1. Introduction

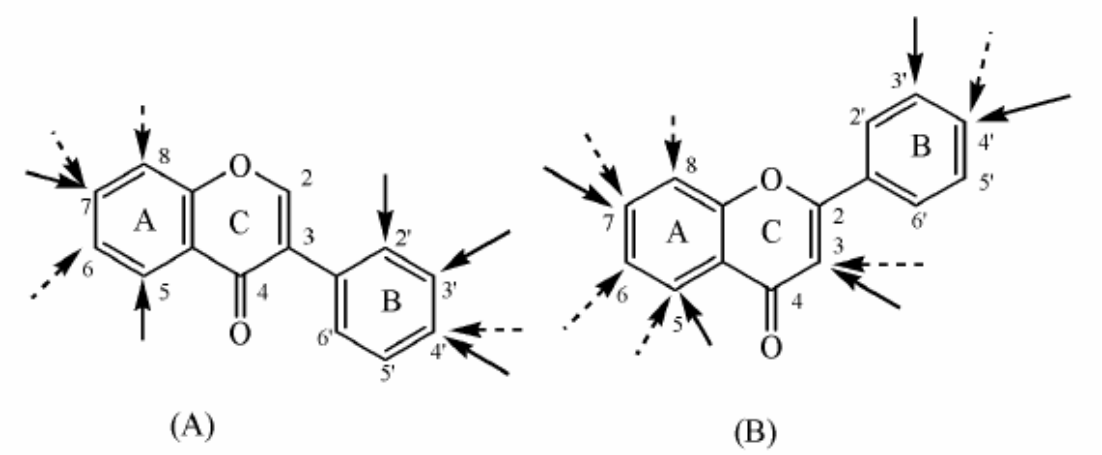
1.1 General approach

The study of flavonoid chemistry has emerged, like that of most natural products, from the search for new compounds with useful physiological properties. Semisynthetic endeavors of oligoflavonoids are in most instances confined to those substitution patterns exhibited by monomeric natural products that are available in quantities sufficient for preparative purposes. In order to alleviate these restrictions, several programs focusing on synthesis of enantiomeric pure flavonoid monomers have been undertaken. However, synthesis of the desired enantiomer in optically pure forms remains a daunting objective and is limited to only a few types of compounds. Chalcone epoxides, α- and β - hydroxydihydrochalcones, dihydroflavonols, flavan-3-ols, flavan-3,4-diols, isoflavans, isoflavanones, and pterocarpans thus far have been synthesized in reasonable yields and purity.

The term "flavonoid" is generally used to describe a broad collection of natural products that include a C6-C3-C6 carbon framework.[1]

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 C6-C3-C6 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.

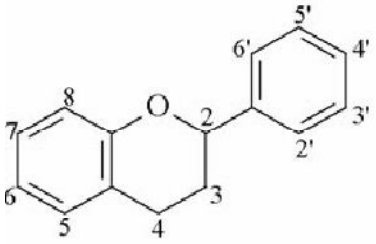
Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. The multiplicity of possible this class were known in the end of the last century and this 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, modifications of flavonoids result in more than 6,000 different compounds from 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[2]. 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[3]. 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. Flavonoids have long sparked the interest of scientists and nonscientists alike, largely because these metabolites account for much of the red, blue, and purple pigmentation found in plants and increasingly for their association with the health benefits of wine, chocolate, and generally with diets rich in fruits and vegetables. Flavonoid compounds are one of the most analyzed groups of secondary metabolites in higher plants. The main reason for the interest in flavonoids is that they are major constituents of plant pigments. Anthocyanins, a flavonoid subclass, have been of special interest because of their ability to confer red, orange, blue, and purple coloration to leaves, flowers, and fruits As pigments, flavonoids have facilitated the testing of hypotheses related to Mendel's law and transposable elements. Flavonoids have been the focus of attempts to modify flower color by genetic engineering . There also is interest in using them as drugs or dietary supplements because of their strong antioxidant

activities[4]. In plants, flavonoids have several functions including attracting insects for pollination and dispersal of seeds, acting in defense systems (e.g., as UV-B protectants and phytoalexins), signaling between plants and microbes, and regulating auxin transport. Many of these functions cannot occur unless flavonoids are properly localized within the cells.[5]

1.2 Nomenclature

The nomenclature of flavonoid ismore specifically a phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: the flavonoids (2-phenylbenzopyrans) 1, isoflavonoids (3-benzopyrans) 2, and the neoflavonoids (4-benzopyrans) 3. These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.



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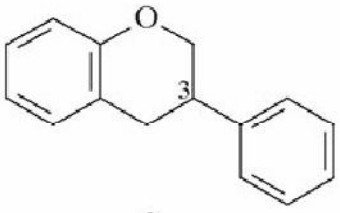

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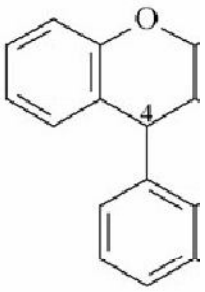

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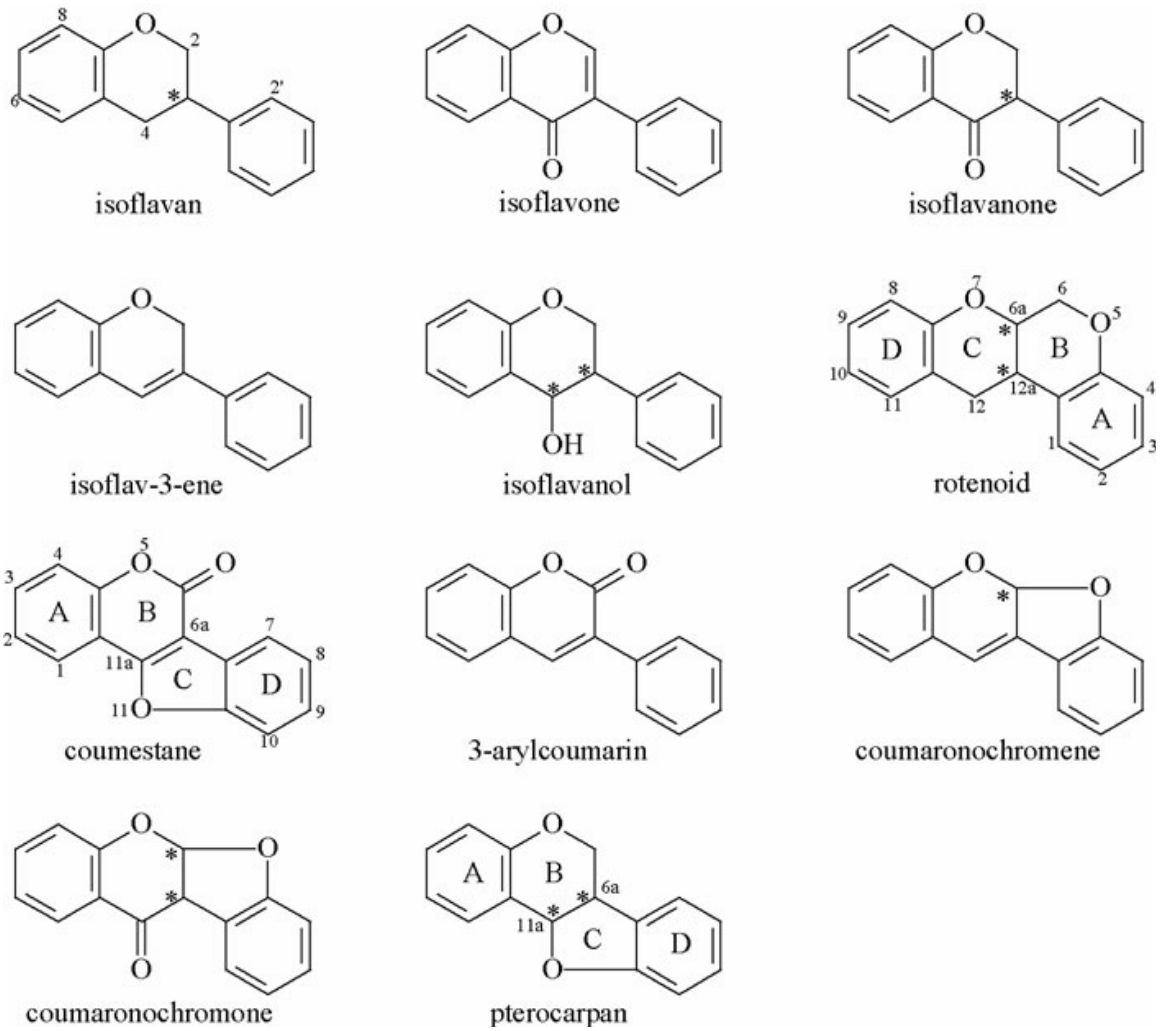
1.2.1 Phenylbenzopyrans (C6-C3-C6 Backbone)

