Polyphénols d'agrumes (flavanones) : extraction de glycosides de la peau d'orange, synthèse de métabolites chez l'homme (glucuronides) et étude physico-chimique de leur interaction avec la sérum albumine

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Polyphénols d'Agrumes (flavanones) : extraction de glycosides de la peau d'orange, synthèse de métabolites chez l'homme (glucuronides) et étude physico-chimique de leur interaction avec la sérum albumine

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Sommaire

Résume

Un groupe d'études épidémiologiques fournit une bonne preuve de la relation inverse associé à la consommation de fruits et légumes et les maladies chroniques important comme maladies cardiovasculaires et certains types de cancers. Après les longues années d'études sur phytomacronutrients, le rôle de phytomicronutrients tels que les polyphénols est désormais très étudiée et appréciée dans le contrôle de ces maladies dégénératives. La présente étude combine les études d'extraction, de synthèse et d'analyse sur les principaux polyphénols des fruits d'agrumes, FLAVANONES.

Connaissance de nutritionnels et de santé a augmenté la production d'agrumes en provenance des dernières décennies. Ces productions plus générer des bye-produits. Pour leur utilisation alternative à des antioxydants extraits riches, l'extraction assistée par ultrasons (UAE) des polyphénols en particulier flavanones de l'orange (*Citrus sinensis* L.) par son peau en utilisant l'éthanol comme solvant de qualité alimentaire a été prouvé son efficacité en comparaison avec la méthode conventionnelle . Un plan composite central (CCD) a révélé que l'approche des conditions optimisées pour UAE ont une température de 40 ° C, une puissance de 150W sonication et un 4:1 (v / v) d'éthanol: ratio de l'eau. En outre, l'activité antioxydante déterminée par les tests DPPH et ORAC a confirmé la pertinence des UAE pour la préparation d'extraits de plantes riches en antioxydants.

Les glucuronides de flavanone sont les principaux métabolites phénoliques détectés dans le plasma humain après la consommation d'agrumes. Jusqu'à maintenant, toutes les études sur les cellules liées au cancer ou les maladies cardiovasculaires ont été réalisées soit sur les aglycones ou sur leurs glycosides. Par conséquent, il ya grand besoin de glucuronides flavanone pure pour démontrer le potentiel réel de flavanones dans la prévention de ces maladies. Dans ce travail, glucuronides de naringénine (4'- et 7-*O*-*β*-D-glucuronides) et de hespérétine (3'- et 7-*O*-*β*-D-glucuronides), les aglycones flavanone majeur dans le pamplemousse et d'orange, respectivement, ont été synthétisés chimiquement par une protection et la déprotection sélective des groupements d'acide glucuronique et de flavanone. La caractérisation structurale complète de composés purifiés a été réalisée par résonance magnétique nucléaire et spectrométrie de masse.

L'affinité des quatre glucuronides pour l'albumine sérum d'humaine (HSA) a été testée par leur capacité à éteindre la fluorescence intrinsèque de HSA (*Trp*, seul résidu de sous-domaine IIA). Leurs constantes de fixation (*K*) ont été estimées de l'ordre de 30 à 60 × 10^3 M^{-1} et comparées à celles de l'aglycones (70 à 90 × 10^3 M^{-1}). Les enquêtes de la liaison compétitive ou non compétitive de la glucuronides dans la présence de sondes fluorescentes (sarcosine dansyl) nous a permis d'obtenir un aperçu dans les sites de liaison. L'étude a également été étendue aux chalcones hespérétine et naringénine (synthétisés en utilisant des conditions alcalines optimisée), qui sont les précurseurs de biosynthèse des flavanones.

Mots-clés: agrumes, les polyphénols, flavanone, l'extraction assistée par ultrasons, la synthèse, l'albumine sérique humaine.

Abstract

A bunch of epidemiological studies provides good evidence on the inverse relationship associated with the consumption of fruits and vegetables and the chronic diseases importantly cardiovascular diseases and some types of cancers. After the long years of study on phytomacronutrients, the role of phytomicronutrients such as polyphenols is now highly studied and appreciated in the control of such degenerative diseases. The present study combines the extraction, synthetic and analytical studies on the major polyphenols of citrus fruits, FLAVANONES.

Awareness of nutritional and health facts has increased the production of citrus fruits from last few decades. These higher productions generate higher by-products. For their alternative utilisation to have antioxidants rich extracts, the ultrasound-assisted extraction (UAE) of polyphenols especially flavanones from orange (*Citrus sinensis* L.) peel by using ethanol as a food grade solvent has been proved its efficiency when compared with the conventional method. A central composite design (CCD) approach revealed that the optimized conditions for UAE were a temperature of 40°C, a sonication power of 150W and a 4:1 (v/v) ethanol:water ratio. Furthermore, the antioxidant activity determined by the DPPH and ORAC tests confirmed the suitability of UAE for the preparation of antioxidant-rich plant extracts.

Flavanone glucuronides are the major phenolic metabolites detected in human plasma after consumption of citrus fruits. Up to now all cell studies related to cancer or cardiovascular diseases were conducted either on the aglycones or on their glycosides. Hence, there is great need of pure flavanone glucuronides to demonstrate the real potential of flavanones in the prevention of these diseases. In this work, glucuronides of naringenin (4′- and 7-*O*-*β*-D-glucuronides) and hesperetin (3′- and 7-*O*-*β*-D-glucuronides), the major flavanone aglycones in grapefruit and orange respectively, have been chemically synthesized by selective protection and deprotection of flavanone and glucuronic acid moieties. The complete structural characterisation of purified compounds were realised by nuclear magnetic resonance and mass spectrometry.

The affinity of the four glucuronides for human serum albumin (HSA) was tested via their ability to quench the intrinsic fluorescence of HSA (single *Trp* residue in sub-domain IIA). Their binding constants (*K*) were estimated in the range of $30 – 60 \times 10^3$ M^{-1} and compared with those of the aglycones ($70 – 90 \times 10^3$ M^{-1}). Investigations of competitive or noncompetitive binding of the glucuronides in the presence of fluorescent probes (dansyl sarcosine) allowed us to get some insight in the binding sites. The study was also extended to the hesperetin and naringenin chalcones (synthesised using optimized alkaline conditions), which are the biosynthetic precursors of flavanones.

Keywords: citrus, polyphenols, flavanone, ultrasound-assisted extraction, synthesis, human serum albumin.

Publications scientifiques

Revues internationales

- **Muhammad Kamran KHAN**, Maryline ABERT-VIAN, Anne-Sylvie FABIANO-TIXIER, Olivier DANGLES, Farid CHEMAT
  Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis L.*) peel. ***Food Chemistry***, 2010, *119*, 851-858

- **Muhammad Kamran KHAN**, Njara RAKOTOMANOMANA, Michèle LOONIS, Olivier DANGLES
  Chemical synthesis of citrus flavanone glucuronides
  Journal of Agricultural and Food Chemistry, 2010, *58*, 8437–8443

- **Muhammad Kamran KHAN**, Njara RAKOTOMANOMANA, Claire DUFOUR, Olivier DANGLES
  Affinity of flavanone aglycones and the corresponding chalcones for human serum albumin (HSA). Influence of HSA on the flavanone – chalcone isomerization
  (soumission imminente)

- **Muhammad Kamran KHAN**, Njara RAKOTOMANOMANA, Claire DUFOUR, Olivier DANGLES
  Binding of flavanone glucuronides to human serum albumin
  (soumission imminente)

- **Muhammad Kamran KHAN** & Olivier DANGLES
  A multidisciplinary review on Flavanones (en projet)

Communications internationales

- 3ème Conférence Internationale sur les Fruits & Légumes (FAV HEALTH), 18-21 oct 2009, Avignon, France
  Présentation orale: Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (citrus sinensis L.) peel

- 4ème Conférence Internationale sur Polyphénols et Santé (ICPH), 7-10 déc 2009, Yorkshire, Angleterre

Poster: Chemical synthesis of flavanone glucuronides and investigation of their affinity for human serum albumin

- 5ème Journées Franco-italiennes de Chimie (GIFC), 26-27 avr 2010, Gênes, Italie
  Présentation orale: Chemical synthesis of flavanone glucuronides and investigation of their affinity for human serum albumin

- 25ème Conférence Internationale sur Polyphénols (ICP), 24-27 août 2010, Montpellier, France
  Présentation orale: Chemical synthesis of flavanone glucuronides and chalcones, and investigation of their affinity for human serum albumin

Communications nationales

- 21ème journée de la Société Chimique de France (SCF-PACA), 16 avr 2009, Marseille, France
  Poster: Synthèse chimique de deux métabolites des polyphénols majeurs (flavanones) d'agrumes

- Journée Ecole doctorale Sciences des Procédés – Sciences des Aliments (SP-SA), 18 juin 2009, Montpellier, France.
  Poster: Synthèse des glucuronides de flavanones, les métabolites principaux obtiennent après l'ingestion d'agrumes

- 6ème Rencontre de Chimie Organique de Marseille (RCOM), 6-7 mai 2010, Marseille, France
  Présentation orale: Chemical synthesis of dietary flavanone metabolites and study of their interaction with human serum albumin.

- Journée Ecole doctorale Sciences des Procédés – Sciences des Aliments (SP-SA), 22 juin 2010, Montpellier, France.
  Poster: Synthèse chimique des glucuronides et chalcones de flavanones et l'étude cinétique de chalcones à cyclisé dans la sérum albumine humaine.

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Présentation de l'Unité Mixte de Recherche « Sécurité et Qualité des Produits d'Origine Végétale »

Directeur : Christophe N'Guyen-Thé (INRA).

Directeur adjoint : Olivier Dangles (Université d'Avignon).

Objectifs généraux

Améliorer ou préserver les caractéristiques organoleptiques, hygiéniques et nutritionnelles des fruits et légumes frais ou transformés. Les travaux de recherche concernent l'ensemble de la filière:

- élaboration de la qualité avant récolte,
- mise au point de technologies de conservation ou de transformation permettant de valoriser au mieux cette qualité,
- intérêt en nutrition préventive et maîtrise du risque microbiologique associé au développement des produits réfrigérés prêts à l'emploi.

Pour répondre à ces objectifs, l'activité est répartie entre trois équipes de recherches :

- Chimie des Antioxydants,
- Propriétés physiques et physiologiques des fruits et légumes,
- Microbiologie et hygiène.

Équipe Chimie des Antioxydants

L'activité de l'équipe Chimie des Antioxydants est centrée sur les microconstituants des plantes d'importance alimentaire, en particulier les polyphénols et les caroténoïdes.

Les microconstituants sont présents :

- dans la plante où leurs fonctions sont généralement bien établies,
- dans l'aliment d'origine végétale où ils peuvent subir diverses transformations chimiques (ex. : oxydation) au cours des procédés technologiques ou des traitements domestiques,

- chez l’homme (après ingestion) où les questions de leur biodisponibilité, métabolisme et effet santé sont reconnues depuis une vingtaine d’années comme un enjeu scientifique majeur.

L’équipe Chimie des Antioxydants déploie son activité sur l’ensemble de ces trois champs (plante, aliment, homme) :

- **Plante** : analyse de la composition en microconstituants, optimisation des procedures d’extraction par le recours aux technologies ultrasons et micro-ondes.
- **Aliment** : analyse de la composition en microconstituants et de son évolution au cours d’opérations technologiques.
- **Homme** : étude des bases physico-chimiques des effets santé potentiels des polyphénols et caroténoïdes (biodisponibilité, interactions, pouvoir antioxydant, oxydation). L’effort de synthèse chimique (accès à des formes conjuguées de polyphénols et à des métabolites oxydés de caroténoïdes) est également dirigé dans ce sens.

Avant – Propos

Le régime méditerranéen, caractérisé par une consommation élevée de fruits et légumes, est associé à un allongement de l'espérance de vie et à une protection de la santé. De nombreuses études épidémiologiques ont suggéré que la consommation régulière de fruits et légumes permettait de lutter contre diverses pathologies dégénératives associées au stress oxydant telles que maladies cardiovasculaires voire les maladies neurodégénératives et certains cancers. Actuellement, grâce au développement des méthodes d'analyses physico-chimiques et biologiques, nous acquérons une meilleure connaissance de la composition des plantes d'importance alimentaire et des aliments qui en dérivent, du devenir de leurs principaux composants après ingestion et des effets nutritionnels qui en découlent.

Les polyphénols sont quantitativement les plus importants métabolites secondaires des plantes. Ils possèdent une grande variété de structures allant de composés contenant un simple noyau phénolique (acide phénoliques) à des composés polymériques complexes comme les tanins (polymères de catéchine et épicatéchine présentant plusieurs dizaines d'unités). Les polyphénols constituent les principes actifs de nombreuses plantes médicinales ; ils ont la capacité de moduler l'activité d'un grand nombre d'enzymes et de certains récepteurs cellulaires. En outre, in vitro, un grand nombre de polyphénols sont reconnus pour leurs propriétés antioxydantes, anti-inflammatoires, antifongiques, antivirales et anticancéreuses. Plus de deux cents études ont été réalisées sur l'impact de la la consommation de végétaux sur la santé. La plupart ont mis en évidence une baisse du facteur de risque pour de nombreuses affections (infarctus, cancers du poumon, du côlon, de l'estomac, du rein, de la prostate et du sein). Les polyphénols présentant une activité antioxydante sont de plus en plus étudiés. En effet, l'oxydation est un phénomène largement répandu aussi bien dans le domaine alimentaire (oxydation des lipides) que physiologique (stress oxydant). L'ingestion de polyphénols par l'intermédiaire des fruits et des légumes pourrait permettre à notre organisme de renforcer ses moyens de défense contre les processus d'oxydation qui menacent quotidiennement nos cellules, même si les mécanismes mis en jeu dépassent sans doute largement la réduction directe des espèces oxygénées réactives par les polyphénols. Un des objectifs de la recherche est de parvenir à établir les preuves des effets de la consommation de polyphénols sur la santé et à identifier, parmi les centaines de polyphénols, ceux qui pourraient jouer un rôle protecteur plus important dans une optique de nutrition préventive.

Aujourd'hui encore, ces molécules n'ont pas livré tous leurs secrets. Notre travail s'inscrit dans un programme de recherche visant à mieux comprendre le devenir des polyphénols chez l'homme après ingestion (biodisponibilité).

Cette étude est centrée sur les FLAVANONES, des polyphénols abondants dans toutes les espèces d'agrumes. Après un rappel des structures et des propriétés des flavanones dans un premier chapitre, une étude de l'extraction des glycosides de flavanones à partir de la peau d'orange sera présentée. Dans une troisième partie, nous exposerons la synthèse de formes conjuguées (glucuronides) de flavanones d'importance alimentaire. Ces formes conjuguées sont typiquement formées du fait de l'activité des enzymes de conjugaison humaines, en particulier dans les cellules intestinales et le foie. En outre, les chalcones sont les précurseurs des flavanones dans la voie de biosynthèse. Les chalcones isomères des flavanones étudiées seront également préparées.

Dans la dernière partie, l'affinité des composés synthétisés pour la sérum albumine, protéine impliquée dans le transport de métabolites de polyphénols dans le plasma, sera étudiée.

Enfin, le manuscrit se terminera par une discussion générale qui permettra de dégager quelques perspectives de prolongement à ce travail.

Chapitre 1

Les Polyphénols d'Agrumes: Les Flavanones

Les Polyphénols d'Agrumes: Les Flavanones

It is now well accepted that a low consumption of fatty foods, regular physical activity and a high consumption of plant-derived foods help maintain a good health status. In particular, there is an association between an increased level of fruits and vegetables in the diet and a reduced risk of some life-threatening diseases such as cardiovascular diseases and cancer (Parr & Bolwell, 2000). There is growing acceptance that many phenolic secondary metabolites (polyphenols) present in foodstuffs may exert beneficial effects in the prevention of these degenerative diseases (Del Rio *et al.*, 2010). Over the last few decades, the worldwide consumption of citrus fruits and juices has been increasing, thereby stimulating the research on the most abundant bioactive citrus phenols, i.e. FLAVANONES.

1.1. Chemistry and Classification:

Polyphenols are classified into two major classes: Flavonoids and NonFlavonoids. The later one includes the structurally simple molecules such as phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) and stilbenes, and complex molecules comprising of stilbene oligomers, tannins and lignins (Cheynier, 2005). The former, the most studied subclass of polyphenols, represents about more than 9000 identified compounds (Marten and Mithöfer, 2005; Pietta, 2000). Flavonoids commonly share the same generic structure, the flavan nucleus, consisting of two aromatic rings (A and B) linked by an oxygen-containing pyran ring (C). Differences in the linkage of aromatic ring (B) to the benzopyran (chroman) moiety (A and C) allow to distinguish between flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans), and neoflavonoids (4-benzopyrans) (Fig. 01). The 2-phenylbenzopyrans are further divided into two groups depending on the presence of a hydroxyl group at position C-3 of C-ring. These include: 3-hydroxyflavonoids, which contain a hydroxyl group (flavonols, flavanols, anthocyanidins, dihydroflavonols), and 3-deoxyflavonoids, which are short of a hydroxyl group (flavanones and flavones). Flavones differ from flavanones by a C2-C3 double bond (Fig. 02) (Marais *et al.*, 2006). The flavanone class encompasses an array of compounds with simple and complex structures referring to their *O*- and/or *C*-substitutions (hydroxy, methoxy, methylenedioxy, *C*-methyl, *C*-hydroxymethyl, *C*-formyl groups), isoprenoid substituents (noncyclic isoprenoid group, furano or dihydrofurano rings, dimethylpyrano or dimethyldihydropyrano rings), *C*-benzyl groups, stilbene and anastatin moieties, conjugations to phenolic acids, and diarylheptanoid attachments (Veitch and Grayer, 2006).

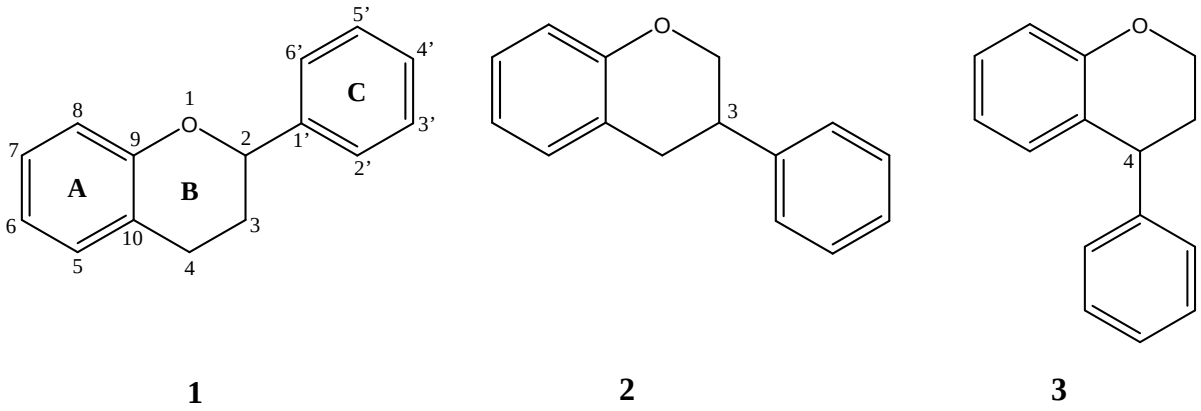

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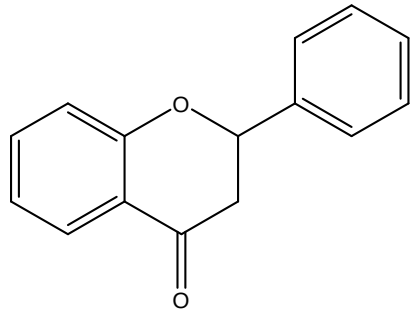
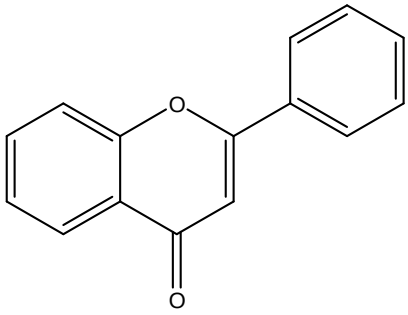
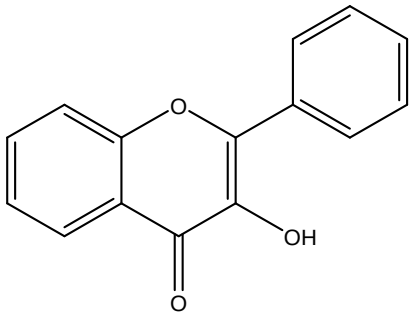
Figure 01: Basic structures of Flavonoids **1**, Isoflavonoids **2**, and Neoflavonoids **3**.



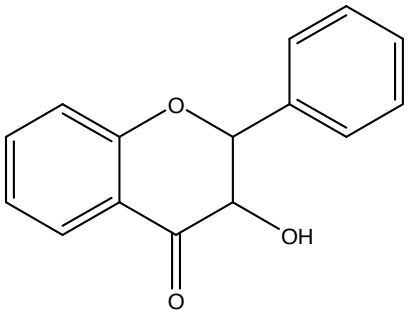
Flavanone



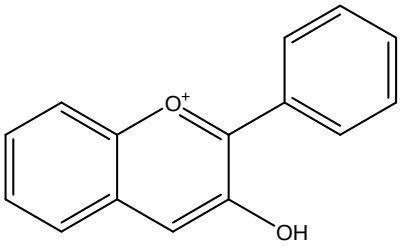
Flavone



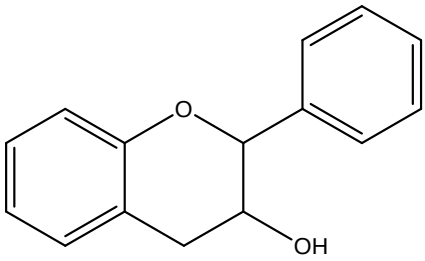
Flavonol



Dihydroflavonol



Anthocyanidin



Flavanol

Figure 02: Basic structures of Flavonoid subclasses.

1.2. Biosynthesis of Flavanones in Plants:

Due to the diverse physiological functions in plants and beneficial nutritional effects, flavonoids are now attractive targets for genetic engineering strategies with aim to produce plants having high nutritional value by modifying the flavonoids biosynthesis. In most of the plant species, the flavonoid biosynthetic pathway has been almost completely elucidated. In general, the biosynthesis of flavonoids is initiated by two precursors named Malonyl-CoA and *p*-Coumaroyl-CoA which are originated from carbohydrate metabolism and phenylpropanoids pathway, respectively. After the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA, yellow coloured chalcones are formed which consist of two phenolic groups attached by an open three carbon bridge. This enzymatic initiated step is catalysed by chalcone synthase. The unstable chalcone form is normally isomerised by the enzyme chalcone isomerase to form the corresponding flavanone. Flavanones are the backbone of this biosynthesis pathway as based on them all other flavonoid classes are generated like flavones, isoflavones, flavanols, flavonols and anthocyanidins (Fig 03) (Schijlen *et al.*, 2004; Marten and Mithöfer, 2005). Moreover, in citrus species, UDP-glucose flavanone-7-*O*-glucosyltransferase (UFGT) and UDP-rhamnose flavanone glucoside rhamnosyltransferase (UFGRT) sequentially convert the flavanone aglycones into their glucosides and rhamnoglucosides (Lewinsohn *et al.*, 1989).

This biosynthetic pathway is highly exploited by agronomists, plant pathologists, soil scientists, and biologists to study the role of phenolic compounds in different plant physiological functions such as insect-plant interaction (Simmonds, 2001), pigmentation (Mato *et al.*, 2000), heavy metal tolerance (Keilig and Ludwig-Müller, 2009), disease resistance and UV-scavenging (Cooper-Driver and Bhattacharya, 1998). Recently, Fowler and Koffas (2009) have reviewed the biotechnological production of flavanones by using various microorganisms. On the other hand, some works deal with trying to produce lower levels of flavanones in plants. For example, an *Agrobacterium*-mediated genetic transformation approach has been used to reduce the naringin contents (due to its bitter taste) in *Citrus paradisi* Macf. (grapefruit). A decrease in leaf naringin levels was obtained by targeting the chalcone synthase (CHS) and chalcone isomerase (CHI) genes (Koca *et al.*, 2009).

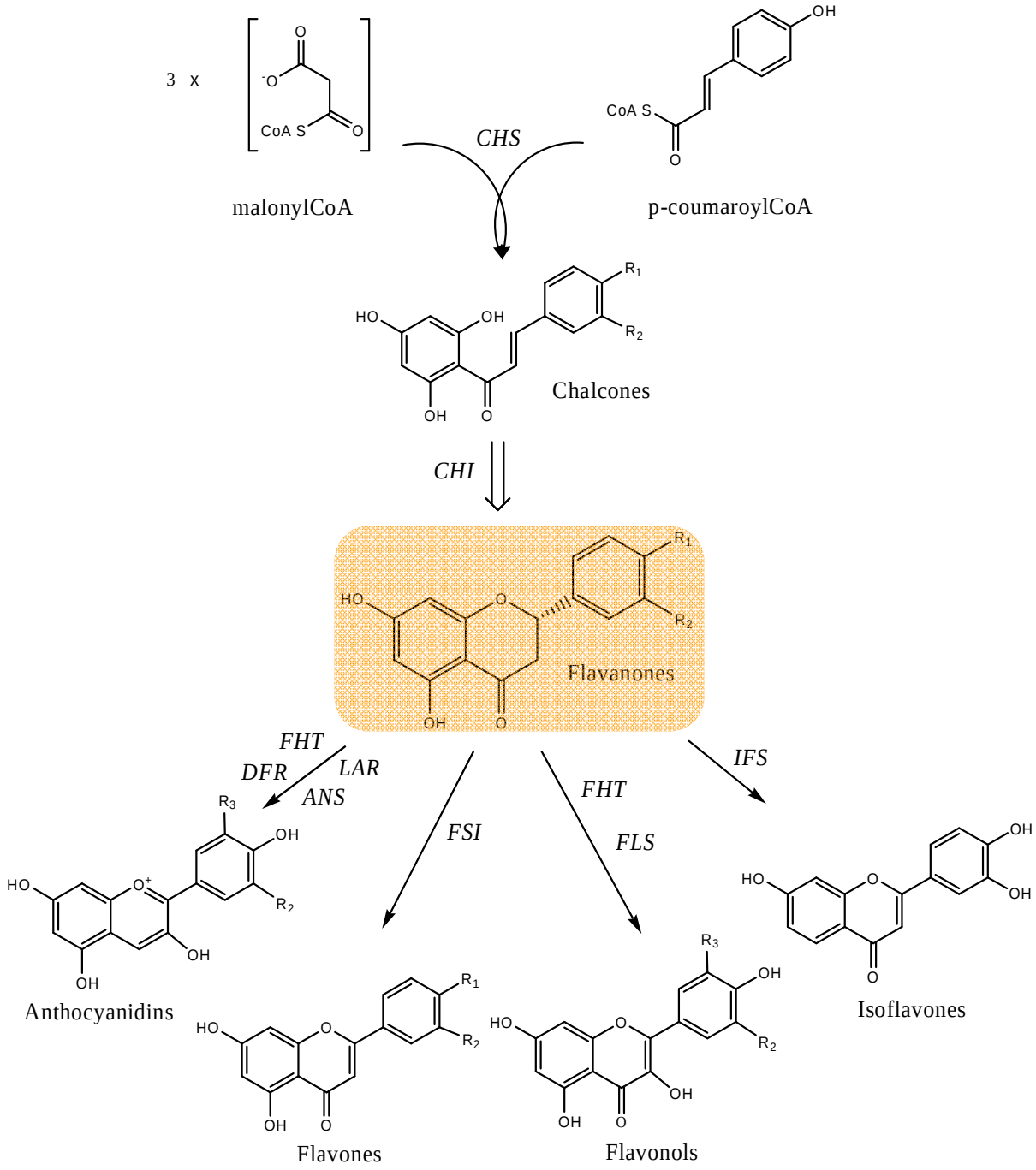


Figure 03: Biosynthesis of flavonoids.
R is generally OH or OMe, although other substitutions can be occurred at these positions.
CHS: Chalcone synthase; *CHI*: Chalcone isomerase; *FHT*: Flavanone 3-hydroxylase; *DFR*: Dihydroflavonol 4′-reductase; *LAR*: Leucoanthocyanidine 4-reductase; *ANS*: Anthocyanidin synthase; *FSI*: Flavone synthase; *FLS*: Flavonol synthase; *IFS*: 2-Hydroxyisoflavone synthase.

1.3. Diversity and Distribution of Flavanones:

1.3.1. Introduction

Fruits and vegetables are rich sources of micronutrients such as vitamins and antioxidants. Among these phytochemicals, flavanones are widely distributed in about 42 higher plant families especially in Compositae, Leguminosae and Rutaceae (Iwashina, 2000). A few decades ago, flavanones were only considered minor flavonoids (see Bohm in the three volumes of "The Flavonoid Advances in Research", Ed. J. B. Harborne, published between 1975 and 1994), like chalcones, dihydrochalcones, dihydroflavonols and aurones. However, during the last 15 years, the total number of known flavanones has become so large that they now appear among the major flavonoid classes like flavones, isoflavones, flavanols, flavonols and anthocyanidins (see Veitch and Grayer in "Flavonoids – Chemistry, Biochemistry and Applications", Eds Andersen and Markham, 2006).

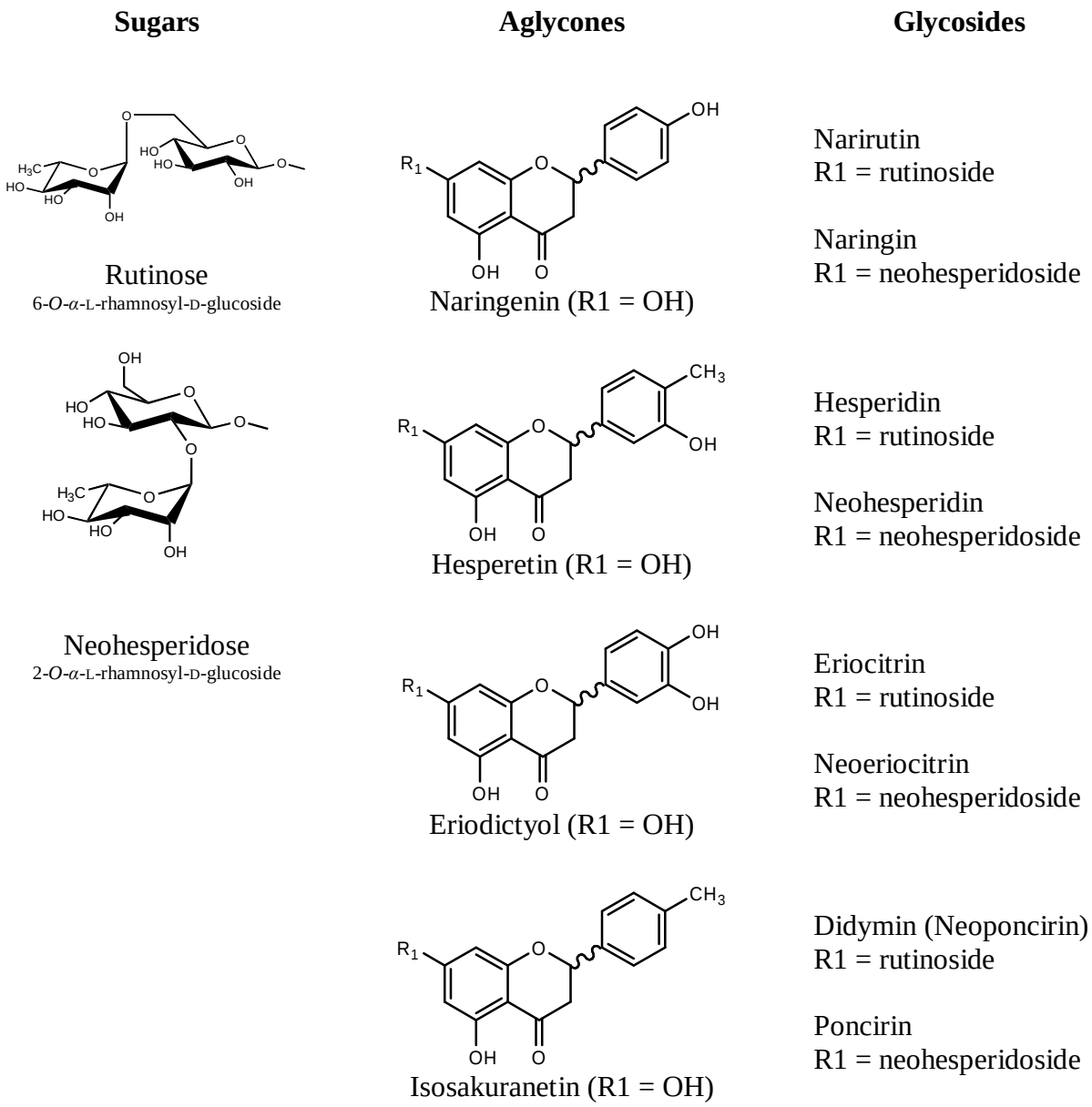
Based on the criterion of flavanone content, citrus plants belonging to the Rutaceae family appear especially important. Depending on the plant type, flavanones can be found in all plant parts, above- and below-ground, from vegetative part to generative organs: stem, branches, bark, flowers, leaves, roots, rhizomes, seeds, fruits, peels etc. Beside the aglycone forms, flavanones are also present along with their conjugates. They can be classified into several subgroups depending on their *O*-substitution (OH and OMe), *C*-methylation, *C*-prenylation and *C/O*-glycosylation (Veith & Grayer, 2008). Up to now about 350 flavanone aglycones and 100 flavanone glycosides have been discovered in nature (Iwashina, 2000). The glycosidic forms represent a significant proportion of the conjugated flavanones (Fig 04).

