra but can be computed from the  $m/z$  differences of the parent ions and corresponding  $X_i$ ,  $Y_i$  and  $Z_i$  fragments (Vukics & Guttman, 2010; Ferreres et al., 2007; Li & Claeys, 1994). The  $m/z$  values for these fragments are presented in Table 2.

Flavonoid glycosides usually contain one or two glycoside residues, but molecules with more residues have been identified in nature. By definition, diglycosides can have the residues attached at different positions (di-*O*-glycosides and di-*C*,*O*-glycosides) or at the same position (*O*-diglycosides and *C*,*O*-diglycosides). The differentiation of the different types can be made from the product ions identified in the spectra, particularly from the  $Z_i$

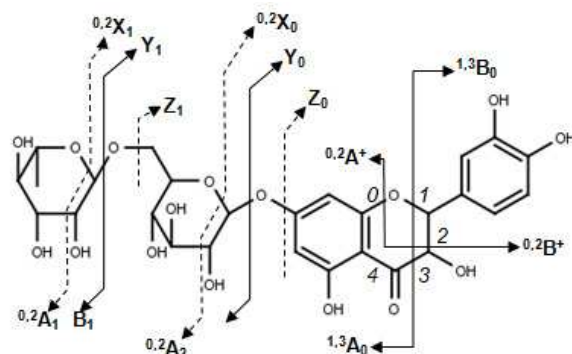


Fig. 2. Ion nomenclature used for flavonoid glycosides. Fragmentation of quercetin-7-O-rutinoside is depicted. Glycoside ions are named according to Domon & Costello, 1988; aglycone ions follow the nomenclature of Ma et al., 1997.

Fragments	- 0,1X	- 0,2X (- H <sub>2</sub> O) (- 2H <sub>2</sub> O)	- 0,3X	- 1,5X	- 2,3X (- 2H <sub>2</sub> O) (- 3H <sub>2</sub> O)	- 0,4X (- 2 H <sub>2</sub> O)	- Y <sub>i</sub>	- X <sub>i</sub>
Hexose	150	120 (138) (156)	90	134	66 (84)	96	162	180
Deoxyhexose	134	104 (122) (140)	74	120	66 (84)	80	146	164
Pentose	120	90 (108) (126)	60	104	- (-)	66	132	150

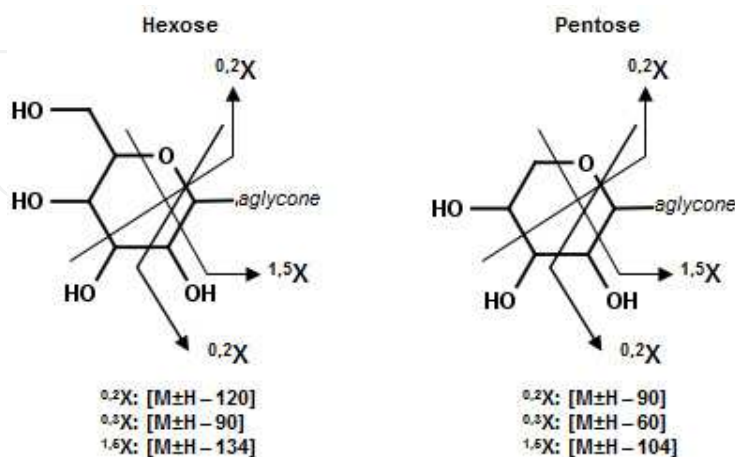


Table 2. Characteristic mass losses of flavonoid glycosides and characteristic cross-ring cleavages in the sugar rings of hexoses and pentoses (Vukic & Guttman, 2010; Cuyckens & Claeys, 2004).



ions – for example,  $Z_1$  fragments do not occur in monoglycosides. Also,  $Y_i$  fragments undergo well-known losses to form radical ions, which can be used to characterize the distribution of sugar residues. It must be noted that in some *O*-glycosides an internal sugar loss may take place, in which the aglycone-bound sugar is released first and, simultaneously, the other residue attaches itself to the aglycone; although this may lead to more complex spectra, this is well described in the literature, and can be overcome by the study of flavonoid-sodium adducts (Vukics & Guttman, 2010; Ma et al., 2000; Brüll et al., 1998; Cuyckens et al., 2001).

Concerning the glycoside sequence of di-, tri- and tetra-glycosides, there is no general procedure to apply for the ordering of the different residues, and it must be done rationally case by case. However, it has been shown that the interglycosidic linkage is easily accessible by MS; although these bonds can be of different types, 1→2 and 1→6 bonds are the preferred ones. For these two bond types, the  $Y_0^+/Y_1^+$  is always larger for a 1→2 bond than for a 1→6 bond – the  $Y_0^+$  ion is more abundant than the  $Y_1^+$  ion for glycosides with a 1→2 bond, and the relative abundance is reversed in the case of a 1→6 bond. The negative ionisation mode may be particularly important in this case as the  $Z_1^-$  appears to be exclusive of 1→2 bonded glycosides. In the case of *C,O*-diglycosides, it is usually necessary to obtain MS<sup>3</sup> spectra to confirm the various interglycosidic linkages, because while 1→2 glycosides yield, by internal cleavage of the *C*-glycosyl moiety,  $^{0,2}X_0^-$  ions, all other possibilities (1→3, 1→4 and 1→6) do not have characteristic fragmentation products (Ma et al., 2000; Cuyckens et al., 2000; Vukics & Guttman, 2010).