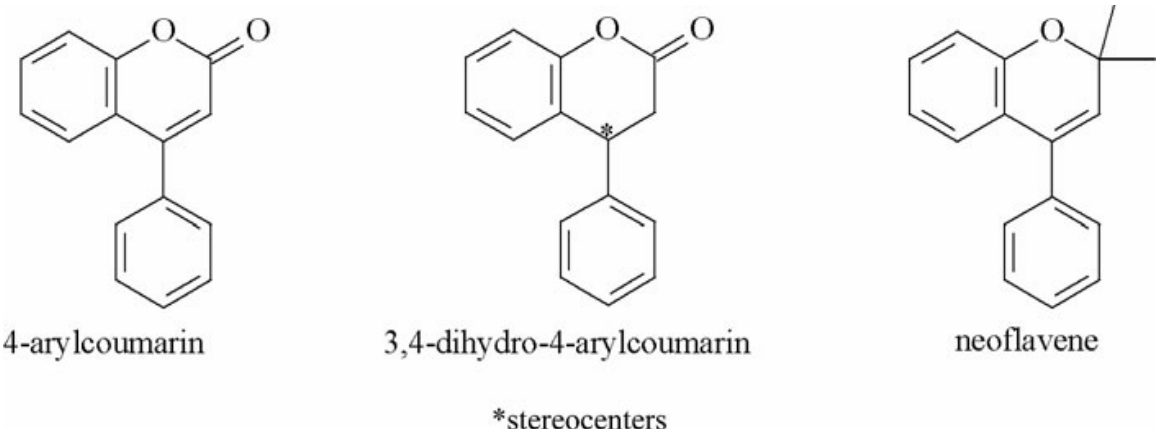
Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups:



1.2.2 Neoflavonoids

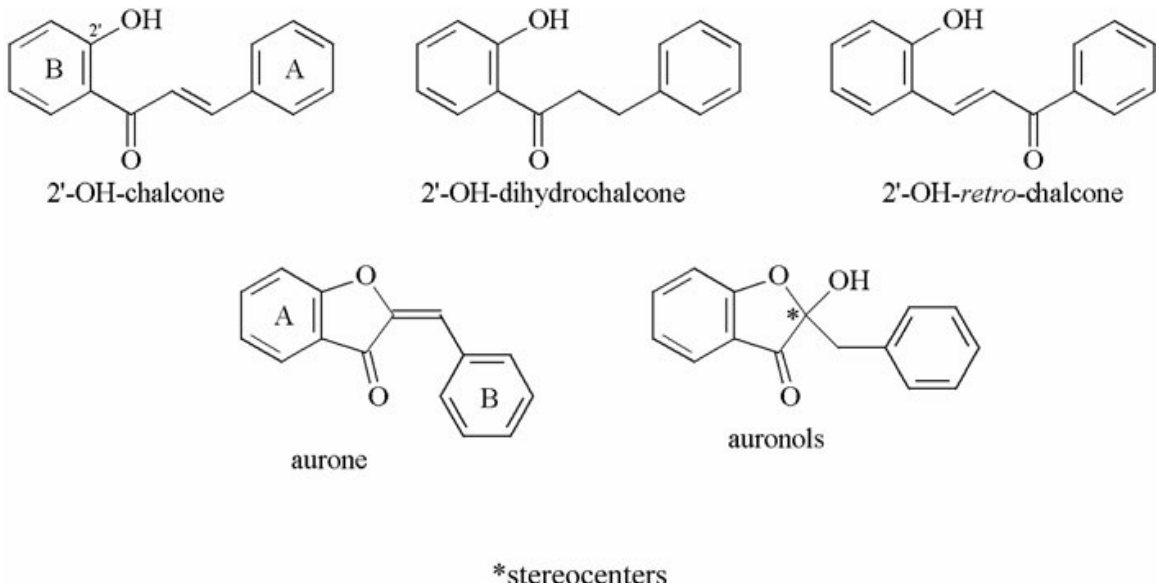
The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1-

benzopyran-2-ones), 3,4-dihydro-4-arylcoumarins, and neoflavenes.



1.2.3 Minor Flavonoids

Natural products such as chalcones and aurones also contain a C6-C3-C6 backbone and are considered to be minor flavonoids. These groups of compounds include the 2′ hydroxychalcones, 2′-OH dihydrochalcones, 2′-OH-retro-chalcone, aurones (2-benzylidenecoumaranone), and auronols[6]

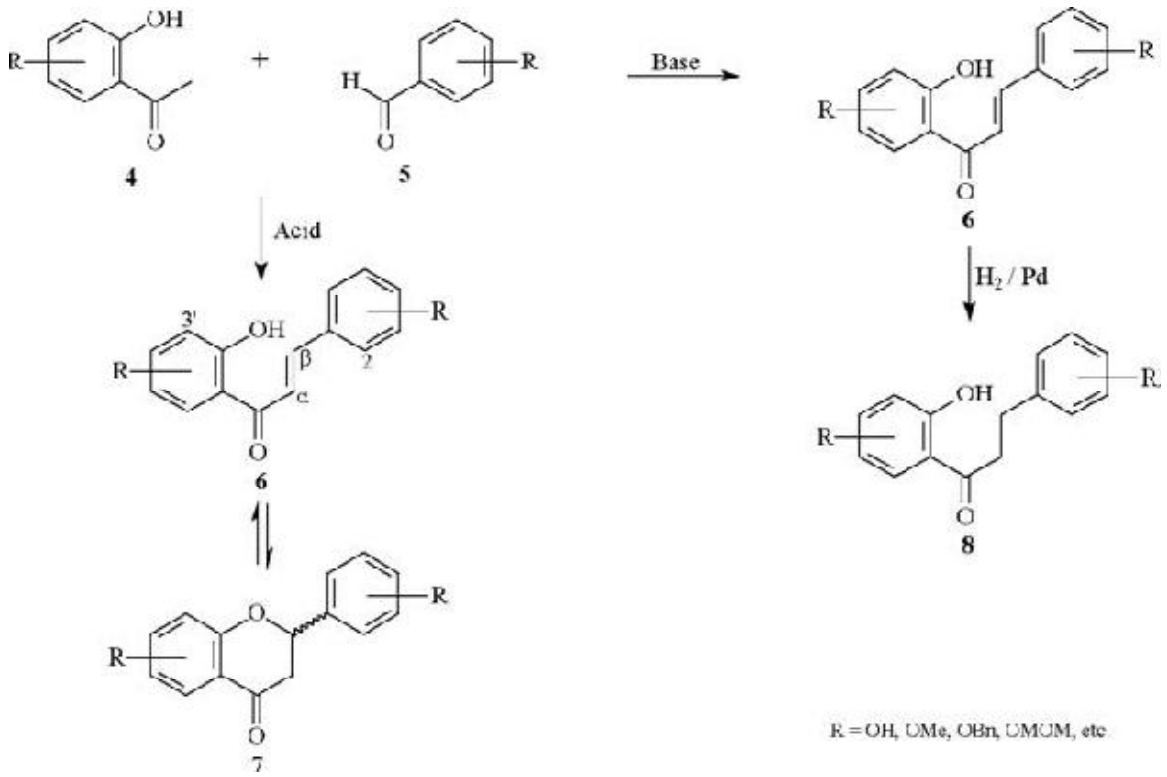


1.3 SYNTHESIS OF FLAVONOIDS

Chalcones, Dihydrochalcones, and Racemic Flavonoids

Chalcones and dihydrochalcones are considered to be the primary C6-C3-C6 precursors and constitute important intermediates in the synthesis of flavonoids.

Chalcones are readily accessible via two well-established routes comprising a base-catalyzed aldol condensation or acid-mediated aldolization of 2-hydroxyacetophenones **4** and benzaldehydes **5** (Scheme 1.1)5. The base-catalyzed aldol condensation is usually the preferred route toward chalcone **6** formation, since under acidic conditions cyclization of the ensuing chalcone leads to formation of corresponding racemic flavanones **7**.6 Dihydrochalcones **8** are generally obtained via reduction (H2/Pd) of the preceding chalcones (Scheme 1.1).



Scheme 1.1 Acid- and base-catalyzed synthesis of chalcones, racemic flavanones, and dihydrochalcones.