It is worth noting that the highest concentrations of flavanones are found in peel as compared to the fleshy part of citrus fruit (Nogata *et al.*, 2006). Of the plant flavanones, the naringenin and hesperetin aglycones and their glycosides are of particular interest because of their high prevalence in foods.

1.3.2. Naringenin

Naringenin (5,7,4′-trihydroxyflavanone) is found in high concentrations in citrus fruits while low concentrations are also found in tomatoes and their products (Erlund, 2004). Naringenin can be found as aglycone and / or as glycosides. Among the latter, naringin and

Figure 4: Some common flavanone aglycones and their respective glycosides



narirutin are especially abundant. Naringin (naringenin-7-neohesperidoside) is the conjugate of naringenin with neohesperidose (rhamnosyl-α-1,2 glucose) and has a bitter taste due to its glucose moiety. Naringin is the major flavonoid of grapefruit and sour orange, which present different naringin contents depending on their varieties (Table 01). Other citrus species like sweet orange, tangelo, lemon and lime exhibit low quantities of naringin. Another major naringenin glycoside, narirutin (naringenin-7-rutinoside) displays a rutinose (rhamnosyl-α-1,6 glucose) moiety and is most abundant in grapefruit although less than naringin. Significant levels of narirutin are also detected in tangor, sweet orange, tangerine and tangelo (Peterson *et*

al., 2006a & 2006b). The naringenin chalcone is found in higher quantities in tomato peels, which also have some other flavanone chalcones (Iijima *et al.*, 2008).

Table 1: Flavanones glycosides in different citrus varieties

Citrus type	Narirutin	Naringin	Hesperidin	Neohesperidin	Eriocitrin	Neoeriocitrin	Didymin	Poncirin
Grapefruit	4.90	16.60	2.78	1.4	0.45	0.35	0.07	0.17
Grapefruit red and pink	3.34	13.87	0.27	0.42	0.00	0.00	0.00	0.00
Grapefruit white	5.36	16.90	3.95	0.25	0.16	0.05	0.09	0.20
Lemon	0.80	0.18	15.78	0.00	9.46	0.00	0.17	0.00
Lime	0.23	0.00	15.64	0.00	1.38	0.04	0.00	0.00
Sour orange	0.08	18.83	0.00	11.09	0.53	14.01	2.89	0.00
Sweet orange	2.33	0.17	15.25	0.00	0.28	0.04	0.45	0.00
Tangelo	2.42	5.60	4.21	13.56	1.69	1.11	0.60	0.00
Tangerine (mandarin)	2.70	0.00	19.26	0.00	0.02	0.00	1.11	0.00
Tangor	7.10	0.00	15.42	0.00	1.01	1.77	0.00	0.00

Mean values are in mg aglycone / 100 g juice or edible fruit (without rind, pith and seeds)
Value are taken from Peterson *et al.*, 2006a & 2006b

1.3.3. Hesperetin

As naringenin, hesperetin (4′-methoxy-5,7,3′-trihydroxyflavanone) and its glycosides are also mainly present in citrus fruits. The aglycone is less dominant in nature than the glycosides. The most widely distributed glycosides of hesperetin are hesperidin and neohesperidin, which are conjugates with rhamnosyl-α-1,6-glucose and rhamnosyl-α-1,2 glucose, respectively. Hesperidin (hesperetin-7-rutinoside) is present in higher extents in lemons, limes, sweet oranges, tangerine and tangor species of citrus fruits (Cano *et al.*, 2008), while neohesperidin (hesperetin-7-neohesperidoside) is absent in them. Significant amounts of both also occur in grapefruits while tangelo and sour orange are especially rich in neohesperidin (Peterson *et al.*, 2006a & 2006b).

1.4. Extraction of flavanones:

Epidemiological studies have suggested the beneficial effects of citrus fruits (rich in flavanones) against many degenerative diseases like cardiovascular diseases and some cancers (Benavente-Garcia *et al.*, 1997; Tripoli *et al.*, 2007). These positive influences on human health has significantly increased the citrus consumption in the last few years and it is continuously increasing with an estimated world production of citrus fruits up to 82 million tons in the session 2009–2010, among which the major commercially important orange fruits accounts for about 50 million tons (USDA, 2010). The domestic and industrial use of these large quantities of citrus fruits, especially for the production of juice, results in the accumulation of high amounts of by-products such as peel, seed, cell and membrane residues which account for about half of the fruit weight. These by-products can be used for the production of molasses, pectins, essential oils, limonene and cattle feed (Bocco *et al.*, 1998; Jeong *et al.*, 2004; Li *et al.*, 2006a, 2006b). In addition, citrus by-products are a good source of phenolic compounds, especially the characteristic flavanone glycosides which mainly include naringin, hesperidin, narirutin, and neohesperidin. Currently, their extraction from citrus peels has attracted considerable scientific interest to use them as natural antioxidants mainly in foods to prevent the rancidity and oxidation of lipids (Anagnostopoulou *et al.*, 2006; Peschel *et al.*, 2006; Zia-ur-Rehman, 2006). Indeed, in recent years, a lot of research has focused on plants and their by-products to extract natural and low-cost antioxidants that can replace synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which might be liver-damaging, carcinogenic (Ak & Gülçin, 2008) and more generally toxic (Moure *et al.*, 2001).

Up to now, several conventional extraction techniques have been reported for the extraction of phenols from citrus peels like solvent extraction (Anagnostopoulou *et al.*, 2006; Jeong *et al.*, 2004; Li *et al.*, 2006a; Manthey & Grohmann, 1996; Xu *et al.*, 2007; Zia-ur-Rehman, 2006), hot water extraction (Xu *et al.*, 2008), alkaline extraction (Bocco *et al.*, 1998; Curto *et al.*, 1992), resin-based extraction (Calvarano *et al.*, 1996; Kim *et al.*, 2007), enzyme-assisted extraction (Li *et al.*, 2006b), electron beam- and c-irradiation-based extractions (Kim *et al.*, 2008; Oufedjikh *et al.*, 2000) and supercritical fluid extraction (Giannuzzo *et al.*, 2003). These conventional or more innovative extraction techniques may either cause the degradation of the targeted compounds due to high temperature and long extraction times as in solvent extractions, or pose some health-related risks due to the unawareness of safety

criteria during irradiation. Furthermore, enzyme-assisted extraction is limited due to problems of enzyme denaturation.

With the increasing energy prices and the drive to reduce CO_2 emissions, chemical and food industries are challenged to find new technologies in order to reduce energy consumption, to meet legal requirements on emissions, product/process safety and control, and for cost reduction and increased quality as well as functionality. Separation technology (such as extraction, distillation, and crystallization) is one of the promising innovation themes that could contribute to sustainable growth of chemical and food industries. For example, existing extraction technologies have considerable technological and scientific bottlenecks to overcome: often requiring up to 50% of investments in a new plant and more than 70% of total process energy used in food, fine chemicals and pharmaceutical industries. These shortcomings have led to the consideration of the use of new "green" techniques in extraction, which typically use less energy and the low costs, such as microwave extraction, ultrasound extraction, ultrafiltration, flash distillation and controlled pressure drop process (Chemat *et al.*, 2009).

With the development of the ‘‘Green Chemistry” concept during the last few years, environment-friendly techniques are becoming more and more attractive. The extraction of bioactive compounds under ultrasound irradiation (20–100 kHz) is one of the upcoming extraction techniques that can offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat, Tomao, & Virot, 2008). During sonication, the cavitation process causes the swelling of cells or the breakdown of cell walls, which allow high diffusion rates across the cell wall in the first case or a simple washingout of the cell contents in the second (Vinatoru, 2001). It will be important to quote that bursting of cavitation bubbles may cause a temperature of 5000°C and the pressure of 1000 atm. However, this extremely high amount of heat produced cannot significantly affect the bulk conditions because the bubbles are very tiny and the heat is dissipated to the medium in very short period of time (Luque-Garcia & Luque de Castro, 2003). UAE highly depends on the destructive effects of ultrasonic waves. Besides the solvent, temperature and pressure, better recoveries of cell contents can be obtained by optimising ultrasound application factors including frequency, sonication power and time, as well as ultrasonic wave distribution (Wang & Weller, 2006). Optimisation of ultrasound-assisted extraction (UAE) has been described recently to extract hesperidin from Penggan

(*Citrus reticulata*) peel (Ma *et al.*, 2008a), phenolic acids and flavanone glycosides from Satsuma Mandarin (*Citrus unshiu* Marc) peel (Ma *et al.*, 2009; Ma *et al.*, 2008b) and total phenolic contents from Penggan peel (Ma *et al.*, 2008a). Some other examples showing the efficiency of UAE in comparison to conventional or other innovative techniques are presented in Table 1 of chapter 2. In these works, methanol came up as a suitable extraction solvent to reach good yields of the above-mentioned phenolic compounds. However, environmentally benign and non-toxic food grade organic solvents like ethanol, n-butanol and isopropanol are recommended by the US Food and Drug Administration for extraction purposes (Bartnick *et al.*, 2006). Using these food grade solvents, UAE was found more efficient for the extraction of polyphenols from orange peel wastes than conventional solvent extraction (Khan *et al.*, 2010). Moreover, in a 'green chemistry' approach, extraction without solvent has been developed using the technique of Microwave Hydrodiffusion and Gravity (Zill-e-Huma *et al.*, 2009).

1.5. Synthesis of Flavanones

1.5.1. Aglycones

Up to now, the most common pathway for the synthesis of flavanone aglycones is the aldol condensation of 2-hydroxyacetophenones with benzaldehydes (Claisen–Schmidt condensation reaction). The reaction is usually performed under heating using acidic or alkaline conditions. The chalcones initially formed undergo cyclisation to their respective flavanones under the same conditions (Krbechek *et al.*, 1968; French *et al.*, 2010). The condensation is still under study to develop efficient and environment-friendly conditions. For instance, strongly alkaline sodium hydroxide and ethoxide were replaced by Mg-Al hydrotalcites (Climent *et al.*, 1995). Furthermore, different derivatives of chalcones and flavanones were also prepared by aldol condensation (Hsieh *et al.*, 1998). Currently, the emphasis is on developing new catalysts that could be effective in aldol condensations and alternative methods (Chandrasekhar *et al.*, 2005). Recently, the introduction of Li was shown to increase the surface basicity and catalytic activity of MgO in the synthesis of flavanone aglycones (Cortes-Concepcion *et al.*, 2010). An alternative method of Claisen-Schmidt condensation was also proposed to prepare flavanone aglycones and their derivatives (Shi *et al.*, 2010). The method was more straightforward than the Claisen-Schmidt condensation and the overall yield was similar (figure 05).

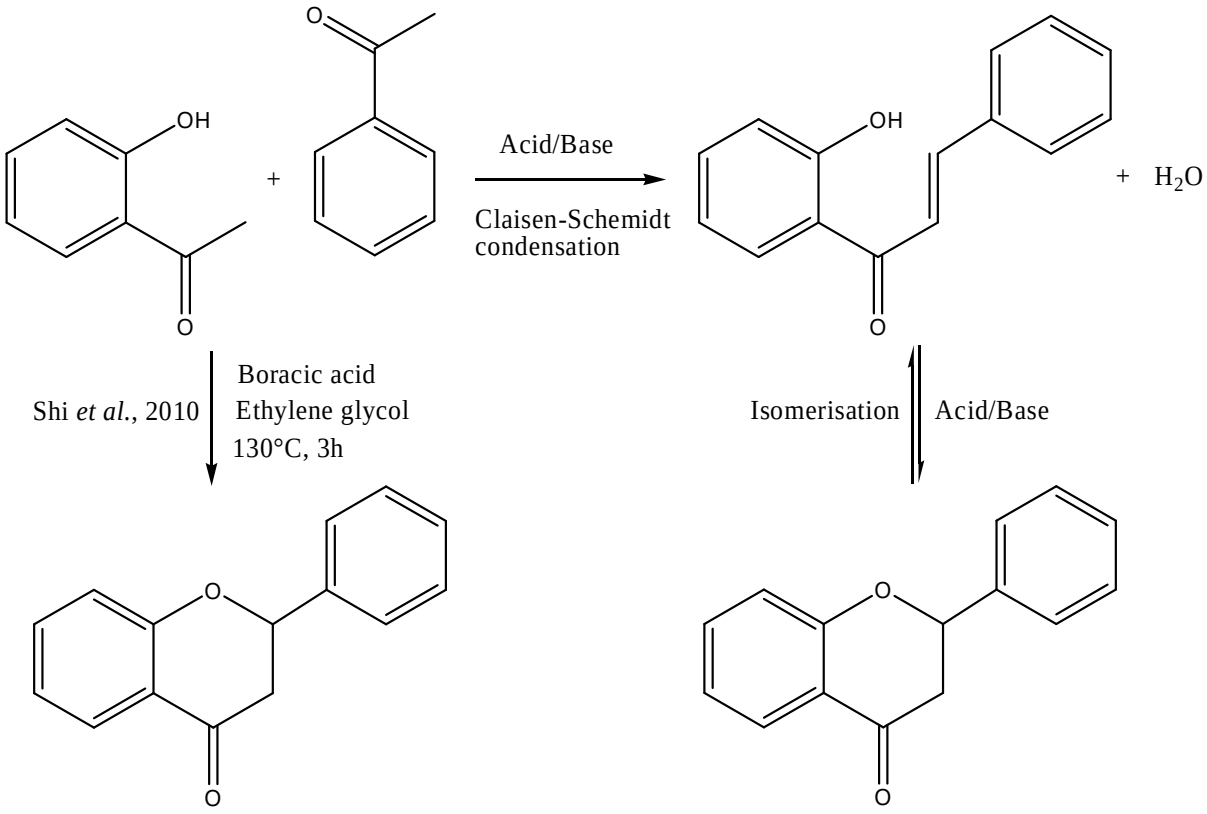


Figure 05: Flavanone synthesis via the Claisen–Schmidt condensation of 2′-hydroxyacetophenone with benzaldehyde followed by the isomerization of the 2′-hydroxychalcone intermediate formed versus the method proposed by Shi et al., 2010.

1.5.2. Chalcones

The scarcity of flavanone chalcones in Nature is primarily due to their instability, as in neutral medium, they undergo cyclisation to the corresponding flavanones. However, chalcones can be simply prepared by opening of the C-ring of flavanones using strongly alkaline conditions (Miles and Main, 1985). The reaction starts by the removal of a weakly acidic hydrogen atom from the flavanone C3 to yield an enolate anion, which opens up into a chalcone anion (Andújar *et al.*, 2003). Upon quick acidification, the chalcone precipitates and can be isolated as a solid. This procedure was successfully used for the preparation of the naringin chalcone (Gonzàlez *et al.*, 2002) and 2′,6′-dihydroxy-4,4′-dimethoxychalcone (Miles and Main, 1985).

1.5.3. Glycosides

The most prevalent flavanone derivatives are the 7-*O*-*β*-glycosides. The selective glycosylation of 7th position OH group flavanone can be performed by using the well known methods of Koenigs and Knorr (silver carbonate and quinoline) (Zemplén and Bognàr, 1943; Oyama and Kondo, 2004) or the method of Zemplén and Farkas (10% aq. sodium or potassium hydroxide and acetone used for synthesis of hesperedin) (Zemplén and Farkas, 1943). With some modifications, these methods are still in use not only for glycosylation of phenolic compounds (Esaki *et al.*, 1994) but also for glucuronidation (Moon *et al.*, 2001). A simple route to flavanone 7-glucoside is the partial hydrolysis of naringin and hesperedin using formic acid in cyclohexanol (Fox *et al.*, 1953). The enzymatic synthesis of flavanone glycosides was also described (Kometani *et al.*, 1996) as well as the synthesis of amino derivatives for use as scaffolds in drug discovery (Hanessian and Kothakonda, 2005) and metal complexes to increase the antioxidant and anti-inflammatory activities (Pereira *et al.*, 2007).

1.5.4. Glucuronides

A better knowledge of the biochemical mechanisms by which dietary flavanones exert their potential health effects requires investigations on appropriate cell models (e.g., endothelial or smooth muscle cells) with the authentic circulating metabolites, of which glucuronides make the largest contribution, instead of the commercially available glycosides and aglycones that are frequently used as a first approach despite the limited biological significance. As an alternative to the expensive, inconvenient and low yielding extraction of conjugates from biological fluids, chemical synthesis appears as the most direct strategy to obtain substantial amounts of these metabolites for bioavailability and in vitro cell studies. Hence, there is a growing interest for the synthesis of polyphenol glucuronides as standards for identification and titration of in vivo metabolites and as biologically pertinent compounds for cell studies aiming at elucidating the potential health effects of polyphenols. Several works have been published about the chemical synthesis of polyphenol glucuronides.

For instance, the popular procedure, based on the Lewis acid-activated coupling of methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-*α*-D-glucuronate (Tomas-Barberan & Clifford, 2000) with partially protected polyphenols, was applied to the synthesis of isoflavone 7-*O*-*β*-D-glucuronides (Al-Maharik & Botting, 2006), quercetin 3-*O*-*β*-D-glucuronide (Needs & Kroon, 2006) and a series of hydroxycinnamic acid *O*-*β*-D-glucuronides (Galland *et al.*, 2008). Catechin *O*-*β*-D-glucuronides were also prepared with

methyl-2,3,4-tri-*O*-acetyl-1-*O*-bromo-α-D-glucuronate as the glucuronyl donor (Gonzàlez-Manzano *et al.*, 2009). Recently, the synthesis of a flavanone glucuronide (persicogenin 3′-*O*-β-D-glucuronide) was carried out with methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trifluoroacetimidoyl)-α-D-glucuronate, followed by a final deprotection step involving pig liver esterase (PLE) for the hydrolysis of the methyl ester of the glucuronyl residue (Boumendjel *et al.*, 2009). A synthesis of quercetin 3-*O*-β-D-glucuronide was also performed by regioselective oxidation of the corresponding 3-*O*-β-D-glucoside (phenolic OH groups protected as benzyl ethers) using TEMPO/NaOCl/NaBr under phase transfer conditions (Bouktaib *et al.*, 2002). Recently, the synthesis of four flavanone glucuronides (naringenin 4′- and 7-*O*-β-D-glucuronides and hesperetin 3′- and 7-*O*-β-D-glucuronides) based on a regioselective protection of the flavanone nucleus was reported (Khan *et al.*, 2010).

1.6. Bioavailability of Flavanones

1.6.1. Introduction

The oral bioavailability of a given nutrient describes its fate once ingested: intestinal absorption, transport in the general circulation, delivery to tisssues, metabolism and excretion. In spite of the high consumption of citrus fruits and juices worldwide, the bioavailability of flavanones is still incompletely known. Their daily intake has not been estimated in different populations but could be quite high compared with the average flavonol intake (25 mg/day) in several European countries (Manach *et al.*, 2003). For instance, the mean dietary intake in Finland has been evaluated to be 8.3 mg/day and 28.3 mg/day for naringenin and hesperetin, respectively (Manach *et al.*, 2003; Erlund, 2004).

After oral intake, flavanone monoglycosides and diglycosides are hydrolysed in the small intestine and in the colon, respectively, and the released aglycones or phenolic acids are converted into their respective glucuronides, sulphates and sulphoglucuronides during their passage across the small intestine and liver. Finally, the bioactive forms (metabolites) are distributed through plasma at various cell sites and significant quantities can also be found in urinary excretions (Matsumoto *et al.*, 2004). The fate of flavanones after ingestion is summarized in figure 6.

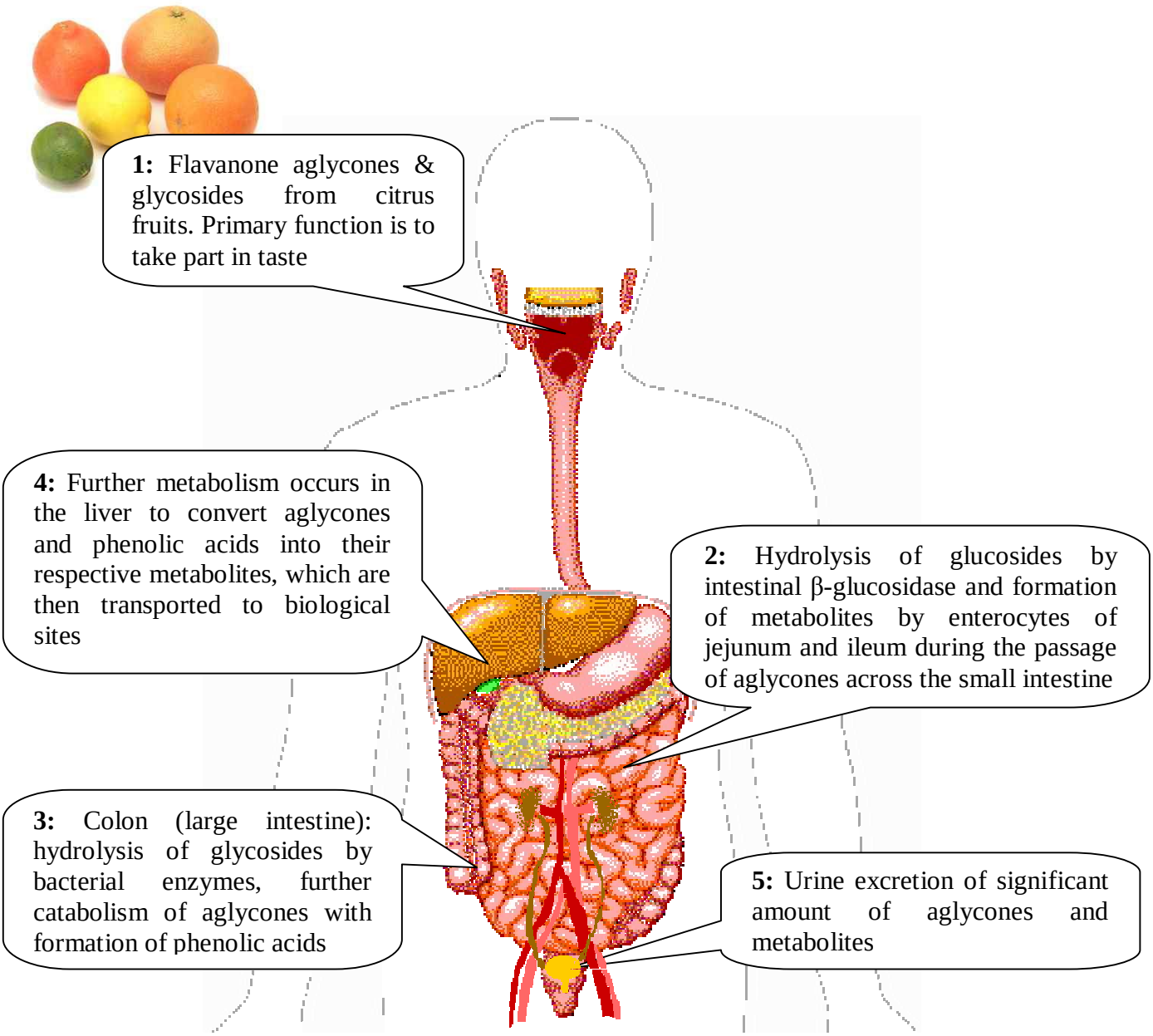


Figure 6: Metabolic fate of flavanones

1.6.2. Metabolism of flavanones and their metabolites

A great part of the bioavailability studies has been devoted to naringenin, hesperetin and their glycosides. Improvements in methods for analyzing flavanone metabolites in human plasma and urine have made possible to estimate flavanone bioavailability in humans.

The the first step in flavanone metabolism is the extensive deglycosylation of flavanone glycosides within the intestinal epithelium by human and bacterial enzymes like β-glucosidase, rhamnoglucosidase, rutinoglucosidase etc. Investigations in rats demonstrated

that the deglycosylation of naringenin-7-glucoside occurred early in the small intestine (Choudhury *et al.*, 1999) while that of naringenin-7-rhamnoglucosides occurred in the colon (large intestine). Indeed, naringenin conjugates (glucurono- and /or sulfo conjugates) appeared within 3h in the plasma of rats fed with naringenin or its 7-glucoside whereas no naringenin metabolites were still detected in rats fed with naringenin-7-rhamnoglucoside. However, 10h after ingestion, similar naringenin concentrations were found regardless of the diet, which clearly showed the delayed intestinal absorption of naringenin rhamnoglucosides (Felgines *et al.*, 2000). It was confirmed in humans that hesperidin and naringin are absorbed in the distal part of the intestine (cecum). Once deglycosylated, the aglycones are glucuronated and/or sulphated during their transfer from the luminal side of the gut to the portal vein by the action of UDP-glucuronosyltransferase and sulphotransferase enzymes (Manach *et al.*, 2003). In cecum, the intestinal microflora not only cleaves the glycosidic bonds but also degrades the aglycones into phenolic acids such as *p*-hydroxyphenylpropionic acid (*p*-HPPA), *p*-coumaric acid (*p*-CA), and *p*-hydroxybenzoic acid (*p*-HBA) (Felgines *et al.*, 2000; Manach *et al.*, 2003). Likewise, eriocitrin (eriodictyol-7-rutinoside) is metabolised by intestinal microflora (Bacteroides distasomis or B. uniformis) to eriodictyol, which is then converted into 3,4-dihydroxycinnamic acid by Clostridium butyricum (Miyake *et al.*, 2000). After intestinal absorption, metabolites, aglycones and phenolic acids reach the liver, the main organ involved in flavanone metabolism, where further glucuronidation, sulfation, and in some cases methylation occur, thus converting the rest of aglycones and phenolic acids into their respective metabolites. Due to lack of catechol groups in hesperetin and naringenin, no methylation by catechol-o-methyltransferase (COMT) was observed which is in contrast to catechin and quercetin (Felgines *et al.*, 2000). Two metabolic pathways are possible with eriocitrin (eriodictyol rutinoside): one is the formation of phenolic acids (3,4-dihydroxycinnamic acid) by the microflora and the second is the formation of eriodictyol, homoeriodictyol (3′-methoxy-4′,5,7-trihydroxyflavanone) and hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone) conjugates due to methylation of the catechol group of the aglycone. The conversion of eriodictyol to homoeriodictyol and hesperetin through methylation in liver was also reported (Miyake *et al.*, 2000).

Recently, a study was conducted to determine the effect of tumor on flavanone metabolism. The similar naringenin concentrations in liver and kidney of healthy and tumor-bearing rats suggested that there was no effect of tumor on intestinal and hepatic metabolism of flavanones (Silberberg *et al.*, 2006).

Moreover, the impact of full-fat yogurt on the bioavailability and metabolism of orange flavanones in human was investigated by analysing the human plasma and urine over different intervals of time. Addition of yogurt into orange juice significantly reduced the quantity of flavanone metabolites excreted up to 5 h after ingestion. However, a statistical analysis over a longer time span (0-24 h) did not show any significant effect of yogurt addition (Mullen *et al.*, 2008).

Glucuronidation and sulfation are the major conjugation pathways of flavanone aglycones. Structural studies on the plasma and urinary metabolites showed that the major metabolites of naringenin are naringenin-7-glucuronide, naringenin-4′-glucuronide, naringenin-7-sulfate-4′-glucuronide, naringenin-7-glucuronide-4′-sulfate and naringenin-7,4′-disulfate (Tripoli *et al.*, 2007; Brett *et al.*, 2009). Similarly, the main hesperetin conjugates are hesperetin-7-glucuronide, hesperetin-3′-glucuronide, hesperetin diglucuronide and hesperetin sulfoglucuronide (Matsumoto *et al.*, 2004; Mullen *et al.*, 2008). Among all these metabolites, glucuronides largely prevail (87%) but the importance of the other metabolites should not be underestimated (Manach *et al.*, 2003). The position at which glucuronidation occurs might influence the resulting bioactivity including the antioxidant activity (Tripoli *et al.*, 2007). Up to now, no data have been reported about the antioxidant activity of flavanone glucuronides. However, since the common flavanones hesperetin and naringenin are devoid of catechol group, which is the critical structural determinant of the antioxidant (reducing) activity for polyphenols, both are weak antioxidants and their glucuronides (with one less free phenolic OH group) are expected to be even less potent. It is thus quite likely that the bioactivity expressed by flavanone glucuronides is largely unrelated to their redox properties and rather reflects their interactions with specific proteins.

1.6.3. Pharmacokinetics

After the oral administration of 500 mg of naringin, urine analysis of healthy volunteers was optimised for the determination of naringenin and its metabolites (Ishii *et al.*, 1997). Moreover, the pharmacokinetics of naringenin and its glucuronides in rat plasma and brain tissue was successfully performed by HPLC (Peng *et al.*, 1998). The study was extended to determine the naringenin levels in rat blood, brain, liver and bile using microdialysis coupled with a HPLC system (Tsai, 2002). Most probably, the first report on the pharmacokinetics of flavanones in human subjects was published by Erlund and co-authors in 2001. After ingestion of orange or grapefruit juice (8 mL/kg of body weight), the plasma

concentration of hesperetin and naringenin aglycones (after deconjugation) was found in the range 0.6 – 6 μmol/L. Moreover, elimination half-lives ($t_{1/2}$) in the range 1.3 – 2.2 h showed a relatively fast clearance. The percentage of flavanones excreted in urine was lower than that of their absorption, which indicated a substantial distribution to tissues for these phenolic compounds (Erlund *et al.*, 2001). In another study, ingestion of hesperetin and naringenin (135 mg of each) under fasting conditions resulted in their appearance as metabolites in blood plasma 20 min later. The peak plasma concentration (C_{max}) of 2.7 μmol/L and 7.4 μmol/L was reached 4.0 and 3.5 h afer ingestion, respectively (Kanaze *et al.*, 2007). Plasma and urine analyses pointed to the higher naringenin bioavailability in comparison to hesperetin (Gardana *et al.*, 2007; Kanaze *et al.*, 2007). An in vitro hydrolysis showed a faster hydrolysis rate for hesperidin and narirutin (flavanone rutinosides) than for naringin and neohesperidin (flavanone neohesperiosides) (Wang *et al.*, 2008). More recently, the same group demonstrated the bioavailability of hesperetin and naringenin after the consumption of *Citrus aurantium* L. and *Citrus sinensis* Osbeck (Cao *et al.*, 2010).

The permeability of epithelial cells to flavanones is a good determinant of their intestinal absorption. Flavanones are transported from apical side (gut lumen) to basolateral side (blood). In in vitro models, hesperetin (aglycone) was found to be efficiently absorbed across Caco-2 cell monolayers in comparison to hesperidin (hesperetin glycoside). The absorption mechanisms involved transcellular passive diffusion along with a newly proposed mechanism of proton-coupled active transport (Kobayashi *et al.*, 2008a). The study was further elaborated to explain the H^+-driven polarised absorption and similar mechanisms were found for naringenin and eriodictyol aglycones (Kobayashi *et al.*, 2008b).

The faster absorption of flavanone aglycones compared to flavanone glycosides was also shown for eriodictyol and eriocitrin in humans (Miyake *et al.*, 2006).

Concentration of flavanone conjugates in plasma and urine is an important criterion to determine the site of absorption and estimate the bioavailability in human. It varies according to glycoside concentration and flavanone structure. After ingestion of 1 L of orange juice containing 444 mg of hesperidin and 96 mg of narirutin, the highest plasma concentration of hesperetin and naringenin (after deconjugation) were 1.28 ± 0.13 μmol/L and 0.20 ± 0.04μmol/L, respectively. The levels of flavanones in urine were expressed as percentage of their intake and amounted to 7.87 ± 1.69% for naringenin and 6.41 ± 1.32 % for hesperetin. The relative urinary excretion of flavanones was not significantly affected by the dose

ingested. The relative urinary excretion of naringenin from grapefruit was found higher (8.9%) (Manach *et al.*, 2003). In another study, high naringenin concentrations of 128 ± 2 μM, 144 ± 8 μM and 139 ± 15 μM were detected at time 10h in the plasma of rats fed with naringenin (0.25 % of total diet), naringenin-7-glucoside (0.38 %) and naringenin-7-rhamnoglucoside (0.5 %), respectively. The urinary excretion of naringenin was two times higher in naringenin-fed rats than in naringenin-7-rhamnoglucoside-fed ones (Felgines *et al.*, 2000). Still the missing part in most bioavailability studies is the availability of authentic conjugates for use as standards. The present study is aimed at bridging this gap by developing chemical syntheses of flavanone glucuronides. Those conjugates are also very much needed for investigating the mechanisms of their bioactivity in cell models.

1.7. Interaction of flavanones with Human Serum Albumin (HSA)

In the last decade, the biological studies performed to explore the possible health effects of flavanones were devoted to assess either their metabolism and bioavailability or their possible therapeutic value as potential drugs. But unfortunately much less attention was given to study the delivery of flavanones to specific biological sites. Flavonoids are transported to their biological sites by the blood plasma. Serum albumin is the major component of blood plasma, occurring there at a concentration of 0.6 mM. Beside the maintenance of colloidal osmotic blood pressure and bodily detoxification, serum albumin transports fatty acids, vast types of drugs and dietary polyphenols (Dangles & Dufour, 2006). The literature on the structural aspects and binding locations of HSA is well described by a number of comprehensive reviews. The determination of the amino acid sequences of HSA (585 amino acids) was the first important step for the determination of the binding properties of albumin (Behrens *et al.*, 1975; Meloun *et al.*, 1975). Then, X-ray crystallography made it possible to elucidate the three-dimensional structure of HSA and precisely characterise the binding domains (He & Carter, 1992). Mainly, HSA consists of three helical domains I (1-195), II (196-383) & III (384-585) and each domain is further subdivided into two subdomains A and B (Fig 7). The protein has an overall shape of heart and its structure is stabilised by 17 disulfide bonds. Subdomains IIA (site I) and IIIA (site II) are most studied because of their involvement in the binding of drugs and other xenobiotics. Both subdomains bind through hydrophobic cavities lined by some positively charged amino acid residues (*Lys*) at the entrance of the pockets (Sugio *et al.*, 1999).