In theory, flavonoids could be glycosylated in any position, but *O*-glycosylation occurs mainly at position 7, as in flavones, isoflavones, flavanones and flavonols; *O*-glycosylation in positions 3 and 5 is also frequent, albeit less than in position 7. *C*-glycosyl flavonoids are usually flavones, and the glycosyl moieties are attached at positions 6 or 8; the literature has two reports of 3-*C*-glycosyl-flavones. Positions can be identified with basis of the product ions of the compounds because each glycosylation site appears to yield specific fragmentations. The  $[Y_0-CO]^-$  is specific of 7-*O*-monoglycosides, while 3-*O*-monoglycosides are characterized by the  $[Y_0-2H-CO]^-$  ion. Also, higher energy fragmentations yield product ions that contain both the glycoside moiety and fragments of the aglycone, particularly B-ring derived fragments. In what concerns *C*-glycosides, 6-*C*-glycosides usually undergo more extent fragmentations than 8-*C*-glycosides, and the former are typically associated with a  $^{2,3}X^+-2H_2O$  fragment and an abundant  $^{0,3}X^-$  ion, while for the latter no typical product ions have been put forward and the  $^{0,3}X^-$  ion usually has a low abundance (Li & Claeys, 1994; Vukics & Guttman, 2010; Ferreres et al., 2007; Cuyckens & Claeys, 2005).

Many flavonoid glycosides possess acylated glycosyl moieties. Various acyl groups have been reported in the literature, and they are usually identifiable by characteristic mass losses, which are shown in Table 3. However, the position of glycoside acylation is not easily accessible by mass spectrometry, except for the cases in which a  $^{0,4}X$  fragment is present in the spectra of a hexose-containing flavonoid as it unequivocally establishes that the hexosyl moiety is acylated in position 6 (Cuyckens & Claeys, 2004).

### 3.5 Aglycones – identification of flavonoid classes

The identification of the diverse flavonoid classes is often achieved by the MS<sup>2</sup> spectra of the various compounds, because, as a general rule, each class of flavonoids is characterized by

specific fragmentation patterns. This is especially true in the case of flavan-derived flavonoids, and, to a lesser extent, in the case of some isoflavan-derived flavonoids. For the remaining classes, qualitative MS spectra are still scarce, and, in particular, systematic studies of compounds of the same class are still to be performed.

### 3.5.1 Flavones, flavonols, flavanones and flavanonols

The most useful fragmentations of flavonoids, in terms of structural elucidation, are those that involve breaking the C-ring bonds, which are termed retro Diels-Alder (RDA) by analogy with the Diels-Alder cycloaddition. These fragmentations (included in Figure 2) give rise to product ions containing the A or B ring and part of the C ring; for example, the  $^{1,3}A^+$  ion derived from a flavone, formed by a 1/3 fragmentation (cleavage of bonds 1 and 3), will contain the whole A ring plus the O1, C4 and O4 atoms.

These RDA fragmentations allow the establishment of diagnostic product ions for the various types of flavonoids. These are valid for a large of experimental MS conditions, but it must be kept in mind that the higher fragmentation energies will lead to increased

Acyl group	Characteristic fragments (mass change, amu)	
Acetyl	[M+H-acetylhexose] <sup>+</sup>	(204)
	[M±H-CO <sub>2</sub> ]	(44)
Malonyl	[M+H-malonyl] <sup>+</sup>	(86)
	[M+H-malonylhexose] <sup>+</sup>	(248)
Benzoyl	[M+H-benzoylhexose] <sup>+</sup>	(266)
	[benzoyl] <sup>+</sup>	<i>m/z</i> =105
	[benzoylhexose] <sup>+</sup>	<i>m/z</i> =267
Galloyl	[M±H-galloyl]	(152)
	[M-H-gallic acid] <sup>-</sup>	(170)
	[M+H-galloylhexose] <sup>+</sup>	(314)
	[Gallic acid - H] <sup>+</sup>	<i>m/z</i> =169
Coumaroyl	[M+H-coumaroyl] <sup>+</sup>	(146)
	[M+H-coumaroylhexose] <sup>+</sup>	(308)
Feruloyl	[M±H-feruloyl]	(176)
	[M±H-feruloylhexose]	(338)
	[feruloyl] <sup>+</sup>	<i>m/z</i> =177
	[ferulic acid] <sup>+</sup>	<i>m/z</i> =194
Sinapoyl	[feruloylhexose] <sup>+</sup>	<i>m/z</i> =339
	[M±H-sinapoyl]	(206)
	[M±H-sinapoylhexose]	(368)
	[sinapoyl] <sup>+</sup>	<i>m/z</i> =207
	[sinapic acid] <sup>+</sup>	<i>m/z</i> =224
	[sinapoylhexose] <sup>+</sup>	<i>m/z</i> =369
	[sinapoylhexose] <sup>-</sup>	<i>m/z</i> =367

Table 3. Characteristic acyl groups found in acylated glycosyl flavonoids and corresponding characteristic fragmentations. ± stands for either + or -. Adapted from Cuyckens & Claeys, 2004.

fragmentation and a higher abundance of ions with lower masses, deviating from these diagnostic ions and fragmentation pathways.

The most important RDA fragmentations in flavones and flavonols (3-hydroxyl-flavones) are the 0/2, the 0/4, the 1/3. While the 0/4 pathway appears to be exclusive of flavones, according to a low-energy CID study, where it leads to the  ${}^0_4\text{B}^+$  ion, which loses water to form the  ${}^0_4\text{B}^+ - \text{H}_2\text{O}$ , the  ${}^0_2\text{A}^+$  is an exclusive of flavonols; also, the  ${}^1_3\text{B}^+$  ion appears to be exclusive of flavones. In common, flavonoids from both classes give rise to the product ions  ${}^1_3\text{A}^+$ , and  ${}^0_2\text{B}^+$  (Cuyckens et al., 2005). This information is essential when analysing, for example, flavonoid metabolites by MS. Quercetin metabolism leads to the formation of, among others, conjugates with sulphate and glucuronic acid, as well as to methoxy-quercetin. Considering a full scan MS of quercetin (molecular weight of 302 g/mol) metabolites collected from rat plasma, an ion at  $m/z = 479$  corresponds to a protonated quercetin glucuronide; the 1/4 RDA fragmentation produces a  ${}^1_4\text{A}^+$  at  $m/z = 303$ , that bears the mass increase, meaning the glucuronosyl moiety is present in this ion; therefore, it must be located at either position 5 or 7 (Justino et al., 2004). Similar rationales for other ions lead to the identification of the positions of metabolism in these compounds.