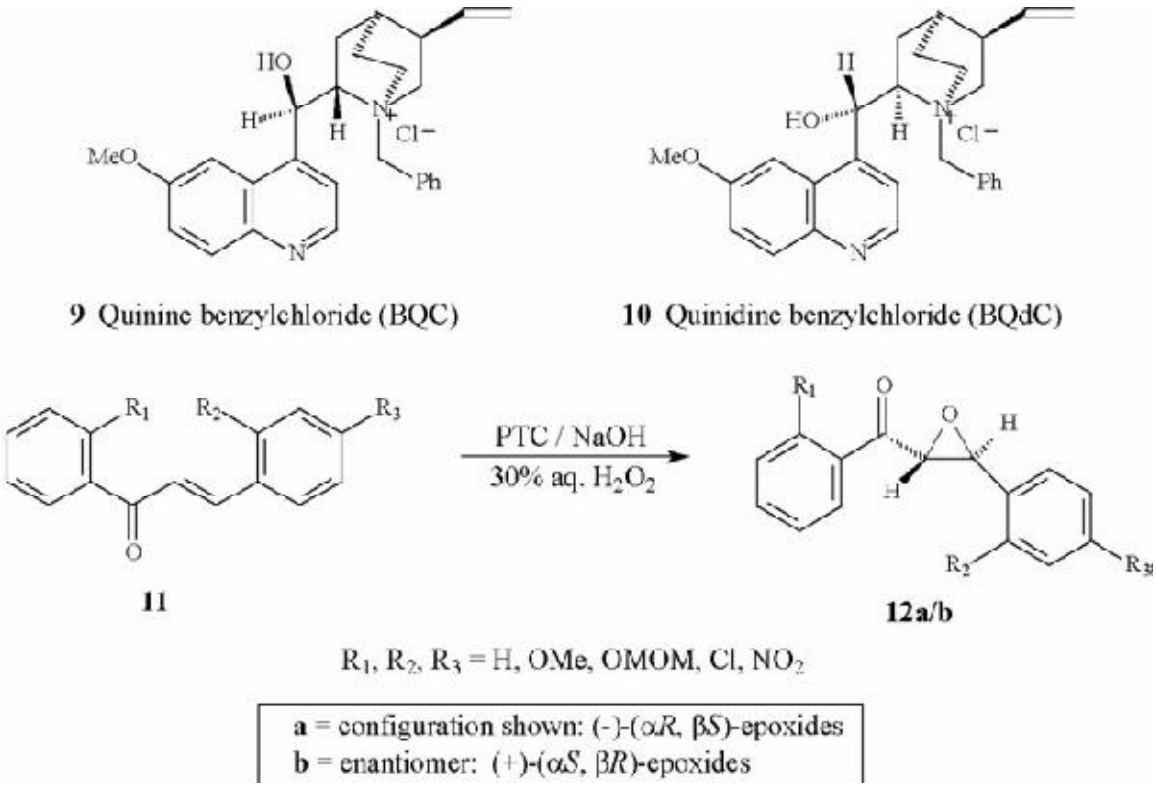
1.3.1 Asymmetric Epoxidation of Chalcones

Asymmetric epoxidation of olefinic bonds plays a crucial role in introducing chirality in the synthesis of several classes of optically active natural compounds.

Sharpless [7]developed viable protocols for the enantioselective epoxidation of allylic alcohols and unfunctionalized olefins. However, attempts regarding the enantioselective epoxidation of α,β-unsaturated ketones, in particular chalcones, have met with limited success.

Wynberg and Greijdanus [8](1978) first reported the utilization of quinine benzylchloride **9** (BQC) and quinidine benzylchloride (BQdC) **10** as chiral

phasetransfer catalysts (PTC). Since then, the use of PTC has emerged as one of the preferred methods for the asymmetric epoxidation of α,β - unsaturated ketones and led to the first stereoselective synthesis of (-)- and (+)-trans-chalcone epoxides 12a/b [yield: 38–92%; enantiomeric excess (ee): 25–48%][9](Scheme 1.2).

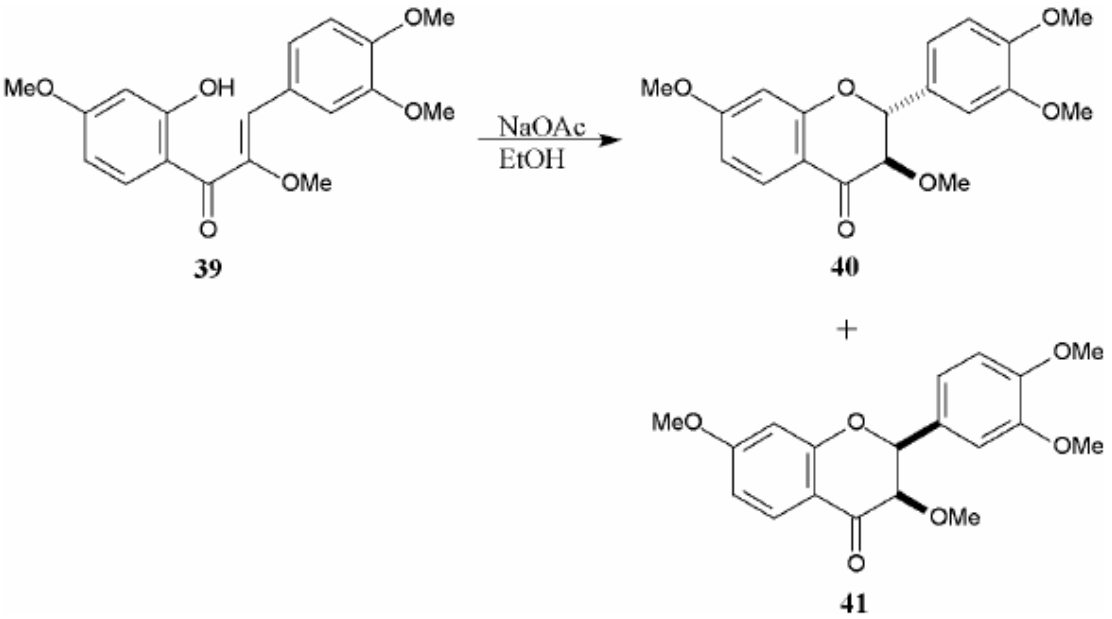


Scheme 1.2 Epoxidation of chalcones **11** with BQC **9** and BQdC **10** as PTC.

1.3.2 Dihydroflavonols

Cyclization of 2′-hydroxy- α,3,4,4′-tetramethoxychalcone 39 with sodium acetate in ethanol furnished both 3,3′,4′,7-O-tetramethyl-2,3-trans-40 and 3,3',4',7-Otetramethyl- 2,3-cis-dihydroflavonols 41 in 22% and 11% yields,

respectively (Scheme 1.3). However, this method was not applicable to cycli-zation of α-OH-chalcones[10]

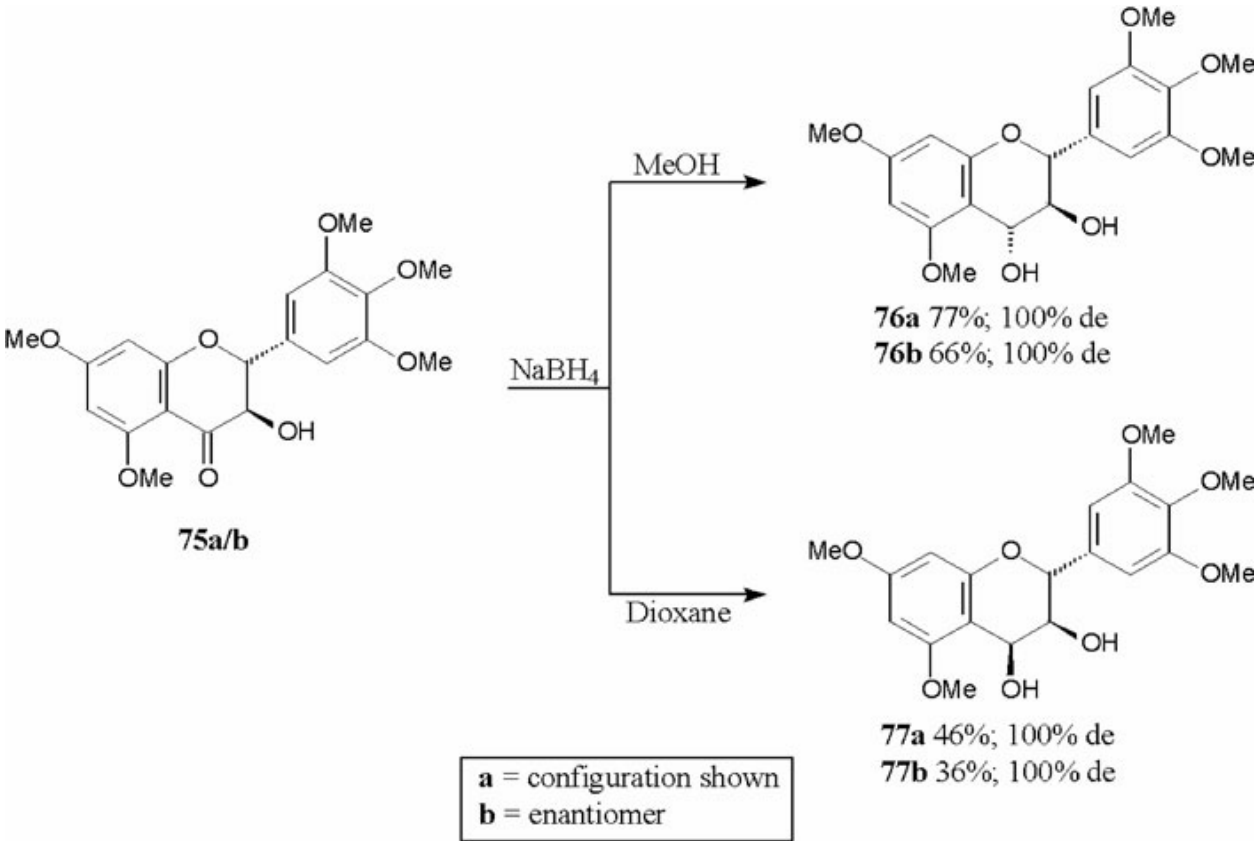


Scheme 1.3 Chalcone cyclization with NaOAc in EtOH to yield trans- and cisdihydroflavonols

Flavan-3-ols and Flavan-3,4-diols one of the most common ways for the synthesis of flavan-3-ols and the closely related flavan-3,4-diol analogues involves the reductive transformation of dihydroflavonols. Reduction of the dihydroflavonols 75a/b with sodium borohydride in methanol affords the