A significant amount of the literature available on albumin-flavonoid interactions reports not only quantitative thermodynamic data (binding constants), but also qualitative analyses aimed at locating the possible binding sites (Dufour & Dangles, 2005; Banerjee *et al.*, 2008; Lu *et al.*, 2007; Rawel *et al.*, 2005). In particular, quercetin and its metabolites were studied for their affinity with HSA (Murota *et al.*, 2007; Zsila *et al.*, 2003). In addition to other conventional techniques, fluorescence spectroscopy is the analytical tool that is most widely used to investigate binding to HSA (Oravcovà *et al.*, 1996). Indeed, there is a fluorescent tryptophan residue (*Trp214*) in site I, which can be excited at 295 nm and emits fluorescence at 340 nm. From the quenching of this fluorescence by a given ligand, the binding constant can be estimated (Sulkowska, 2002).

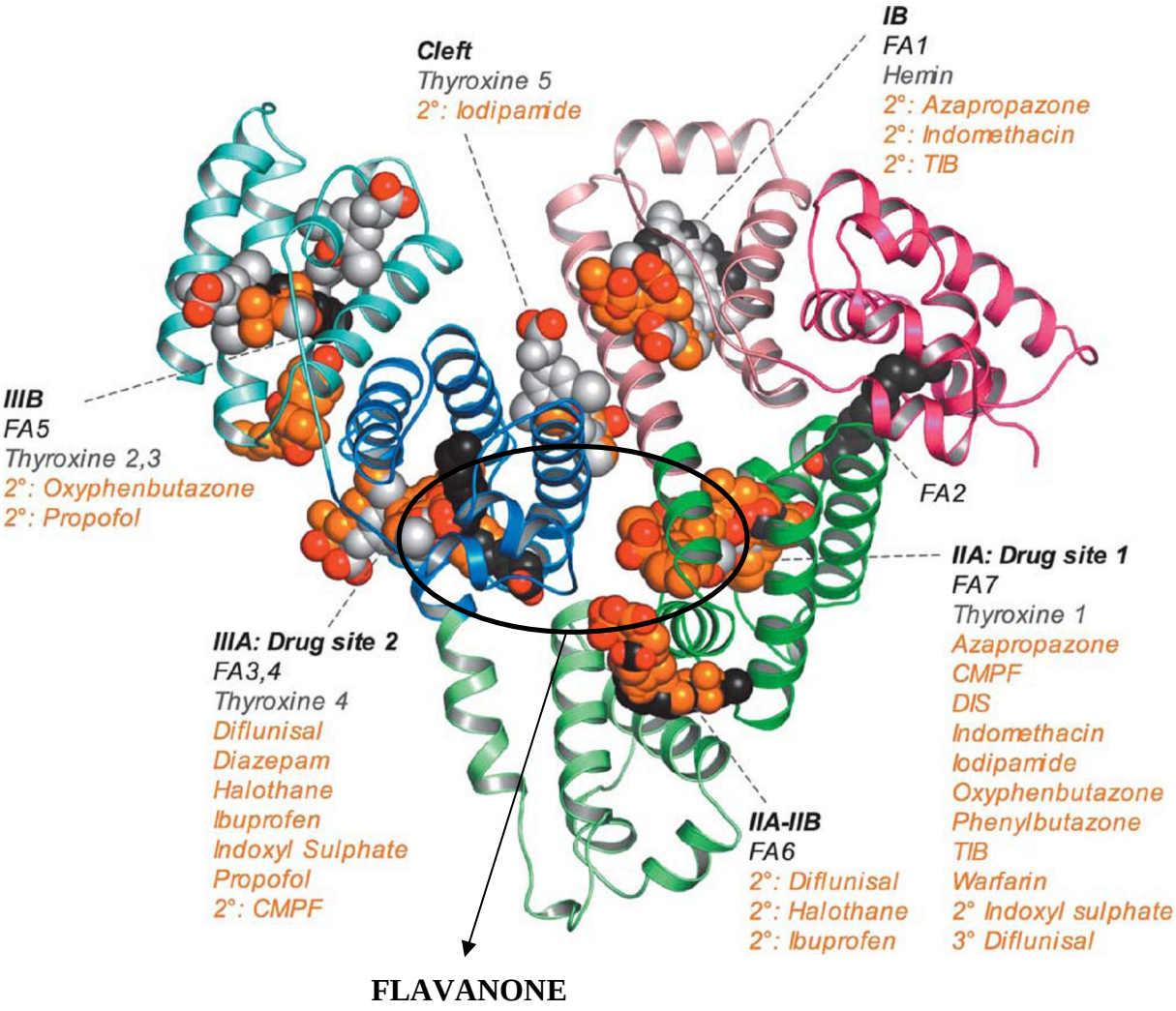


Figure 7: Three dimensional structure of HSA with common ligands bound at different binding sites. (adapted from Ghuman *et al.*, 2005)

The affinity of flavanones to HSA has been more recently investigated. In an original study, piezoelectric quartz crystal impedance (PQCI) analysis was performed to measure the affinity of hesperidin for immobilised HSA. The association constant calculated was 2×10^3 M^{-1} using Scatchard analysis (Liu *et al.*, 2004). The interaction of hesperidin with bovine serum albumin (BSA) was also investigated by fluorescence spectroscopy (Wang *et al.*, 2007). Xie and co-authors used fluorescence spectroscopy with support of Fourier-transformed infrared (FT-IR) and UV-visible spectroscopies to determine the binding constant, binding site and binding mechanism of hesperetin to HSA. From the Stern-Volmer equation, a binding constant of about 81×10^3 M^{-1} was estimated at pH 7.4. The *K* value decreased with increasing the pH from 6.4 to 8.4 due to a) conformational changes of HSA which affect the shape of the hydrophobic binding cavities and b) the increased dissociation of the phenolic hydroxyl groups of hesperetin. Moreover, FT-IR spectroscopy suggested that hesperetin binds to subdomain IIA. The main mechanisms involved in the interaction include the hydrophobic effect (van der Waals interactions between the ligand and hydrophobic amino acid residues with concomitant desolvation), electrostatic interactions between Lys residues and the flavanone phenolate ion, formed after deprotonation of the most acidic OH group at position C7, and hydrogen bonding between the phenolic OH and keto groups of hesperetin and the polypeptide chain or other polar amino acid residues (Xie *et al.*, 2005a). The study was further extended to investigate the association of naringenin ($K = 127\times10^3$ M^{-1}) with HSA (Xie *et al.*, 2005b). Conjugation of flavanone aglycones may affect their affinity for has. For instance, the binding constant of naringin, a naringenin diglycoside, was found lower ($K = 18\times10^3$ M^{-1}) than that of naringenin (Zhang *et al.*, 2008). So far, the affinity for HSA of true circulating flavonoid metabolites has not been investigated.

1.8. Bioactivity of Flavanones

1.8.1. Introduction

Over the last few decades, extensive research has been conducted on dietary compounds that could be protective against lethal diseases, in particular cardiovascular diseases and some types of cancers. These potentially bioactive compounds include phytoestrogens, carotenoids, ascorbic acid, citrus limonoids, organosulfur compounds and a good number of polyphenols. The basic mechanisms implicated in the potential health effects of polyphenols are mainly the inhibition of lipid and DNA oxidation (antioxidant activity) and the regulation of gene expression (Kris-Etherton *et al.*, 2002; Patil *et al.*, 2009). Like other

polyphenols, flavanones are also studied for their effects on specific cells. However, the missing part remains the investigation of true flavanone metabolites. Examples of in vitro / in vivo studies conducted to explore the beneficial effects of flavanones and the mechanisms involved are discussed below.

1.8.2. Radical-Scavenging Effect

Reactive oxygen species (ROS) / reactive nitrogen species (RNS) in biological systems are typically unstable and oxidizing species that are produced in low concentration for physiological signalling pathways and in larger concentration (oxidative stress) to destroy viruses and bacteria in leucocytes during infection (inflammatory response). The chronic exposure to oxidative stress is considered an initiating event in the development of degenerative diseases (Brown & Borutaite, 2006; Forman *et al.*, 2008). ROS/RNS include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\bullet}$), the hypochlorite ion (ClO^-), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), lipid oxyl and peroxyl radicals ($RO^{\bullet}$, $ROO^{\bullet}$) produced during the autoxidation of polyunsaturated fatty acids... Phenolic compounds are extensively studied for their ability to reduce ROS/RNS (antioxidant activity, AA), thereby preventing the oxidative damage they cause to the host's biomolecules.

The antioxidant activity of flavanones depends upon the number and spatial arrangement of phenolic OH groups (Cai *et al.*, 2006; Sadeghipur *et al.*, 2005). Up to now, in vitro and in vivo investigations have been performed to determine the antioxidant potential of flavanone aglycones, chalcones, and glycosides. No literature is available about the antioxidant capacity of flavanone glucuronides.

A comparative study on the antioxidant properties of nine different flavanones (naringin, neohesperidin, neoeriocitrin, hesperidin, narirutin, naringenin, hesperetin, heridictyol and isosakuraternin) using the crocin bleaching inhibition assay has shown that the presence of a catechol nucleus (3′,4′-dihydroxy substitution on the B-ring) and its *O*-methylation have no significant effect on the AA of aglycones, which is surprising. By contrast, an increase in AA was observed with the glycosides having a catechol nucleus while *O*-methylation of the catechol has an opposite effect (Di Majo *et al.*, 2005). *O*-glycosylation often reduces the AA, which points to the participation in the radical-scavenging reaction of the OH group involved in the glycosidic bond (Acker *et al.*, 1996). The different glycosydic

moieties may also have a small effect on AA. For instance, the glycosylation of hesperetin on the C7-OH group by neohesperidose affects the AA while the glycosylation by rutinose has no effect (Di Majo *et al.*, 2005). While substitution on a hydroxyl group typically decreases the AA, addition of a hydroxyl group can strongly increase it. For example, 3′,5′-dihydroxynaringin (pyrogallol B-ring) is ca. 70 times as potent as naringenin (Ye *et al.*, 2009). Flavanones present a higher AA in a hydrophilic environment. In a lipophilic environment, some flavanones (neohesperidin, hesperetin, isosakuranetin) show a reduced antioxidant potential while others (naringin, narirutin, naringenin, neoeriocitrin, heridictyol) even become prooxidant (Finotti and Di Majo, 2003). Overall, common dietary flavanones being devoid of a catechol nucleus are only poor antioxidants and their metabolites are expected to be even less potent. Hence, the most significant mechanisms involved in their health effects must be unrelated to their antioxidant activity.

1.8.3. Anti-Inflammatory Effect

The phenomena of inflammation have been well described in literature through many reviews. Inflammation is the most obvious manifestation of immune defence. It is manifested by swelling, pain, heat, and redness in the affected tissue and helps eliminate the sources of damage (viruses, bacteria…) and initiate healing. Inflammation is produced by immune cells within the tissue, releasing specific mediators which control local circulation and cell activities (Silverstein, 2009). Inflammation response to external stimuli may arise from the action of amines (histamine and 5-hydroxytryptamine), short peptides (bradykinin), long peptides (interleukin-1 (IL-1)), lipids (prostaglandins (PGs) and leukotrienes (LTs)), and many regulatory enzymes (protein kinase C, phosphodiesterase, lipoxygenase, and cyclooxygenase) (Vane & Botting, 1987). Many of the chronic and uncured diseases which plague our civilization are due to a dysfunctioning of the immune response.

Hesperidin (hesperetin 7-rutinoside) was found to inhibit kinases and phosphodiesterases responsible for cellular signal transduction and activation during an inflammation response (Manthey *et al.*, 2001). An inhibitory effect of hesperidin on pleurisy (chronic inflammation of lungs) induced by carrageenan was investigated in rats. The results showed a reduction in the volume of exudates and the number of migrating leucocytes by 48% and 34%, respectively, which makes hesperidin a mildly anti-inflammatory agent. Furthermore, this research group observed that hesperidin can reduced yeast-induced hyperthermia in rats (Emim *et al.*, 1994). In another study, hesperidin showed an inhibitory

effect on lipopolysaccharide (LPS)-induced overexpression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), overproduction of prostaglandin E2 and nitric oxide (NO) (Sakata *et al.*, 2003). Similar anti-inflammatory effects were also found for poncirin in RAW 264.7 macrophage cells (Kim *et al.*, 2007). A study also showed the anti-inflammatory activity of hesperidin by inhibiting arachidonic acid and histamine release (Galati et al, 1994).

Examples of naringenin and its glycosides as strong anti-inflammatory agents are:

a) the inhibition of pro-inflammatory cytokine induced by lipopolysaccharide in macrophages and *ex vivo* human whole-blood models to prevent Periodontitis (Bodet *et al.*, 2008); b) the attenuation of LPS / IFN (interferon)-γ-induced TNF (tumour necrosis factor)-α production in glial cells by inhibiting iNOS (inducible nitric oxide synthase) expression and nitric oxide production, p38 mitogen-activated protein kinase (MAPK) phosphorylation, and downstream signal transducer and activator of transcription-1 (STAT-1) to protect neuroinflammatory injury (Vafeiadou *et al.*, 2009); c) the reduced production of nitrate and nitrite (indicators of inflammatory process) in DSS (dextran sodium sulphate)-induced ulcerative colitis mice models to control the formation of intestine edema (Amaro *et al.*, 2009).

1.8.4. Anti-Cancer Effect

Advances in cancer research have been spectacular during the past decade. However, it is very unfortunate that the rate of cancer incidence is increasing at an alarming rate. The more recent estimation on cancer in France has given the figure of 320 000 cases diagnosed in 2005 in which 180 000 were in man and 140 000 in woman (INC report). While it cannot be concluded that technological progress is promoting cancer rate, it is clear that serious research in combating cancer is still essential.

Cancer is a complex family of diseases. In terms of molecular and cell biology, cancer is a disease of abnormal gene expression. This altered gene expression occurs through a number of mechanisms, including direct insults to DNA (such as gene mutations, translocations, or amplifications) and abnormal gene transcription or translation. When the cellular DNA of one or more normal cells has been exposed to carcinogens and substances or agents that can damage genetic material, the cell undergoes genetic alterations that result in malignant transformations and the process is known as carcinogenesis. Carcinogens include

chemical agents (from industrial pollutants, tobacco etc…), viruses (Human papilloma virus, hepatitis B & C virus), ionising (X-rays) and ultraviolet radiations, physical substances (asbestos, wood dust) and many others. ROS (superoxide, hydrogen peroxide, and hydroxyl radical) were also found major causes of not only DNA damage but also protein and lipid damages which lead to aging (Ames & Gold, 1998).

Unlike other flavonoids, flavanones have not been extensively studied for their bioactivities. Moreover, the studies have remained limited to aglycones and glycosides. Recently, hesperetin 7-glucuronide (Hp7G) was demonstrated to affect osteoblast differentiation (Trzeciakiewicz *et al.*, 2010). The major citrus flavanones can be effective in fighting carcinogenesis by minimizing DNA damage, tumor development and proliferation.

1.8.4.1. Antimutagenic effect

Flavanones can protect DNA damage by their capacity to absorb UV light. The results from a UV irradiated model of plasmidic DNA showed a considerable protecting effect of naringenin against UV-induced damage of DNA (Kootstra, 1994). The moderate antioxidant capacity of flavanones can also be useful in protecting against mutation by free radicals generated near DNA. Furthermore, naringin also inhibits H_2O_2-induced cytotoxicity and apoptosis, possibly via its effect on H_2O_2-induced expression of an apoptosis-associated gene (Kanno *et al.*, 2003). Naringenin may exhibit anti-mutagenic changes by stimulating DNA repair, following oxidative damage in human prostate cancer cells (Gao *et al.*, 2006).

1.8.4.2. Inhibition of tumor development

The pharmacological importance of flavanones can also be evaluated by their action against tumor development. So *et al.* (1996) studied the effect of hesperetin and naringenin on the development of breast cancer induced by 7,12-dimethylbenz[a]anthracene in female rats. The results showed that tumor development was delayed in rats fed with orange juice / naringin-supplemented diet (So *et al.*, 1996). Later on, concerning the anti-angiogenic effect of flavanones, an enzyme-linked immunosorbent assay (ELISA) was used to measure the vascular endothelial growth factor (VEGF) release from mammary adenocarcinoma human breast cancer cells. Naringin appeared more potent than rutin, apigenin, kaempferol and chrysin (Schindler *et al.*, 2006). 8-Prenylnaringenin, a derivative of naringenin, inhibits angiogenesis induced by basic fibroblast growth factor, VEGF, or the synergistic effect of two

cytokines in combination, in an in vitro and in vivo study (Pepper *et al.*, 2004). Eight flavanones, including flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, naringenin, naringin, and taxifolin, were investigated for their antitumor effects in colorectal carcinoma cells (HT29, COLO205, and COLO320HSR). 2′-Hydroxyflavanone came up as the most potent chemopreventive agent and thus showed a significant inhibitory effect on tumor formation. A recent study on hesperetin supplementation in male albino Wistar rats showed its inhibition of 1,2-demithylhydrazine (DMH)-induced colon carcinogenesis. The investigation suggested that hesperetin may inhibit phase I enzymes (involved in carcinogen activation), induce phase II xenobiotic metabolising enzymes and scavenge the electrophilic carcinogenic species (Aranganathan *et al.*, 2009).

1.8.4.3. Anti-proliferation

Naringenin was successfully investigated for its cell antiproliferation effect on an HT-29 colon cancer cell line. Cell proliferation measured by a colorimetric assay was significantly inhibited especially when HT-29 cells are exposed to naringenin at doses greater than 0.7 mM. The results suggested a potential role for citrus fruits as a source of chemoprotective agents against colon cancer (Frydoonfar *et al.*, 2003). In a comparative study, flavanones showed a significant anti-proliferative activity against lung, colon, breast, prostate and melanoma cancerous cell lines, although less efficiently than flavones. Moreover, glycosylation reduced the anti-proliferative activity in both flavonoid classes (Manthey & Guthrie, 2002). A C2-C3 double bond seems important for the anti-proliferative activity as results from different studies clearly showed the greater anti-proliferative activity of flavones compared to flavanones (Agullo *et al.*, 1996; Kawaii *et al.*, 1999; Manthey & Guthrie, 2002; Rodriguez *et al.*, 2002). Another structural element that may influence anti-proliferative activity is the substitution of the flavonoid nucleus. For instance, methylation of hesperetin and eriodictyol at C7-OH increased the anti-proliferative capacity (Benavente-Garcia and Castillo, 2008). Several mechanisms have been put forward to explain the antiproliferative activity of flavonoids. The most accepted hypothesis is the inhibition of several kinases involved in signal transduction such as protein kinases C, tyrosine kinases, PI 3-kinases or S6 kinase (Casagrande & Darbon, 2001).

The effects of 17β-estradiol (E2) hormone cover a wide range of physiological processes in mammals such as reproduction, cardiovascular health, bone integrity, cognition

and behaviour etc. Besides these roles in human physiology, E2 is also involved in the development of many diseases, including various types of cancers. The mechanism proposed to explain the chemoprotective activity of naringenin suggests that the flavanone binds to ERα (estrogen receptor α) as an antagonist, thereby limiting the effect of E2 in promoting cellular proliferation (Bulzomi *et al.*, 2010).

1.8.5. Cardiovascular Effects

Cardiovascular diseases (CVD) affect the heart and surrounding blood vessels and can take many forms, such as high blood pressure, coronary artery disease, heart disease and stroke. CVD are the largest cause of death in the EU and account for approximately 40% of deaths (2 million deaths per year). Increased oxidative stress was found one of the major factors causing CVD and its control by antioxidants including polyphenols is of great biological significance.

1.8.5.1. Vasorelaxant & Vasoprotective effects

The vascular endothelial cells are very important in normal coronary functions. The regulation of vascular tone and blood flow to organs is controlled by endothelial cells, which synthesize and release a number of factors such as prostacyclin, nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and endothelin. Among these factors, NO is critical in the preservation of normal vascular functions and there is a clear relationship between coronary artery disease and NO dysfunctioning (Benavent-Garcia, 2008).

Flavonoids promote endothelial NO synthase (eNOS) and at the same time inhibit the inducible NOS (iNOS) (Olszanecki *et al.*, 2002). In vitro, inhibition of iNOS in LPS-activated RAW 264.7 cells is not significant with flavanones (naringenin) in comparison to flavones and flavonols (apigenin, luteolin, quercetin), which may demonstrate the significance of a C2-C3 double bond (Kim *et al.*, 1999). Recently, the vasorelaxant potential of hesperetin and hesperidin (Orallo *et al.*, 2004) and naringenin and naringin (Orallo *et al.*, 2005) was demonstrated in rats. These vasorelaxant effects are probably due to the inhibition of different phosphodiesterase isoenzymes.

1.8.5.2. Effect on Coronary Heart Disease (CHD)

There is compelling evidence that CHD is principally related with an elevation of LDL cholesterol. Normally, cholesterol, cholesterol esters and triglycerides are transported by LDL (especially Apolipoprotein B (ApoB)) from their sites of absorption or synthesis to sites of bioactivity. So any malfunctioning of these lipoproteins causes the accumulation of cholesterol in circulatory system and ultimately generates CHD.

The anti-atherosclerosis potential of citrus flavanones, hesperetin and naringenin, was attributed to their ability to regulate apoB secretion and cellular cholesterol homeostasis in human hepatoma cells line HepG2. A decrease in apoB accumulation was observed in the media following 24h incubation with hesperetin and naringenin. This reduced apoB secretion was related to a reduced cellular concentration of cholesteryl ester (Wilcox *et al.*, 2001). The mechanism involved in naringenin anti-atherosclerosis activity was a reduced apoB secretion primarily due to the inhibition of microsomal triglyceride transfer protein and the enhancement of LDL receptor (LDLr)-mediated apoB-containing lipoprotein uptake (Borradaile *et al.*, 2003).

1.8.5.3. Anti-atherosclerotic effects

Atherosclerosis consists in the hardening and narrowing of arteries, which progressively takes place and blocks arteries, putting blood flow at risk. Atherosclerosis is the usual cause of heart attacks, strokes, and peripheral vascular disease. Hesperetin was shown to limit the rise of hepatic lipid contents and enzymes activities involved in triacylglycerol synthesis in rats fed with 1% orotic acid (Cha *et al.*, 2001). Moreover, a hypolipidemic effect of hesperetin was also reported even during the high lipid concentrations (Kim *et al.*, 2003).

In rats fed a high-cholesterol diet, 0.1% naringenin reduced the levels of plasma cholesterol and hepatic triacylglycerols. This effect was accompanied by a decrease in the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl-CoA cholesterol acyltransferase (Lee *et al.*, 1999; Lee *et al.*, 2003). In another study on rabbits fed with cholesterol-rich diet, naringin showed an increase in superoxide dismutase and catalase activities to cooperate in the detoxification of free radicals produced. Moreover, naringin was also shown to regulate gene expression of superoxide dismutase, catalase and glutathione peroxidase (Jeon *et al.*, 2001).

1.8.6. Other biological effects

Anti-microbial Effects: the antiviral activity of hesperidin was demonstrated against herpes simples, polio, parainfluenza, and syncytial viral infections while naringin, was inactive (Kaul et al, 1985). In a recent study, hesperetin showed a moderate antimicrobial activity against Salmonella typhi and S. typhimurium (Kawaguchi *et al.*, 2004).

Anti-allergic activity: A study by Matsuda *et al.* (1991) suggests that hesperidin has an antiallergic activity via the inhibition of histamine release from pertinent mast cells in rats.

Miscellaneous: Knekt *et al.* (2002) found an association between a high intake of hesperetin and naringenin and a lower incidence of cerebrovascular disease and asthma.

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Publication N° 1

L'extraction des glycosides

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Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel

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Abstract

The present study reports on the extraction of polyphenols especially flavanones from orange (*Citrus sinensis* L.) peel by using ethanol as a food grade solvent. After a preliminary study showing that the best yield of extraction was reached for a particle size of 2 cm^2, a response surface methodology (RSM) was launched to investigate the influence of process variables on the ultrasound-assisted extraction (UAE) followed by a central composite design (CCD) approach. The statistical analysis revealed that the optimized conditions were a temperature of 40°C, a sonication power of 150W and a 4:1 (v/v) ethanol:water ratio. The high total phenolic content (275.8 mg of gallic acid equivalent / 100g FW), flavanone concentrations (70.3 mg of naringin and 205.2 mg of hesperidin / 100g FW) and extraction yield (10.9 %) obtained from optimized UAE proved its efficiency when compared with the conventional method. Furthermore, the antioxidant activity determined by the DPPH and ORAC tests confirmed the suitability of UAE for the preparation of antioxidant-rich plant extracts.

Keywords: Ultrasound, extraction, antioxidant, by-product, orange peel.

1. INTRODUCTION

Epidemiological studies have suggested the beneficial effects of citrus fruits (rich in flavanones) against many degenerative diseases like cardiovascular diseases and some cancers (Benavente-Garcia, Castillo, Marin, Ortuno, & Rio, 1997; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). These positive influences on human health has significantly increased the citrus consumption in the last few years and it is continuously increasing with an estimated world production of citrus fruits up to 72 million tons in the session 2007-08, among which the major commercially important orange fruits accounts for about 45 million tons (USDA, 2008). The domestic and industrial use of these large quantities of citrus fruits, especially for the production of juice, results in the accumulation of high amounts of by-products such as peel, seed, cell and membrane residues which account for about half of the fruit weight. These by-products can be used for the production of molasses, pectins, essential oils, limonene and cattle feed (Bocco, Cuvelier, Richard, & Berset, 1998; Jeong et al., 2004; Li, Smith, & Hossain, 2006). In addition, citrus by-products are a good source of phenolic compounds, especially the characteristic flavanone glycosides which mainly include naringin, hesperidin, narirutin, and neohesperidin. Currently, their extraction from citrus peels has attracted considerable scientific interest to use them as natural antioxidants mainly in foods to prevent the rancidity and oxidation of lipids (Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Boskou, 2006; Peschel et al., 2006; Zia-ur-Rehman, 2006). Indeed, in recent years, a lot of research has focused on plants and their by-products to extract natural and low-cost antioxidants that can replace synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which might be liver-damaging, carcinogenic (Ak & Gülçin, 2008) and more generally toxic (Moure et al., 2001).

Up to now, several conventional extraction techniques have been reported for the extraction of phenols from citrus peels like solvent extraction (Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Boskou, 2006; Jeong et al., 2004; Li, Smith, & Hossain, 2006a; Manthey & Grohmanu, 1996; Xu, Ye, Chen, & Liu, 2007; Zia-ur-Rehman, 2006), hot water extraction (Xu et al., 2008), alkaline extraction (Bocco, Cuvelier, Richard, & Berset, 1998; Curto, Tripodo, Leuzzi, Giuffrè, & Vaccarino, 1992), resin-based extraction (Calvarano, Postorino, Gionfriddo, Calvarano, & Bovalo, 1996; Kim, Kim, Lee, & Kim, 2007), enzyme-assisted extraction (Li, Smith, & Hossain, 2006b), electron beam- and γ-irradiation-based extractions (Kim, Lee, Lee, Nam, & Lee, 2008; Oufedjikh, Mahrouz, Amiot,

& Lacroix, 2000) and supercritical fluid extraction (Giannuzzo, Boggetti, Nazareno, & Mishima, 2003). These conventional or more innovative extraction techniques may either cause the degradation of the targeted compounds due to high temperature and long extraction times as in solvent extractions, or pose some health-related risks due to the unawareness of safety criteria during irradiation. Furthermore, enzyme-assisted extraction is limited due to problems of enzyme denaturation. With the development of the "Green Chemistry" concept during the last few years, environment-friendly techniques are becoming more and more attractive. The extraction of bioactive compounds under ultrasound irradiation (20-100 kHz) is one of the upcoming extraction techniques that can offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat, Tomao, & Virot, 2008).

During sonication, the cavitation process causes the swelling of cells or the breakdown of cell walls, which allow high diffusion rates across the cell wall in the first case or a simple washing-out of the cell contents in the second (Vinatoru, 2001). Besides the solvent, temperature and pressure, better recoveries of cell contents can be obtained by optimizing ultrasound application factors including frequency, sonication power and time, as well as ultrasonic wave distribution (Wang & Weller, 2006). Optimization of ultrasound-assisted extraction (UAE) has been described recently to extract hesperidin from Penggan (*Citrus reticulata*) peel (Ma et al., 2008a), phenolic acids and flavanone glycosides from Satsuma Mandarin (*Citrus unshiu* Marc) peel (Ma et al., 2008b; Ma, Chen, Liu, & Ye, 2009) and total phenolic contents from Penggan peel (Ma, Chen, Liu, & Ye, 2008c) (see table 1). In these works, methanol came up as a suitable extraction solvent to reach good yields of the above-mentioned phenolic compounds. However, environmentally benign and non-toxic food grade organic solvents like ethanol, n-butanol and isopropanol are recommended by the US Food and Drug Administration for extraction purposes (Bartnick, Mohler, & Houlihan, 2006).

A literature search did not yield any reference about earlier reports on the UAE of phenolic compounds from orange peels by using food grade solvents. The objective of this work is to outline the potentiality of UAE in the fast preparation of extracts rich in polyphenols (especially flavanone glycosides) from orange peels in good yields. Several parameters that could potentially affect the extraction efficiency were evaluated and optimized using a statistical experimental design approach. Finally, the optimized UAE

results obtained were compared with those achieved by using a conventional extraction method.

2. MATERIALS AND METHODS

2.1. Plant material

About 10 kg of orange (*Citrus sinensis* L. Osbeck from Valencia late cultivar, Spain) peels after juice extraction were collected locally from a citrus juice industry (Vaucluse, France). They were stored in a freezer at -20°C.

2.2. Chemicals

The solvents used were of analytical grade and supplied by VWR International (Darmstadt, Germany). Flavanone glycosides (naringin, hesperidin) were purchased from Extrasynthese (Genay, France), caffeic acid from Sigma-Aldrich (Steinhaus, Germany), and trolox from Acros Organics (Slangerup, Denmark). DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2′-azobis (2-methyl)propionamidine dihydrochloride) and fluoresceine were obtained from Alfa Aesar (Karlsruhe, Germany), Sigma-Aldrich (Steinhaus, Germany) and Acros Organics (Morris Plains, USA), respectively.

2.3. Instrumentation

Sonication apparatus: Ultrasound-assisted extraction (UAE) was performed with a PEX 3 Sonifier (R.E.U.S., Contes, France) composed of an inox jug having 23 ×13.7 cm internal dimensions with a maximal capacity of 3 L, and a transducer, in the base of jug, operating at a frequency of 25 kHz with maximum input power of 150 W. The double layered mantle allowed us to control the temperature of the medium by cooling/heating systems. The output power of the generator is 150 Watts while the power dissipated in the medium is about 60 Watts, as measured by calorimetry. The detailed diagram of the apparatus has shown in the figure 1.

HPLC analysis: HPLC analyses were performed using a Waters (Milford, MA) HPLC system consisting of a Waters 600E pump, a Waters 717 autosampler, a Waters 2996 photodiode array detector. The HPLC pumps, autosampler, column temperature, and diode array system were monitored and controlled by using Waters Empower 2 Chromatography

Data software program. The wavelength used for the quantification of the flavanones glycosides with the diode detector was 280 nm. The chromatographic separation was carried out on a Purospher Star RP-18 end-capped column (250 mm × 4 mm I.D.; 5 μm particle size from VWR), with a RP18 guard column (4 mm×4mm I.D.; 5μm particle size also from VWR). The end-capped column and guard column were held at 37°C and the flow rate was set at 1mL/min. The mobile phase consisted of two solvents: 0.5% acetic acid (A) and 100% acetonitrile (B). The solvent gradient in volume ratios was as follows: 10–30% B over 20 min. The solvent gradient was increased to 35% B at 25 min and it was maintained at 35% B for 5 min. The injection volume was 20 μL. Analyses were performed at least three times and only mean values were reported. Quantification was carried out by using the external standard method and the final concentrations were calculated in mg/100g FW.

Spectrophotometers: Absorbance measurements were carried out on a Spectronic GENESYS 5 UV-Visible spectrophotometer (wavelength range: 200-1100 nm) equipped with a eight-position multicell holder. Measurements of fluorescence intensity were carried out on a SPEX-Fluoromax 2 spectrofluorimeter from Jobin Yvon.

2.4. Extraction procedure

A comparative study has been conducted between the conventional and ultrasound-assisted techniques after the optimisation of the latter. *UAE*: in experiments aimed at optimizing the extraction temperature, ultrasound power, and ethanol percentage, orange peels (0.25 g/mL) were sonicated in the solvent (ethanol-water mixture) for 30 min. The optimal parameters were further used to investigate the extraction time required for maximal yield. *Solvent extraction (SE)*: a control extraction was run by using the temperature and ethanol percentage that were found optimal for UAE.

2.5. Particle size study

A series of five experiments with five different particle sizes (0.5, 1.0, 1.5, 2.0 and 2.5 cm^2) was carried out by using the conventional solvent extraction procedure with central point conditions (25°C, 1:1 ethanol-water solution, stirring, 30 min). Peel particles having a thickness of about 0.5 cm were cut out randomly with help of calibrated steel cubes.

2.6. Total phenolic content (TPC)

The TPC of samples was measured with a kit (SEPPAL (Isitec-lab), France) especially suitable for TPC measurement of foods and drinks. This kit includes reagent A (modified Folin-Ciocalteu reagent), reagent B (alkaline buffer) and a gallic acid solution (3 g/L). A small volume (20 µL) of H_2O (blank), gallic acid solution (standard) or the extract (sample) was mixed with reagent A (2 mL). After 1 min, 1 mL of reagent B was added to each sample. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their absorbance was measured at 760 nm. TPCs were calculated by using the following formulae: TPC = 3×(sample absorbance – blank absorbance)/(standard absorbance – blank absorbance). TPC measurements were performed thrice and mean values, expressed as mg gallic acid/100 g of fresh weight (mg GA/100 g FW), were reported.