More recently, a mixed ESI-MS and quantum chemical approach has analysed the fragmentation pathways of flavones and flavanols, and has proposed structures for other ions, other than RDA-derived, that are also informative in terms of structural elucidation. In particular, losses of one and two  $\text{C}_2\text{H}_2\text{O}$  moieties from the precursor ion, involving all the rings, and the formation of  ${}^1_3\text{A}^+ - \text{C}_2\text{H}_2\text{O}$  ion, are the most useful (Justino et al., 2009). Similar methodology has been applied in other cases (Madeira et al; 2010).

Flavones and flavonols constitute the vast majority of the flavan-based flavonoids studies. Flavanones, which lack the C ring 2-3 double bond, are less abundant than those two and have received less attention. In terms of MS fragmentations, in particular RDA pathways, flavanone itself undergoes cleavages to yield the  ${}^1_3\text{A}^+$  and  ${}^1_4\text{B}^+$  ions; these same ions are also observed for other flavanones, such as 5,7,4'-tri-hydroxy-flavanone (Nikolic & van Breemen, 2004). Studies on flavanone derivatives have evidenced that the positive mode CID spectra of isoprenylated flavanones is dominated by isoprenyl loss and  ${}^1_3\text{A}^+$  ion formation, while the negative mode is dominated by  ${}^1_4\text{A}^-$ , [ ${}^1_3\text{A}^-$  - isoprenyl] and [ ${}^1_4\text{A}^-$  - isoprenyl] ions. (Zhang et al., 2008) Derived ions, such as [ ${}^1_3\text{A} + \text{H}$ ] $^+$  and [ ${}^1_4\text{B} - \text{H}_2 + \text{H}$ ] $^+$ , have also been identified. (Zhou et al., 2007). Negative mode ESI has also allowed dividing flavanones into two groups, one with a 2'-OH group, with a spectra dominated by the  ${}^1_4\text{A}^-$  ion (for 5-OH-containing molecules) or the  ${}^1_4\text{B}^-$  (otherwise), and the other with no 2' substituents, for which the spectra were dominated by an intense  ${}^1_3\text{A}^-$  ion and a low abundant  ${}^1_3\text{B}^-$  ion. (Zhang et al., 2008) a 0/3 fragmentation, origination a  ${}^0_3\text{B}^-$  ion, has also been observed (Xu et al., 2009); an equivalent fragmentation has also been observed in the positive mode, to produce a  ${}^0_3\text{B}^+$  derived ion (Kéki et al., 2007). Flavanols (3-hydroxy-flavanones) show fragmentation patterns similar to those of flavanones; the main difference is that positive ESI spectra of flavanols are dominated by loss of water and homolytic H loss from the 3-hydroxy group to generate the  $[\text{M}-\text{H}]^{\bullet+}$  ion (Zhang et al., 2008).

### 3.5.2 Isoflavones and Isoflavonols

Unlike flavan derived flavonoids, the fragmentation pathways of isoflavan derived flavonoids have only been explored more recently, but a good set of systematic studies are

published. Besides common neutral losses, isoflavones undergo RDA fragmentation to yield the  ${}^0_4\text{B}^+-\text{H}_2\text{O}$  and the  ${}^1_3\text{A}^+$  ions; a full B ring loss, yielding the  $[\text{M}-\text{B ring}-\text{CO}]^+$  ion, is also frequently reported. Also characteristic is the loss of a CO group from Ring C oxo group, leading to ring contraction (Madeira et al., 2010; Heinonen et al., 2003; Borges et al., 2001; Simons et al., 2011).

In the negative mode, the 1/3 and 0/3 RDA fragmentations are predominant, and the 0/4 fragmentation is also observed sometimes, accompanied by extensive losses of CO, CO<sub>2</sub> and C<sub>3</sub>O<sub>2</sub> moieties. C ring expansion is also commonly observed (Vesecchi et al., 2011; Kang et al., 2007; March et al., 2004; Ablajan, 2011). For isoflavones, rare RDA fragmentations have been described in the literature, such as a 2/3 fragmentation that has also been described for various acylated 7,2'-hydroxy-3',4'-dimethoxyisoflavan glycosides (Qi et al., 2008) and a 2/4 fragmentation of daidzein (Wei et al., 2000).

The main difference in the fragmentation pathways of flavan- and isoflavan-derived flavonoids appears to be that in the latter the 0/3 RDA fragmentation occurs frequently while it appears not to occur for flavan-compounds; also, the 1/3 pathway appears to be much more important in isoflavan-compounds, in particular in those without a 2-OH group. Differentiation of isomeric aglycones of flavones and isoflavones is also possible based on a double neutral loss of CO (Kuhn et al., 2003).

### 3.5.3 Chalcones

Chalcones exist in nature with a variety of substituents. The MS spectra of these compounds are characterized by substituent loss, fragmentations of the substituents and chalcone fragmentations. Chalcone fragmentations are dominated by cleavage of a single bond, yielding a B ring derived ion with the attached C=O group in the charged form ( $\text{C}\equiv\text{O}^+$ ), from which a CO loss yield the free B ring (Nowakowska & Pankiewicz, 2008). Besides that, 2'-OH-chalcones, the most common ones, with an OH group in ring B adjacent to the propenone chain, are known to be converted to the corresponding flavanones by various processes, and that has also been observed to occur in ESI MS; the patten of chalcone fragmentation will then be the same of flavanone fragmentation (Zhang et al., 2008).