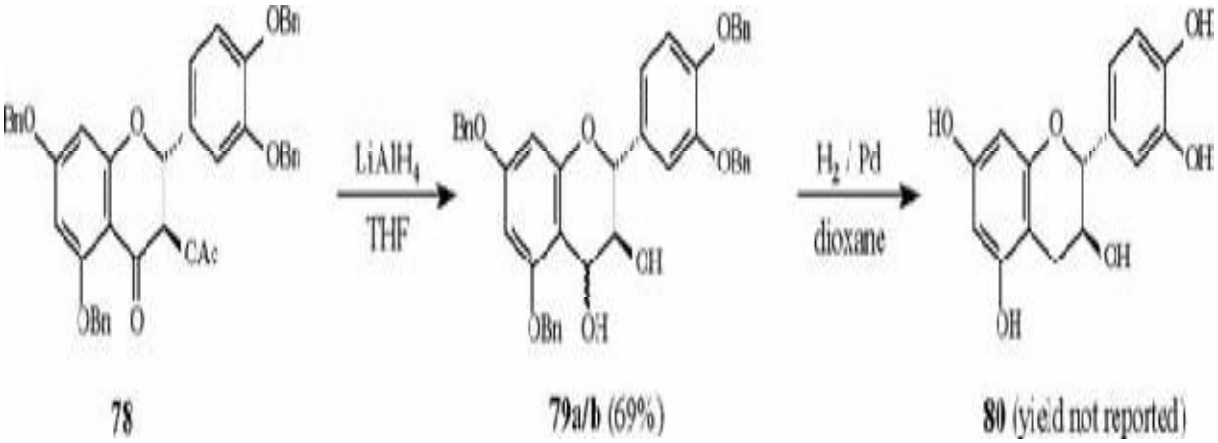
2,3-trans-3,4-trans-flavan-3,4-diols 76a/b, while reduction in an aprotic solvent like dioxane yielded the C4-epimers 77a/b exclusively (Scheme 1.6).

Such reversal in the direction of the hydride attack could probably be explained in terms of the presence of hydrogen bonding in aprotic solvents.11



Scheme 1.6 Reduction of dihydroflavonols with NaBH4 to afford flavan-3,4-diols

Consecutive treatment of 2,3-trans-3-O-acetyldihydroquercetin tetra-O-benzyl ether 78 with LiAlH4 and H2/Pd gave the free phenolic flavan-3-ol 79 in optically pure form (Scheme 1.7).



Scheme 1.7 Reduction of 2,3-trans-3-O-acetyldihydroquercetin tetra-O-benzylether 78 to yield catechin 80.7

1.3.3 Isoflavonoids

the reduction and cyclization of the corresponding 2'-hydroxyisoflavanones, cycloaddition reactions of 2H-chromenes with 2-alkoxy-1,4-benzoquinones and 1,3-Michael–Claisen annulation Only two methods, i.e., asymmetric dihydroxylation of an isoflav-3- ene and subsequent "hydrogenative cyclization" or 1,4- benzoquinone cyclo-addition reactions utilizing chiral Ti(IV) complexes permitted enantioselective access to this class of compounds.[12]

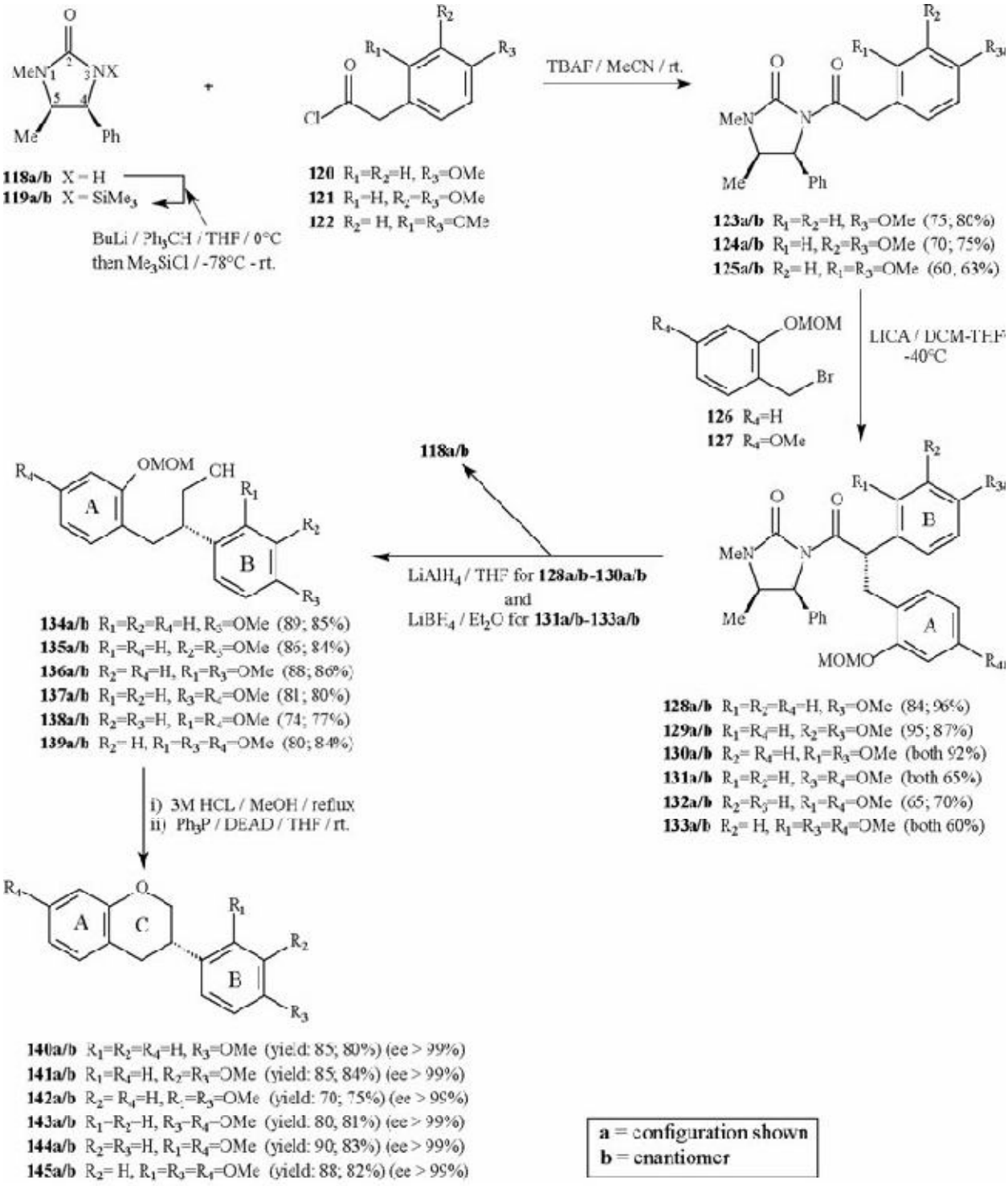
1.3.4 Isoflavans

The protocol involved the stereoselective α-benzylation of phenylacetic acid derivatives, subsequent reductive removal of the chiral auxiliary, and cyclization into the isoflavans (Scheme 1.9).

Owing to the efficiency of the asymmetric alkylation reactions of chiral imide enolates, (4S,5R)-(+)- and (4R,5S)-(-)-1,5-dimethyl-4-phenyl-2-

imidazolidinones **118a** and **118b** were used as chiral auxiliaries in the benzylation reactions [13]. The basicity of the imidazolidinones was decreased by utilizing the trimethylsilyl ethers **119a** and **119b** in the acylation step using the phenylacetyl chlorides **120-122**. The ensuing N-acyl imidazolidinones **123a/b-125a/b** were then alkylated with the appropriate 2-O-methoxymethylbenzyl bromides **126** and **127** in good to excellent yields with only one diastereomer isolated (de > 99%). Removal of the chiral auxiliary was effected by reductive deamination using LiAlH4 in THF for imides **128a/b-130a/b** and a saturated solution of LiBH4 in ether for analogues **131a/b-133a/b** to give the 2,3-diarylpropan-1-ols **134a/b-139a/b** [14]. Acidic deprotection (3M HCl in MeOH), followed by cyclization under Mitsunobu conditions [15] afforded the target >96- 99%).