2.7. Yield determination

Ethanol was removed from the extracts by evaporation under vacuum at 40°C on a rotary evaporator. Then, the samples were frozen and lyophilised to remove water. Finally, the yield of each extract was calculated from its weight and expressed in percentage.

2.8. Design of Experiment

Box-Wilson design, also called Central Composite Design (CCD), is used to achieve maximal information about the process from a minimal number of possible experiments. The type of CCD used in this study was central composite face-centred (CCF) experimental design to determine the optimal conditions of UAE. The application of a CCF design is a convenient way to optimize a process with three levels (-1, 0 and +1) for each factor. In this design, the star points are at the centre of each face of the factorial space, thus $\pm\alpha = \pm1$. This design is needed to evaluate the effects and interactions of three independent variables, namely temperature (°C) (X1), power (W) (X2) and ethanol:water ratio (%v/v) (X3). The coded levels and the natural values of the factors used in this experimental design are shown in Table 2 in parallel. A total of 20 different combinations, including six replicates of centre point, each designated by the coded value 0, were chosen in random order according to a CCF configuration for three factors. The selected optimization parameters were TPC after 30 min (mg gallic acid/100g fresh weight) (Y1), naringin concentration (mg/100g FW) (Y2), hesperidin concentration (mg/100g FW) (Y3), yield of extracts (%) (Y4) and extraction rate constant (min^{-1}) (Y5).

The experimental designs used were constructed and the experimental results were processed by using the software STATGRAPHICS PLUS (Version 5.1, Statistical Graphics Corporation, Rockville, USA, 2000). An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable in order to test the model significance and suitability. The F-value in ANOVA is the ratio of mean square error to the pure error obtained from the replicates at design centre and the P-value defines the significance of the different variables. A description of significant effects obtained from ANOVA for TPC (30 min) was presented by a Standardized Pareto Chart.

2.9. Kinetic studies

Extraction rate constant (k): During each extraction process, uptake of 1 mL from the mixture was performed at 5, 10, 20 and 30 min to determine the corresponding TPC values. Assuming a first-order accumulation of total phenols in solution (Fig. 4), we can write:

$TPC_t = TPC_\infty (1 - e^{-kt})$

TPC_t: TPC value at time t, TPC_∞: final TPC value (determined at t = 8h), k: apparent first-order rate constant of extraction. Thus, from the linear plots of $-Ln(1-(TPC_t/TPC_\infty))$ against time (correlation coefficient in the range 0.90-0.99), the k values could be determined.

Activation energy (E_a): From the Arrhenius equation, the activation energies for total phenol extraction by UAE and SE were determined from plots of Ln*k* against 1/T, where T is the absolute temperature (283, 298 and 313 K).

2.10. Antioxidant tests

As there is no standardised method to evaluate the antioxidant potential of foods and biological systems, it is recommended to evaluate the antioxidant activity by various methods (Frankel & Meyer, 2000).

***DPPH assay*:** DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable highly coloured free radical that can abstract labile hydrogen atoms from phenolic antioxidants with concomitant formation of a colourless hydrazine (DPPH-H) (Diouf , Stevanovic, & Cloutier, 2009). The free radical-scavenging activity (FRSA) of an extract can be expressed as the percentage of DPPH reduced by a given amount of extract. The FRSA of the extracts was evaluated

according to the method described by Mimica-Dukic, Bozin, Sokovic, & Simin (2004), with some modifications. The extract was dissolved in 50% (v/v) aqueous methanol with a final concentration of 5 g/L. A small volume (0.1 mL) of the extract solution was mixed with 2.0 mL of a 0.1 mM DPPH solution in MeOH and the mixture was left in the dark at room temperature for 60 min. The absorbance was measured at 517 nm. The total FRSA of each extract was expressed as the percentage of DPPH reduced and was calculated by the following equation: FRSA = 100x(initial absorbance – final absorbance)/initial absorbance. The initial absorbance and final absorbance are the absorbance values of DPPH at time zero and after 60 min, respectively.

***ORAC (Oxygen Radical Absorbance Capacity) assay*:** In this method, the hydrophilic peroxyl radicals ($ROO^{\bullet}$) generated by the thermal decomposition of the diazo compound AAPH oxidize the fluorescent probe FL, thus causing a fluorescence quenching. Hence, inhibition of this quenching by an antioxidant is a measurement of its ability to reduce $ROO^{\bullet}$ (Gomes, Fernandes, & Lima, 2005). The ORAC method employed was adapted from a method previously described by Ou, Hampsch-Woodill, & Prior (2001). All reagents were prepared in a 75 mM phosphate buffer at pH 7.4. Trolox (0-75 μM) was used as the standard. A mixture of the fluorescent probe fluorescein (FL, 2 mL of a 26 nM solution in phosphate buffer) and extract (15 μL of a 5 g/L solution in MeOH) was pre-incubated for 10 min at 37°C. Then, 1 mL of a 664 mM AAPH solution in the phosphate buffer was added. The fluorescence intensity was measured every 2 min during 40 min with excitation and emission wavelengths set at 490 nm and 511 nm, respectively. Its decay refers to FL oxidation by the AAPH-derived peroxyl radicals. The ORAC value is calculated from the area under the curve expressing the quenching of FL fluorescence in the presence of the extract in comparison with curves constructed with known trolox concentrations. The measurements were taken in triplicate. The area under the curve (AUC) was calculated as $AUC = 1 + f_2/f_0 + f_4/f_0 ... + f_i/f_0$ where f_i is the fluorescence reading at time i (in sec). The net AUC was obtained by subtracting the AUC of the blank (no antioxidant). The results were expressed as millimoles of trolox equivalents (TE) per gram of sample on a fresh weight basis (mmol TE/100g FW). Both antioxidant tests (DPPH and ORAC) were performed at least three times for each extract and only mean values were reported.

3. Results and discussion

3.1. Influence of particle size

From previous studies (Garcia-Ayuso & Luque de Castro, 1999; Cuoco, Mathe, Archier, Chemat, & Vieillescazes, 2008; Vilkhu, Mawson, Simons, & Bates, 2008; Wang & Weller 2006), the particle size was considered one of the important factors that can affect the efficiency of polyphenol extraction from orange peels. Thus, preliminary experiments on orange peels of 0.5, 1.0, 1.5, 2.0 and 2.5 cm^2 gave final yields of 3.44, 3.65, 4.32, 4.41 and 4.38%, respectively. A size of 2 cm^2 was selected as an optimum for our extraction experiments. The slightly lower yield observed with particles of smaller size could be due to the particles staying at the surface of the solvent during extraction, thereby limiting their exposition to ultrasonic waves.

3.2. Central composite design results

The coded and decoded values of independent variables and the responses obtained in the multivariate study for each experiment are shown in Table 2. In this second part of the study, the effect of temperature (°C) X1, ultrasonic power (W) X2 and ethanol:water ratio (% v/v) X3 on UAE of orange peel polyphenols in terms of TPC (mg GA/100g FW) Y1, naringin concentration (mg/100g FW) Y2 and hesperidin concentration (mg/100g FW) Y3 was evaluated by response surface methodology. Identification of naringin and hesperidin was achieved by comparing their retention times and UV spectra with standards (Fig. 2). The yield (%) Y4 and the extraction rate constant (k) (min^{-1}) Y5 were also determined. ANOVA for TPC determination (30 min) gave a coefficient of determination (R^2) of 98.3%, which indicates a close agreement between experimental and predictive values. ANOVA data for TPC are also shown on a Pareto Chart (Fig. 3), which represents the significant effects of all variables (linear and quadratic) and their interactions. The length of the bars is proportional to the absolute magnitude of the estimated effects coefficients while the dashed line represents the minimal magnitude of statistically significant effects (95% of the confidence interval) with respect to response. It can be seen that ultrasound power has the most important influence on TPC followed by temperature, ethanol:water ratio, interaction of power and ethanol:water ratio, squared term of temperature and interaction of power and temperature. The lack of significance of the cross-product terms suggests the absence of interactions between variables in the studied range.

The experimental data built after running 20 trials allowed us to fit all the responses as a function of temperature, power and ethanol:water ratio. The second-order polynomial equations of the response surfaces obtained are as follow:

$$Y1\ (\text{mg GA/100g}) = 184.782 + 19.471T + 25.2149P + 13.4689E - 9.09455T^2 + 5.01087TP + 1.76187TE + 3.69195P^2 - 8.22213PE - 0.730045E^2 \quad (1)$$

$$Y2\ (\text{mg/100g}) = 33.2623 + 3.6048T + 5.7951P + 3.664E - 1.7605T^2 + 1.1415TP + 1.557TE - 0.458P^2 - 0.20425PE - 0.5945E^2 \quad (2)$$

$$Y3\ (\text{mg/100g}) = 112.432 + 10.5262T + 15.159P + 9.6949E - 4.7805T^2 + 2.09637TP + 1.54313TE - 1.0805P^2 - 0.035875PE - 2.247E^2 \quad (3)$$

$$Y4\ (\%) = 7.72445 + 0.763T + 0.857P + 0.397E - 0.123636T^2 + 0.35375TP + 0.03625TE + 0.306364P^2 - 0.07375PE - 0.0936364E^2 \quad (4)$$

$$Y5\ (\text{min}^{-1}) = 0.0204645 + 0.00303T + 0.00903P + 0.00063E - 0.00113636T^2 + 0.0017375TP + 0.0013375TE + 0.00516364P^2 - 0.0000875PE - 0.00153636E^2 \quad (5)$$

where T is the temperature (°C), P the ultrasound power (W) and E the ethanol:water ratio (% v/v).

3.3. Optimal conditions

Response surface optimization can be found depending on the three key variables, namely, temperature, power and ethanol:water ratio. The optimal conditions obtained from the first derivatives of the second-order polynomial equation were derived a second time. The derivatives were then equalled to 0 and solved in an equation system. The coded values obtained from these equations were thus decoded and rounded in order to be applied to the device. The obtained natural values corresponding to optimal conditions for each response were as follows: Y1 = 40°C, 150 W, 80%; Y2 = 40°C, 150 W, 80%; Y3 = 40°C, 150 W, 80%; Y4 = 40°C, 16 W, 80%; Y5 = 39°C, 50 W, 69%. As expected and according to the response surfaces, the extraction efficiency in terms of TPC, naringin and hesperidin concentrations increases by increasing all the three factors. In all these responses, the optimal values were beyond the limits that we selected. Thus, the values finally selected correspond to the

maximal values chosen to define the experimental domain (Lucchesi, Smadja, Bradshaw, Louw, & Chemat, 2007). On the basis of our principle responses (Y1, Y2, Y3), the temperature of 40°C, ultrasound power of 150 W and ethanol:water ratio (v/v) of 80% were chosen as optimal values to go on with our experiments. A repeatability study was conducted by using these optimal conditions to assess the predictive ability of the models and the results were found in accordance with those obtained in the second trial {+1 (40°C), +1 (150W), +1 (80% ethanol)} of experimental design. Several recent investigations on the extraction of phenolic contents from citrus peel have also suggested operating conditions similar to those recommended in this study (Li, Smith, & Hossain, 2006a; Ma, Chen, Liu, & Ye, 2009; Ma et al., 2008a).

3.4. Comparison of UAE vs. SE

TPC extracted from orange peels by UAE (40°C, 150 W, 80% ethanol, stirring) and SE (idem except sonication) is shown on Fig. 4. The TPC obtained by UAE during 15 min was significantly higher than by SE during 60 min. Due to mechanical effects on cell walls evidenced by scanning electron microscopy (Balachandran, Kentish, Mawson, & Ashokkumar, 2006; Li, Pordesimo, & Weiss, 2004), UAE permits higher extraction yields in shorter periods of time, thereby reducing the energy input.

The main flavanone glycosides found in orange (*C. sinensis*) are naringin and hesperidin, the latter being more abundant than the former (Wang, Chuang, & Hsu, 2008). Both were simultaneously titrated by HPLC from the samples obtained by UAE and SE after 60 min. The quantities of naringin and hesperidin from UAE (70.3 and 205.2 mg/100g of fresh weight, respectively) were considerably higher than those obtained from SE (50.9 and 144.7 mg/100g FW, respectively). No evidence for flavanone degradation under sonication could be found. Indeed, the ultrasonic degradation of phenols is typically slow in comparison with more volatile aromatics that diffuse more readily into the cavitation bubble for pyrolysis (Chowdhury & Viraraghavan, 2009). In addition, phenol degradation is favoured at higher frequencies (required for the generation of the hydroxyl radical by water homolysis) than the one selected in this work (20 kHz). The extraction yield is an important response factor for evaluating an extraction process. It was estimated to be 10.9% and 8.6% for UAE and SE, respectively. This is consistent with UAE having a potential to extract natural products in better yields than conventional techniques, not only at the lab-scale but also at the pilot-plant scale (Boonkird, Phisalaphong, & Phisalaphong, 2008).

Extraction of total phenols was found *ca*. 3 times as fast under ultrasounds (k = 0.10 (± 0.01) min^{-1}) as in the conventional procedure (k = 0.03 (± 0.01) min^{-1}). Consistently, the activation energy (6.34 kJ/mol for UAE *vs.* 34.21 kJ/mol for SE) is smaller. Similar kinetic effects were evidenced by Chemat et al. (2004) for the UAE of caraway seeds in hexane.

3.5. Antioxidant capacities

UAE and SE were finally evaluated by comparing the antioxidant potential of the corresponding extracts. Phenolic antioxidants are typically able to quickly reduce reactive oxygen species (ROS) including free radicals, thereby protecting biomolecules (*e.g.*, polyunsaturated fatty acids) against oxidation (Dangles, 2006). The FRSA value was 54% and 42% for the extracts obtained by UAE and SE, respectively. The increase in FRSA observed with UAE, although modest, is in agreement with the higher total phenol concentration estimated by UAE and confirms the usual correlation between antioxidant activity and TPC (Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Boskou, 2006; Ma et al., 2008b). The ORAC values were estimated to be 712 mmol TE/100g FW and 509 mmol TE/100g FW for the UAE and SE extracts, respectively.

While UAE during 60 min results in a 35-40% increase in TPC vs. SE, the FRSA estimated by the DPPH assay increases by less than 30% and the ORAC value by 40%. Hence, the ORAC assay appears more consistent with the increase in TPC than the DPPH radical assay. Indeed, the major orange flavanones naringenin and hesperetin are relatively weak antioxidants since they do not display a catechol group (1,2-dihydroxybenzene), which is the critical structural determinant of strong phenolic antioxidants (Goupy, Loonis, Dufour, & Dangles, 2003). As a consequence, they are expected to react very slowly with the stable DPPH radical. Thus, much more reactive radicals, such as the peroxyl radicals delivered in the ORAC test, are required to fully express the electron/H-donating activity of orange flavanones (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009). It must also be pointed out that nonphenolic antioxidants such as ascorbate (which is responsive to the test for TPC determination) can be partially responsible for the overall antioxidant activity of the extracts, especially in the DPPH assay where the flavanones are expected to make a minor contribution.

Conclusion

The UAE of phenolic antioxidants from orange peels with ethanol-water mixtures appeared very effective in comparison to conventional procedure. The results from CCD pointed out the sonication power as the most influential factor in the UAE process followed by temperature and ethanol:water ratio. Although the same volumes of solvent were used in both extraction processes, the duration of the ultrasound-assisted process and consequently the energy input were drastically reduced without affecting the overall yield. Hence, UAE can be called an ‘environment-friendly’ or ‘green’ technique. Overall, ultrasound-assisted extraction of polyphenols from abundant food by-products such as orange peels and by using food grade solvents has a strong potential of industrial development as an efficient and environment-friendly process for the preparation of extracts rich in natural antioxidants aimed at replacing synthetic antioxidants.

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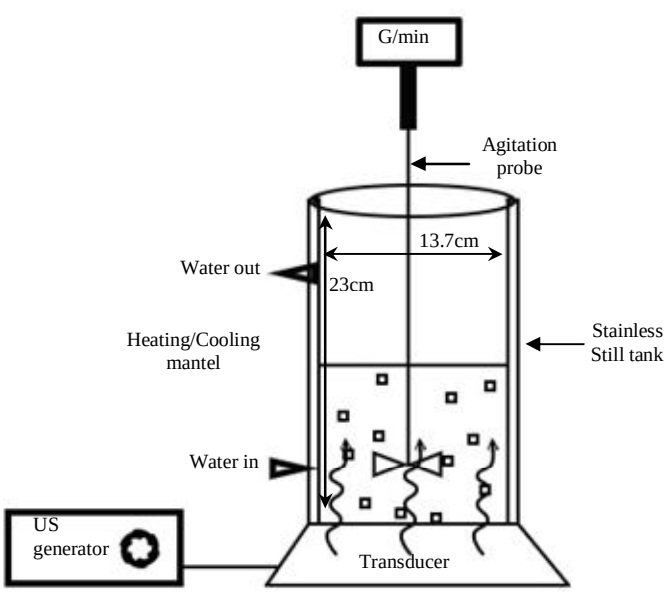
Table 1: Recent publications on the extraction of polyphenols under ultrasound irradiation

Plant Material	Analytes	Comments	Reference
Satsuma Mandarin *Citrus unshiu* Marc	Phenolic acids (PA)	UAE time = 10-40min; maceration for 8h for similar yields of PA.	Ma et al., 2009
Du Zhong Ye *Folium eucommiae*	Flavonoids	UAE was found more efficient than heating, microwave- & enzyme-assisted extractions	Huang et al., 2009
Wheat bran *Triticum aestivum*	Phenolics-rich heteroxylans	Extraction time reduced from 60 min (conventional extraction) to 5 min (UAE).	Hromádková et al., 2008
Penggan *C. reticulata*	Hesperidin	Comparable yields with UAE but less degradation of hesperidin compared with soxhlet extraction.	Ma et al., 2008a
Satsuma mandarin *C. unshiu* Marc	Phenolic acids and flavanone glycosides	Increase in polyphenol content and antioxidant activity of extracts obtained by UAE in comparison with maceration.	Ma et al., 2008b
Penggan *C. reticulata*	Total phenolic content (TPC)	TPC increased on increasing irradiation time and temperature	Ma et al., 2008c
Winged burning bush *Euonymus alatus*	Flavonols rutin & quercetin	UAE efficiency monitored by microscopy	Yang & Zhang, 2008

Table 2: Central Composite Design of three variables with their observed responses

Exp. No	Coded variables			Decoded Variables			Responses				
	X1	X2	X3	T*	P*	E*	TPC 30min (mg GA /100g)	Naringin (mg/100g)	Hesperidin (mg/100g)	Yield (%)	Extraction rate constant (min^{-1})
1	0	0	0	25	100	50	185.493	36.193	119.290	7.8	0.0260
2	1	1	1	40	150	80	233.460	48.610	146.729	10.03	0.0402
3	0	0	0	25	100	50	185.068	32.721	112.853	7.77	0.0197
4	1	-1	1	40	50	80	197.646	33.347	113.332	8.09	0.0170
5	-1	-1	-1	10	50	20	121.259	17.831	71.692	6.27	0.0126
6	0	-1	0	25	50	50	162.480	28.247	93.183	6.97	0.0150
7	0	0	0	25	100	50	183.531	35.694	118.834	7.81	0.0215
8	0	0	0	25	100	50	185.994	33.295	113.186	7.76	0.0210
9	-1	1	1	10	150	80	187.276	34.007	117.123	7.93	0.0253
10	1	-1	-1	40	50	20	140.352	21.640	86.740	6.81	0.0152
11	0	0	-1	25	100	20	174.472	30.860	99.236	7.14	0.0153
12	0	0	1	25	100	80	192.352	32.260	110.866	7.96	0.0189
13	0	0	0	25	100	50	184.666	32.051	112.310	7.78	0.0208
14	-1	1	-1	10	150	20	169.918	29.345	96.847	7.09	0.0292
15	-1	0	0	10	100	20	159.217	29.531	98.749	6.89	0.0164
16	1	1	-1	40	150	20	225.302	36.469	124.489	9.59	0.0353
17	1	0	0	40	100	50	190.878	31.257	106.286	8.15	0.0186
18	-1	-1	1	10	50	80	155.258	24.561	87.903	6.86	0.0125
19	0	0	0	25	100	50	186.496	34.051	118.650	7.75	0.0211
20	0	1	0	25	150	50	213.188	35.146	119.252	8.93	0.0326

*T=X1=Temperature (°C); P=X2=Power (W); E=X3=Ethanol:water ratio (% v/v)



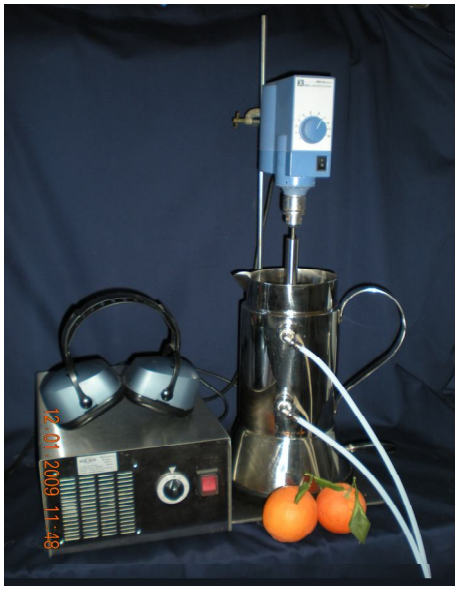


Figure 1: Sonication apparatus used for UAE

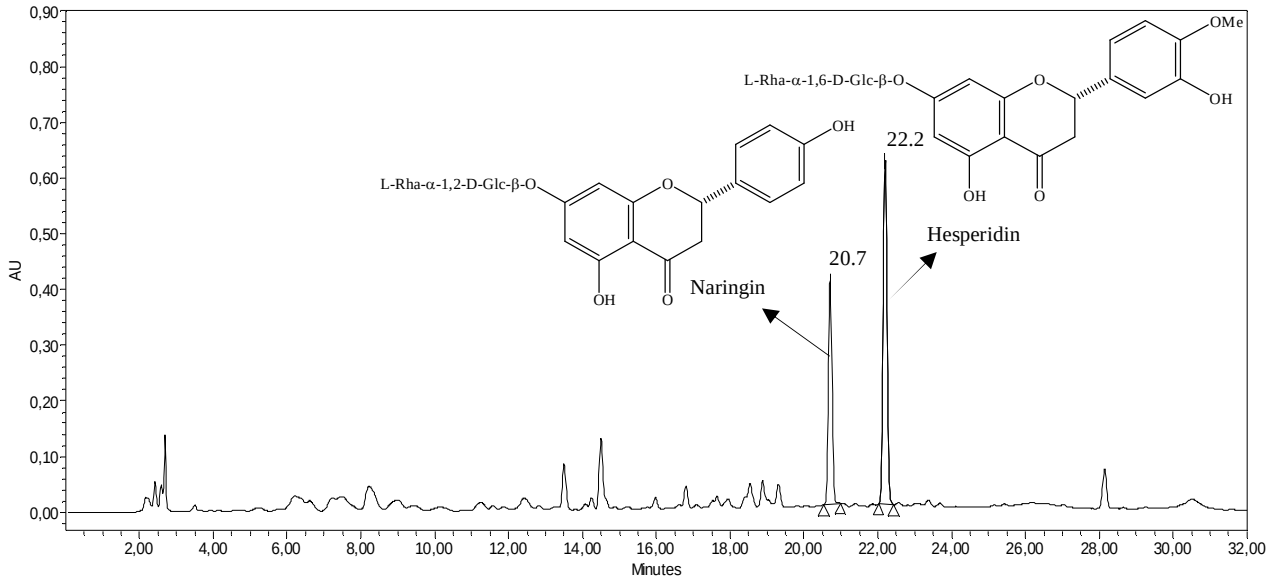


Figure 2: HPLC analysis of an extract obtained by ultrasound-assisted extraction of orange peels

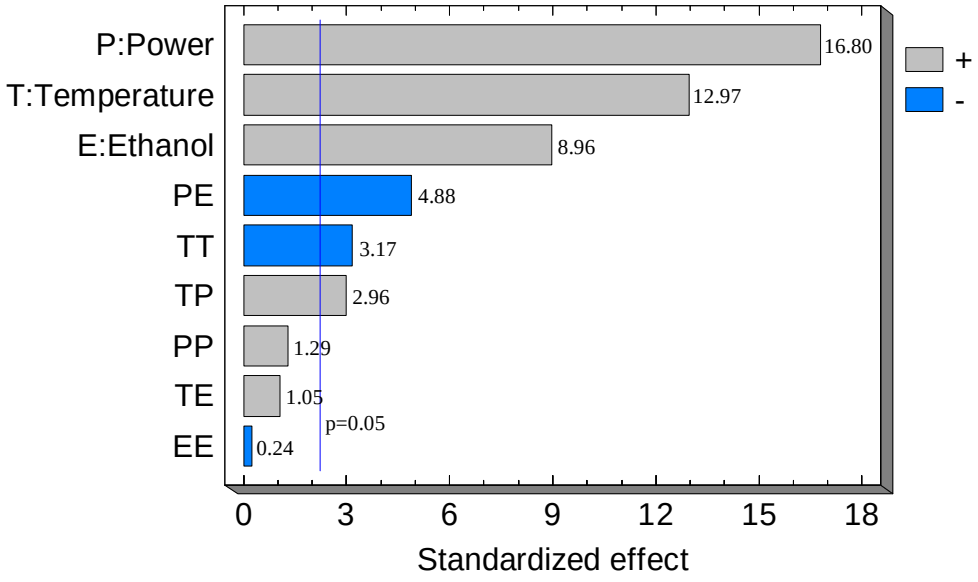


Figure 3: Pareto Chart for Total phenolic content (mg GA/100g) at 30 min

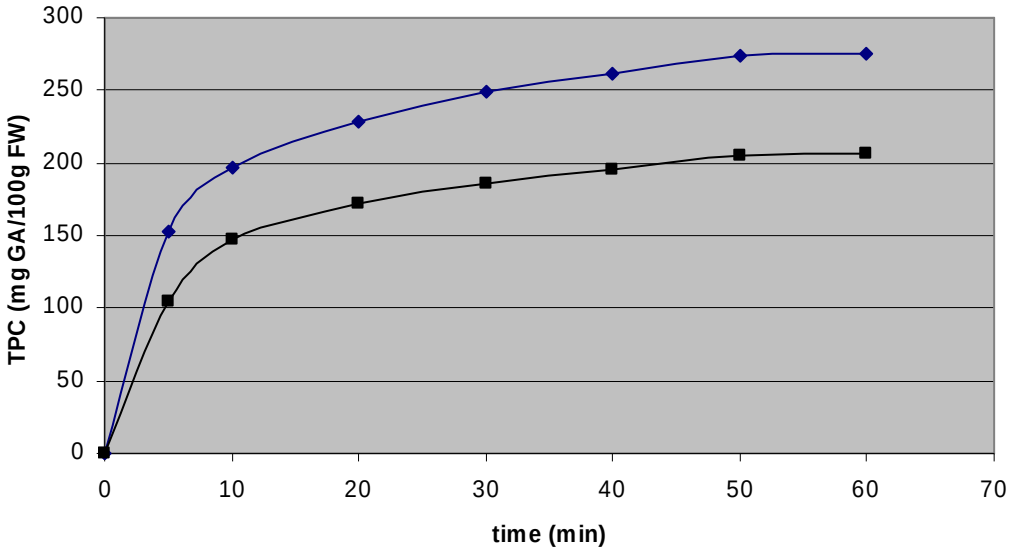


Figure 4: Comparison of total phenolic contents (mg GA/100g) from ultrasound-assisted extraction (UAE) —◆— and Solvent extraction (SE) —■—

Publication N° 2

La synthèse des glucuronides

La synthèse des glucuronides

Chemical Synthesis of Citrus Flavanone Glucuronides

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ABSTRACT

Flavanone glucuronides are the major phenolic metabolites detected in human plasma after consumption of citrus fruits. As such, they might display significant cardioprotective effects. In this work, glucuronides of naringenin (4′- and 7-*O*-*β*-D-glucuronides) and hesperetin (3′- and 7-*O*-*β*-D-glucuronides), the major flavanone aglycones in grapefruit and orange respectively, have been chemically synthesized. On the one hand, the most reactive hydroxyl group C7-OH was protected by selective benzoylation to allow subsequent glucuronidation of C4′-OH (naringenin) or C3′-OH (hesperetin) (B-ring). On the other hand, the selective debenzoylation at C7-OH of the perbenzoylated flavanone aglycones allowed glucuronidation at the same position (A-ring). After careful deprotection, the target compounds were purified and characterized by nuclear magnetic resonance and mass spectrometry.

KEYWORDS: flavanone, glucuronide, bioavailability, conjugation, chemical synthesis

INTRODUCTION

Because of the large and increasing worldwide consumption of citrus fruits and juices, potentially bioactive citrus polyphenols, which mainly belong to the flavanone class, are receiving increasing attention from nutrition biologists. Among flavanones, the naringenin and hesperetin glycosides are of particular interest because of their high prevalence in grapefruit and orange respectively (*1*, *2*). Citrus flavanones may play a beneficial role in the prevention of cardiovascular diseases and cancers due to their anti-inflammatory, antioxidant, anti-mutagenic and anti-tumor activities (*2-4*). After ingestion, the flavanone glycosides typically reach the colon where they are hydrolyzed by microflora glycosidases into aglycones (naringenin, hesperetin), which are partially absorbed through the intestinal barrier (*5*, *6*). In contrast, the partial absorption of flavanone aglycones and *O*-*β*-D-glucosides (e.g. naringenin 7-*O*-*β*-D-glucoside) can occur earlier in the small intestine through passive diffusion for the former and with preliminary hydrolysis of the latter by endothelial *β*-D-glucosidases (*6-8*). In the intestinal and hepatic cells, flavanone aglycones are extensively converted into glucuronides and sulphates so that the entire fraction of dietary flavanone that crosses the intestinal barrier finally enters the general blood circulation as conjugates, mainly glucuronides (*5*, *9-11*). In particular, naringenin 4′- and 7-O-*β*-D-glucuronides and hesperetin 3′- and 7-O-*β*-D-glucuronides were all detected in the plasma and urine of human volunteers having consumed a single portion of orange fruit or juice (*11*).

Despite the detailed information now available on the bioavailability of flavanones, there is still a great need of purified metabolites for accurate titration and identification in biological fluids. In addition, a better knowledge of the biochemical mechanisms by which dietary flavanones exert their potential health effects requires investigations on appropriate cell models (e.g., endothelial or smooth muscle cells) with the authentic circulating

metabolites instead of the commercially available glycosides and aglycones that are frequently used as a first approach despite the limited biological significance. As an alternative to the expensive, inconvenient and low yielding extraction of conjugates from biological fluids, chemical synthesis appears as the most direct strategy to obtain substantial amounts of these metabolites for bioavailability and in vitro cell studies. In this paper, we wish to report the synthesis of the four circulating glucuronides of hesperetin and naringenin.

MATERIALS AND METHODS

All starting materials were obtained from commercial suppliers, mainly Sigma-Aldrich (Steinheim, Germany), and were used without purification. Solvents were distilled over CaH_2. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 obtained from Merck KGaA (Darmstadt, Germany). Detection was achieved by UV light (254 nm) and by charring after exposure to a 5% H_2SO_4 solution in EtOH. Purifications were performed by column chromatography on silica gel 60 (40-63 μm) (Merck KGaA). Dowex 50WX4-50 ion-exchange resin was used for acidification. Melting points were measured on a Barnstead Electrothermal 9100 apparatus and are uncorrected. Glucuronyl donor (**1**) (methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate) was synthesized from glucurono-3,6-lactone according to a 3-step procedure already described in the literature (*12, 13*).

NMR. 1D ^{1}H and ^{13}C NMR spectra of synthetic intermediates were recorded at 300 MHz on a Bruker Advance DPX-300 apparatus. 1D ^{1}H and ^{13}C NMR spectra and 2D ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HMBC, HSQC) spectra of the final products were recorded at 500 MHz on a Bruker Advance DRX-500 apparatus. NMR chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane using the deuterium signal of the solvent ($CDCl_3$, CD_3OD) for calibration. ^{1}H-^{1}H coupling constants (J) are in hertz (Hz).

HR-MS. High-resolution mass analyses were carried out on Qstar Elite instrument (Applied Biosystems SCIEX, Foster City, CA) equipped with atmospheric pressure ionisation (API) interface. Mass detection was performed in the positive electrospray ionization mode in the following conditions: ion spray voltage, 5.5 kV; orifice voltage, 40 V; nebulising gas (air) pressure, 20 psi. The mass spectra were obtained with a time of flight (TOF) analyzer. The accurate mass measurement was performed in triplicate along with internal calibration. The ions chosen for internal references were the ammonium adducts $[M+NH_4]^+$ of two oligomers of polypropylene glycol (PPG425) (m/z 442.3374 and 500.3792).

Analytical HPLC. HPLC analyses were performed on a Waters 600 Controller HPLC system equipped with a Waters 2996 photodiode array detector and monitored by the Waters Empower 2 chromatography data software. The wavelength used for the HPLC analyses was 280 nm. The chromatographic separation was carried out on a LiChrospher 100 RP-18 end-capped column (250 × 4 mm; 5 µm particle size) held at 37°C. The flow rate was set at 1 mL min^{-1}. A mixture of 0.05% aqueous HCO_2H and MeCN (7/3) was used for the elution.

Semi-preparative HPLC. Purifications were carried out on a Waters 600 chromatograph coupled to a UV-vis Water™ 486 detector and equipped with a Waters Atlantis PrepT3OBD™ 5 µm (19 mm × 150 mm) column. The solvent was 0.05% aqueous HCO_2H / MeCN (7/3). The flow rate was 18 mL min^{-1} Detection was carried out at 280 nm. Fractions obtained were concentrated under vacuum and freeze-dried. Their purity was checked by UPLC-MS analysis.