### 3.5.4 Other classes

Many other flavonoid classes exist. Many of those have been isolated and characterized, but no useful fragmentation pathways have been established.

For aurones, a positive mode of two aurone glycosides was identified as main product ions the ones formed by the loss of the glycosyl moiety, followed by a CO loss involving the heterocyclic O atom in the 5-member ring, and, from the  $[\text{M}-\text{glycosyl}+\text{H}]^{++}$  ion, the  ${}^1_3\text{A}^+$  ion, which may lose a further CO group, and the  ${}^1_3\text{B}^+$  ion (Kesari et al., 2004).

Coumarins show a wide diversity of substituents, and many even have fused rings attached. This variability does not allow overall fragmentation patterns, but many studies observe that in many cases the heterocyclic ring undergoes a contraction by CO loss (from the C=O group) to yield an ion containing a five membered ring (for example, Nowakowska & Pankiewicz, 2008; Zhang et al., 2007; Zhang et al., 2008).

Pterocarpan, isoflavonoid derivatives, show much more complex fragmentation pathways than the above analysed classes of flavonoids; MS studies of different deuterated pterocarpan derivatives, as well as of pterocarpan glycosides, points out that these are dominated by various and successive ring openings and/or contractions (Tóth et al., 2000; Zhang et al., 2007). A rare 2/4 RDA fragmentation, like that of isoflavan-derived flavonoids, has also been observed for pterocarpan in the negative mode (Simons et al., 2011).

Neoflavonoids, in which the B ring is attached in position 4, and include 4-aryl-coumarins, have been poorly studied by MS. The few reports available indicate, however, that neoflavonoid fragmentation is dominated by loss of the CH<sub>3</sub> radical in methoxylated compounds and by B ring loss, to yield the [M+H-B ring] fragment (Charles et al., 2005; Hulme et al., 2005; Liu et al., 2005). Figure 3 summarizes the characteristic RDA fragmentation pathways for flavonoid classes for which there is reliable information.

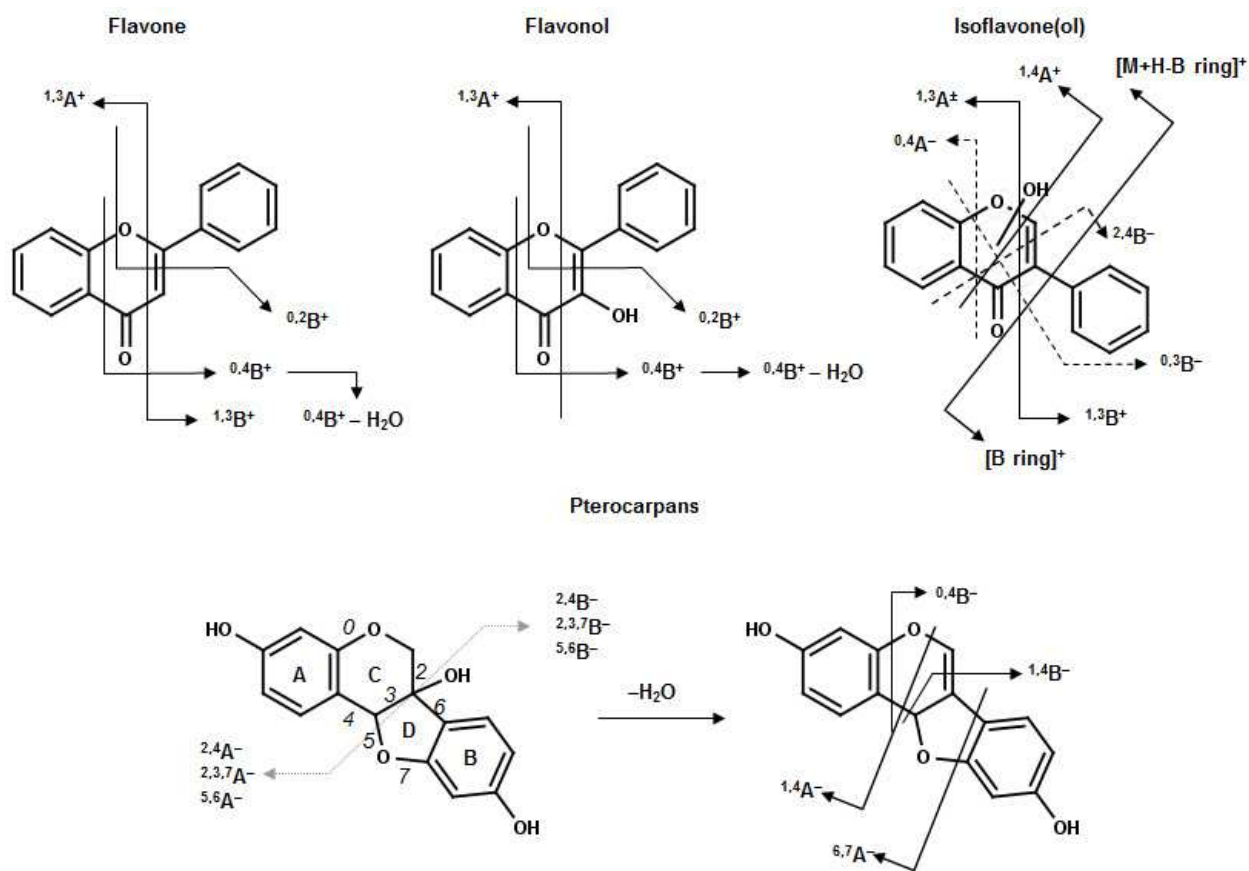


Fig. 3. Diagnostic products of flavones, flavonols, isoflavones, isoflavonols and pterocarpan. ± stands for either + or -. Adapted from Cuyckens et al, 2000; Kuhn et al., 2003; Madeira et al, 2010; Wei et al., 2000; Kang et al., 2007 and Simons et al., 2011.