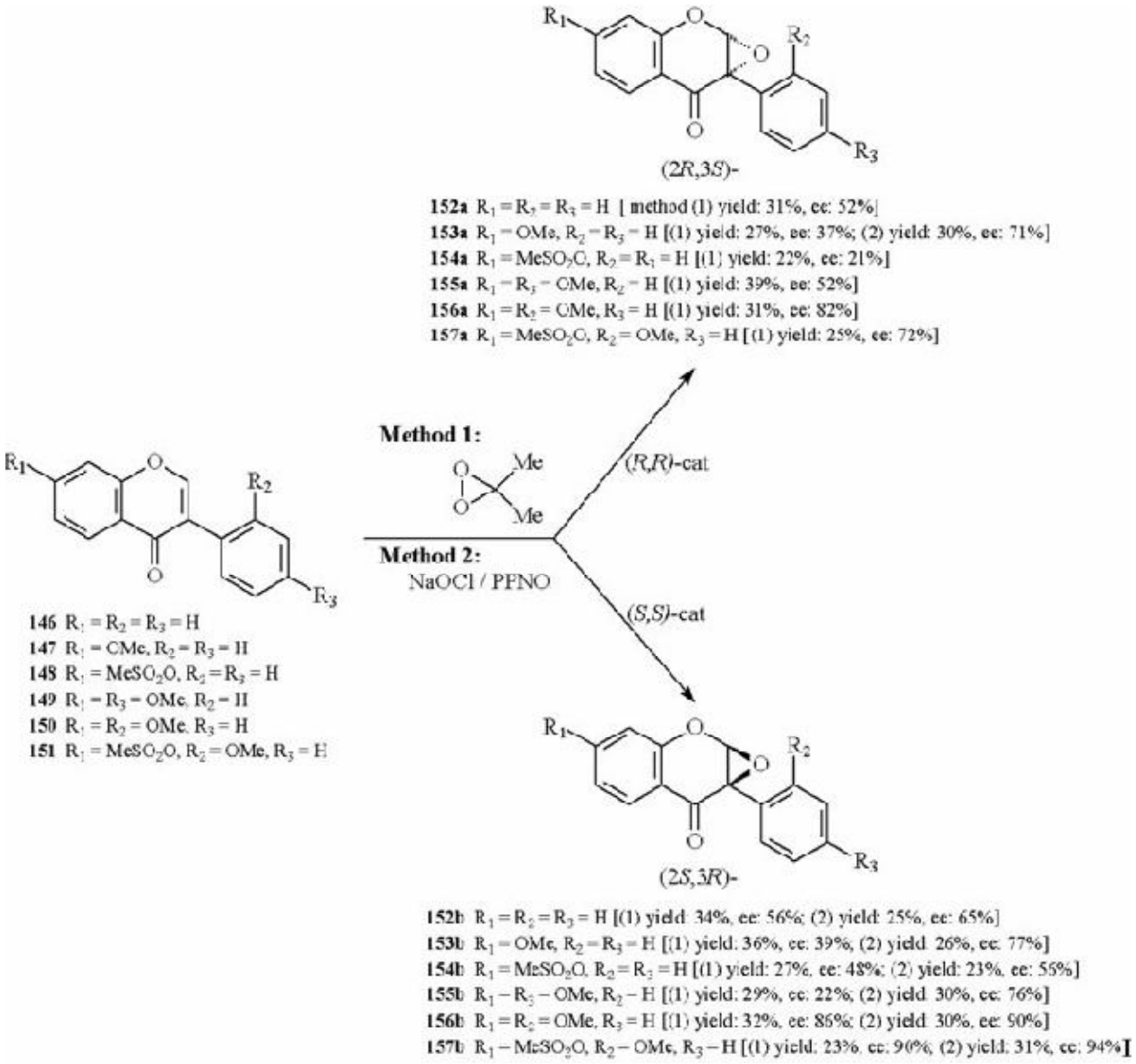
The stereochemistry of the alkylation step is explicable in terms of the preferential formation of a Z-enolate .Attack of the electrophile is then directed to the face of the enolate opposite the phenyl moiety on the chiral auxiliary. The chiral auxiliary with 4S-configuration led to propanols exhibiting positive optical rotations and those from 4R-N-acyloxazolidinones showing negative values, in accordance with observations .[16] Alkylation of (4S,5R)-(+)-N-phenylacetylimidazolidinones resulted in (+)- propanols and (3S)-isoflavans and (4R,5S)-(-)-N phenylacetylimidazolidinones in (-)-propanols and (3R)-isoflavans



Scheme 1.9 Stereoselective synthesis of (R)- and (S)-isoflavans

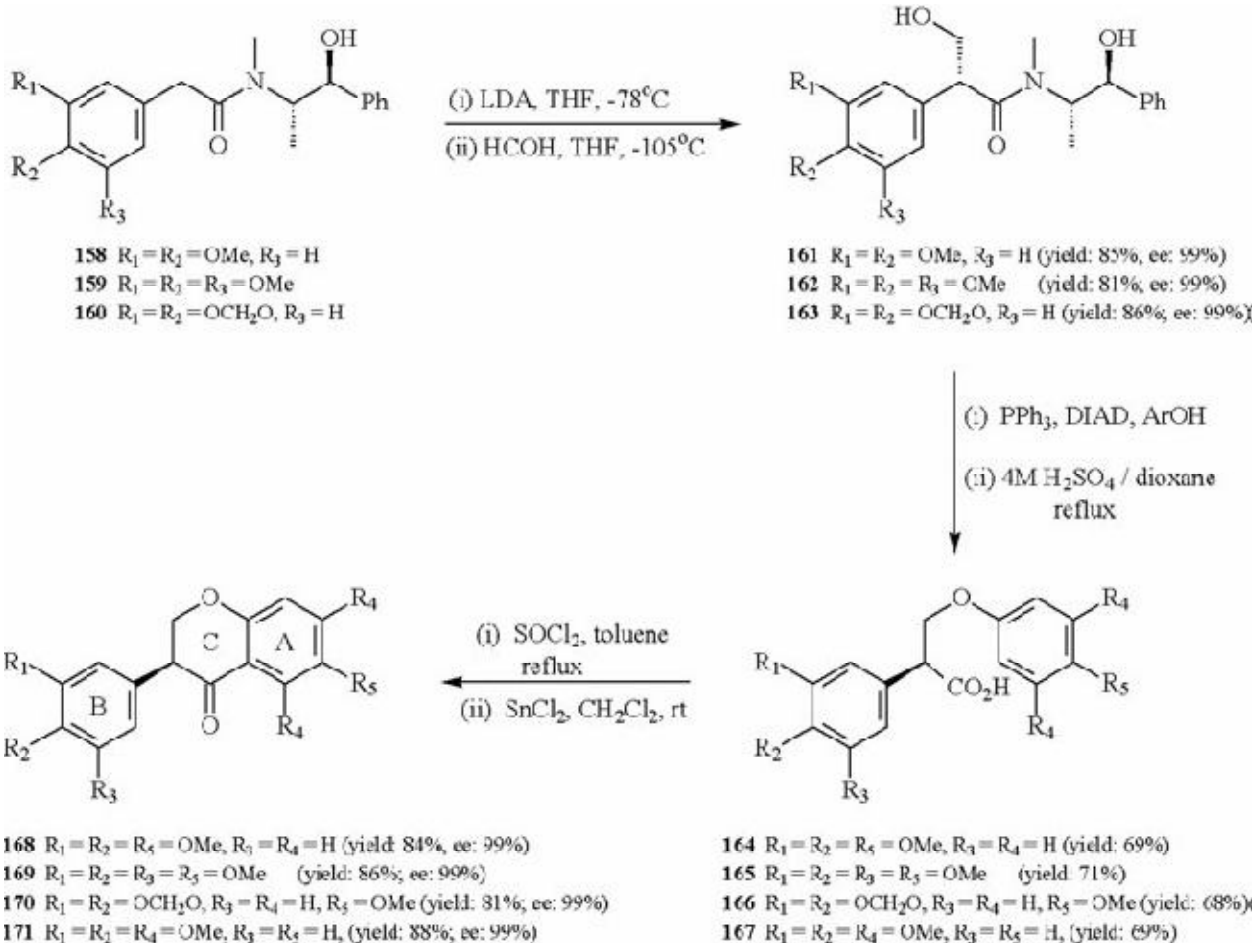
1.3.5 Isoflavone Epoxides

The first representatives of flavone epoxides were prepared either by alkaline hydrogen peroxide epoxidation of isoflavones or by an intramolecular Darzens reaction of α-bromo-O-acyloxyacetophenones. [17].demonstrated that dimethyldioxirane (DMDO) is a convenient and effective reagent for the epoxidation of various substituted isoflavones and subsequently prepared isoflavone glycoside epoxides in high yields by utilizing this versatile oxidizing agent. However, attempts to synthesize enantiomeric isoflavone epoxides with DMDO and a chiral auxiliary demonstrated that the sugar chiral auxiliary did not exercise enantiofacial selectivity and epoxides were isolated as 1:1 diasteromeric mixtures. The Jacobsen's Mn(III)salen complexes have proved to be highly efficient catalyst for the enantioselective epoxidation of olefins by using various oxygen donors. It was demonstrated that epoxidation of 2,2-dimethyl-2H-chromenes, in the presence of optically active Mn(III)salen complexes and DMDO, proceeded enantioselectively. (R,R)- chloride as catalysts and DMDO or NaOCl as oxygen donors, afforded for the first time the optically active isoflavone epoxides **152a/b–157a/b** (Scheme 1.10).



Scheme 1.10 Enantioselective synthesis of isoflavone epoxides **152a/b 157a/b**. Isoflavanones By employing a stereocontrolled aldol reaction as the key step, optically active isoflavones **168–171** were synthesized for the first time by Vicario et al. (2000) in good yields and excellent ee's (Scheme 1.11). This sequence included an asymmetric aldol reaction between (S,S)-

(+)-pseudoephedrine arylacetamide and formaldehyde to introduce chirality in the isoflavanone carbon framework at C-3. This was followed by the introduction of the B-ring as a phenol ether under Mitsunobu conditions and subsequent removal of the chiral auxiliary. Acids **164– 167** were then converted by an intramolecular Friedel–Crafts acylation, yielding the isoflavanones **168–171** in good yields and essentially enantiopure.