UPLC-MS. UPLC-MS analyses were performed on the Acquity Ultra Performance LC™ (UPLC™) apparatus from Waters, equipped with an UV-visible diode array detector (DAD) and coupled with a Bruker Daltonics HCT ultra ion trap mass spectrometer with a negative electrospray ionization (ESI) mode. The separation was conducted on a 1.7 µm (2.1-50 mm) Acquity UPLC BEH C18 column thermostated at 30 °C with an isocratic elution

(0.05% aqueous HCO_2H / MeCN (7/3)) at a flow rate of 0.2 mL min^{-1}. The mass spectra were generated in the Ultrascan mode in the m/z range 100-900. The ion source parameters were: nebuliser pressure, 40 psi; drying gas flow, 9 L min^{-1}; drying gas temperature, 350 °C.

Molecular modelling. Semi-empirical quantum mechanics calculations were performed in vacuum with the Hyperchem software (Autodesk, Sausalito, USA) using the PM3 program.

Synthesis. *Naringenin / Hesperetin (**2a / 2b**).* Commercially available compounds. **2a:** Mol wt, 272.25 $gmol^{-1}$; light-brown powder; mp, 252-253°C; R_f (cyclohexane (cHex):EtOAc, 6:4) 0.36; ^{1}H NMR (CD_3OD), δ 7.33 (2H, d, J = 8.5, H2′, H6′), 6.82 (2H, d, J = 8.5, H3′, H5′), 5.90 (1H, d, J = 2.2, H6), 5.89 (1H, d, J = 2.2, H8), 5.33 (1H, dd, J = 13.0, 3.0, H2), 3.12 (1H, dd, J = 13.0, 17.0, H3a), 2.69 (1H, dd, J = 3.0, 17.0, H3b); ^{13}C NMR (CD_3OD) δ 198.19 (C4), 168.76 (C7), 165.88 (C5 or C9), 165.30 (C5 or C9), 159.43 (C4′), 131.50 (C1′), 129.45 (2C, C2′, C6′), 116.74 (2C, C3′, C5′), 103.77 (C10), 97.47 (C6 or C8), 96.58 (C6 or C8), 80.89 (C2), 44.44 (C3); **2b:** Mol wt, 302.28 $gmol^{-1}$; light yellow powder; mp, 235-236°C; R_f (cHex/EtOAc, 6:4) 0.27; ^{1}H NMR (CD_3OD), δ 6.93 – 6.92 (3H, m, H2′, H5′, H6′), 5.92 (1H, d, J = 2.2, H6), 5.89 (1H, d, J = 2.2, H8), 5.32 (1H, dd, J = 12.6, 3.1, H2), 3.87 (3H, s, OCH_3), 3.07 (1H, dd, J = 12.6, 17.1, H3a), 2.72 (1H, dd, J = 3.1, 17.1, H3b); ^{13}C NMR (CD_3OD) δ 198.01 (C4), 168.77 (C7), 165.87 (C5 or C9), 165.17 (C5 or C9), 149.76 (C3′ or C4′), 148.19 (C3′ or C4′), 133.55 (C1′), 119.40 (C6′), 114.94 (C5′), 112.97 (C2′), 103.78 (C10), 97.47 (C6 or C8), 96.59 (C6 or C8), 80.69 (C2), 56.83 (OCH3), 44.49 (C3).

*7-O-Benzoylnaringenin (**3a**).* Benzoyl chloride (2.15 mL, 18.5 mmol) was added dropwise to an ice-cold solution of **2a** (1 equiv) in pyridin (15 mL) and the reaction mixture was stirred overnight at room temperature. The mixture was extracted with EtOAc (300 mL), then washed with water (200 mL), 2 M HCl (3x100 mL), saturated aqueous $NaHCO_3$ (100 mL) and saturated aqueous NaCl (100 mL). The combined organic extract was dried over

Na_2SO_4, filtered, and concentrated. The product was purified by precipitation from EtOAc/n-hexane to give **3a** with a yield of about 90%. Light brown powder; mp, 138-139 °C; R_f (cHex/EtOAc, 6:4) 0.58; ^{1}H NMR (CD_3OD), δ 8.18 (2H, d, J = 7.2, H2Bz, H6Bz), 7.71 (1H, t, J = 7.2, H4Bz), 7.56 (2H, t, J = 7.2, H3Bz, H5Bz), 7.36 (2H, d, J = 8.6, H2′, H6′), 6.85 (2H, d, J = 8.6, H3′, H5′), 6.47 – 6.45 (2H, broad s, H6, H8), 5.50 (1H, dd, J = 13.0, 2.8, H2), 3.2 (1H, dd, J = 13.0, 17.2, H3a), 2.90 (1H, dd, J = 2.8, 17.2, H3b); ^{13}C NMR (CD_3OD) δ 197.24 (C4), 164.10, 163.41, 162.46 (O=C-Bz, C5, C9), 158.75 (C4′ or C7), 156.17 (C4′ or C7), 134.01 (C1′, C4Bz), 130.32, 128.69 (C1Bz, C2Bz, C3Bz, C5Bz, C6Bz), 127.97 (C2′, C6′), 115.73 (C3′, C5′), 106.31 (C10), 103.41 (C6 or C8), 101.91 (C6 or C8), 79.06 (C2), 43.45 (C3).

*7-O-Benzoylhesperetin (**3b**)***:** Same procedure as for **3a** by starting from **2b**. Yield, 90%; light brown powder; mp, 146-147 °C; R_f (cHex/EtOAc, 6:4) 0.50; ^{1}H NMR (CD_3OD), δ 8.20 (2H, d, J = 7.2, H2Bz, H6Bz), 7.70 (1H, t, J = 7.2, H4Bz), 7.55 (2H, t, J = 7.2, H3Bz, H5Bz), 6.93 – 6.92 (3H, m, H2′, H5′, H6′), 6.46 (1H, d, J = 2.1, H6), 6.44 (1H, d, J = 2.1, H8), 5.49 (1H, dd, J = 12.7, 3.0, H2), 3.87 (3H, s, OMe), 3.07 (1H, dd, J = 12.7, 17.0, H3a), 2.82 (1H, dd, J = 3.0, 17.0, H3b); ^{13}C NMR (CD_3OD) δ 197.22 (C4), 164.05, 163.37, 162.42 (O=C-Bz, C5, C9), 158.74 (C7), 147.08 (C3′ or C4′), 145.97 (C3′ or C4′), 133.98 (C1′ or C4Bz), 131.15 (C1′ or C4Bz), 130.32, 128.68 (C1Bz, C2Bz, C3Bz, C5Bz, C6Bz), 118.20 (C6′), 112.64 (C2′ or C5′), 110.70 (C2′ or C5′), 106.32 (C10), 103.39 (C6 or C8), 101.91 (C6 or C8), 79.07 (C2), 56.06 (OCH3), 43.43 (C3).

*4′,5,7-Tri-O-benzoylnaringenin (**4a**)*. Compound **2a** (10 g, 37 mmol) and benzoyl chloride (12 mL, 3 equiv) were dissolved in 700 mL of THF and the mixture was cooled down in an ice-water. Then, 50 mL of triethylamine were added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. Then it was diluted with EtOAc (500 mL), washed with saturated aqueous $NaHCO_3$ (100 mL) and NaCl (100 mL), dried over

Na_2SO_4, filtered and evaporated. The crude solid was triturated in n-hexane for several times to give **4a**. Yield, 85%; light brown powder; mp, 112-113 °C; R_f (cHex/EtOAc, 6:4) 0.76; ^{1}H NMR ($CDCl_3$), δ 8.27 – 8.19 (6H, m, 3H2bz, 3H6bz), 7.71 - 7.66 (3H, m, 3H4Bz), 7.66 - 7.48 (8H, m, 3H3Bz, 3H5Bz, H2′, H6′), 7.31 (2H, d, J = 8.6, H3′, H5′), 7.02 (1H, d, J = 2.2, H6), 6.86 (1H, d, J = 2.2, H8), 5.61 (1H, dd, J = 13.5, 2.7, H2), 3.12 (1H, dd, J = 13.5, 16.7, H3a), 2.84 (1H, dd, J = 2.7, 16.7, H3b); ^{13}C NMR ($CDCl_3$) δ 188.85 (C4), 165.05, 164.99, 163.91, 163.29 (3O=C-Bz, C5, C9), 156.33 (C7), 151.30 (C4′), 135.78, 134.14, 133.76, 133.59 (3C4Bz, C1′), 130.36, 128.64 (3C1Bz, 3C2Bz, 3C3Bz, 3C5Bz, 3C6Bz), 127.44 (C2′, C6′), 122.26 (C3′, C5′), 112.10, 111.13, 109.45 (C6, C8, C10), 79.18 (C2), 45.24 (C3).

*3′,5,7-Tri-O-benzoylhesperetin (**4b**)*. Same procedure as for **4a** by starting from **2b** rather than from **2a**. Yield, 85%; light-brown powder; mp, 101-102 °C; R_f (cHex/EtOAc, 6:4) 0.68; ^{1}H NMR ($CDCl_3$), δ 8.21 – 8.17 (6H, m, 3H2Bz, 3H6Bz), 7.80 - 7.75 (3H, m, 3H4Bz), 7.75 - 7.50 (6H, m, 3H3Bz, 3H5Bz), 7.43 (1H, dd, J = 8.5, 2.1, H6′), 7.37 (1H, d, J = 2.1, H2′), 7.19 (1H, d, J = 8.5, H5′), 7.07 (1H, d, J = 2.2, H6), 6.90 (1H, d, J = 2.2, H8), 5.66 (1H, dd, J = 13.0, 2.6, H2), 3.87 (3H, s, OMe), 3.07 (1H, dd, J = 13.0, 17.1, H3a), 2.82 (1H, dd, J = 2.6, 17.1, H3b); ^{13}C NMR ($CDCl_3$) δ 188.98 (C4), 164.98, 164.58, 163.89, 163.32 (3O=C-Bz, C5, C7, C9), 151.57 (C4′), 140.19 (C3′), 134.11, 133.59 (3C4Bz, C1′), 130.70, 128.56 (3C1Bz, 3C2Bz, 3C3Bz, 3C5Bz, 3C6Bz), 124.94 (C2′ or C6′), 121.21 (C2′ or C6′), 112.64, 112.07, 111.03, 109.42 (C5′, C6, C8, C10), 78.97 (C2), 56.12 (OCH3), 44.98 (C3).

*4′,5-Di-O-benzoylnaringenin (**5a**)*. Compound **4a** (5 g, 8.5 mmol) and imidazole (0.465 g, 0.7 equiv) were dissolved in N-methyl-2-pyrrolidinone (NMP, 5 mL). Then, thiophenol (0.87 mL, 1 equiv) was slowly added at 0 °C. The resulting mixture was stirred for 1h at room temperature, then diluted with EtOAc (50 mL) and washed with saturated aqueous NaCl (3x100 mL) and with 1M HCl (100 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude solid was triturated with cyclohexane and

precipitated from a n-hexane/AcOEt mixture to give **5a** (4 g). Yield, 95%; white powder; mp, 218-219 °C; R_f (cHex/EtOAc, 6:4) 0.50; ^{1}H NMR (CD_3OD), δ 8.30 – 8.16 (4H, m, 2H2Bz, 2H6Bz), 7.70 - 7.66 (2H, m, 2H4Bz), 7.64 - 7.50 (6H, m, 2H3Bz, 2H5Bz, H2′, H6′), 7.32 (2H, d, J = 8.6, H3′, H5′), 6.42 (1H, d, J = 2.3, H6), 6.32 (1H, d, J = 2.3, H8), 5.59 (1H, dd, J = 12.9, 2.9, H2), 3.06 (1H, dd, J = 12.9, 16.7, H3a), 2.70 (1H, dd, J = 2.9, 16.7, H3b); ^{13}C NMR (CD_3OD) δ 192.63 (C4), 178.79, 174.58 (2O=C-Bz, C5 or C7, C9), 155.13 (C5 or C7), 151.82 (C4′), 133.28, 132.28 (2C4Bz, C1′), 130.61, 129.22 (2C1Bz, 2C2Bz, 2C3Bz, 2C5Bz, 2C6Bz), 128.74 (C2′, C6′), 122.50 (C3′, C5′), 103.61, 100.13, 96.92 (C6, C8, C10), 84.34 (C2), 39.62 (C3).

*3′,5-Di-O-benzoylhesperetin (**5b**)*. Same procedure as for **5a** by starting from **4b**. Yield, 95%; white powder; mp, 197-198 °C; R_f (cHex/EtOAc, 6:4) 0.35; ^{1}H NMR (CD_3OD), δ 8.21 – 8.17 (4H, m, 2H2Bz, 2H6Bz), 7.80 - 7.75 (2H, m, 2H4Bz), 7.75 - 7.50 (4H, m, 2H3Bz, 2H5Bz), 7.43 (1H, dd, J = 8.5, 2.1, H6′), 7.37 (1H, d, J = 2.1, H2′), 7.19 (1H, d, J = 8.5, H5′), 6.41 (1H, d, J = 2.4, H6), 6.32 (1H, d, J = 2.4, H8), 5.51 (1H, dd, J = 13.0, 2.8, H2), 3.87 (3H, s, OMe), 3.07 (1H, dd, J = 13.0, 17.0, H3a), 2.82 (1H, dd, J = 2.8, 17.0, H3b); ^{13}C NMR ($CDCl_3$) δ 188.79 (C4), 165.56, 164.92, 164.12, 163.79 (2O=C-Bz, C5, C7, C9), 151.65 (C4′), 140.04 (C3′), 133.67, 133.44, 131.29 (2C4Bz, C1′), 130.38, 128.53 (2C1Bz, 2C2Bz, 2C3Bz, 2C5Bz, 2C6Bz), 125.08 (C2′ or C6′), 121.18 (C2′ or C6′), 112.59 (C5′), 107.48, 105.92, 101.96 (C6, C8, C10), 78.65 (C2), 56.09 (OCH3), 44.86 (C3).

*7-O-Benzoylnaringenin 4′-O-[methyl (2″,3″,4″-tri-O-acetyl)-β-D-glucopyranosyl uronate] (**6a**)*. Compound **3a** (0.5 g, 1.3 mmol) was added to a solution of glucuronyl donor (**1**) (0.45 g, 1 equiv.) in dry CH_2Cl_2 (15 mL) containing 4Å molecular sieves. The reaction mixture was cooled down in an ice-water bath for 15 min under N_2 atmosphere. Then, $BF_3.Et_2O$ (170 µL, 1 equiv.) was added and the mixture was stirred at room temperature for about 30 min. Then, the mixture was diluted with EtOAc, filtered on celite, concentrated and

purified by column chromatography (cHex/EtOAc; 6:4) to provide **6a**. Yield, 50%; white solid; mp, 149-150°C; R_f (cHex/EtOAc, 6:4) 0.31; ^{1}H NMR ($CDCl_3$), δ 11.89 (1H, s, C5-OH), 8.18 (2H, d, *J* = 7.3, H2Bz, H6Bz), 7.67 (1H, t, *J* = 7.3, H4Bz), 7.53 (2H, t, *J* = 7.3, H3Bz, H5Bz), 7.43 (2H, d, *J* = 8.7, H2′, H6′), 7.08 (2H, d, *J* = 8.7, H3′, H5′), 6.47 (1H, broad d *J* = 2.0, H6 or H8), 6.45 (1H, m, H6 or H8) 5.48 (1H, dd, *J* = 13.0, 2.8, H2), 5.39 – 5.30 (3H, m, H2″, H3″, H4″), 5.20 (1H, d, *J* = 7.1, H1″), 4.23 (d, *J* = 7.0) plus 4.22 (d, *J* = 7.0, H5″) (1H, H5″), 3.75 (3H, s, OMe), 3.15 (1H, dd, *J* = 13.0, 17.0, H3a), 2.9 (1H, dd, *J* = 2.8, 17.0, H3b), 2.09, 2.08, 2.07, 2.06 (9H, 4bs, 3Ac); ^{13}C NMR ($CDCl_3$) δ 196.90 (C4), 170.10, 169.34, 169.22 ($3OCOCH_3$, C6″), 166.80, 164.04, 163.40, 162.25 (O=C-Bz, C5 , C9), 158.79 (C4′ or C7), 157.04 (C4′ or C7), 134.02, 133.08 (C4Bz, C1′), 130.31, 128.70 (C1Bz, C2Bz, C3Bz, C5Bz, C6Bz), 127.75 (C2′, C6′), 117.38 (C3′, C5′), 106.29, 103.53, 101.91, 98.97 (C1″, C6, C8, C10), 78.76 (C2), 72.71, 71.82, 71.05, 69.07 (C2″, C3″, C4″, C5″), 53.02 (OCH_3 GlcU), 43.45 (C3), 20.62 ($3OCOCH_3$).

*7-O-Benzoylhesperetin 3′-O-[methyl (2″,3″,4″-tri-O-acetyl)-β-D-glucopyranosyl uronate] (**6b**)*. Same procedure as for **6a** by starting from **3b**. Yield, 50%; light-yellow powder; mp, 115-116 °C; R_f (cHex/EtOAc, 6:4) 0.19; ^{1}H NMR ($CDCl_3$), δ 11.89(s) plus 11.88 (s) (1H, C5-OH), 8.18 (2H, d, *J* = 7.3, H2Bz, H6Bz), 7.67 (1H, t, *J* = 7.3, H4Bz), 7.53 (2H, t, *J* = 7.3, H3Bz, H5Bz), 7.28 (1H, broad s, H2′), 7.19 (dd, *J* = 8.5, 2.3) plus 7.18 (dd, *J* = 8.5, 2.3) (1H, H6′), 6.96 (d, *J* = 8.5) plus 6.96 (d, *J* = 8.5) (1H, H5′), 6.44 - 643 (2H, m, H6, H8) 5.51- 5.20 (4H, m, H4″, H2″, H3″, H2), 5.10 (d, *J* = 6.5) plus 5.08 (d, *J* = 6.5) (1H, H1″), 4.15 (d, *J* = 9.2) plus 4.12 (d, *J* = 9.2) (1H, H5″), 3.87 (3H, s, OMe hesperetin), 3.74 (3H, s, OMe GlcU), 3.07 (1H, dd, *J* = 12.6, 17.0, H3a), 2.82 (1H, dd, *J* = 2.9, 17.0, H3b), 2.1 (3H, s, Ac), 2.06 (3H, s, Ac), 2.04(s) plus 2.01(s) (3H, Ac); ^{13}C NMR ($CDCl_3$) δ 196.99 (C4), 170.16, 169.37, 169.29 ($3OCOCH_3$, C6″), 166.87, 163.40, 162.21 (O=C-Bz, C5, C9), 158.76 (C7), 151.24 (C4′), 145.75 (C3′), 134.02, 130.50, 130.37, 130.32, 128.70 (C1′, C1Bz, C2Bz, C3Bz,

C4Bz, C5Bz, C6Bz), 123.07 (C5′ or C6′), 118.81 (C5′ or C6′), 112.76 (C2′), 106.32, 103.50, 101.93, 100.58 (C1″, C6, C8, C10), 78.62 (C2), 72.56, 71.87, 71.12, 69.27 (C2″, C3″, C4″, C5″), 56.14 (OCH_3 hesperetin), 52.98 (OCH_3 GlcU), 43.03 (C3), 20.66 ($3OCOCH_3$).

*4′,5-Di-O-benzoylnaringenin 7-O-[methyl (2″,3″,4″-tri-O-acetyl)-β-D-glucopyranosyl uronate] (**7a**)*. Same procedure as for **6a** by starting from **5a**. Yield, 50%; white solid; mp, 165-166 °C; R_f (cHex/EtOAc, 6:4) 0.32; ^{1}H NMR ($CDCl_3$), δ 8.14 (2H, broad d, J = 7.0, H2Bz, H6Bz), 8.06 (2H, m, H2Bz, H6Bz), 7.60 – 7.50 (2H, m, H4Bz), 7.46 - 7.40 (6H, m, H3Bz, H5Bz, H2′, H6′), 7.22 (2H, d, J = 8.6, H3′, H5′), 6.10 (d, J = 2.0, H6 or H8) plus 6.09 (d, J = 2.0) (1H, H6 or H8), 6.07 (d, J = 2.0, H6 or H8) plus 6.07 (d, J = 2.0) (1H, H6 or H8), 5.40 (dd, J = 13.0, 2.9, H2), 5.30 – 5.13 (4H, m, H1″, H2″, H3″, H4″), 4.14 (d, J = 8, H5″) plus 4.13 (d, J = 8, H5″), 3.75 (3H, s, OMe GlcU), 3.0 (1H, dd, J = 13.0, 18.0, H3a), 2.8 (1H, dd, J = 2.9, 18.0, H3b), 1.18 (3x3H, bs, 3Ac); ^{13}C NMR ($CDCl_3$) δ 196.07 (C4), 170.04, 169.35, 169.10 ($3OCOCH_3$, C6″), 166.61, 165.06, 163.96 (2O=C-Bz, C5 or C7 , C9), 154.72 (C5 or C7), 151.33 (C4′), 133.80, 133.70 (2C4Bz, C1′), 130.24, 129.20 (2C1Bz, 2C2Bz, 2C3Bz, 2C5Bz, 2C6Bz), 127.43 (C2′, C6′), 122.30 (C3′, C5′), 110.55, 104.53, 97.58, 96.32 (C1″, C6, C8, C10), 78.80 (C2), 72.74, 71.55, 70.70, 68.91 (C2″, C3″, C4″, C5″), 53.09 (OCH_3 GlcU), 43.40 (C3), 20.50 ($3OCOCH_3$).

*3′,5-Di-O-benzoylhesperetin 7-O-[methyl (2″,3″,4″-tri-O-acetyl)-β-D-glucopyranosyl uronate] (**7b**)*. Same procedure as for **6a** by starting from **5b**. Yield, 50%; white powder; mp, 119-120 °C; R_f (cHex/EtOAc, 6:4) 0.25; ^{1}H NMR ($CDCl_3$), δ 8.21 – 8.17 (4H, m, H2Bz, H6Bz), 7.78 (2H, m, H4Bz), 7.75 - 7.50 (4H, m, H3Bz, H5Bz), 7.33 (1H, dd, J = 2.1, 9.0, H6′), 7.37 (1H, d, J = 2.1, H2′), 7.19 (1H, d, J = 9.0, H5′), 6.59 (1H, d, J = 2.4, H8), 6.50 (1H, d, J = 2.4, H6), 5.51 (1H, dd, J = 13.0, 2.9, H2), 5.40 - 5.20 (4H, m, H1″, H2″, H3″, H4″), 4.23 (1H, broad d, J = 7.6, H5″), 3.87 (3H, s, OMe hesperetin), 3.74 (3H, s, OMe GlcU), 3.07 (1H, dd, J = 13.0, 17.0, H3a), 2.82 (1H, dd, J = 2.9, 17.0, H3b), 2.08, 2.08, 2.07 (3x3H, broad

s, 3Ac); ^{13}C NMR ($CDCl_3$) δ 188.61 (C4), 169.97, 169.34, 169.11 ($3OCOCH_3$, C6″), 166.62, 164.60, 163.85, 161.69 (2O=C-Bz, C5, C7 , C9), 152.07 (C4′), 140.21 (C3′), 133.63, 130.67 (2C4Bz, C1′), 130.36, 128.58 (2C1Bz, 2C2Bz, 2C3Bz, 2C5Bz, 2C6Bz), 124.98 (C2′ or C6′), 121.21 (C2′ or C6′), 112.63 (C5′), 109.98, 106.39, 102.57, 97.71 (C1″, C6, C8, C10), 79.02 (C2), 72.73, 71.48, 70.67, 68.82 (C2″, C3″, C4″, C5″), 56.12 (OCH_3 hesperetin), 53.06 (OCH_3 GlcU), 43.73 (C3), 20.59 ($3OCOCH_3$).

*Naringenin 4′-O-β-D-glucuronide (**8a**)*. To a solution of **6a** (0.85 mmol) in MeOH (30 mL) under N_2 atmosphere at 0 °C was slowly added a 0.5 M aqueous solution of Na_2CO_3 (13 mL). The mixture was stirred at room temperature with HPLC monitoring at 280 nm every hour. After 5h, **8a** was detected as a single product (estimated analytical yield ca. 60%). Then, Dowex 50WX4-50 ion-exchange resin (H^+ form) was added under stirring to lower the pH to ca. 6. The resin was filtered off and the filtrate concentrated at 50 °C under vacuum. Purification was carried out by semi-preparative HPLC. Overall yield, 30%; white amorphous powder; R_f (n-BuOH/CH_3CO_2H/H_2O, 10:1:1) 0.18; HPLC (0.05% aq. HCO_2H/MeCN (7/3)), t_R = 2.41 min, λ_{max} = 290, 330 nm; ^{1}H NMR (D_2O), δ 7.37 (2H, d, *J* = 7.9, H2′, H6′), 7.07 (2H, d, *J* = 7.9, H3′, H5′), 5.91 (2H, broad s, H6, H8), 5.53 (1H, broad d, *J* = 11.2, H2), 5.05 (1H, broad d, *J* = 6.9, H1″), 3.90 (1H, d, *J* = 8.4, H5″), 3.69 - 3.61 (3H, m, H2″, H3″, H4″), 3.26 (dd, *J* = 17.0, 11.2) plus 3.23 (dd, *J* = 17.0, 11.2) (1H, H3a), 2.86 (1H, broad d, *J* = 17.0, H3b); DEPTQ ^{13}C NMR (D_2O) δ 196.56 (C4), 174.03 (C6″), 170.22, 167.13, 164.22 (C5, C7, C9), 134.05 (C1′), 129.65 (C2′, C6′), 118.30 (C3′, C5′), 103.74 (C10), 101.47 (C1″), 97.76, 96.97 (C6, C8), 79.96 (C2), 76.45, 76.24, 73.93, 72.64 (C2″, C3″, C4″, C5″), 42.98 (C3). HRMS-ESI, m/z $[M+H]^+$ calcd for C_{21} $H_{21}O_{11}$, 449.1078; found, 449.1082. Molar ratio of epimers ca. 1/1 (based on H_{3a} integration).

*Hesperetin 3′-O-β-D-glucuronide (**8b**)*. Same procedure as for **8a** by starting from **6b**. Overall yield, 30%; white amorphous powder; R_f (n-BuOH/CH_3CO_2H/H_2O, 10:1:1) 0.21; HPLC

(0.05% aq. HCO_2H/MeCN (7/3)), t_R = 4.67 min, λ_{max} = 288, 333 nm; ^{1}H NMR (D_2O), δ 7.30 (d, J = 8.4) plus 7.28 (d, *J* = 8.4) (1H, H6′), 7.23 (1H, broad s, H2′), 7.19 (d, *J* = 8.4) plus 7.16 (d, *J* = 8.4) (1H, H5′), 6.02 (1H, broad s, H6 or H8), 6.00 (1H, broad s, H6 or H8), [5.55 (1H, dd, *J* = 3.1, 12.0) plus 5.47 (dd, *J* = 3.1, 12.0)] (1H, H2), 4.7 (broad d, *J* = 5.5, partly masked by the water peak), 3.78 (3H, s, OMe), 3.88 (d, *J* = 9.8) plus 3.83 (d, *J* = 9.8) (1H, H5″), 3.48 – 3.23 (3H, m, H2″, H3″, H4″), 3.27 (dd, *J* = 12.0, 17.0) plus 3.19 (dd, *J* = 12.0, 17.0) (1H, H3a), 2.85 (dd, *J* = 3.1, 17.0) plus 2.84 (dd, *J* = 3.1, 17.0) (1H, H3b); DEPT ^{13}C NMR (D_2O) δ 200.48 (C4), 167.08, 164.16 (C5, C7, C9, C6″), 146.59 (C3′, C4′), 131.93 (C1′), 123.22 (C6′), 117.08 (C2′), 114.38 (C5′), 103.63 (C10), 101.72 (C1″), 97.92, 97.00 (C6, C8), 80.34 (C2), 76.67, 76.56, 73.78, 72.76 (C2″, C3″, C4″, C5″), 57.20 (OCH_3), 42.86 (C3). Molar ratio of epimers ca. 7/3 (based on H_2 integration). HRMS-ESI, m/z $[M+H]^+$ calcd,for $C_{22}H_{23}O_{12}$ 479.1184; found, 479.1185.

*Naringenin 7-O-β-D-glucuronide (**9a**)*. Same procedure as for **8a** by starting from **7a**. Overall yield, 30%; white amorphous powder; R_f (n-BuOH/CH_3CO_2H/H_2O, 10:1:1) 0.18; HPLC (0.05% aq. HCO_2H/MeCN (7/3)), t_R = 2.38 min, λ_{max} = 283, 329 nm; ^{1}H NMR (D_2O), δ 7.43 (2H, d, *J* = 8.0, H2′, H6′), 6.96 (2H, d, *J* = 8.0, H3′, H5′), 6.27 (1H, broad s, H6 or H8), 6.24 (1H, broad s, H6 or H8),5.49 (1H, broad d, *J* = 14.1, H2), 5.12 (1H, m, H1″), 3.77 (1H, broad d, *J* = 9.3, H5″), 3.67 – 3.54 (3H, m, H2″, H3″, H4″), 3.27 (1H, broad dd, *J* = 16.7, 14.1, H3a), 2.85 (1H, broad d, *J* = 16.7, H3b); ^{13}C NMR (D_2O) δ 198.32 (C4), 172.15 (C6″), 164.63, 163.27, 162.59, 162.59 (C5, C7, C9), 156.69 (C4′), 134.13 (C1′), 129.98 plus 129.06 (C2′, C6′, 2 epimers), 115.91 (C3′, C5′), 104.13 (C10), 99.02 (C1″), 97.47, 96.27 (C6, C8), 79.20 (C2), 75.28, 74.87, 72.53, 71.37 (C2″, C3″, C4″, C5″), 42.63 plus 41.59 (C3, 2 epimers). HRMS-ESI, m/z $[M+H]^+$ calcd,for $C_{21}H_{21}O_{11}$ 449.1078; found, 449.1078.

*Hesperetin 7-O-β-D-glucuronide (**9b**)*. Same procedure as for **8a** by starting from **7b**. Overall yield, 30%; light-brown amorphous powder; R_f (n-BuOH/CH_3CO_2H/H_2O, 10:1:1)

0.17; HPLC (0.05% aq. HCO_2H/MeCN (7/3)), t_R = 4.51 min, λ_{max} = 284, 328 nm; ^{1}H NMR (D_2O), δ 6.83 – 6.77 (3H, m, H5′, H6′, H2′), 6.07 (1H, broad s, H8), 6.03 (1H, broad s, H6), 5.04 (1H, broad d, *J* = 14.8, H2), 4.80 (1H, d, *J* = 7.5, H1″), 3.70 (3H, s, OMe), 3.59 – 3.34 (4H, m, H2″,H3″, H4″, H5″), 2.83 (1H, broad t (dd), *J* = 16.0, 14.8, H3a), 2.54 (1H, broad d, *J* = 16.0, H3b); ^{13}C NMR (D_2O) δ 199.31 (C4), 165.78, 165.62, 164.22, 163.62 (C5, C7, C9, C6″), 146.35 (C3′ or C4′), 144.07 (C3′ or C4′), 132.37 (C1′), 120.21 (C6′), 114.94 (C2′ or C5′), 113.50 (C2′ or C5′), 105.17 (C10), 100.11 (C1″), 98.46, 97.17 (C6, C8), 80.24 (C2), 76.52, 76.36, 73.59, 72.51 (C2″, C3″, C4″, C5″), 57.18 (OCH_3 hesperetin), 43.59 (C3). HRMS-ESI, m/z $[M+H]^+$ calcd for $C_{22}H_{23}O_{12}$ 479.1184; found, 479.1185.

RESULTS AND DISCUSSION

Investigations over the last three decades have shown that dietary polyphenols are only moderately bioavailable and that the fraction crossing the intestinal barrier is typically extensively metabolized in the intestinal and hepatic cells. Thus, the potential cell effects of dietary polyphenols must be mainly mediated by their metabolites, of which glucuronides make the largest contribution. Hence, there is a growing interest for polyphenol glucuronides as standards for identification and titration of in vivo metabolites and as biologically pertinent compounds for cell studies aiming at elucidating the potential health effects of polyphenols. Several works have been published about the chemical synthesis of polyphenol glucuronides. For instance, the popular procedure, based on the Lewis acid-activated coupling of methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-*α*-D-glucuronate (**1**) with partially protected polyphenols, was applied to the synthesis of isoflavone 7-*O*-*β*-D-glucuronides (*14*), quercetin 3′-*O*-*β*-D-glucuronide (*15*) and a series of hydroxycinnamic acid *O*-β-D-glucuronides (*16*). Catechin *O*-*β*-D-glucuronides were also prepared with methyl-2,3,4-tri-*O*-acetyl-1-*O*-bromo-*α*-D-glucuronate as the glucuronyl donor (*17*). Recently, the synthesis of a flavanone

glucuronide (persicogenin 3′-*O*-*β*-D-glucuronide) was carried out with methyl-2,3,4-tri-*O*-acetyl-1-*O*-(trifluoroacetimidoyl)-*α*-D-glucuronate, followed by a final deprotection step involving pig liver esterase (PLE) for the hydrolysis of the methyl ester of the glucuronyl residue (*18*). A synthesis of quercetin 3-*O*-*β*-D-glucuronide was also performed by regioselective oxidation of the corresponding 3-*O*-*β*-D-glucoside (phenolic OH groups protected as benzyl ethers) using TEMPO/NaOCl/NaBr under phase transfer conditions (*19*).

In this particular work, an efficient chemical synthesis of the four major metabolites of citrus flavanones (hesperetin 7- and 3′-O-*β*-D-glucuronides, naringenin 7- and 4′-O-β-D-glucuronides) is reported. The glucuronides were completely characterized by NMR, HRMS, and UPLC-MS.