### 3.6 Flavonoid-metal complexes

Flavonoids are good chelating agents towards metal ions and, in the case of iron and copper, the favoured places of chelation are catechol groups, hydroxyl groups adjacent to oxo groups, and 1-oxo-3-hydroxyl-containing moieties (Ren et al., 2008; Fernandez et al., 2002). This ability to chelate metals has been used to enhance the capabilities of MS; it has been

used to assist the elucidation of flavonoid glucuronides (Davis et al., 2006; Davis & Brodbelt, 2007) and various diglycosides (Pikulski et al., 2007). This is a result of the spectral changes observed when flavonoids are complexed with metals, giving rise to simpler yet more intense spectra (Satterfield & Brodbelt, 2000).

#### 4. Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance spectroscopy, hereafter simply designated by NMR, is one of the most powerful research techniques used to investigate the structure and some properties of molecules. One of the main applications of NMR in flavonoid research is the structural elucidation of novel compounds, for which nothing is known; although NMR traditionally requires large amounts of sample, which is not easy to obtain when analysing novel compounds, the technical developments in the last decade, both in NMR instrumentation, pulse programs and in computing power, have allowed the complete assignment of all proton and carbon signals using amounts in the order of 1 mg (Fossen & Andersen, 2005).

The major goal of this section is to summarize the applications of the various NMR experiments to flavonoid research, together with the information (other than atom connectivity) that can be taken from these experiments. Flavonoid NMR data are not presented here, as these are easily accessible, for a vast number of compounds, from the literature (reviewed in Fossen & Andersen, 2005).

##### 4.1 One Dimensional NMR: $^1\text{H}$ and $^{13}\text{C}$

The two most basic NMR experiments are the  $^1\text{H}$  and the  $^{13}\text{C}$  NMR experiments, which are aimed at the determination of the resonance frequency of each  $^1\text{H}$  or  $^{13}\text{C}$  nucleus in the molecule.

$^1\text{H}$  NMR experiments register the chemical shifts ( $\delta$ ) and spin-spin couplings, the latter described by the coupling constants ( $J$ ). This provides valuable information about the relative number of hydrogens and also their type, by comparison of the recorded chemical shifts with compiled data. This is particularly useful in establishing the aglycone type and the acyl groups attached to it, as well as in identifying the number and the anomeric configuration of the glycoside moieties attached to the aglycone.  $^{13}\text{C}$  NMR data is used to complement  $^1\text{H}$  NMR data, and is particularly useful at establishing the type of groups present in the samples' molecules by comparison with compiled data; however, it must be noted that  $^{13}\text{C}$  NMR is much less sensitive due to the abundance of  $^{13}\text{C}$  (1.1 %) when compared to  $^1\text{H}$  (99.9 %) (Claridge, 1999).

Together, these two 1D experiments are used primarily to identify aglycone types and substituent groups, but a definite structural elucidation, which the accurate location of the various groups, requires various 2D experiments.

##### 4.2 Homonuclear 2D NMR

2D NMR experiments generate contour maps that show the correlations between different nuclei in the molecules, and can be either homonuclear or heteronuclear, depending on whether the interacting nuclei are of the same or different elements (Claridge, 1999). COSY (COrrelation SpectroscopY) was one of the first multidimensional systems. COSY

crosspeaks are between protons that are coupled to each other, usually two bonds apart ( $^2J_{\text{HH}}$ ), but sometimes also three and four bonds apart ( $^3J_{\text{HH}}$  and  $^4J_{\text{HH}}$ ); the intensity of coupling affects the intensity of the peak. DQF-COSY (Double Quantum Filter COSY) is an improvement of the COSY experiment in which non-coupled proton signals, such as those from solvent, are eliminated as they may overlap signals from the analyte (Claridge, 1999). A further improvement is the TOCSY (TOtal Correlation Spectroscopy) experiment, which creates correlations between *all* protons in a given spin system, as long as there are couplings between every intervening protons; this is extremely useful to identify protons on sugar rings – every proton from one sugar ring will have a correlation with all other protons from the same ring but not with those of other rings. Magnetization is transferred over up to 5 or 6 bonds, and is interrupted by small or null  $^1\text{H}$ - $^1\text{H}$  couplings and hetero-atoms; also, the number of transfer steps can be adjusted by changing the spin-lock time (Fossen & Andersen, 2005). A good reference for the TOCSY transfer in various sugars is Gheysen's work (Gheysen et al., 2008). Selective 1D TOCSY (also known as HOHAHA, homonuclear Hartman-Hahn) is particularly useful in compounds with more than one sugar moiety, in which overlap occurs; in this experiment, one peak is selected and that magnetization is transferred stepwisely to the protons in the same spin system; instead of crosspeaks, transfer is shown by increased multiplet intensity (Fossen & Andersen, 2005).

#### 4.3 Heteronuclear 2D NMR

Heteronuclear 2D NMR experiments correlate nuclei of different elements. The most powerful techniques of all are undoubtedly the 2D proton-carbon experiments HMQC/HSQC (Heteronuclear Multiple Quantum Coherence/Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) as they provide an opportunity to dovetail proton and carbon NMR data directly.