158 $R_1 = R_2 = OMe$, $R_3 = H$
159 $R_1 = R_2 = R_3 = OMe$
160 $R_1 = R_2 = OCH_2O$, $R_3 = H$

161 $R_1 = R_2 = OMe$, $R_3 = H$ (yield: 85%, ee: 99%)
162 $R_1 = R_2 = R_3 = OMe$ (yield: 81%, ee: 99%)
163 $R_1 = R_2 = OCH_2O$, $R_3 = H$ (yield: 86%, ee: 99%)

168 $R_1 = R_2 = R_4 = OMe$, $R_3 = R_5 = H$ (yield: 84%, ee: 99%)
169 $R_1 = R_2 = R_3 = R_5 = OMe$ (yield: 86%, ee: 99%)
170 $R_1 = R_2 = OCH_2O$, $R_3 = R_4 = H$, $R_5 = OMe$ (yield: 81%, ee: 99%)
171 $R_1 = R_2 = R_4 = OMe$, $R_3 = R_5 = H$, (yield: 88%, ee: 99%)

164 $R_1 = R_2 = R_5 = OMe$, $R_3 = R_4 = H$ (yield: 69%)
165 $R_1 = R_2 = R_3 = R_5 = OMe$ (yield: 71%)
166 $R_1 = R_2 = OCH_2O$, $R_3 = R_4 = H$, $R_5 = OMe$ (yield: 68%)
167 $R_1 = R_2 = R_4 = OMe$, $R_3 = R_5 = H$, (yield: 69%)

Scheme 1.11 Stereoselective synthesis of isoflavanones **168–171**.

1.4 Isolation and Identification Of Flavonoids

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 (1H and 13C) 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.[18]

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.

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[19] or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60oC to 200oC .[20]

Supercritical fluid extraction with carbon dioxide also may be used 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 (2H or 13C) 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[21] (reviewed in 21. 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 .[22]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 [23]. However, in some cases, flavonoid conjugates can be enzymatically hydrolyzed with external glucuronidases and sulfatases prior to the isolation and analysis of products.

1.5 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 1H hydrogen and/or 13C 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 recordingof 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. 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 [24]. An example of such instruments enabling multistage tandem MS (MSn) are instruments with ion trap analyzers. In these analyzers, the fragment ions created with CID may be further studied using additional MSn stages[25]

1.6 Structural Characterization of Individual Flavonoids and Their Conjugates

1.6.1 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 1H-NMR were initiated in 1960s [26]and along with 13C-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 [27].

1.6.2 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 [28]. 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 [29]. 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 X 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 [30].

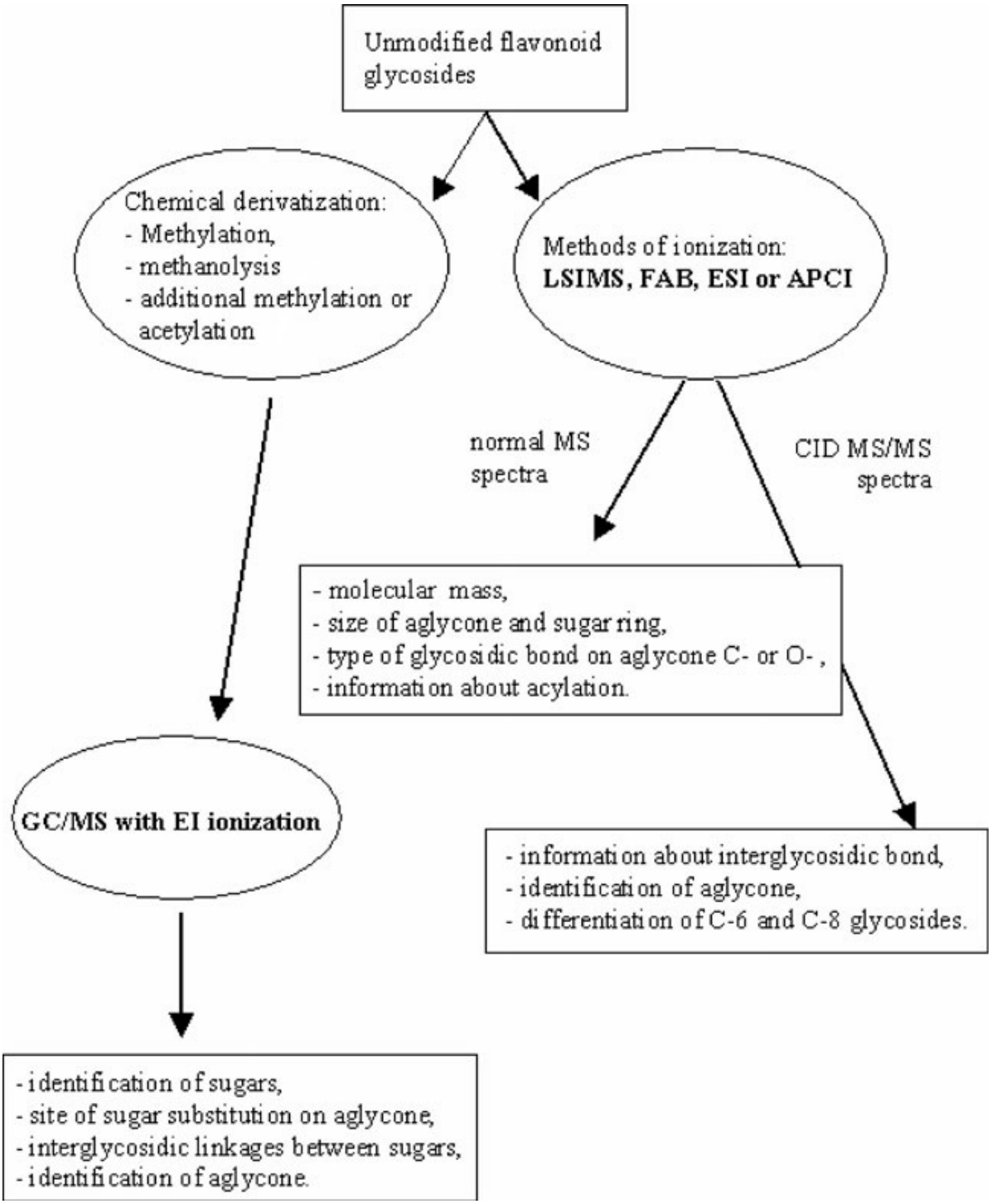


Figure 2.2 Structural information obtainable with different mass spectrometric methods.

1.7 Flavonoids in Foods

A ‘‘poor’’ diet is a major contributing factor to the etiology of chronic diseases such as heart disease and cancer. However, defining what constitutes a ‘‘healthy’’ diet remains contentious, as it is difficult to definitively ascribe beneficial and detrimental properties to thediverse components of the many foods we consume. Nevertheless, considerable evidence indicates that adequate fruit and vegetable consumption has a role in maintaining health and preventing disease. Some of these protective effects may be due to flavonoids, which are widely distributed in plant-based foods at varying levels. For example, numerous in vitro investigations have demonstrated potent effects of flavonoids in mammalian systems that are potentially anticarcinogenic and antiatherogenic. These include antioxidant protection of DNA and low-density lipoprotein, modulation of inflammation, inhibition of platelet aggregation, estrogenic effects, and modulation of adhesion receptor expression.From a nutritional perspective, the actual importance of flavonoids to health and disease remains unclear. Unlike the recognized micronutrients that can be obtained from plantbased diets, such as vitamin E and vitamin C, a lack of dietary flavonoids does not result in obvious deficiency syndromes. Consequently, the initial classification of some citrus flavonoids as ‘‘vitamin P’’ was later revoked.10 In addition, epidemiological studies relating intake of flavonoids to disease incidence or risk do not give consistent results. For example, the average combined intake of flavonols and flavones in a cross-cultural correlation study composed of 16 cohorts followed up for 25 years after initial baseline measurements collected around 1960.was found to be inversely associated with coronary heart disease mortality, statistically

explaining 25% of the variability in rates across the cohorts. Early analysis and identification of flavonoids in plant materials and products led to estimated intakes of up to 1 g/day in the United States. This approximation included flavanones, flavonols, and flavones (160 to 175 mg/day), anthocyanins (180 to 215 mg/day), catechins (220 mg/day), and biflavans (460 mg/day). More recent estimates focusing on flavonol and flavone intake indicate that these early intake levels may be too high. One explanation for this is that flavonoid analysis initially employed semiquantitative spectrophotometric measurement.Analytical methodology has since progressed with the development of more sensitive and specific techniques. Optimized and better-validated sample preparation and hydrolysis techniques are now commonly used. For example, Hertog et al. optimized and tested the completeness of acid hydrolysis and solvent extraction of flavonol glycosides to their free (aglycone) form before quantifying flavonol and flavones concentrations of freeze-dried fruit and vegetable samples. Reversed-phase high-pressure liquid chromatography (RP-HPLC) with ultraviolet (UV) detection has improved isolation and separation of compounds superseding thin layer chromatography isolation and quantification by measurement of UV spectral shifts in response to addition of colorimetric reagents.[31]