In a preliminary study, the partial protection of the flavanone OH groups was achieved by acetylation. However, the low stability of the arylacetate groups in the glucuronidation step resulted in low yields and tedious purifications. By contrast, partial protection by benzoylation was found satisfactory. Among the three phenolic OH groups of hesperetin and naringenin, C7-OH is the most acidic because of the stabilization of the corresponding phenolate with the keto group. Such an activation is absent for the B-ring OH groups and is cancelled out for C5-OH by the strong hydrogen bond it forms with the keto group. Hence, both hesperetin and naringenin can be selectively benzoylated at C7-OH in the presence of one equivalent of benzoyl chloride at 0 °C (**Figure 1**). As expected, benzoylation at C7-OH induced a deshielding of the C6, C8 and C10 NMR signals. A strong shielding of the C7 signal was also noted. This first step allowed us to carry out the regioselective glucuronidation of the B-ring phenolic OH by compound **1** in the presence of the boron trifluoride – diethylether complex as the Lewis acid. In the ^{1}H NMR spectra of the protected glucuronides **6a** and **6b**, most B-ring protons (H3′ and H5′ in **6a**, H2′ and H5′ in **6b**) appeared

strongly deshielded with respect to the corresponding protons in **3a** and **3b** as a consequence of the weakening of the electron-donating effect of the O-atom by the attached glucuronyl group. In the glucuronidation step, the unprotected strongly H-bonded C5-OH group remained inactive.

For A-ring conjugation, hesperetin and naringenin were first converted into the corresponding tri-O-benzoates (**Figure 2**). Then, the selective thiolysis of the most reactive 7-O-benzoate group was successfully achieved using thiophenol as the nucleophile. Displacements of NMR signals opposite to those recorded upon selective benzoylation at C7-OH were observed. The flavanone dibenzoates thus formed are convenient substrates for glucuronidation at C7-OH, which was performed under the same conditions as for B-ring conjugation.

In this work, the final deprotection step consisted in the alkaline hydrolysis of one or two benzoate(s) (flavanone nucleus) along with three acetates and one methyl ester (sugar moiety). It was found particularly difficult to complete without degradation of the flavanone nuclei (probably starting by a retro-Michael reaction leading to opening of the pyrane ring and concomitant formation of chalcones). In particular, strongly alkaline conditions (NaOH/EtOH or KOH/EtOH) and even the milder methanolysis of the acetate and benzoate groups catalyzed by MeONa led to extensive degradation. Among the deprotection reagents tested, the most satisfactory one was a solution of sodium carbonate (1.2 equiv. per ester group) in water/methanol (2/5). Such conditions were successfully used for the synthesis of 7-hydroxycoumarin O-β-D-glucuronide (*20*). The ^{1}H and ^{13}C NMR signals of the deprotected glucuronides were assigned from 2D COSY, HMBC and HSQC experiments. Our results are consistent with the literature (*9, 21*).

The major citrus flavanones are naringin (7-O-β-D-(L-rhamnosyl-α-1,2-D-glucosyl)-(2*S*)-naringenin) and hesperidin (7-O-β-D-(L-rhamnosyl-α-1,6-D-glucosyl)-(2*S*)-hesperetin). In both compounds, the configuration of C2 is (*S*) (*1*) as a consequence of the enantioselectivity of the chalcone isomerase enzyme catalyzing the formation of the flavanone nucleus by intramolecular Michael addition within the chalcone precursor (*22*). However, mixtures of (2*R*)- and (2*S*)-hesperidin epimers in an approximate molar ratio of 1/6 can be detected in orange juice (*23*), possibly because of the propensity of flavanones to undergo epimerization at C2 via the chalcone form. Interestingly, the minor (2*R*) epimer seems ca. twice as much bioavailable as the (2*S*) epimer based on the excreted rates in urine of (2*R*) and (2*S*) hesperetin conjugates (*23*). In this work, the commercially available racemic naringenin and hesperetin were used. After the glucuronidation step, the ^{1}H-NMR spectra of the glucuronides (except **7b**) are complicated by the splitting of several signals in two signals of near equal intensity, thus confirming that the compounds are equimolar mixtures of epimers. For instance, the NMR spectrum of **6b** in $CDCl_3$ displays two strongly deshielded singlets at ca. 11.9 ppm for the C5-OH proton, which is strongly hydrogen-bonded to the neighboring O4 atom. This splitting is also frequently observed, not only for other protons of the flavanone nucleus but also for some benzoyl, acetyl and GlcU protons, in particular H5″. After deprotection, only the B-ring glucuronides displayed some split signals confirming the presence of the two epimers. The fraction of hesperetin 3′-O-β-D-glucuronide (**8b**) purified by semi-preparative HPLC was enriched in one of them (7/3 molar ratio) during this step. The UPLC-MS analysis did not permit to distinguish between the two epimers. The molecular ion of each pure glucuronide (MS^1) was sequentially fragmented into aglycone and D-glucuronic acid (MS^2), and finally into the aglycone fragments (MS^3) (**Figure 3**). No significant differences were observed between regioisomers, thus indicating that they possess the same fragmentation patterns. Furthermore, the aglycone fragmentation patterns were consistent

with the literature (*24-26*). Unfortunately, it is impossible to differentiate between the A-ring and B-ring glucuronides on the basis of their mass fragmentation pattern as both MS^2 spectra give the aglycone and D-glucuronic acid as the main fragments. By contrast, the A-ring and B-ring glucuronides can be distinguished from their UV spectra. Indeed, C7-OH is conjugated with the keto group through the A-ring and as such contributes to the main UV absorption band. Hence, glucuronidation of the A-ring causes a hypsochromic shift of 4-5 nm in comparison to the parent aglycones ($\lambda_{max} \approx 288$ nm) while glucuronidation of the B-ring leaves the spectra unchanged. Molecular modelling experiments with the deprotected glucuronides yielded low-energy conformations showing a pseudo-equatorial position for the B-ring (vs. C-ring) and an anti arrangement of H2 and one of the H3 protons, which is consistent with the observed coupling constants in the NMR spectra.

In conclusion, simple and reasonably efficient procedures (10 - 15% overall yield from the starting aglycones) have been developed for the chemical synthesis of four major citrus flavanone metabolites. After a one- or two-step partial protection of the flavanone moiety by benzoyl groups, the glucuronidation step was carried out either on the A- or B-ring and followed by the removal of all protecting groups in optimized mild alkaline conditions, thereby avoiding significant degradation of the glucuronides. The affinity of the flavanone glucuronides for serum albumin, their likely carrier in the blood plasma, and their cell effects in relation with the protection against cardiovascular diseases are currently investigated.

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Supporting Information Available: Optimized conformations of the naringenin glucuronides and high-resolution mass spectra of the citrus flavanone glucuronides.

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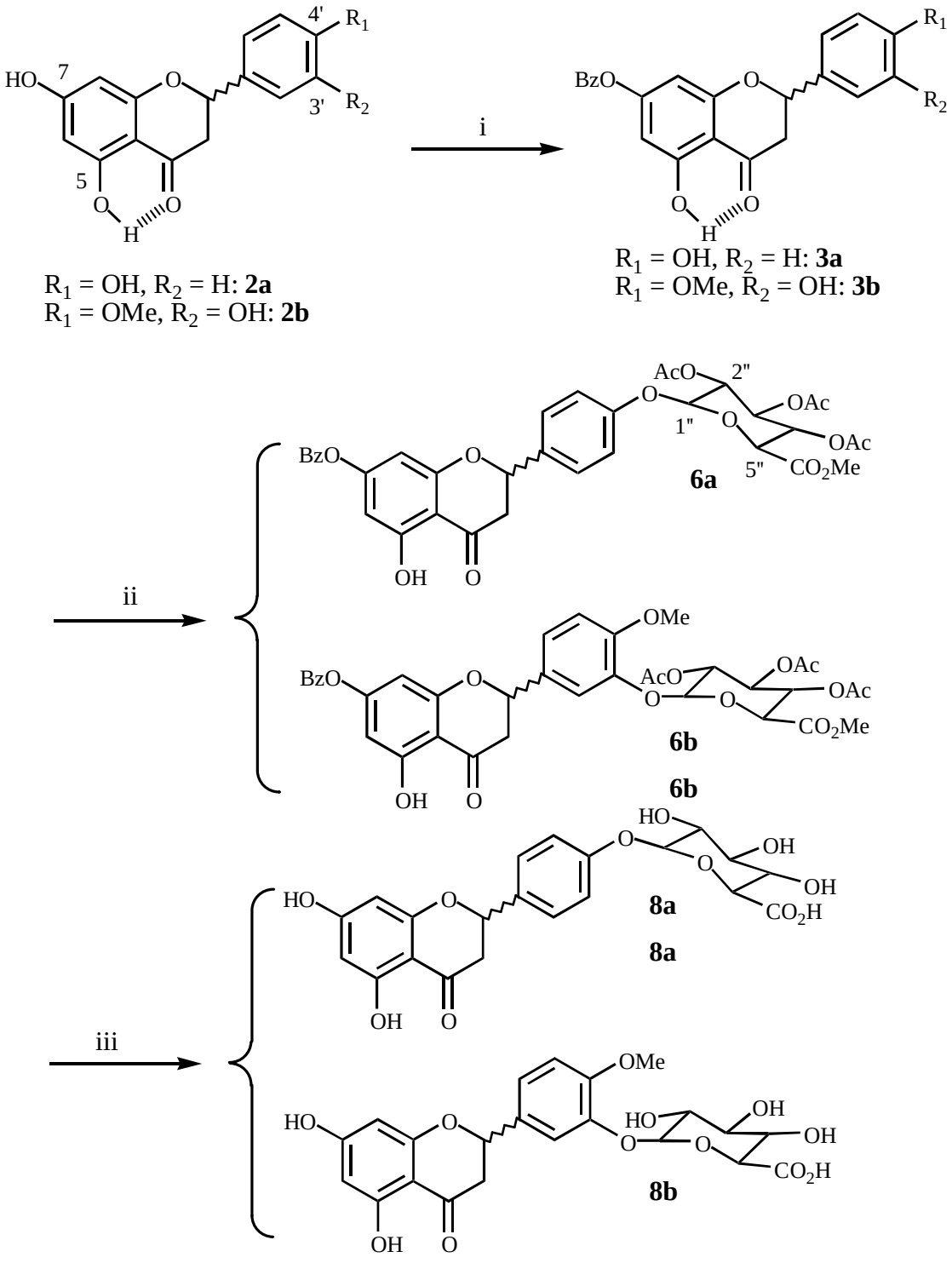


Figure 1: Glucuronidation of citrus flavanones on the B-ring

i) BzCl (1.1-1.5 equiv.), pyridin ii) Methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate (**1**), BF_3-OEt_2 (2 equiv.), CH_2Cl_2 iii) Na_2CO_3 (1.2 equiv. per ester group), H_2O/MeOH (2/5), then acidification to pH 6 by Dowex resin (H^+ form).

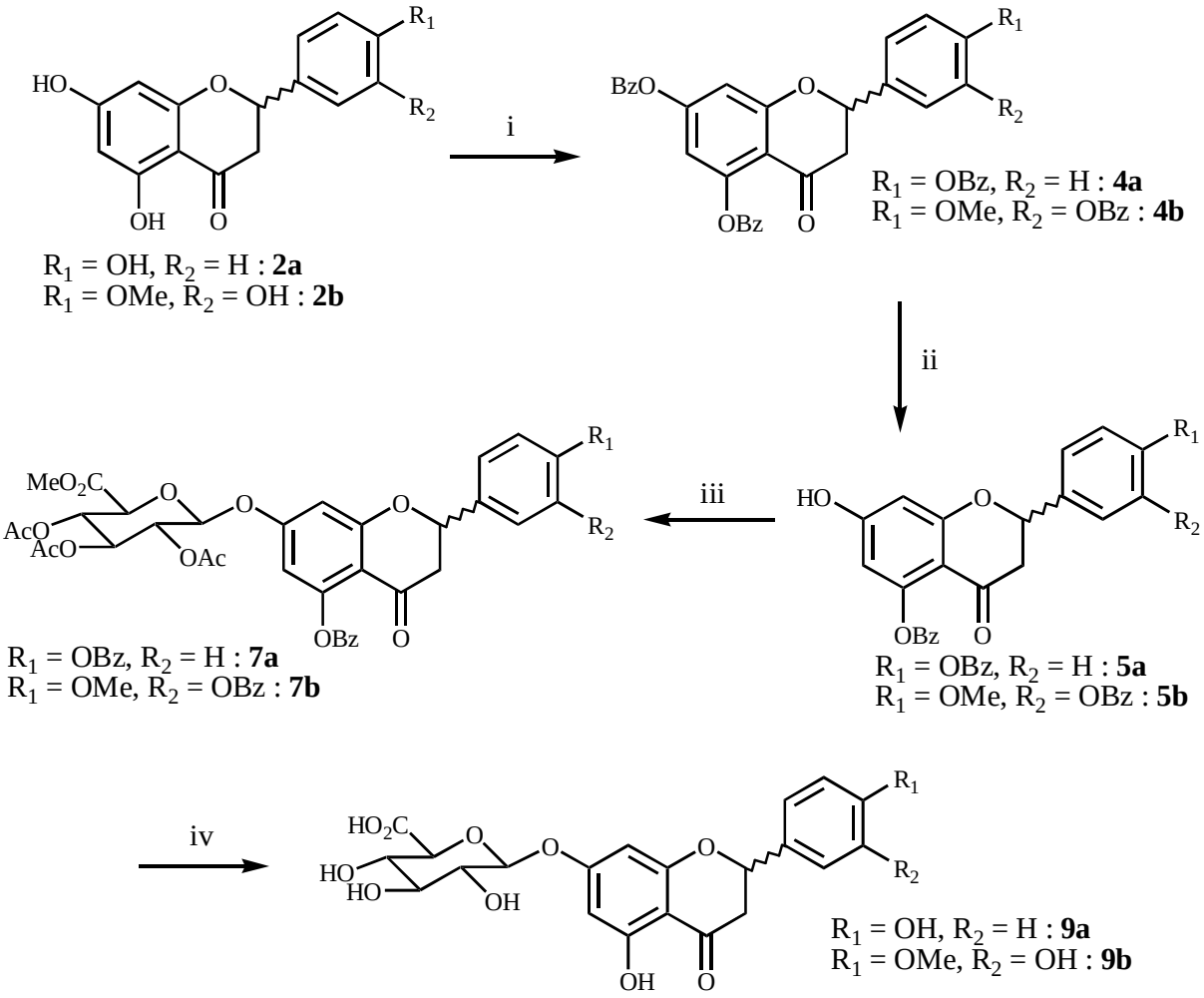


Figure 2: Glucuronidation of citrus flavanones on the A-ring

i) BzCl (excess), NEt_3, THF, 0°C ii) PhSH (1 equiv.), imidazole, NMP iii) Methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)-α-D-glucuronate (**1**), BF_3-OEt_2 (2 equiv.), CH_2Cl_2 iv) Na_2CO_3 (1.2 equiv. per ester group), H_2O/MeOH (2/5), then acidification to pH 6 by Dowex resin (H^+ form).

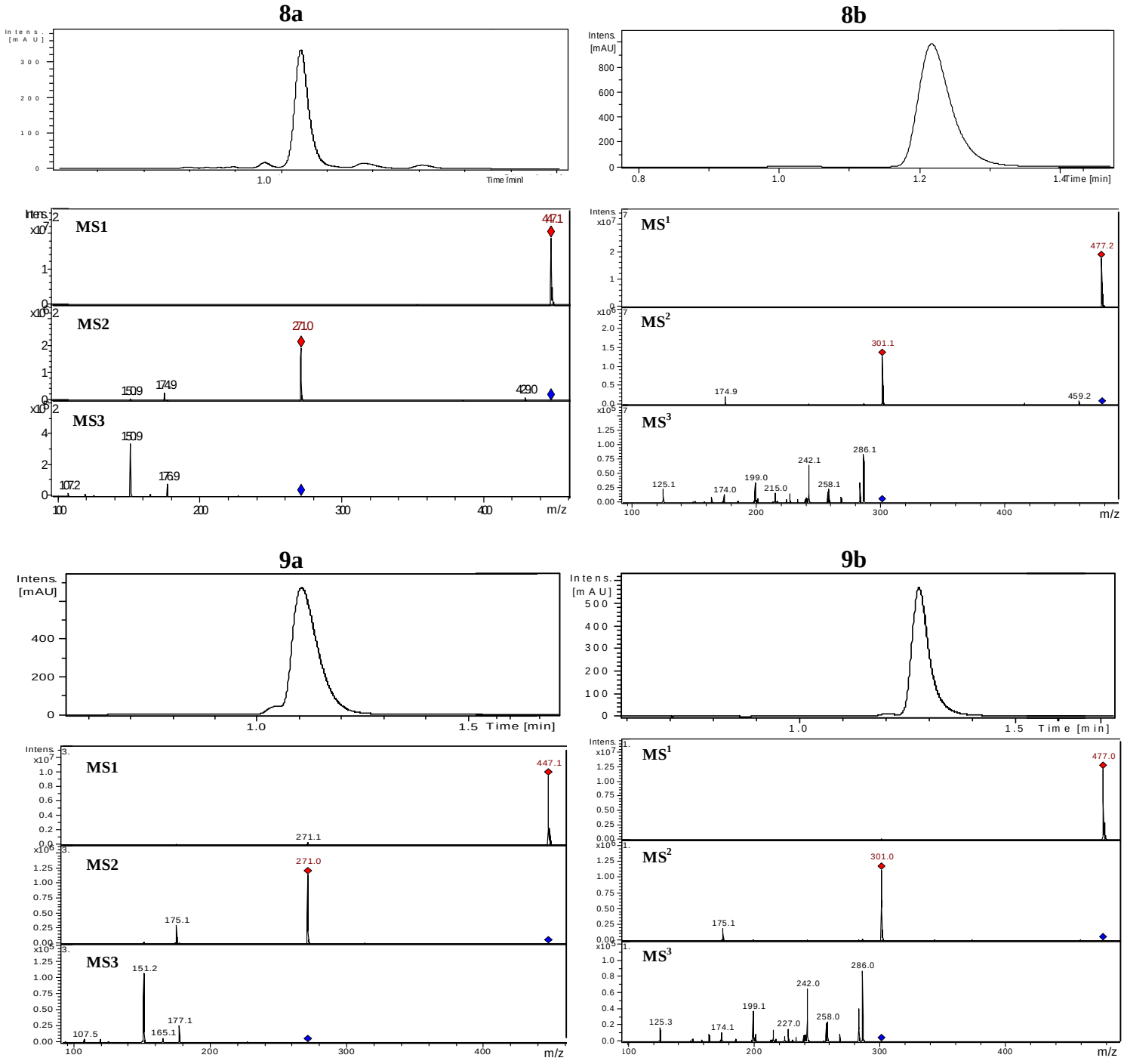
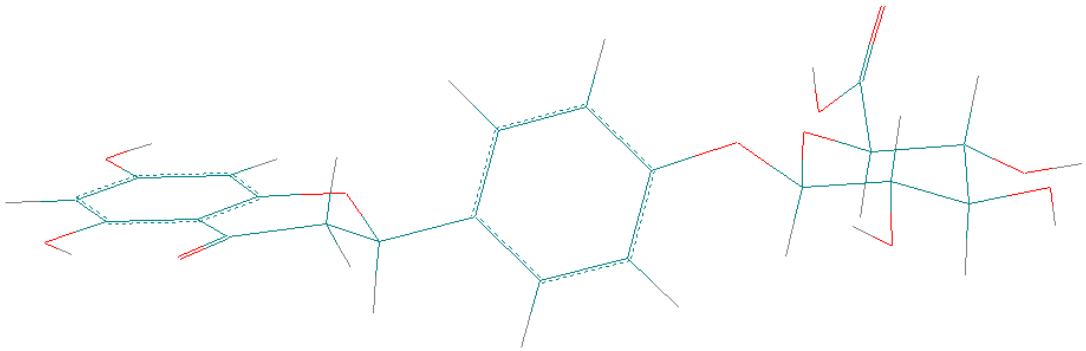
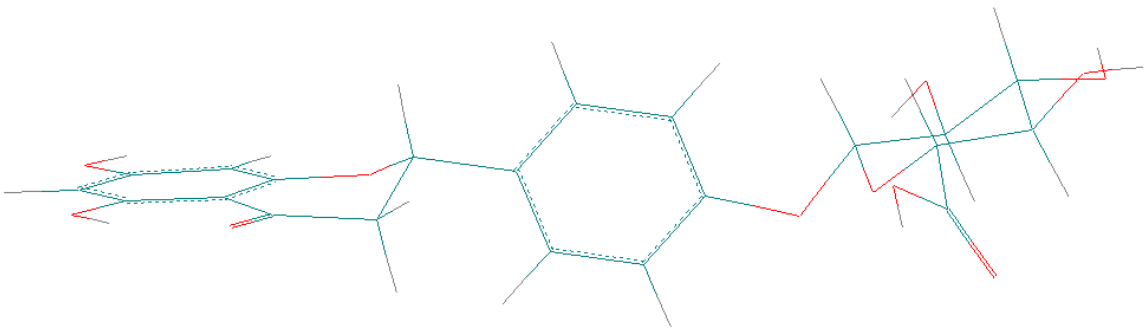


Figure 3: UPLC-MS analyses of flavanone glucuronides.



(2*R*)-Naringenin 4'-O-β-D-glucuronide



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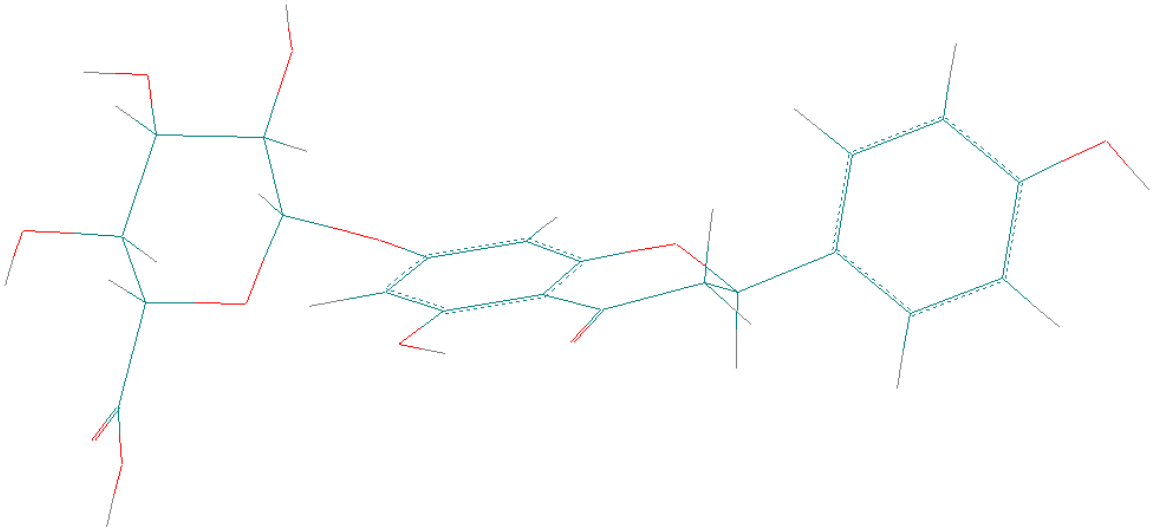
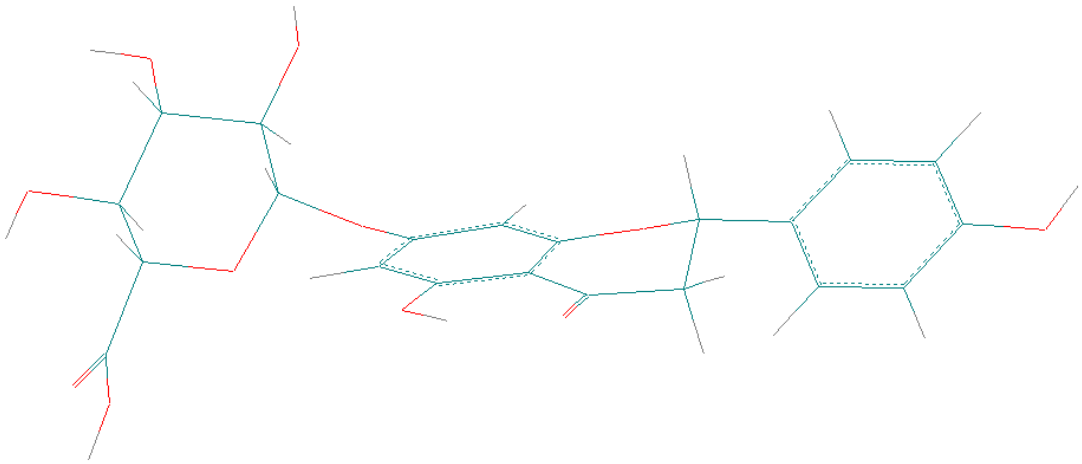


(2*R*)-Naringenin 7-O-β-D-glucuronide



(2*S*)-Naringenin 7-O-β-D-glucuronide

Figure 1: Optimized conformations of the naringenin glucuronides.

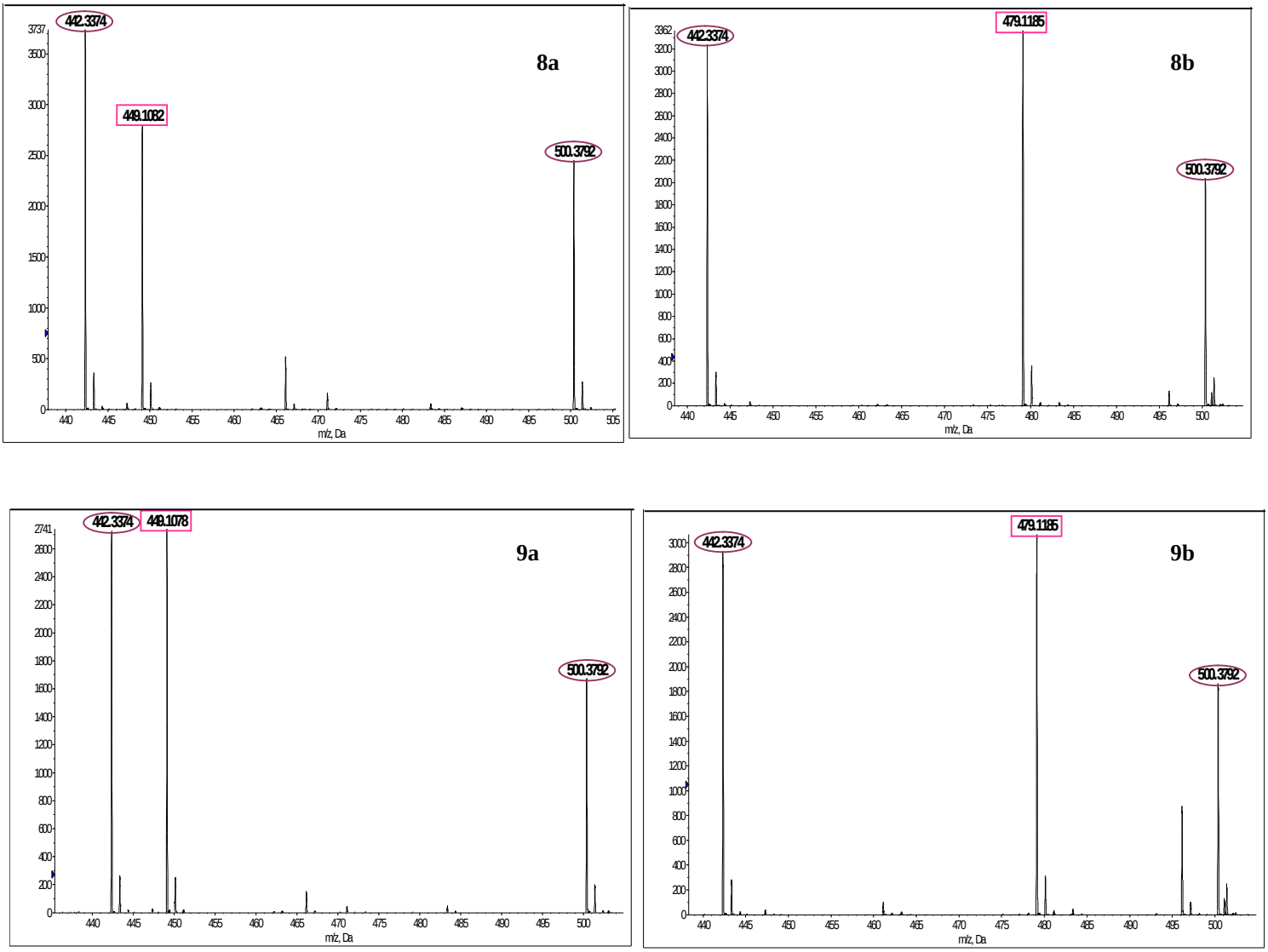


Figure 2: High-resolution mass spectra of the citrus flavanone glucuronides (positive ESI)

Publication N° 3

Les chalcones

Les chalcones

Abstract

Chalcones, the biosynthetic precursors of flavanones, have interesting biological properties as antioxidants and anti-inflammatory agents. We now report the chemical synthesis of naringenin and hesperetin chalcones under optimised alkaline conditions and their structural characterisation by UV-visible spectroscopy and NMR. We found that chalcones spontaneously cyclise back to their parent flavanone aglycones under neutral aqueous conditions. The cyclisation kinetics of chalcones showed significant differences in their first order rate constants in the presence ($K_{cy\text{-}2a}$ = 1.80 × 10^{-4} s^{-1} / $K_{cy\text{-}2b}$ = 2.47 × 10^{-4} s^{-1}) or absence ($K_{cy\text{-}2a}$ = 1.74 × 10^{-3} s^{-1} / $K_{cy\text{-}2b}$ = 1.38 × 10^{-3} s^{-1}) of HSA (chalcone – HSA ratio = 1 : 2) at a temperature of 298 K. The influence of HSA (human serum albumin) on the stereochemistry of the cyclisation was studied by HPLC on a chiral column. Moreover, fluorescence spectroscopy was used to calculate the binding constants of chalcones and flavanones to HSA.

Introduction

Flavanones, the major polyphenols in citrus and tomato species, may be potentially useful in the prevention of human diseases attributed to free radical damage. The observation that flavanones display antioxidant, anti-inflammatory and anti-mutagenic activities that may be protective against cancer and cardiovascular diseases, has stimulated the research about flavanones and their derivatives (Benavente-Garcia & Castillo, 2008; Erlund, 2004; Shen et al., 2007; Tripoli et al., 2007). Among them, flavanone chalcones are of great importance because of their high prevalence as precursors in the biosynthetic pathway of flavonoids and isoflavonoids (Heller & Forkmann, 1994; Schijlen *et al.*, 2004; Marten and Mithöfer, 2005). Chalcones are 1,3-diphenyl-2-propene-1-ones, in which two phenolic rings are linked by a three carbon α,β-unsaturated carbonyl system. It is well known that naringenin chalcone is one the major flavonoids of tomato fruit and normally accumulates in the peel of the fruit (Gall et al., 2003; Capanoglu et al., 2008; Slimestad et al., 2008). Like their parent flavanone aglycones, chalcones have also proved their effectiveness against many deadly diseases. Recently, an in vivo study showed that naringenin chalcone reduces asthmatic symptoms in mice. Furthermore, the study suggests the anti-allergic potential of naringenin chalcone in human (Iwamura et al., 2010). Before that, the same research group demonstrated the in vitro anti-allergic (Yamamoto et al., 2004) and in vitro anti-inflammatory (Hirai et al., 2007) actions of naringenin chalcone. Yet, the metabolism and bioavailability of flavanone chalcones in humans is uncompletely known. However, a study in rats pointed to the formation of naringenin chalcone-2′-O-β-D-glucuronide, naringenin-7-O-β-D-glucuronide, and naringenin-4′-O-β-D-glucuronide after the ingestion of naringenin chalcone (Yoshimura et al., 2009). Moreover, chemically synthesised neohesperidin dihydrochalcone (NDHC) was shown to undergo in vitro catabolism by human intestinal microbiota. NDHC was firstly hydrolysed into hesperetin dihydrochalcone 4′-β-D-glucoside and hesperetin dihydrochalcone

and then converted into 3-(3-hydroxy-4-methoxyphenyl)propionic acid or 3-(3,4-dihydroxyphenyl)propionic acid (Braune et al., 2005). The absence of biological study on hesperetin chalcones is probably due to its scarcity in plant species. However, the chemical synthesis of dihydrochalcones of hesperetin (Esaki et al., 1994) and neohesperidin (Montijano et al., 1997), which are excellent sweetener additives, was reported. Moreover, flavanones (hesperidin, narirutin, didymin) can be converted to their respective chalcones during the industrial processing of fruits and in gastrointestinal conditions, which may affect intestinal absorption (Gil-Izquierdo et al., 2003).

Polyphenols are transported to tissues by the blood plasma. Serum albumin is the major protein in blood plasma, occurring there at a concentration of 0.6 mM. Beside its role in the maintenance of colloidal osmotic blood pressure and detoxification, serum albumin transports fatty acids, a large variety of drugs and dietary polyphenol metabolites (Sugio et al., 1999). In particular, serum albumin-flavonoid interactions have been extensively studied and the binding constants and possible binding sites have been determined (Dufour & Dangles, 2005; Banerjee et al., 2008; Lu et al., 2007; Rawel et al., 2005). Usually, fluorescence spectroscopy is the preferred analytical tool for investigating ligand – SA binding (Oravcovà et al., 1996). Indeed, HSA displays a single tryptophan residue (Trp-214, site I, sub-domain IIA), which can be excited at 295 nm and emits fluorescence at 340 nm. From the quenching of this fluorescence by a ligand, the binding constant can normally be estimated (Sulkowska, 2002). This approach was used to study the binding of hesperidin to bovine serum albumin (BSA) (Wang et al., 2007). A combination of fluorescence spectroscopy with Fourier-transformed infrared (FT-IR) and UV-visible spectroscopies was used to determine the binding constants and binding sites of naringenin (Xie et al., 2005a) and hesperetin (Xie et al., 2005b) within HSA. Hesperidin-HSA binding was also investigated by piezoelectric quartz

crystal impedance (PQCI) analysis (Liu et al., 2004). Up to now, no work has been reported on the binding of flavanone chalcones to HSA.