HMQC and HSQC establish one bond correlations between the protons of a molecule and the carbons to which they are attached ( $^1J_{\text{CH}}$ ). Both these are much more sensitive than the correspondent 1D  $^{13}\text{C}$  experiments; while in 1D experiment the low abundance of the isotope leads to a low signal-to-noise ratio, in the heteronuclear 2D experiment the initial magnetization occurs on the highly sensitive  $^1\text{H}$  nuclei and is then transferred to the  $^{13}\text{C}$  atoms that are connected to each proton. A similar NMR experiment is  $^1\text{H}$ - $^{13}\text{C}$  HMBC (Heteronuclear Multiple Bond Correlation), in which long-range interactions (typically  $^2J_{\text{CH}}$  and  $^3J_{\text{CH}}$ ) are analyzed; HMBC is usually more sensitive to 3-bond correlations than to 2-bond correlations, but this depends on the overall signal-to-noise ratios and on the adjustable parameters of each experiment. A newer experiment,  $^2J_{\text{CH}}, ^3J_{\text{CH}}$ -HBMC, has been designed to differentiate these two types of correlations (Claridge, 1999; Krishnamurthy, 2000; Fossen & Andersen, 2005).

HBMC application to flavonoids usually addresses assignment on nonprotonated C atoms, from both the aglycones and acyl groups. Unlike TOCSY, HMBC transfer is not stopped by heteroatoms, and so it can also be used to determine the linkage points of heteroatom-containing groups such as sugar residues. HMBC is also useful to distinguish some classes of flavonoids, as flavones from aurones, which have similar  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra but very different HMBC spectra. Currently, only enhanced variants of the HSQC and HMBC experiments, namely gradient enhanced (*ge*) ones, are used, due to their higher sensitivity and capacity. These have been used to establish strong intramolecular H bonding between the 4-oxo and 5-hydroxy groups in flavonoids (Exarchou et al., 2002; Kozerski et al., 2003).

Further developments in NMR experiments, using new 2D and 3D techniques, have been developed in recent years, and are starting to be used for flavonoid analysis. In particular, 2D and 3D HSQC-TOCSY experiments are capable of assigning all  $^{13}\text{C}$  signals of individual glycosides in polyglycosylated flavonoids (Fossen & Andersen, 2005).

#### 4.4 Connectivity through space – the nuclear overhauser effect

While the above mentioned 2D techniques are useful to establish the connectivities between atoms through bonds, the Nuclear Overhauser Effect (NOE), which can be summarized as “A change in the intensity of an NMR signal from a nucleus, observed when a neighboring nucleus is saturated”, is useful at establishing non-bonded connectivities, or connectivities through space. The crosspeaks in a  $^1\text{H}$ - $^1\text{H}$  NOESY (NOE Spectroscopy) spectrum correspond to correlations between protons that are close to each other in space (up to 4 Å) but not necessarily connected through bonds; these correlations may arise from both intramolecular and intermolecular proton interactions, and has been successfully used to establish rotational conformers and restrictions, establish intermolecular associations and even solve protein-ligand and DNA-ligand structures. A 2D NOE experiment, ROESY (Rotating Overhauser Effect Spectroscopy), has been used in flavonoid research mainly to establish the stereochemistry of various flavonoids (Claridge, 1999; Fossen & Andersen, 2005). Figure 4 presents sample NMR spectra of anthocyanidine glucosides (Jordheim et al., 2006).

#### 4.5 Solid state NMR

X-ray crystallography depends on the ability to obtain flavonoid crystals, which has only been achieved for a small number of flavonoids; in alternative, the  $^1\text{H}$  CP-MAS (Cross Polarization Magic Angle Spinning) NMR techniques have been used to elucidate the solid state conformation of flavonoids, either pure or, for example, in tissues, providing enough sample is available (typically in the 10 mg scale). In particular, such an experiment had provided information on the planarity of the flavonoid rings, on the intramolecular H bonding between the 4-oxo group and the 3- and 5-hydroxy groups, and on intermolecular association. (Fossen & Andersen, 2005; Olejniczak & Potrzebowski, 2004)

### 5. UV-vis spectrophotometry

Ultraviolet and visible spectroscopy was one of the earliest techniques routinely used for flavonoid analysis due to the existence of two characteristic UV/Vis bands in flavonoids, band I in the 300 to 550 nm range, arising from the B ring, and band II in the 240 to 285 nm range, arising from the A ring. For examples, while the band I of flavones and flavonols lies in the 240 – 285 nm range, that of flavanone (no C ring instauration) lies in the 270 – 295 nm range; conversely, the band II of flavones and flavanones (no 3-OH group) lies around 303 – 304 nm, and that of 3-hydroxylated flavonols is centred around 352 nm.

Shift reagents, such as sodium methoxide and aluminium chloride, lead to shifts in the maximum wavelength of these bands due to methoxide-induced deprotonation of OH groups or  $\text{Al}^{3+}$  complexation by OH groups, were also routinely used to study flavonoid structure. Nowadays these techniques are not routinely used but still continue to be applied in some cases, in particular to HPLC eluates - a hyphenated LC-UV-MS has been developed using post-column UV shift reagents for the flavonoid analysis of crude extracts.