1.8 DATABASE OF FLAVONOIDS IN FOODS

The database currently contains entries for 35 types of fruits, 31 vegetables, 26 beverages, eight different jams, three types of chocolate, and 12 herbs. Data are presented as average, minimum, and maximum values (mg/100 g) for each of the flavonoid subclasses; i.e., flavonols, flavones,

proanthocyanidins, catechins, and flavanones. All the flavonol, flavone, and flavanone content data have been determined after optimized hydrolysis of their respective glycosides to the aglycone. Catechin monomers such as epicatechin gallate occur naturally in their free form and are presented as mg catechin/100 g. Procyanidins are presented as their monomeric unit (_)-epicatechin.[32]

1.8.1 FRUITS

Catechins, flavonols, and proanthocyanidins are abundant in fruits. In contrast, flavanones and flavones are restricted to citrus varieties such as oranges and lemons In some fruits (e.g., apples), flavonols are principally present in the skin and hence peeling significantly reduces levels unlike catechins which are found in the flesh of fruits. Overall, catechins were the most abundant flavonoid, ()-catechin (C) and (_)-epicatechin (EC) being particularly prevalent. Black grapes (4.9 and 4.7 mg/100 g, C and EC, respectively) are one of the richest fruit sources of catechins followed by apples (0.8 and 6.3 mg/100 g, C and EC, respectively). Catechins are also relatively abundant in stone fruits, such as blue plums (4.3 and 3.6 mg/100 g, C and EC, respectively) and apricots (2.6 and 3.0 mg/100 g, C and EC, respectively). The gallic acid esters of catechin, (_)-epigallocatechin, (_)-epigallocatechin gallate, (_)-epicatechin gallate, and ()-gallocatechin, are relatively uncommon in fruits, with only berries, currants, and grapes containing small amounts. Strawberries were found to contain the most complex mixture of catechins, comprising catechin (75% of total catechins), ECG (18% of total catechins), EGC (5% of total catechins), and GC (3% of total catechins). Catechin esters have been characterized, but not measured,

in some fruits such as nectarines and mangos. Type B procyanidins are also present in fruits, with apples (3.8 to 15.4 mg/100 g), plums (16.1 mg/100 g), and peaches (3.2 mg/100 g) containing the highest concentrations. However, citrus fruits do not appear to contain detectable levels of catechins or type B procyanidins. Quercetin is the most common flavonol in fruits, elderberries (17.0 mg/100 g), lingonberries (12.6 mg/100 g), and cranberries (13.0 mg/100 g) being particularly rich sources. Berries and currants are also the fruits containing most kaempferol and myricetin. For example, these two flavonols account for 29 and 18%, respectively, of the total flavonol content of the bilberry. Although kaempferol and myricetin have also been identified in fruits such as peaches and pears, concentrations are generally too low to be readily quantified in the whole fruit. The skin of these fruits contains these flavonols in significant amounts; however, their flesh, which constitutes >70% of the fresh weight, does not. Consequently, when analyzed as normally eaten only trace levels are present.

Often termed the citrus flavonoids, flavanones are only found in citrus fruits such as oranges, grapefruit, and lemons. Although naringenin is present at greater concentrations than hesperetin in grapefruit (39.2 and 1.4 mg/100 g, respectively), the latter is the dominant form in oranges, lemons, and limes. Although citrus fruit also contains low levels of flavones, the olive is by far the richest source of luteolin and apigenin (12.4 and 4.6 mg/100 g, respectively).[33]

1.8.2 VEGETABLES

Allium (e.g., onions), Brassica (e.g., broccoli and kale), and Lactuca (e.g., lettuce) varieties of vegetables and tomatoes (Lycopersicon species) are

abundant sources of flavonols, primarily quercetin and kaempferol. Flavones are also found in some vegetables such as celery, sweet peppers, and lettuce. Catechins and type B procyanidins, however, have not been found in leafy green or root vegetables but have been detected in legumes such as broad and green beans. The tomato is the only vegetable (although taxonomically a fruit) to possibly contain the flavanones naringenin and hesperetin.

Of the Allium species, shallots and red onions represent the richest potential source of quercetin containing 95 and 64 mg/100 g, respectively. Brassica vegetables including broccoli, kale, cabbage, and 32russels sprouts tend to contain complex mixtures of flavonols, with significant quantities of kaempferol and myricetin glycosides present in addition to quercetin conjugates. Kale is a good example of this with mean levels of 11.5 mg quercetin/100 g and 34.1 mg kaempferol/100 g. Legumes such as green and broad beans also contain complex mixtures, mainly of flavonols and catechins. For example, broad beans contain (_)-epicatechin (30.0 mg/100 g), ()-catechin (14.5 mg/100 g), (_)-gallocatechin (4.8 mg/100 g), and quercetin, myricetin, and kaempferol at concentrations below 3.0 mg/100 g.

Green chilli pepper is one of the few vegetables to contain both flavonols (quercetin, 11.39 mg/100 g) and flavones (luteolin, 2.7 mg/100 g) at detectable levels. Celery and sweet ball peppers are the main food sources of flavones independent of flavonols.[34]

1.8.3 BEVERAGES

Catechins are often the most common flavonoids in beverages such as fruit juice, tea, and wine These tend to contain complex mixtures of simple catechins and their gallated esters. Type B procyanidins have frequently been characterized in beverages such as fruit juices; however, reliable quantitative data are limited. Flavonols are also present in most beverages while flavanones are again restricted to citrus juices such as grapefruit and orange.

The presence of flavones in beverages is not well described with only some characterization information available in the literature.

Fruit juice contains both catechins and flavonols. Apple juice is one of the richest juice sources of catechins (containing 6.3 mg (_)-epicatechin/100 ml and 0.8 mg ()-catechin/ 100 ml) whereas cranberry juice contains the most flavonols, mainly in the form of quercetin and myricetin (17.5 mg/100 ml and 4.7 mg/100 ml, respectively).

Tea is the only analyzed beverage to contain (_)-epigallocatechingallate (EGCG) in quantifiable amounts. EGCG and (_)-epicatechingallate (ECG) are the most abundant forms, each contributing 27% to the total catechin content (22.2 mg/100 ml) of black tea.

Three flavonols (quercetin, kaempferol, and myricetin) are also found in tea. For example, 100 g of decaffeinated tea contains 5.2 mg quercetin, 2.4 mg kaempferol, and 0.1 mg myricetin.

Wine also contains a complex mix of catechins, flavonols, procyanidins, and flavanones. Red wine contains higher flavonoid levels than white or rose´ wines. Procyanidins usually represent 50% of the flavonoids found in red wine, followed by catechins (37%). A similar profile is observed with beer where again procyanidins dominate accounting for 42% of total flavonoid content.[35]

1.9 Dietary Flavonoids and Health — Broadening

The Perspective Toward the end of the 20th century, epidemiological studies and associated meta-analyses suggested strongly that long-term consumption of diets rich in plant foods offered some protection against chronic diseases, especially cancer. Because uncontrolled production of free radicals was thought to be significantly implicated in the etiology of cancer, these observations focused attention on the possible role of radical scavenging and radical suppressing nutrients and nonnutrients in explaining the apparent benefit of such diets.The realization that free radicals were similarly implicated in the etiology of many other chronic diseases, along with the recognition of the ‘‘French Paradox’’ and the seminal papers from Hertog et al., immediately focused attention on flavonoids and the foods and beverages rich therein. An unfortunate, but unintended side effect of these papers was the tendency of many investigators to think of dietary phenols, polyphenols, and tannins (PPT) as encompassing only the flavonoids, and the flavonoids per se to encompass only the three flavonols and two flavones that featured in those studies, but this is misleading and was never intended. This particular combination of events almost certainly resulted in many subsequent investigations adopting a too narrow focus.

Subsequent epidemiological studies have supported the association between better health and long-term consumption of diets rich in foods of plant origin. However, whether this is because such diets minimize exposure to deleterious substances (e.g., oxidized cholesterol, pyrolysis mutagens, salt, saturated fat, etc.), or maximize intake of certain beneficial nutrients (e.g., isothiocyanates and other sulfur-containing plant constituents, mono-unsaturated fatty acids, and poly-unsaturated fatty acids, PPT, polyacetylenes, selenium, terpenes, etc.) or some combination as advocated in the ‘‘Polymeal’’ concept, remains unknown. An in vitro study indicates that there may be mechanistic basis for true synergy between PPT and isothiocyanates.

In contrast, more recent studies seeking to assess the suggested link between the consumption of flavonols and flavones, or other flavonoids, have given much less consistent results. Some studies have suggested a possible protective effect of flavonoids against vascular diseases or certain (but not all) cancers, whereas other studies have suggested no protective effect or even an increased risk in certain populations. Interestingly, an investigation of the relationship between the consumption of broccoli and other cruciferous vegetables and the risk of breast cancer in premenopausal women attributed the beneficial effects to isothiocyanates and not to the phenolic components, although these crops are good sources of dietary phenols including flavonoids,54–56 and a potential for synergy in vivo has been demonstrated.

In the same time period, various studies have suggested beneficial effects associated with raised consumption of other classes of dietary phenols. For

example, increased coffee consumption has been linked with reduced incidence of type II diabetes. Similarly, increased consumption of lignans (or at least greater plasma concentrations of their metabolites) has been linked with reduced incidence of estrogen-related cancers in some64–66 but not all studies, and a prospective study was equivocal. It has been suggested that this inconsistency might have a genetic basis. Increased consumption of isoflavones has also been associated with decreased risk of estrogen-related cancers and vascular diseases.