Substantial literature is available on the chemical synthesis of chalcones, which is normally performed by base- or acid-catalysed Claisen-Schmidt condensation of aldehydes and methylarylketones followed by a dehydration step to afford chalcones (Geissman & Clinton, 1946; Krbechek et al., 1968). More recently, efficient catalysts and conditions were proposed (Climent et al., 1994; Patil et al., 2009). Regarding the chalcone precursors of naturally occurring flavanones, a convenient synthesis simply consists in the opening of the C-ring of flavanones using highly alkaline conditions (Miles and Main, 1985). The reason of the scarcity of flavanone chalcones in Nature is primarily their instability in physiological conditions, as they rapidly cyclise back to their parent flavanones. Kinetic and spectroscopic studies of the flavanone-chalcone isomerisation were reported in different conditions (Gonzàlez et al., 2002; Nudelman & Furlong, 1991).

To the best of our knowledge, no work has been published on the cyclisation of chalcones to flavanones in the presence of human serum albumin (HSA). In this work, this point is investigated by UV-visible spectroscopy and chiral HPLC to check the possible influence of HSA on the configuration of the chiral C2 atom formed upon cyclisation. Furthermore, the binding constants of chalcones and flavanones to HSA were estimated by fluorescence spectroscopy and appropriate data treatments.

Materials and Methods

Materials

Naringenin (Nar), hesperetin (Hesp) and human serum albumin (HSA) were purchased from Sigma-Aldrich (Steinheim, Germany) and were used as received. Ethanol (EtOH),

cyclohexane (cHex) and ethylacetate (EtOAc) were from VWR International (Darmstadt, Germany) and water used was distillated in laboratory. Iso-propanol and cHex (used for HPLC) were of HPLC grade and supplied by Fischer Scientific (Illkirch, France). Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 obtained from Merck KGaA (Darmstadt, Germany). Detection was achieved by UV light (254 nm) and by charring after exposure to a 5% H_2SO_4 solution in EtOH. Purifications were performed by column chromatography (CC) on silica gel 60 (40-63 μm) (Merck KGaA).

NMR spectroscopy

1D ^{1}H and ^{13}C NMR spectra of synthetic intermediates were recorded at 300 MHz on a Bruker Advance DPX-300 apparatus. NMR chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane using the deuterium signal of the solvent (CD_3OD) for calibration. ^{1}H-^{1}H coupling constants (J) are in hertz (Hz).

Synthesis

*Naringenin / Hesperetin (**1a / 1b**).* Commercially available compounds. **1a:** Mol wt, 272.25 gmol^{-1}; light-brown powder; R_f (cyclohexane (cHex):EtOAc, 6:4) 0.36; ^{1}H NMR (CD_3OD), δ 7.33 (2H, d, J = 8.5, H2′, H6′), 6.82 (2H, d, J = 8.5, H3′, H5′), 5.90 (1H, d, J = 2.2, H6), 5.89 (1H, d, J = 2.2, H8), 5.33 (1H, dd, J = 13.0, 3.0, H2), 3.12 (1H, dd, J = 13.0, 17.0, H3a), 2.69 (1H, dd, J = 3.0, 17.0, H3b); ^{13}C NMR (CD_3OD) δ 198.19 (C4), 168.76 (C7), 165.88 (C5 or C9), 165.30 (C5 or C9), 159.43 (C4′), 131.50 (C1′), 129.45 (2C, C2′, C6′), 116.74 (2C, C3′, C5′), 103.77 (C10), 97.47 (C6 or C8), 96.58 (C6 or C8), 80.89 (C2), 44.44 (C3); **1b:** Mol wt, 302.28 gmol^{-1}; light yellow powder; mp, 235-236°C; R_f (cHex/EtOAc, 6:4) 0.27; ^{1}H NMR (CD_3OD), δ 6.93 – 6.92 (3H, m, H2′, H5′, H6′), 5.92 (1H, d, J = 2.2, H6), 5.89 (1H, d, J = 2.2, H8), 5.32 (1H, dd, J = 12.6, 3.1, H2), 3.87 (3H, s, OCH_3), 3.07 (1H, dd, J =

12.6, 17.1, H3a), 2.72 (1H, dd, J = 3.1, 17.1, H3b); ^{13}C NMR (CD_3OD) δ 198.01 (C4), 168.77 (C7), 165.87 (C5 or C9), 165.17 (C5 or C9), 149.76 (C3′ or C4′), 148.19 (C3′ or C4′), 133.55 (C1′), 119.40 (C6′), 114.94 (C5′), 112.97 (C2′), 103.78 (C10), 97.47 (C6 or C8), 96.59 (C6 or C8), 80.69 (C2), 56.83 (OCH3), 44.49 (C3).

*Naringenin chalcone (**2a**).* Compound **1a** (1g, 0.4 M) was added to a 1:1 ethanol-water mixture containing NaOH (2 g, 2M). After heating at 80°C on a water bath for about 5 min, the deep red solution was cooled to room temperature and neutralised by ice-cold 2 M HCl saturated with NaCl. The yellow precipitate thus obtained was filtered and dissolved in EtOAc for purification by chromatography on silica gel using cHex – EtOAc (4:6) as the eluent. Yield, 20%; bright yellow powder; R_f (cHex/EtOAc, 4:6) 0.47; ^{1}H NMR (CD_3OD), δ 8.09 (1H, broad d, J = 15.6, Hα), 7.71 (1H, broad d, J = 15.6, Hβ), 7.51 (2H, d, J = 8.6, H2, H6), 6.84 (2H, d, J = 8.6, H3, H5), 5.86 (2H, s, H3′, H5′); ^{13}C NMR (CD_3OD) δ 194.56 (C=O), 168.82, 166.42, 165.30 (C2′, C4′, C6′), 159.44 (C4), 144.03 (Cβ), 131.71, 129.44 (C1, C2, C6), 125.99 (Cα), 117.24, 116.73 (C3, C5), 103.75 (C1′), 96.41 (C3′, C5′).

*Hesperetin chalcone (**2b**).* Same procedure as for **2a** by starting from **1b**. Yield, 20%; deep orange powder; R_f (cHex/EtOAc, 4:6) 0.38; ^{1}H NMR (CD_3OD), δ 8.08 (1H, broad d, J = 15.6, Hα), 7.66 (1H, broad d, J = 15.6, Hβ), 7.11 (2H, m, H2, H6), 6.97 (1H, d, J = 8.3, H5), 5.87 (2H, s, H3′, H5′), 3.91 (3H, s, OCH_3); ^{13}C NMR (CD_3OD) δ 194.46 (C=O), 171.06, 166.72, 166.47 (C2′, C4′, C6′), 151.73, 148.37 (C3, C4), 143.95 (Cβ), 130.67 (C1), 126.89 (Cα), 123.58, 115.16, 112.94 (C2, C5, C6), 106.32 (C1′), 97.47, 96.42 (C3′, C5′), 56.79 (OCH_3).

UV-spectroscopy

An Agilent 8453 UV-visible spectrometer equipped with a 1024-element diode-array detector was used to record the absorption spectra over a wavelength range 190 – 1100 nm. A water thermostated bath was used to control the cell temperature with an accuracy of ± 0.1°C. The spectroscopic measurements were carried out with a quartz cuvette of 1 cm optical pathlength.

For kinetic analyses, concentrated solutions of flavanone chalcones (2 mM) were prepared in MeOH to give final concentrations of about 50 μM in the cell. The cyclisation was performed in a pH 7.4 phosphate buffer (50 mM Na_2HPO_4 and 100 mM NaCl) in the presence or absence of HSA (0 – 2 equiv.). Changes in the absorption spectra of chalcone and flavanone were monitored at 370 and 280 nm, respectively (maximal absorption) and recorded as a function of time (cycle time 30 s). Furthermore, the effect of temperature (298, 304, 311 K) and HSA concentration (0 – 2 equiv) on the cyclisation rate was determined. All experiments were performed thrice.

Data treatment. To calculate the first-order rate constants of cyclisation, kinetic data were analysed according to the following equation:

$$A_t = A_\infty + (A_0 - A_\infty) \exp(- k_{cy} t)$$

Where A_t is absorbance at time t; A_0 and A_∞ are the absorbance values at the initial and final time of the reaction; and k_{cy} is the apparent first-order rate constant. The half-life of chalcone was calculated as: $t_{1/2} = \ln 2 / k_{cy}$.

Chiral-UPLC

Chiral separation of the flavanone enantiomers was performed on the Acquity Ultra Performance LC™ (UPLC™) apparatus from Waters, equipped with an UV-visible diode

array detector (DAD). The chromatographic separation was conducted on a ChiralPAK AD-H (Amylose tris-(3,5-dimethylphenylcarbamate) coated on 5μM silica-gel) column with internal dimensions of 250 × 4.6 mm. The column was thermostated at 25 °C and an isocratic elution (Hexane / Iso-propanol (8/2)) at a flow rate of 0.5 mL min^{-1} was used. The wavelengths selected for the UPLC analyses were 280 nm and 370nm.

Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded on a thermostated Safas Xenius spectrofluorometer. The excitation and emission slit widths were set at 10 nm. All studies were performed at 25 (± 1) ◦C. Two types of excitation–emission conditions were used: a) excitation at 470 nm (naringenin chalcone – HSA), emission light collected between 530 and 590 nm; or b) excitation at 295 nm (HSA Trp residue), emission light collected between 270 and 410 nm (310 and 370 nm in case of chalcones). Solutions were prepared daily by dissolving HSA in a pH 7.4 buffer (50 mM phosphate – 100 mM NaCl). Small aliquots (1 – 150 μL) of a concentrated ligand solution in MeOH were added via syringe to 2 mL of 0.5 – 2 μM HSA solutions placed in a quartz cell (path length: 1 cm). In all experiments, the maximal cosolvent concentration was no more than 10%.

Binding data treatment. All calculations were carried out with the least-square regression program Scientist (MicroMath, Salt Lake City, USA). Standard deviations and correlation coefficients are reported. Beside the expression of the fluorescence intensity (I_F), the typical relationships used in the curve-fitting procedures were combinations of the mass law for the complexes and mass conservation for the ligand (L) and protein (P) (see below).

a). Ligands that form fluorescent complexes with albumin. The excitation wavelength was selected so as to maximize the fluorescence of the bound ligand (naringenin chalcone).

Assuming 1:1 binding, optimized values for the binding constant (K_1) and the molar fluorescence intensity of the complex (f_{PL}) were estimated by fitting the I_F vs. L_t curves against eqns (1)–(3) where L_t is the total ligand concentration and C the total protein concentration. The molar fluorescence intensity of the free ligand (f_L) was estimated from a linear plot of the fluorescence intensity vs. ligand concentration in the absence of HSA. The weak fluorescence intensity of free HSA (molar fluorescence intensity f_P) detected in the absence of ligand was taken into account.

$$I_F = f_L [L] + f_P [P] + f_{PL} K_1 [L] [P] \quad (1)$$

$$L_t = [L] (1 + K_1 [P]) \quad (2)$$

$$C = [P] (1 + K_1 [L]) \quad (3)$$

b). Quenching of the intrinsic fluorescence of HSA by the ligands. The excitation wavelength was selected so as to maximize the fluorescence of the single Trp residue of HSA. However, ligands substantially absorb light at the excitation (295 nm) or the emission (340 nm) wavelengths so that an inner filter correction is necessary. Hence, the protein fluorescence intensity is expressed as eqn (4), which is used with eqn (2) and eqn (3) in the curve-fitting procedure. The constant obtained from this method is noted K_2.

$$I_F = f_P [P] \exp (-\varepsilon_L l L_t) \quad (4)$$

$$L_t = [L] (1 + K_2 [P]) \quad (2)$$

$$C = [P] (1 + K_2 [L]) \quad (3)$$

In eqn (4), ε_L stands for the sum of the molar absorption coefficients of the ligand at the excitation and emission wavelengths, and has been checked to be identical for the bound or

free ligand. Its value is determined independently by UV-visible spectroscopy from a Beer's plot. Finally, *l* is the mean distance travelled by the excitation light at the site of emission light detection. For the spectrometer used in this work, *l* is estimated to be 0.65 cm.

Results and Discussion

Structural analysis

Two flavanone chalcones were initially prepared (Fig 1). During the highly alkaline conditions, the phenolic OH groups of flavanones are first removed with formation of flavanone phenolate anions. Then, a hydroxide ion abstracts one of the two H-atoms at C3 to form an enolate anion, which undergoes ring-opening (retro Michael addition) with formation of the chalcone phenolate anions (Andújar et al., 2003). The strong electron delocalization achieved in these anions is probably the main driving force in the ring opening reaction. Upon acidification, chalcone anions are rapidly protonated and neutral chalcones precipitate. Their isolation in the solid state prevents their cyclisation back to flavanones. In weakly basic conditions, flavanones only forms phenolate anions but ring opening does not take place. Under mildly alkaline conditions, 2'-hydroxychalcones cyclise to flavanones through intramolecular Michael addition involving an enolate intermediate. The mechanism was studied with naringin (Gonzàlez et al., 2002) and 2',6'-dihydroxy-4,4'-dimethoxychalcone (Miles and Main, 1985).

UV spectra of flavanones and chalcones in methanol showed a λ_{max} at 280 nm and 370 nm, respectively. Their deprotonation in neutral / weakly alkaline conditions causes red shifts resulting in a λ_{max} of 323 nm and 382 nm for flavanone and flavanone anions, respectively, in agreement with the literature (Petrov et al., 2008). The UV absorption band of flavanones is characteristics of a $\pi - \pi^*$ transition in the carbonyl group conjugated with the A-ring. On the

other hand, the characteristic band of chalcones (360-390nm) is due to a $\pi - \pi^*$ transition in the enone (α,β-unsaturated ketone) moiety conjugated with the B-ring (Li et al., 2007; Rijke et al., 2006).

The NMR data of the chalcones are consistent with the literature (Yoshimura et al., 2009) or others (Esaki et al., 1994; Miles and Main, 1985).

Cyclisation kinetics

The kinetics of the chalcone – flavanone isomerisation has been studied in details (Gonzàlez et al., 2002; Miles and Main, 1985; Montenegro et al., 2007; Nudelman & Furlong, 1991). However, the influence of HSA, the typical carrier of dietary polyphenols in the blood circulation, on this reaction is not known. Thus chalcones, obtained from the opening of the flavanone C-ring in highly alkaline conditions, were cyclized in a pH 7.4 phosphate buffer in the absence or presence of HSA. The reactions were followed by UV-visible spectroscopy at different temperatures (298 K, 304 K, 310 K) and for different chalcone – HSA molar ratios (1:0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, 1:2).

Spectroscopic monitoring (fig 2) shows a gradual increase in the characteristic flavanone band (323 nm) and concomitant decrease in the characteristic chalcone band (382 nm). It is noteworthy that the final absorbance at 382 nm is essentially zero in the absence or presence of HSA, which means that the chalcones are completely converted into the corresponding flavanones. Hence, no equilibrium is achieved in neutral conditions.

The first-order rate constants of cyclisation were estimated (table 1). Based on the selective monitoring of chalcone depletion at 382 nm, cyclisation of the naringenin chalcone appears slower by a factor 7 – 10 in the presence of HSA (2 equiv.) depending on the selected temperature. As for the hesperetin chalcone, the slowing down is smaller (a factor 4 – 6).

Moreover, the rate constants increase by a factor 3 – 4 when the temperature is raised from 298 to 310 K. As expected, the rates of chalcone depletion and flavanone formation are close to each other.

A gradual decrease in the cyclisation rate constants was observed with increase in HSA concentration (table 2). This reflects the increase in the fraction of bound chalcones, which appear less prone to cyclisation than free chalcones. Hence, HSA slightly protects chalcones from cyclisation in flavanones. However, flavanones by far remain the most stable isomers, either in their free or bound form. On the basis of their half-life at 37°C in the presence of HSA (~ 10 - 15 min), it can be concluded that dietary chalcones are probably converted to flavanones during absorption and transport, i.e. before reaching tissues to express biological activity.

The kinetic data were also analysed according to the Eyring equation so as to estimate the activation enthalpy and entropy of cyclisation (Table 1bis).

$$\mathrm{Ln}(k_{cy}/T) = \Delta S^{\neq}/R + \mathrm{Ln}(k_B/h) - \Delta H^{\neq}/RT$$

With R = gas constant, k_B = Boltzmann constant, h = Planck constant

From Table 1bis, it is clear that the HSA environment slows down the cyclisation of chalcones by markedly raising the activation enthalpy. It can thus be suggested that the HSA matrix impedes the distortions of the enone moiety that are required to reach the transition state, possibly by maintaining the chalcones in a planar conformation. This hypothesis of a HSA-induced restriction in flexibility is also supported by a more unfavourable activation entropy for the cyclisation in the presence of HSA.

Chiral separation

In the present study, flavanones resulting from the cyclisation of chalcone – HSA complexes were analyzed by chiral UPLC to look for possible enantioselective ring closure promoted by the chiral environment of the chalcone binding site. The chromatographic conditions used (cHex/isopropanol 4:1) were already described for flavanones (Cacamesse et al., 2005). It is well known that during flavonoid biosynthesis, chalcone isomerase (CHI) catalyses the intramolecular cyclization of chalcones into the corresponding (2S)-flavanones. It was demonstrated that electrostatic interactions between chalcones and amino acid residues lock the substrates into a constrained conformation (Jez et al., 2000). The CHI mechanism was also studied for its pH dependence, rate constant and binding mode for different products, which was confirmed by X-ray crystallography of CHI-flavanone complexes at 2.1 – 2.3 Å (Jez and Noel, 2002). As for HSA, the chiral separation of the flavanone enantiomers showed that the cyclisation was not enantioselective and actually resulted in a racemic mixture (Fig 3). The reasons might be that the binding of chalcones to HSA does not induce distortions in the planar enone moiety that would orient the attack of the A-ring OH group on one of its two sides. This is consistent with our kinetic analysis (see above).

Binding affinity for HSA

Chalcones–HSA binding was investigated by a combination of fluorescence techniques. The most critical point in this study was to operate rapidly enough to avoid substantial ring closure during data acquisition. Hence, fluorescence emission spectra were collected in a very narrow range (410 – 430 nm when Trp is excited, 530 – 560 nm when the chalcone ligand is excited).

One method consists in monitoring the fluorescence of the chalcone ligands in the presence or absence of HSA at different excitation and emission wavelengths. This approach turned out to be disappointing for several reasons. First, ligands in their free or HSA-bound

form are poorly fluorescent, so that the relatively large ligand concentrations required make the data treatment more complicated because of lack of linearity in the fluorescence intensity *vs.* concentration plots and possible multiple bindings. Second, substantial photo-induced Z–E isomerization of the chalcone carbon–carbon double bond takes place that makes the fluorescence intensity unstable, as already observed with hydroxycinnamic acids (Galland et al., 2008). In fact, only naringenin chalcone (**2a**) could be investigated by this method. Indeed, this ligand is much less prone to isomerization and its excitation spectrum (Fig. 4) makes it possible to set the irradiation at a wavelength (470 nm) where absorption is weak (a condition for proportionality between fluorescence intensity and concentration (Valeur, 2002)). Consequently, a saturatable binding isotherm could be constructed from which the binding constant (pure 1:1 binding assumed) was extracted (Fig. 5, Table 3): $K_1 \approx 45 \times 10^3$ M^{-1}. The value is in accordance with binding constants previously reported for the cardamonin chalcone (He et al., 2005), which in addition was shown to bind HSA in site I (sub-domain IIA). A similar binding mode can be assumed for the hesperetin and naringenin chalcones.

For the other ligands, we had to consider the HSA intrinsic fluorescence due to its single Trp residue (Trp-214), which is located in sub-domain IIA (rich in aromatic and positively charged Lys residues) where small negatively charged aromatic ligands are most likely to bind (Ghuman et al., 2005). The signal intensity and its sensitivity to quenching by sub-domain IIA binders make it possible to use small protein and ligand concentrations. However, all the selected ligands absorb at the excitation wavelength (295 nm) so that a correction of the fluorescence intensity at 340 nm for this inner filter effect has to be applied in the data treatment (see experimental part and reference Epps et al., 1999). Acceptable curve-fittings were achieved within the hypothesis of pure 1:1 binding (fig 6). In the

calculations, the 1:1 complexes were assumed to be nonfluorescent (table 3). With **2a**, the K_2 values thus obtained, $K_2 \approx 86 \times 10^3$ M^{-1}, can be compared with the one deduced from the enhancement of ligand fluorescence, $K_1 \approx 45 \times 10^3$ M^{-1}. We assume that the K_1 value is most reliable because no inner filter correction is required. For the other ligands, such cross-control cannot be carried out since K_1 cannot be independently determined from the ligand fluorescence method. However, for hesperetin and its chalcone, reasonable values for the binding constants were obtained by including the molar absorption coefficient at the excitation wavelength in the calculations. The mechanisms normally involved in the binding of chalcones to HSA include van der Waals interactions with apolar residues, hydrogen bonding including possible ionic interactions between Lys residues and chalcone anions (Dangles and Dufour, 2006).

The order of magnitude for the K_1 (He et al., 2005) and K_2 (Wang et al., 2007; Xie et al., 2005a; Xie et al., 2005b) values of flavanones and chalcones is in general agreement with previous reports. Taking the K_1 values as an acceptable scale of relative affinity for HSA, it can be noted that opening the C-ring of flavanones does not significantly affect the affinity to HSA.

Conclusion

The aim of the present study was to outline the influence of HSA on the rate of chalcone cyclisation. HSA was shown to bind the chalcones and slow down the cyclisation without influencing the stereochemical outcome. Indeed, a racemic mixture of flavanones is produced. Moreover, chalcones and their flavanone products roughly have the same affinity for HSA.

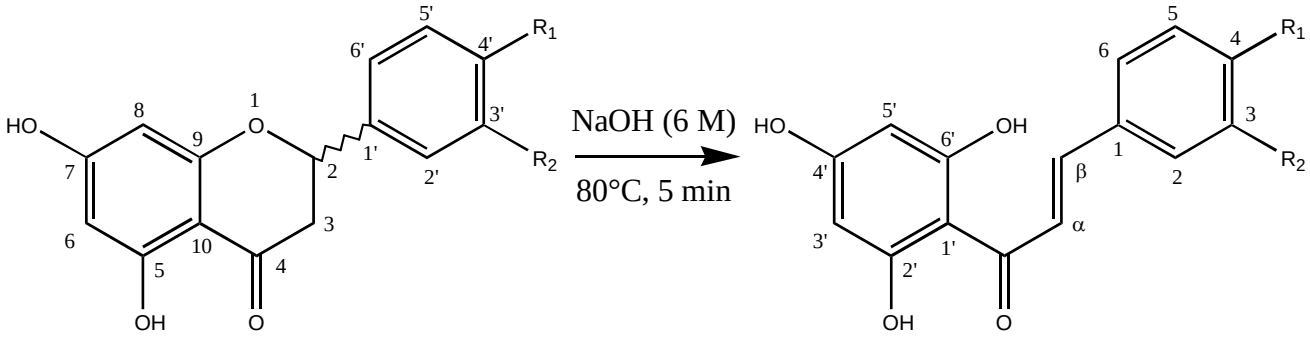


Figure 01: Synthesis of flavanone chalcones

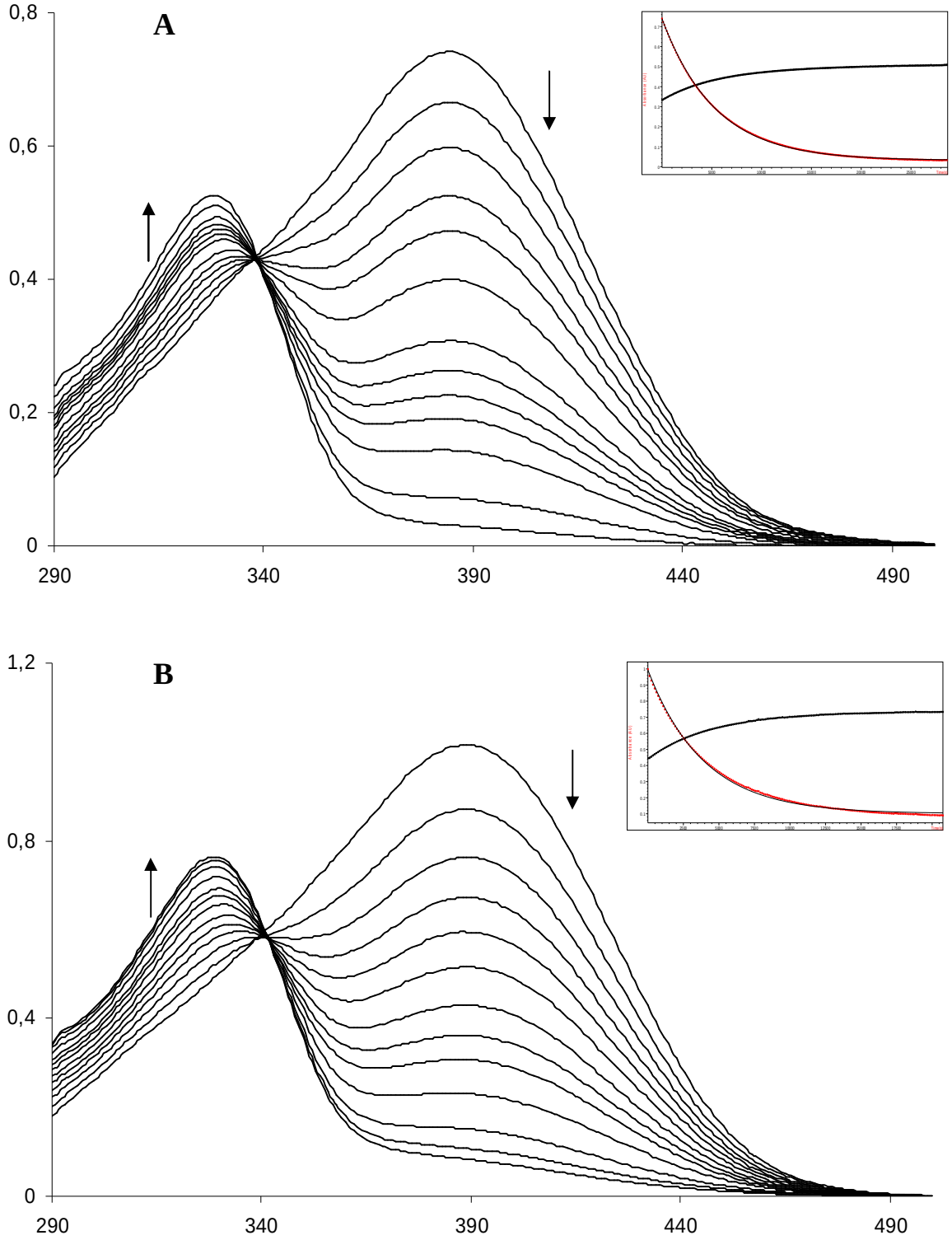
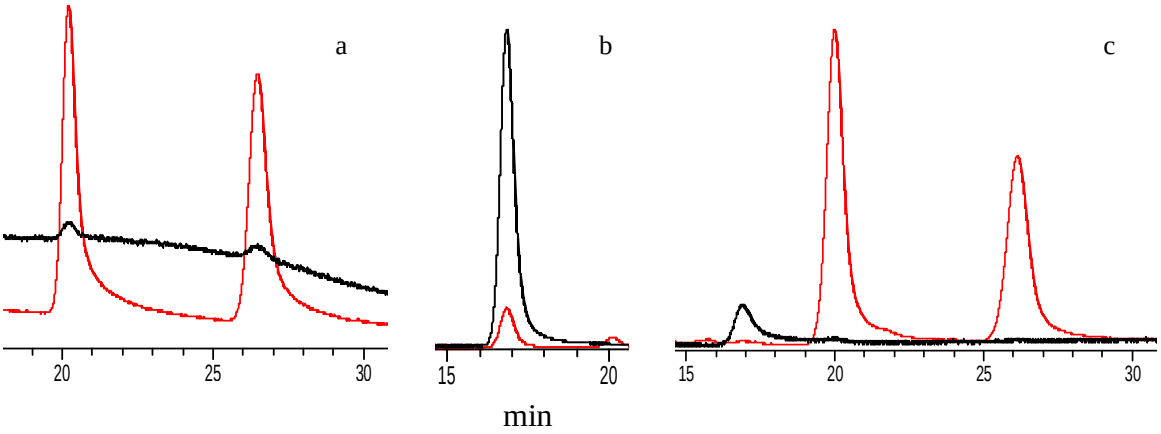


Figure 02: UV-visible spectral changes during the cyclisation of naringenin chalcone (**A)** and hesperetin chalcone (**B)**. Inset: kinetic monitoring at 382 nm and 323 nm. Chalcone-HSA molar ratio = 1:2; T = 25 °C.

Naringenin



Hesperetin

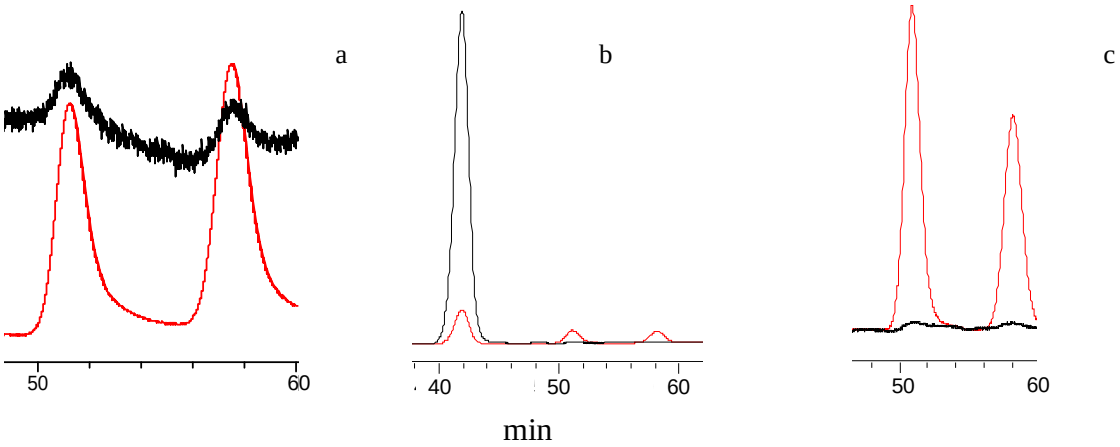


Figure 03: chiral-UPLC analysis at 280 nm (red) and 370 nm (black). a) flavanone enantiomers, b) chalcone (contaminated by small concentrations of flavanones), and c) flavanone enantiomers produced by chalcone cyclisation.

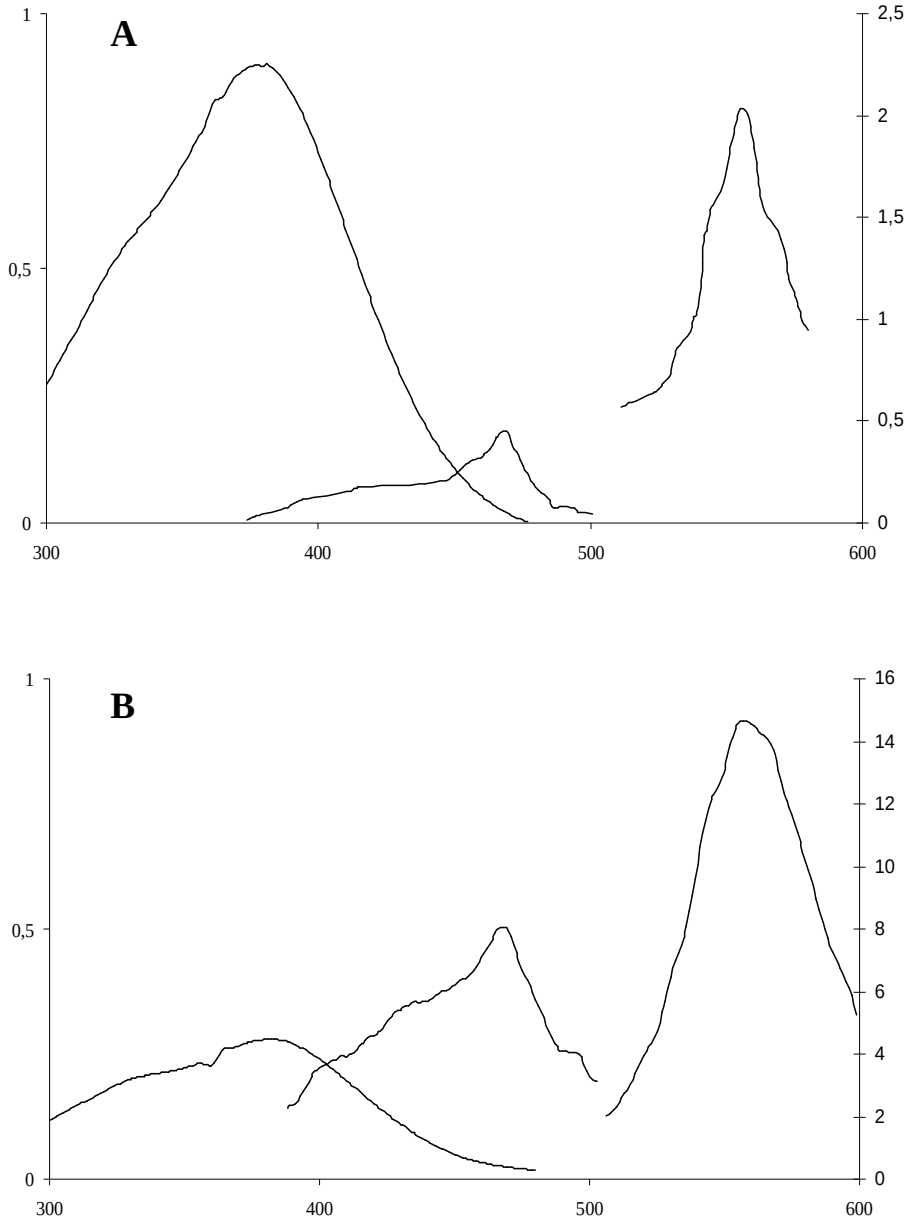


Figure 04: Absorption (1), excitation (2) and emission (3) spectra of naringenin chalcone (**2a**) in the absence (**A**) or presence (**B**) of HSA (pH 7.4 phosphate buffer, 25 °C). Excitation and emission wavelengths are set at 470 and 560 nm, respectively.