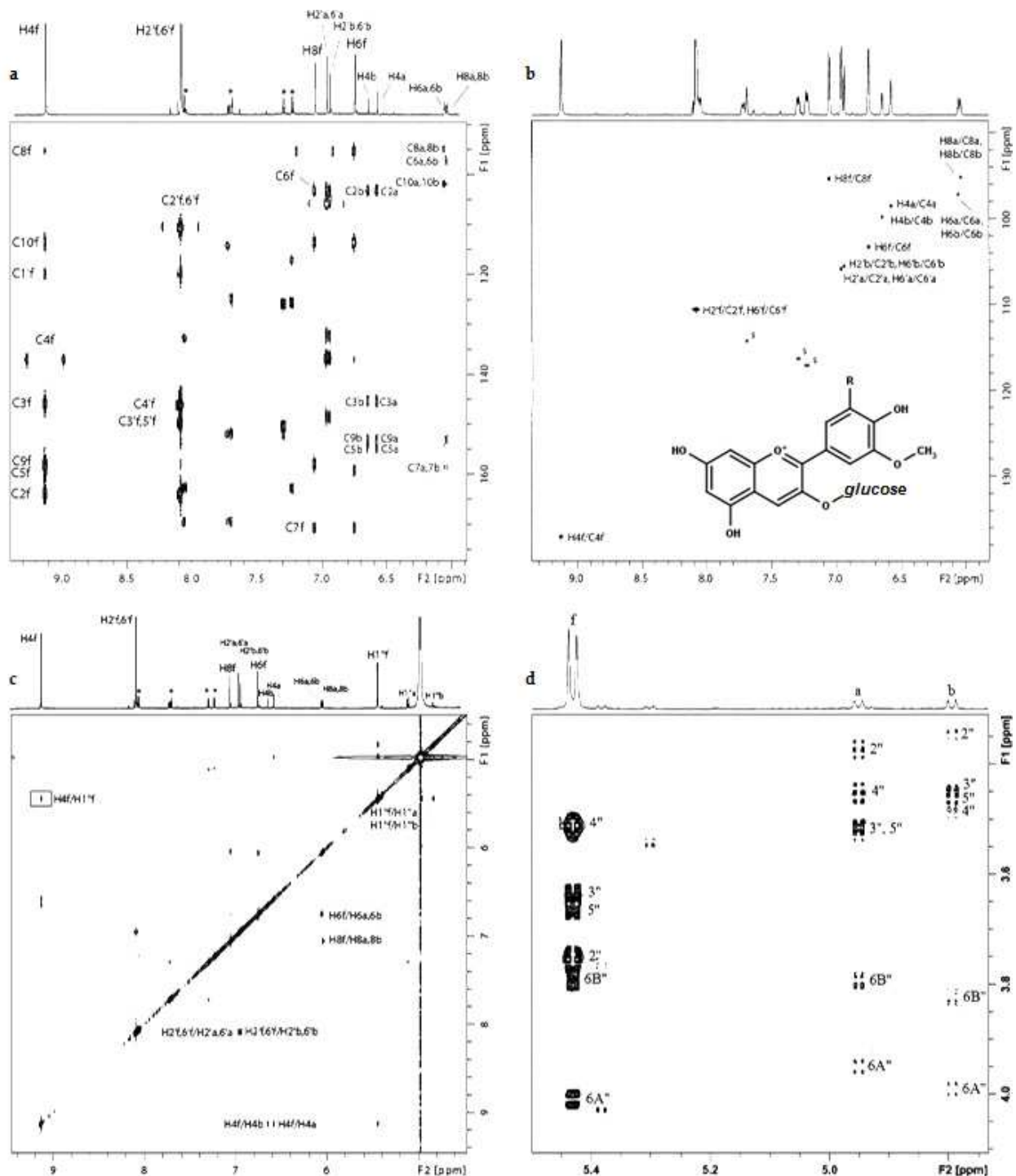


Fig. 4. Sample NMR 2D spectra of malvidin 3-O-β-glucopyranoside (R=OCH<sub>3</sub>) (a, b and c) and of petunidin 3-O-β-glucopyranoside (R=OH) (d) obtained at 600.13 MHz, at about 11 mM at 25 °C in CD<sub>3</sub>OD. a) <sup>1</sup>H-<sup>13</sup>C HMBC spectrum; b) <sup>1</sup>H-<sup>13</sup>C HSQC spectrum; c) NOESY spectrum; A negative cross-peak due to NOE correlation between H-4 and H-1'' of the flavylium cation, locating the position of the monosaccharide to the aglycone, is enclosed in a box. Other labelled cross-peaks are positive and are caused by chemical exchange; d) <sup>1</sup>H-<sup>1</sup>H TOCSY NMR spectrum of petunidin 3-O-β-glucopyranoside. f = assignment for the flavylium; a = assignment for the hemiacetal a (major); b = assignment for the hemiacetal b (minor); \*/s = impurities. Adapted from Jordheim et al., 2006.

UV/Vis spectrophotometry is still widely used to study anthocyanidins, which change their form and colour depending on pH, concentration, metal ions and copigmentation (Giusti & Wrolstad, 2001). This multistate behaviour has been used to derive molecular machines based on flavonoids, particularly flavylum containing ones like anthocyanins (Melo et al., 2000; Moncada et al., 2004).

## 6. Other techniques

MS, NMR and UV/Vis are the most commonly used techniques to elucidate the structure of flavonoids. Three other techniques are also used: X-ray crystallography, although with the potential to solve complete structures, is hampered by the difficulty to obtain good crystals; circular dichroism and vibrational spectroscopies are used to solve specific structural details.

### 6.1 X-ray crystallography

X-ray crystallography is able to detect the arrangement of atoms within a crystal by the atom-induced diffraction of X-rays. Many materials form crystals, such as salts, metals and organic and biological molecules, in particular proteins. Flavonoids, however, only form crystals in sporadic conditions, and the number of reported flavonoid crystals is very low (Fossen & Andersen, 2005).

Nevertheless, traditional X-ray crystallography has been used to identify the intermolecular  $\pi$ - $\pi$  interactions that guide the stacking of parallel aglycones to form supramolecular layers, and to identify aminoacyl residues involved in the formation of protein-flavonoid complexes, which are critical to the circulation of flavonoids in mammals (Rolo-Naranjo et al., 2010); this is one of the strongest applications of X-ray crystallography to the flavonoid area.

More recently, X-ray powder diffraction has been used, either alone or in association with solid-state NMR, to obtain structures of flavonoids, in particular of catechins (Harper et al., 2010).

### 6.2 Circular dichroism spectroscopy

Circular dichroism (CD) is a spectroscopic technique that allows the analysis of the differential absorption of left and right circularly polarized light. The major advantage of CD over optical rotation measurements is that CD absorption is confined to the narrow absorption range of each individual chromophore, and so it can be used to determine the contribution of individual chromophores and to access their possible substitution patterns (Wallace & Janes, 2009).