This brief introduction demonstrates that the relationships between diet and health are far from simple and most certainly far from fully understood, but for a critical and detailed review of epidemiological data the reader is referred to an excellent paper by Arts and Hollman. The objective of the review that follows is to record recent changes in the perceived role of flavonoids as health-promoting dietary antioxidants and place these observations in a broader context embracing other dietary phenols, and mechanisms other than simple radical scavenging and radical suppression.[36]

1.10 Flavonoid Functions in Plants

The flavonoids are a remarkable group of plant metabolites. No other class of secondary product has been credited with so many—or such diverse—key functions in plant growth and development. Many of these tasks are critical for survival, such as attraction of animal vectors for pollination and seed dispersal, stimulation of Rhizobium bacteria for nitrogen fixation, promotion of pollen tube growth, and the resorption of mineral nutrients from senescing leaves. Others provide a competitive edge to plants that grow under suboptimal environments. Flavonoids, for example, are known

to enhance tolerance to a variety of abiotic stressors, they are employed as agents of defense against herbivores and pathogens, and they form the basis for allelopathic interactions with other plant species. The flavonoids are evidently extremely useful to plants, and it is not surprising, therefore, that species from all orders of the plant kingdom, from the basal liverworts to the most advanced angiosperms, invest significant amounts of metabolic energy into the production of these compounds.

The past decade has witnessed resurgence in research activity on the functions of flavonoids in plants. There are several reasons for this. First, advances in molecular biology, coupled with an improved knowledge of the pathway for flavonoid biosynthesis, have led to the production of plant mutants that are deficient or superabundant in one or more flavonoid pigments. Comparisons of mutant and wild-type phenotypes have permitted hypotheses for flavonoid function to be tested directly. Second, improvements in analytical techniques (e.g., high-performance liquid chromatography, liquid chromatography–mass spectrometry, and nuclear magnetic resonance spectroscopy) for flavonoid compounds have stimulated the search for novel compounds useful for the manipulation of flower color. These, in turn, prompted the discovery of hitherto unknown functions of flavonoids in plant reproduction.

Third, concerns about the enlarging ozone hole and the increased exposure of biota to ultraviolet (UV) radiation led to the quest for sunscreens — and to the knowledge that some flavonoids play an important role in protecting plants from harmful UV-B levels. Fourth, there has been an explosive interest in flavonoids, particularly the anthocyanins, as potential nutritional

supplements for humans. This contributed to the discovery of their antioxidant roles in planta. Finally, advances in field-portable instrumentation have enabled hypotheses for flavonoid function to be tested directly in the field.

In this chapter, we review experimental and theoretical evidence for the main hypotheses for flavonoid functions in plants. Our discussion distinguishes between the functions of the red and blue flavonoids (anthocyanins and 3-deoxyanthocyanins) and those of the colorless (or yellow) remainder. Recent evidence indicates that these two subsets differ markedly both in range and type of functions and, for many species, in their cellular location within the plant tissue. The property of anthocyanin molecules to absorb green light, for example, affords unique capabilities, such as the protection of chloroplasts from the damaging effects of strong

irradiance, and as a visible cue to some animals. Flavonols and flavones, on the other hand, do not directly affect photosynthesis, but they can act as chemical signals or UV guides to attract or deter insects, and are highly effective UV filters. Thus, the comparison of the ‘‘colorful’’ versus ‘‘colorless’’ flavonoids provides an instructive insight into the divergent

evolution of the roles of flavonoids from a common biosynthetic pathway.[37]

1.11 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.

A comprehensive and critical review of food flavonoid literature has led to the development of a food composition database for flavonols, flavones, procyanidins, catechins, and flavanones.

This database can now be used and continuously updated to estimate flavonoid intake of populations, to identify dietary sources of flavonoids, and to assess associations between flavonoid intake and disease. However, there is a need for better food composition data for flavones, procyanidins, and flavanones as current literature is sparse particularly for citrus fruits,

fruit juices, and herbs. In addition, anthocyanin food composition data are lacking although validated methods of determination are becoming available.

2- Materials and Methods

The plant was identified by my project supervisor (Mrs M-abd alkareem).

2-1 Sample Collection:

The plant was collected from North Kordofan state.

2-2 Preparation of the Sample:

The leaves of Tepharosia Apollinea were air-dried after collection . The dried roots were ground into fine powder and then weighed

.**2-3 Extraction of the Sample:**

Solvent-solid extraction was carried out on the weighed, air-dried and roots of Tepharosia Apollinea The weighed sample was soaked with methanol (95%) for two days , with continuose shaking . The separation of the residue from filtrate was done by using filter paper. It was followed by the concentration of the filtrate by using distillation method .

2-4 Chromatography:

The type of chromatographic method was used to separate the constituents that were present in the extract is thin-layer chromatography.

2-4-1 Thin-Layer Chromatography (TLC):

TLC was used to ascertain the number of constituents present in the extract and to determine their purity. TLC was also used to determine the solvent mixture that will affect the separation of the components. The TLC were eluted with butanol :acetic acid : water (4 : 1.5 : 6) .

2-4-2 Preparation of Silica Gel Plates:

50g of silica gel powder was weighed into a conical flask, 100ml of distilled water was added and the resulting solution was shaken vigorously in order to avoid lumps. The white smooth paste mixture was spread over the glass plate and was allowed to solidify. The coated glass plates were put inside oven for 1-2 hours at 1100C to ensure further solidification.

-Spotting of the Plates:

This is done with aid of capillary tubes to introduce few drops of the dissolved sample extract unto the coated plate, allowing each drop to dry before adding another drop.

-Developing of the Plates:

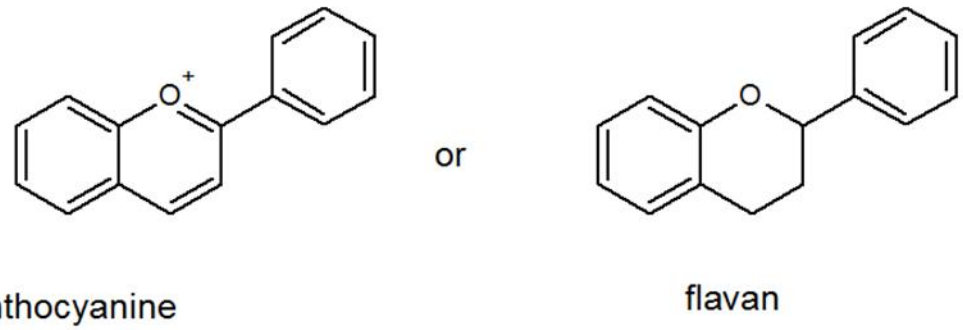
After the solvent had travelled some distance across the plate, the plate was removed and allowed to dry and then spray a clouration reagent which is vaanalin solution with concentrated sulfuric acid . The separated components appeared as dark braown-red spots .

3- Result and Discussion

The flavonoid of the leaves of the tephrosia apollinea were extracted by ethanol .after removing the solvent acurde product was obtained which was fractionated by TLC (silica gel , 4% butanol , 1.5% acetic acid and water 6%) after the usual work up apure flavonoid compound (A) was isolated .

In the IR spectrum (fig I) it gave V(kBr) 700 (C-H Ar) 1050 (C-O),1450 (C=C Ar) 3020, (O-H).

Since the IR revealed absence of C=O structure , then this flavonoid is either an anthocyanine or flavan



anthocyanine flavan

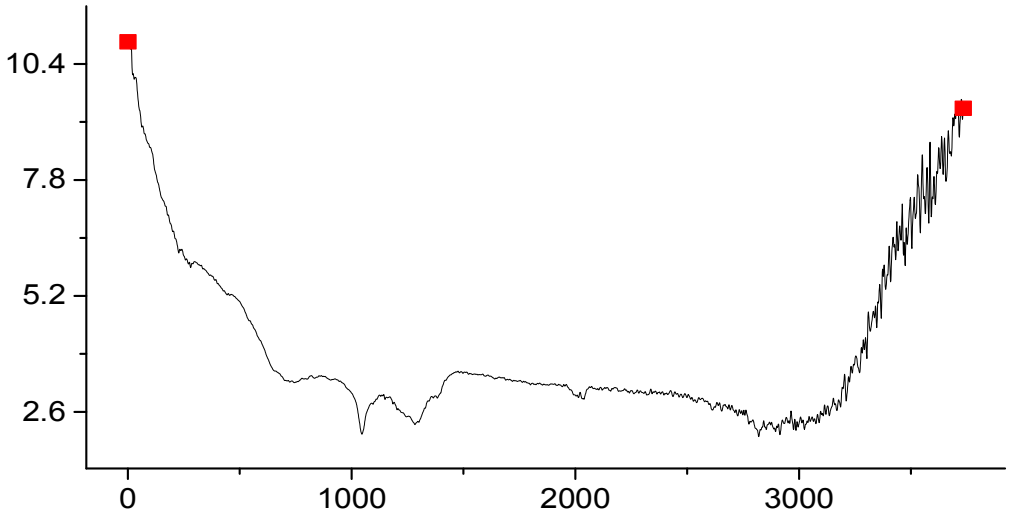


Figure (1): the IR spectraum of Compound (a)

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