One of the most immediate applications of electronic CD and vibrational CD is the determination of the absolute configuration of quiral flavonoid molecules, such as isoflavan-4-ols (Kim et al., 2010). Slade et al., 2005, have reviewed the CD configuration characterization of most classes of flavonoids, and in particular of flavan-3,4-diols, which bear three quiral centers (Ferreira et al., 2004), and more recently proanthocyanidins analysis has also been reviewed, with a focus on CD results (Hümmer & Schreier, 2008).

CD is also routinely used to study the interaction of many flavonoids with biomolecules, providing valuable information on biomolecule-drug interaction, such as DNA binding of

quercetin (Ahmadi et al., 2011), binding to serum albumin (di Bari et al., 2009) and hemoglobin (Chauduri et al., 2011), inhibition of  $\beta$ -amyloid toxicity and fibrillogenesis (Thapa et al., 2011). It must be noted that many of the CD studies of protein-flavonoid association studies are usually accompanied by UV-VIS and/or fluorescence studies, such as the probing of kaempferol interaction with human serum albumin (Matei & Hillebrand, 2010).

### 6.3 Vibrational spectroscopy

Vibrational spectroscopy, in its infra-red and Raman variants, is a spectroscopic technique that analyses the vibrational modes of molecules and molecular groups, allowing bond characterization, and, by comparison with known tabulated data, identification of functional groups; in the case of flavonoids, vibrational spectroscopy has been systematically used to study hydroxyl and carbonyl groups, but more recent technical developments have allowed its application to a broader set of research goals. Raman spectra are much less complex than the IR spectra of the same molecules, and for that reason Raman spectroscopy has been gradually taking over IR spectroscopy, although it is common to use both techniques as complement of each other. Vibrational spectroscopy is seldom used alone, and most studies are accompanied by other spectroscopic approaches and/or quantum chemical computations (Siebert & Hildebrandt, 2007).

Both these spectroscopies are routinely applied to study the effects of substituents on the geometry of the molecule, in particular of dihedral angles, and also on the analysis of intramolecular (Li et al., 2011; Erdogdu et al., 2010) and intermolecular H bonding, either to other flavonoid molecule or to solvent molecules. Similarly, metal complexation by flavonoids is also routinely assessed by vibrational spectroscopy (O'Coinceanainn et al., 2004).

## 7. Conclusion

The role of flavonoids in biological systems appears yet to be far from definitively determined, involving a large number of research groups all over the world. Interestingly, although many new actions of flavonoids *in vivo* have been put forward, the previously proposed actions are never dismissed, only relegated to secondary ways of flavonoid action, usually considered to be important in pathological conditions (Gomes et al., 2008).

As described above, various physical-chemistry techniques have been used as means of characterization of flavonoids. The great amount of work developed since the 1940s yielded a vast library of structural and spectroscopic information about these compounds, making the identification of new isolated species an easy and quick task. However, some limitations have yet to be overcome. For instance, the maximum molecular size allowed in mass spectroscopy (*ca.* 10 kDa) and in NMR spectroscopy (*ca.* 30 kDa) limits the role of these techniques in the characterization of some more complex molecules and molecular complexes, nevertheless, these two techniques have led to great breakthrough in terms of structure elucidation that could not be achieved with the classical spectroscopic techniques like UV/Vis and Vibrational (Raman and Infra-Red) spectroscopy or by X-ray diffraction.

It must be noted that, in many cases, information obtained by NMR or MS needs to be correlated with data from other structural analysis techniques, such as CD, in order to confirm some feeble data. Nevertheless, it has been demonstrated that MS and NMR are the most suitable techniques to determine the chemical structure of flavonoids and its

derivatives. While NMR spectroscopy returns information about atoms and bonding between them, MS gives data about molecular and ion/fragment masses, leading to a more complex and laborious data analysing. This problem can be overcome with the construction of structural databases, which allow an easier and quicker data annotation, as well for NMR spectroscopy. NMR has yet a relevant advantage in the biological studies – the ability of studying them in their natural media. Solid-state NMR can be used to observe flavonoid behaviour in tissues, while solution NMR is useful to determine ligand-acceptor interactions through 2D-NMR experiments such as NOESY, being this one of the greatest tools to undergo protein activity inhibition that can be in the base of the flavonoid biological activity.

It is fair to conclude that although MS methods rarely provide a full molecular determination they are, due to their intrinsic characteristics, the best approach to study flavonoid structures, in complement, when possible, with NMR experiments. For faster cruder screenings, UV absorption data can be used to develop appropriate methods to achieve initial flavonoid class identification.

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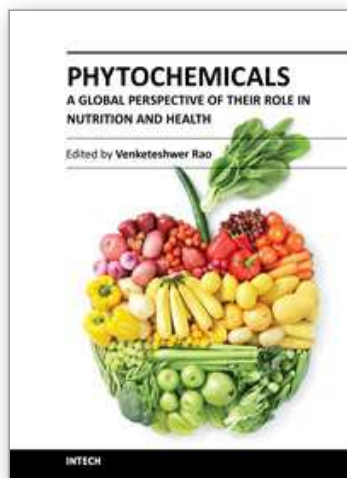
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## **Phytochemicals - A Global Perspective of Their Role in Nutrition and Health**

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Phytochemicals are biologically active compounds present in plants used for food and medicine. A great deal of interest has been generated recently in the isolation, characterization and biological activity of these phytochemicals. This book is in response to the need for more current and global scope of phytochemicals. It contains chapters written by internationally recognized authors. The topics covered in the book range from their occurrence, chemical and physical characteristics, analytical procedures, biological activity, safety and industrial applications. The book has been planned to meet the needs of the researchers, health professionals, government regulatory agencies and industries. This book will serve as a standard reference book in this important and fast growing area of phytochemicals, human nutrition and health.

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