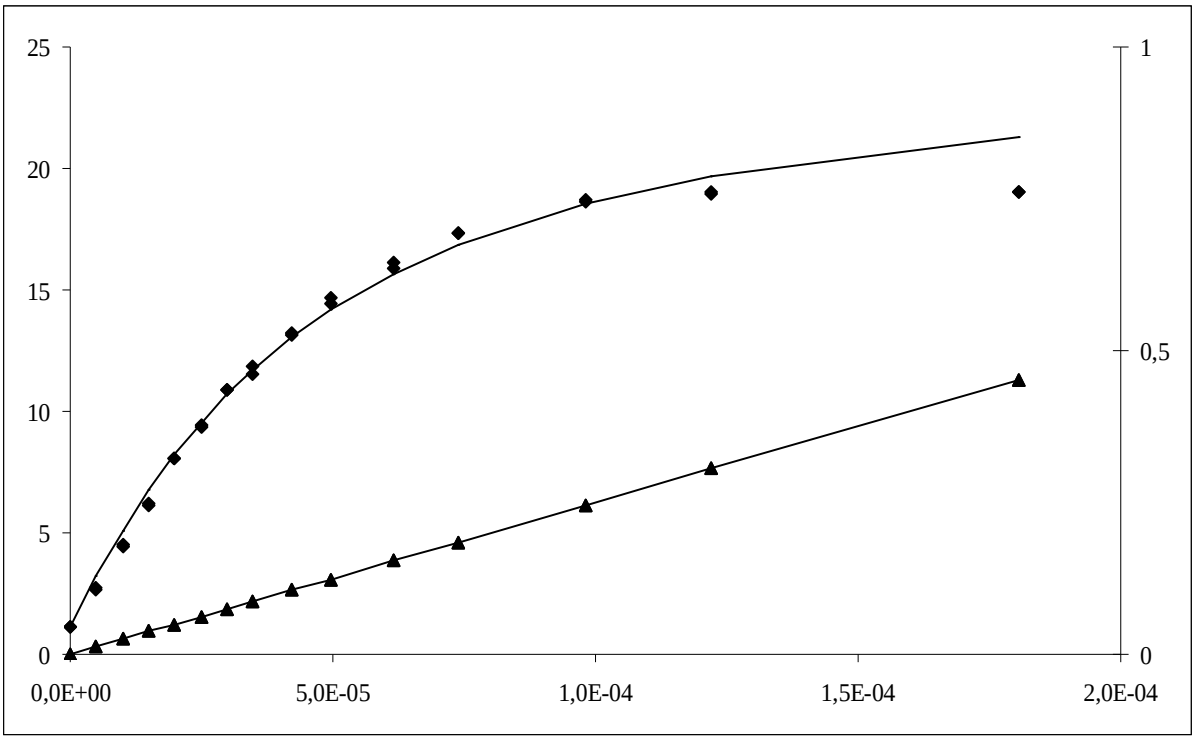


Figure 05: Changes in the fluorescence intensity of naringenin chalcone (**2a**) at 560 nm (λ_{ex} = 470 nm) as a function of its concentration. Initial HSA concentration = 20 μM (pH 7.4 phosphate buffer, 25 °C). Fluorescence of HSA-bound ligand experimental (◆) and calculated (—), absorbance of free ligand at 470 nm (—▲—).

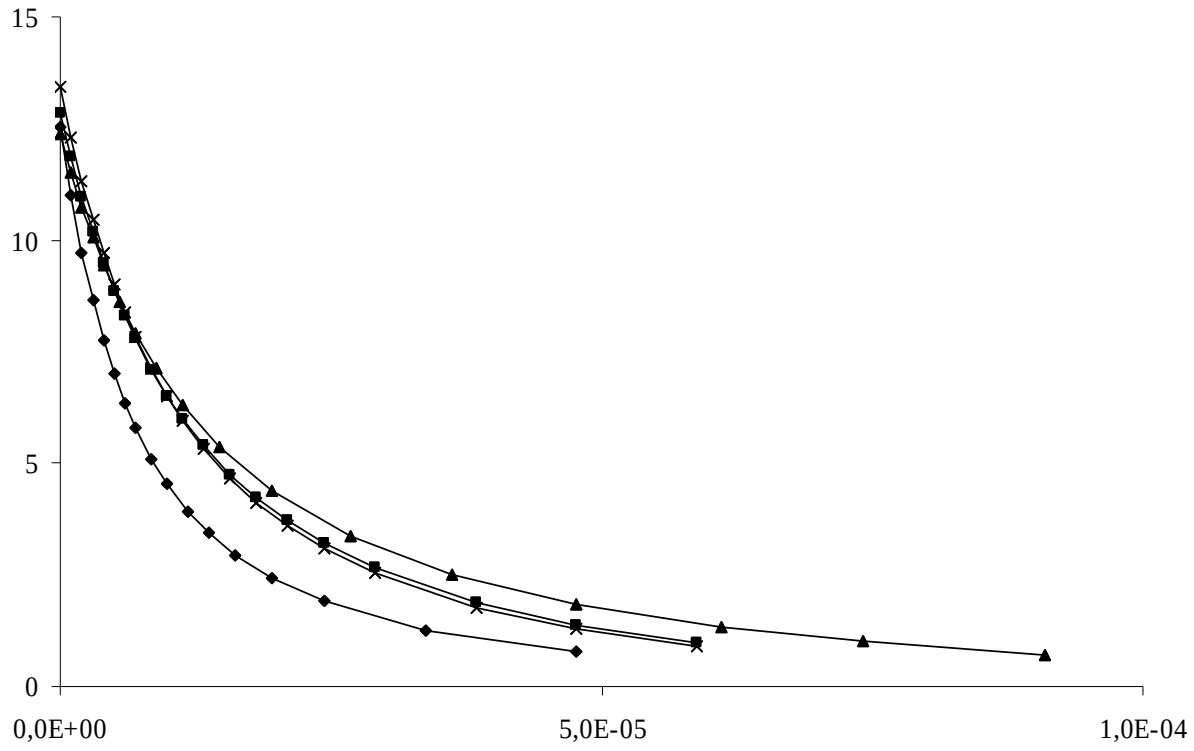


Figure 06: Changes in the fluorescence intensity of HSA at 340 nm (λ_{ex} = 295 nm) as a function of ligands concentration (pH 7.4 phosphate buffer, 25 °C). Initial HSA concentration = 2 μM. Ligands are Naringenin (■), Naringenin chalcone (◆), Hesperetin (×) and Hesperetin chalcone (▲).

Table 01: First-order rate constants (k_{cy}) for chalcones cyclisation at different temperatures in the presence or absence of HSA.

temperature	Naringenin chalcone, 2a (s^{-1})				Hesperetin chalcone, 2b (s^{-1})			
	buffer		buffer + HSA		buffer		buffer + HSA	
	Chalcone depletion (382 nm)	Flavanone formation (323 nm)	Chalcone depletion (382 nm)	Flavanone formation (323 nm)	Chalcone depletion (382 nm)	Flavanone formation (323 nm)	Chalcone depletion (382 nm)	Flavanone formation (323 nm)
25°C	1.74×10^{-3}	1.77×10^{-3}	1.80×10^{-4}	1.66×10^{-4}	1.38×10^{-3}	1.72×10^{-3}	2.47×10^{-4}	2.27×10^{-4}
31°C	2.79×10^{-3}	2.88×10^{-3}	3.86×10^{-4}	3.54×10^{-4}	2.56×10^{-3}	2.84×10^{-3}	5.06×10^{-4}	4.79×10^{-4}
37°C	5.00×10^{-3}	4.94×10^{-3}	7.46×10^{-4}	6.88×10^{-4}	4.25×10^{-3}	4.27×10^{-3}	9.71×10^{-4}	9.34×10^{-4}

***** Chalcone-HSA molar ratio was 1:2 in all experiments

Table 1bis: Activation enthalpy and entropy of chalcone cyclisation in the presence or absence of HSA (2 equiv.)

Studied compounds	$\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$ (J K^{-1} mol^{-1})
Naringenin	65.0 (± 4.9)	-80 (± 16)
Naringenin - HSA	88.5 (± 2.8)	-19.3 (± 9.3)
Hesperetin	69.5 (± 3.3)	-66 (± 11)
Hesperetin - HSA	85.1 (± 1.4)	-28.3 (± 4.7)

Table 02: First-order rate constants (k_{cy}) of chalcone cyclisation at different chalcone – HSA molar ratios.

Chalcone-HSA ratio	Naringenin chalcone 2a (s^{-1})		Hesperetin chalcone 2b (s^{-1})	
	Chalcone depletion (382 nm)	Flavanone formation (323 nm)	Chalcone depletion (382 nm)	Flavanone formation (323 nm)
1 : 0	1.74×10^{-3}	1.77×10^{-3}	1.38×10^{-3}	1.72×10^{-3}
1 : 0.4	6.02×10^{-4}	6.05×10^{-4}	6.91×10^{-4}	7.26×10^{-4}
1 : 0.8	3.40×10^{-4}	2.95×10^{-4}	4.87×10^{-4}	3.83×10^{-4}
1 : 1.2	2.81×10^{-4}	2.63×10^{-4}	3.60×10^{-4}	3.20×10^{-4}
1 : 1.6	2.16×10^{-4}	1.87×10^{-4}	2.73×10^{-4}	2.37×10^{-4}
1 : 2	1.80×10^{-4}	1.66×10^{-4}	2.47×10^{-4}	2.27×10^{-4}

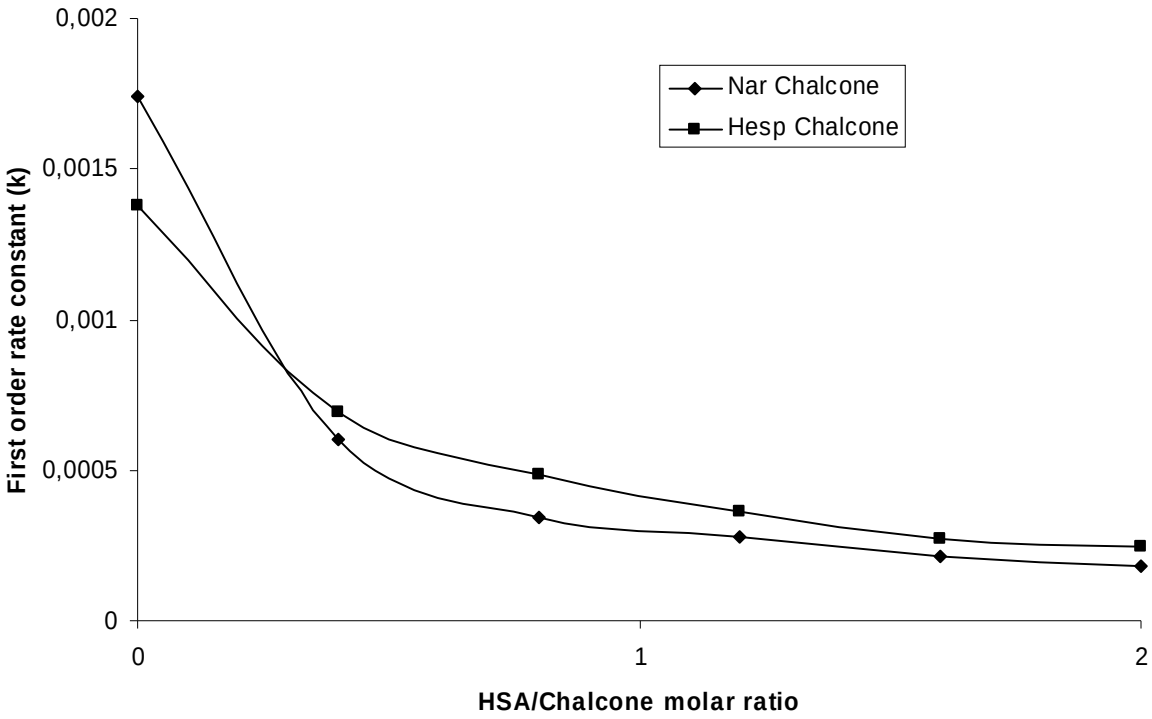


Table 03: Binding constants (K_2) of selected ligands to human serum albumin (2 μM) in a pH 7.4 phosphate buffer at 25 °C. Fluorescence monitoring at 340 nm following excitation of HSA (single Trp residue) at 295 nm.

Ligand	K_2 **(M^{-1})**	**R**	ε_L **(M^{-1} cm^{-1})** 295 nm
1a	77 (± 1) x10^3	0.9997	9910
1b	85 (± 1) x10^3	0.9997	10570
2a	86 (± 5) x10^3	0.9931	3970
2b	73 (± 2) x10^3	0.9992	3710

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Publication N° 4

Liaison des glucuronides de flavanones à la HSA

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ABSTRACT

Naringenin and hesperetin, the major polyphenols (flavanones) present in citrus fruits and juices, mainly circulate in plasma as glucuronides. The binding of flavanone glucuronides to human serum albumin (HSA) has been investigated by fluorescence spectroscopy. Binding constants (*K*) in the range of $3 – 6 \times 10^4$ M^{-1} have been estimated. No significant difference was found in the binding ability of FGs in comparison to their parent aglycones. Competitive or noncompetitive binding experiments in the presence of DNSS (dansyl sarcosine, site IIIA probe) have been used to gain information on the FG binding pocket in HSA. Fluorescence quenching of Trp214 and HSA – DNSS complex suggest that FGs bind to both subdomains IIA & IIIA of HSA.

INTRODUCTION

Due to their possible role in the prevention of cardiovascular diseases and cancers, flavanones from citrus fruits and juices have been investigated for their metabolism and bioavailability (Manach et al., 2003; Felgines et al., 2000; Choudhury et al., 1999; Serra et al., 2008; Matsumoto et al., 2004) as well as for their biological effects (Benavente-Garcia & Castillo, 2008; Erlund, 2004; Tripoli et al., 2007). However, the delivery of flavanones (especially human metabolites) to specific biological sites is still poorly documented. The interaction of flavonoid metabolites with human serum albumin (HSA) is an important factor in their transport to biological sites. Indeed, serum albumin is the major component of blood plasma, occurring there at a concentration of 0.6 mM. Beside its role in the maintenance of colloidal osmotic blood pressure and bodily detoxification, HSA transports fatty acids and a large variety of drugs and dietary components including polyphenols (Dufour & Dangles, 2005). The literature on the structural aspects and binding locations of HSA is well described by a number of comprehensive reviews. The determination of the amino acid sequences of HSA (585 amino acids) were the remarked achievements to present the structural framework which helped a lot to justify the binding of different molecules with albumin (Behrens et al., 1975; Meloun et al., 1975). And now with the advancement of analytical tools, crystallography has made possible to show the three-dimensional structure (X-ray structure) of HSA to characterise it completely and to elaborate the binding domains (He & Carter, 1992). Mainly, it consists of three helical domains I (1-195), II (196-383) & III (384-585) and each domain is further subdivided into two subdomains A and B (Fig 6). The overall shape of protein represents the form of heart and whole structure is stabilised by 17 disulfide bonds. The subdomains IIA (site I) and IIIA (site II) are the highly studied subdomains because of their involvement in the interaction of drugs and other compounds. The fact behind these

types of affinities is the presence of some hydrophobic cavities along with some positive charged amino acid residues at the entrance of the pockets (Sugio et al., 1999).

A significant amount of literature available on the albumin-flavonoid interactions consists of not only the quantitative data, representing the binding constants, but also qualitative analyses to find the possible binding sites of ligands on albumin (Dufour & Dangles, 2005; Banerjee et al., 2008; Lu et al., 2007; Rawel et al., 2005). Like in other domains, quercetin and its metabolites are also greatly studied for their affinity with HSA (Murota et al., 2007; Zsila et al., 2003). Despite the existence of some conventional techniques, fluorescence spectroscopy is most widely applied approach to elucidate the binding affinities and locations of ligands attach to HSA (Oravcovà et al., 1996). Normally, there is a fluorescent tryptophan residue (Trp214) in site I who excites at 295nm and emits energy in the form of fluorescence at 340nm. The measurement of this fluorescence quenching by any ligand using fluorescence spectrophotometer helps to calculate the binding constants after some statistical analyses (Sulkowska, 2002). The statistical analyses are discussed in experimental part.

It is not too longer that scientists have taken the consideration on the affinity of flavanone with HSA and most of these studies are presented by some Chinese research units. A good amount of literature is available to elucidate the binding affinity, binding sites and binding mechanism of flavanones with HSA. For instance, the piezoelectric quartz crystal impedance (PQCI) analysis to find the binding ability of hesperidin (Liu et al., 2004); the molecular interaction of hesperidin with bovine serum albumin (BSA) by fluorescence spectroscopy (Wang et al., 2007); and the fluorescence spectroscopy with support of fourier transformed infrared (FT-IR) and UV-visible spectroscopies to investigate the binding

constants, position and mode of naringenin (Xie et al., 2005a) and hesperetin (Xie et al., 2005b) with HSA.

Reports about the binding to HSA of flavanone aglycones and their glycosides are only a first step toward the understanding of flavanone transport in the blood, since circulating flavanones are actually neither aglycones nor glycosides but glucuronides. Thus, there is still a great need of purified metabolites for accurate determination of binding constants and binding sites. As an alternative to the expensive, inconvenient and low yielding extraction of conjugates from biological fluids, chemical synthesis appears as the most direct strategy to obtain substantial amounts of these metabolites for in vitro cell studies and other biological studies. In our last paper, we have reported the chemical synthesis of the four major flavanone glucuronides circulating in blood after citrus consumption, hesperetin 3'- and 7-O-β-D-glucuronides and naringenin 4'- and 7-O-β-D-glucuronides (Khan et al., 2010). In the present work, we wish to report the binding to HSA of these conjugates in comparison with the parent aglycones. Moreover, some competitive experiments are also carried out to locate their binding site(s).

MATERIALS AND METHODS

2.1. Materials

HSA (96-99%, MM = 66500 g mol^{-1}) was used as received from Sigma – Aldrich (St Quentin Fallavier, France). Quercetin (98%), naringenin, hesperetin, sodium dihydrogenphosphate (99%) and dansyl sarcosine (DNSS, 99%) were also purchased from Sigma – Aldrich (St Quentin Fallavier, France). All solutions were prepared in deionized water or HPLC grade methanol. Glucuronides of naringenin (4′- and 7-*O*-*β*-D-glucuronides)

and hesperetin (3′- and 7-*O*-*β*-D-glucuronides) were chemically synthesized as already described (Khan et al., 2010).

2.2. UV-spectroscopy

An Agilent 8453 UV-visible spectroscopy system was used to record the absorption spectra over a wavelength range 190 – 1100 nm which combined with a 1024-element diode-array detector. A filter was embedded in the system to reduce the stray light in UV-range. A thermostat bath was attached to control the cell temperature with accuracy of ± 0.1°C. Quartz cuvette of 1 cm was used for measurements in solution. The UV-spectroscopy was used to analyse the molar absorption coefficient (ε_L) of ligands. The calculation is explained in the next section 2.4.

2.3. Fluorescence measurements

Albumin was daily prepared in a pH 7.4 buffer (50 mM phosphate – 100 mM NaCl). Steady-state fluorescence was recorded on a thermostated BioLogic fluorometer. All studies were performed at 25 °C ± 1 using 10-nm excitation and emission slit widths. It will be important to quote that the values of binding constants reported were the average of three replications with different HSA – ligand concentrations. Two types of excitation–emission conditions were used after equilibration of the solutions for a few minutes:

– excitation at 370 nm (DNSS probe), emission collected between 350 and 600 nm

– excitation at 295 nm (albumin Trp214 residue), emission collected between 270 and 410 nm.

2.4.1. Interaction of albumin with a single ligand

Aliquots of a 1 – 5 mM ligand solutions in MeOH were added via syringe to 2 mL of a 2 – 5 μM albumin solutions placed in a quartz cell (path length: 1 cm). In all experiments, the maximal cosolvent concentration was 10 %.

2.4.2. Interaction of albumin – DNSS complex with a ligand

To 2 mL of a 75 μM HSA solution were successively added 20 μL of a 7.5 mM solution of DNSS in MeOH (1:1 HSA–DNSS molar ratio) and aliquots (1 – 150 μL) of a concentrated (1 – 3 mM) solution of the second ligand in MeOH.

2.4.3. Binding data analysis

All calculations were carried out with the least-square regression program Scientist (MicroMath, Salt Lake City, USA). Standard deviations and correlation coefficients are reported. Beside the expression of the fluorescence intensity I_F, the typical relationships used in the curve-fitting procedures were combinations of the mass law for the complexes and mass conservation for the ligand L, protein P and dansyl probes D (see below).

2.4.3.1. Fluorescence of the ligands in the presence of HSA

The excitation wavelength was selected so as to maximize the fluorescence of the bound ligands or DNSS probe. Assuming 1:1 binding, optimized values for the binding constant (K_1) and the molar fluorescence intensity of the complex (f_{PL}) were estimated by fitting the I_F vs. L_t curves against eqns (1)–(3) where L_t is the total ligand concentration and C the total protein concentration. The molar fluorescence intensity of the free ligand (f_L) was estimated from a linear plot of the fluorescence intensity vs. ligand concentration in the absence of HSA. The weak fluorescence intensity of free HSA (molar fluorescence intensity f_P) detected in the absence of ligand was taken into account.

$$I_F = f_L [L] + f_P [P] + f_{PL} K_1 [L] [P] \quad (1)$$

$$L_t = [L] (l + K_1 [P]) \quad (2)$$

$$C = [P] (1 + K_1 [L]) \quad (3)$$

2.4.3.2. Quenching of the intrinsic fluorescence of HSA by the ligands

The excitation wavelength was selected so as to maximize the fluorescence of the single Trp residue of HSA. However, most ligands substantially absorb light at the excitation wavelength (295 nm) so that an inner filter correction is necessary. Hence, the protein fluorescence intensity is expressed as eqn (4), which is used with eqn (2) and eqn (3) in the curve-fitting procedure.

$$I_F = f_P [P] \exp (-\varepsilon_L l L_t) \quad (4)$$

In eqn (4), ε_L stands for the sum of the molar absorption coefficients of the ligand at the excitation and emission wavelengths, and has been checked to be identical for the bound or free ligand. Its value is determined independently by UV-visible spectroscopy from a Beer's plot. Finally, l is the mean distance travelled by the excitation light at the site of emission light detection. For the spectrometer used in this work, l is estimated to be 0.65 cm. When necessary, additional 1:2 protein-ligand binding (stepwise binding constant K_2) was taken into account, leading to eqns (5)–(7):

$$L_t = [L] + K_1[P][L] + K_1K_2[P][L]^2 \quad (5)$$

$$C = [P] (1 + K_1[L] + K_1K_2[L]^2) \quad (6)$$

$$I_F = [P] (f_P + f_{PL} K_1[L]) \exp(-\varepsilon_L l L_t) \quad (7)$$

In eqn (7), the fluorescence of complex PL_2 is neglected.

2.4.3.3. Quenching of the fluorescence of dansyl probe–HSA complexes by the ligands

The excitation wavelength was selected so as to maximize the fluorescence of the bound probe. Assuming competition between the dansyl probe D and ligand L and pure 1:1 binding, eqns (8) and (9) can be derived and used in the curve-fitting of the I_F vs. L_t curve for the determination of optimized values for parameters K_1 and f_{DP} (after preliminary determination of K_D in the absence of L and of f_D in the absence of L and P).

$$I_F = D_t \frac{f_D + f_{DP} K_D[P]}{1 + K_D[P]} \exp(-\varepsilon_L l L_t) \qquad (8)$$

$$C = [P]\left(1 + \frac{K_D D_t}{1 + K_D[P]} + \frac{K_1 L_t}{1 + K_1[P]}\right) \qquad (9)$$

where f_D and f_{DP} are the molar fluorescence intensities of the dansyl probe and dansyl probe–albumin complex, respectively, D_t is the total concentration of the dansyl probe, K_D the probe–HSA binding constant and ε_L the sum of the molar absorption coefficient of the ligand at the excitation and emission wavelengths.

RESULTS AND DISCUSSION

The transportation of food molecules in the circulatory system is an important step in the overall process of delivering dietary components to tissues for biological action. Serum albumin, the major protein in blood plasma, carries fatty acids, drugs, polyphenols and other useful components. Thus, investigating interactions between HSA and polyphenol metabolites can provide a pertinent insight in the fate of circulating polyphenols in terms of rate of excretion or delivery to tissues. For instance, following the ingestion of 50 mg of polyphenols

(aglycone equivalent), the mean elimination half-life of catechins and flavanones is 2-3 h, whereas it is 5-8 h for isoflavones and 18-20 h for flavonols (Manach et al. *Am. J. Clin. Nutr.* 2005, 81: 230S – 242S). This ranking is likely to reflect an increasing affinity of the corresponding metabolites for plasma proteins, in particular HSA.

Polyphenol–HSA binding can be investigated by a combination of fluorescence techniques (Dangles et al., 2001). One method consists of monitoring the fluorescence of the polyphenolic ligand in the presence and absence of HSA. This approach turned out to be disappointing with flavanone glucuronides. Indeed, flavanones in their free or HSA-bound form are poorly fluorescent, so that the relatively large ligand concentrations required make the data treatment more complicated because of lack of linearity in the fluorescence intensity vs. concentration plots and possible multiple bindings.

For all ligands, we had to consider the HSA intrinsic fluorescence due to its single Trp residue (Trp-214), which is located in sub-domain IIA where small negatively charged aromatic ligands are most likely to bind (Ghuman et al., 2005). The signal intensity and its sensitivity to quenching by sub-domain IIA binders make it possible to use small protein and ligand concentrations. However, all the selected ligands absorb at the excitation wavelength (295 nm) so that a correction of the fluorescence intensity at 340 nm for this inner filter effect has to be applied in the data treatment (see experimental part and reference – Epps et al., 1999). Acceptable curve-fittings were achieved within the hypothesis of pure 1:1 binding of HSA – flavanone glucuronides (Figure 1, table 1).

The binding constants of naringenin 4′-*O*-*β*-D-glucuronide ($K_1 = 34 \times 10^3$ M^{-1}) and hesperetin 3′-*O*-*β*-D-glucuronide ($K_1 = 37 \times 10^3$ M^{-1}) are slightly lower than those of naringenin 7-*O*-*β*-D-glucuronide ($K_1 = 47 \times 10^3$ M^{-1}) and hesperetin 7-*O*-*β*-D-glucuronide ($K_1 = 60 \times 10^3$ M^{-1}), respectively. In comparison, naringenin and hesperetin bind to HSA with K_1

= 77 × 10^3 M^{-1} and K_1 = 85 × 10^3 M^{-1}, respectively. Thus, glucuronidation only weakly destabilizes the flavanone – HSA complexes, the effect being slightly stronger with B-ring glucuronidation. Moreover, a bathochromic shift in Trp214 emission is observed with the A-ring glucuronides, which suggests structural changes in HSA conformation upon binding (figure 2).

The highly sensitive fluorescent probe dansylsarcosine (DNSS) was used in competitive experiments with the flavanones to gain information about the binding sites. DNSS is reported to bind in sub-domain IIIA of HSA (Epps et al., 1995; Mathias & Jung, 2007). The fluorescence of the DNSS–HSA complex was gradually quenched by increasing flavanone concentrations. The quenching curves were analyzed within the hypothesis of pure competitive 1:1 binding for both the probe and ligand and by taking into account a very weak inner filter effect due to absorption of the ligand at the excitation wavelength (Fig. 3). Our data suggest that competitive binding is not a general rule. Indeed, the quenching of DNSS–HSA fluorescence by all ligands gave K_1 values significantly weaker than those determined from the quenching of HSA fluorescence, which is indicative of noncompetitive binding. In particular, hesperetin binds to HSA slightly more tightly than naringenin whereas the reverse is true in the presence of DNSS. This suggests that even a minor structural change such as an additional methoxyl group is enough to induce a substantial move of the ligand within the binding site. Moreover, DNSS lowers the affinity of flavanones for HSA according to the ranking: aglycones ≈ A-ring glucuronides > B-ring glucuronides. In other words, the binding of B-ring glucuronides to HSA appears less affected by DNSS than the binding of aglycones or A-ring glucuronides. Finally, experiments in the presence of the flavonol quercetin, a sub-domain IIA ligand (Dufour & Dangles, 2005), were conducted (data not shown). It was observed that flavanone glucuronides did not significantly affect the fluorescence of the quercetin - HSA complex.

From the fluorescence quenching of Trp214 located in sub-domain IIA and the noncompetitive binding experiments in the presence of the DNSS probe bound in sub-domain IIIA, it can be suggested that flavanone aglycones and glucuronides bind to slightly different HSA binding sites. In particular, binding to a sub-domain IIA site distinct from that of quercetin is possible (Dufour & Dangles, 2005) with aglycones and A-ring glucuronides closer to the DNSS binding site in sub-domain IIIA. The primary binding site of flavonoids is generally assumed to be sub-domain IIA due to the structural similarity between flavonoids and the coumarin warfarin, whose binding site in sub-domain IIA was well established by X-ray cristallography (Yamasaki et al., 1996). However, fluorescence and FT-IR spectroscopies suggested the binding of hesperetin in both subdomains IIA and IIIA (Xie et al., 2005). The principal mechanisms involved in the interaction of flavanone glucuronides with protein could be the hydrophobic effect and van der waals interactions between the ligand and hydrophobic amino acid residues, electrostatic interactions between positively charged Lys residues and the GlcU carboxylate group, hydrogen bonding between the phenolic hydroxyl groups and the polypeptide chain or the polar amino acid residues (Zhang et al., 2008).

Conclusion

Our study shows that flavanone glucuronides are moderate HSA ligands in agreement with their relatively high elimination rate (Manach et al. 2005). As aglycones, flavanone glucuronides likely bind to both sub-domains IIA and IIIA. The moderate affinity of flavanone glucuronides for HSA should be large enough to ensure substantial in vivo binding given the high concentration of HSA in plasma (ca. 0.6 mM) and the low concentrations of circulating flavanone metabolites (< 1 μM).

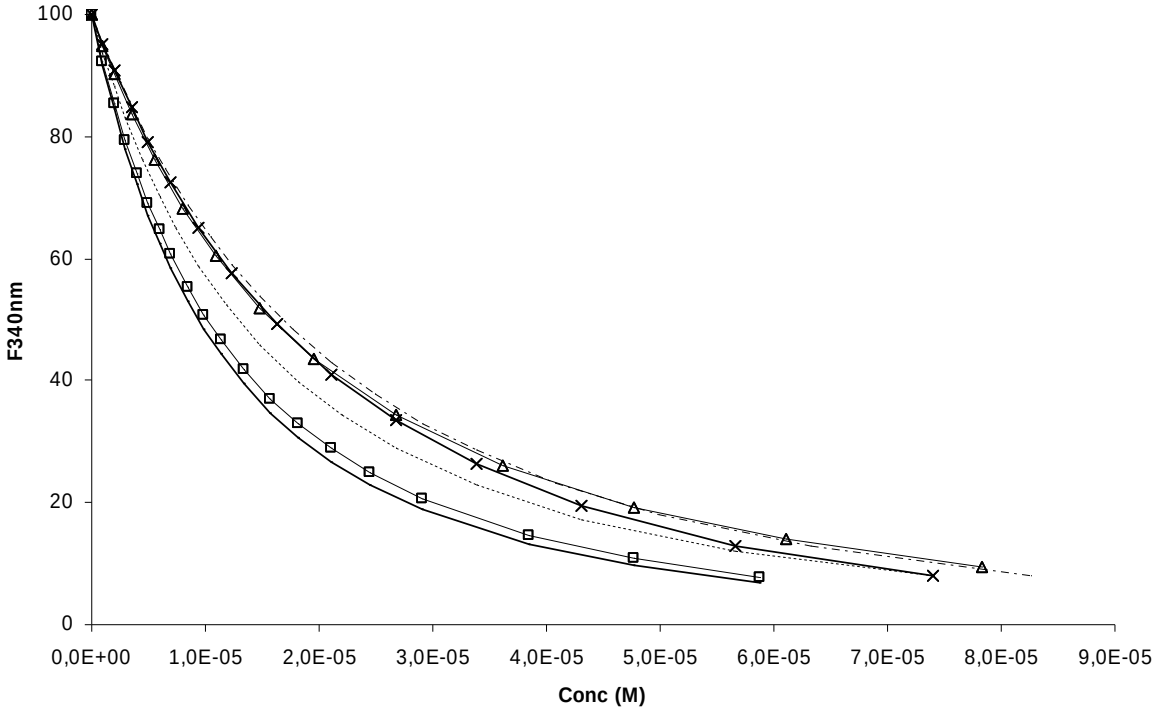

Figure 1: Changes in the relative fluorescence intensity of HSA at 340 nm (λ_{ex} = 295 nm) as a function of the total flavanone concentration (pH 7.4 phosphate buffer, 25 °C). Initial HSA concentration = 2 µM. Nar (—□—), N7G (—△—), N4'G (—X—), Hesp (——), H7G (------), H3'G (-·-·-·-).

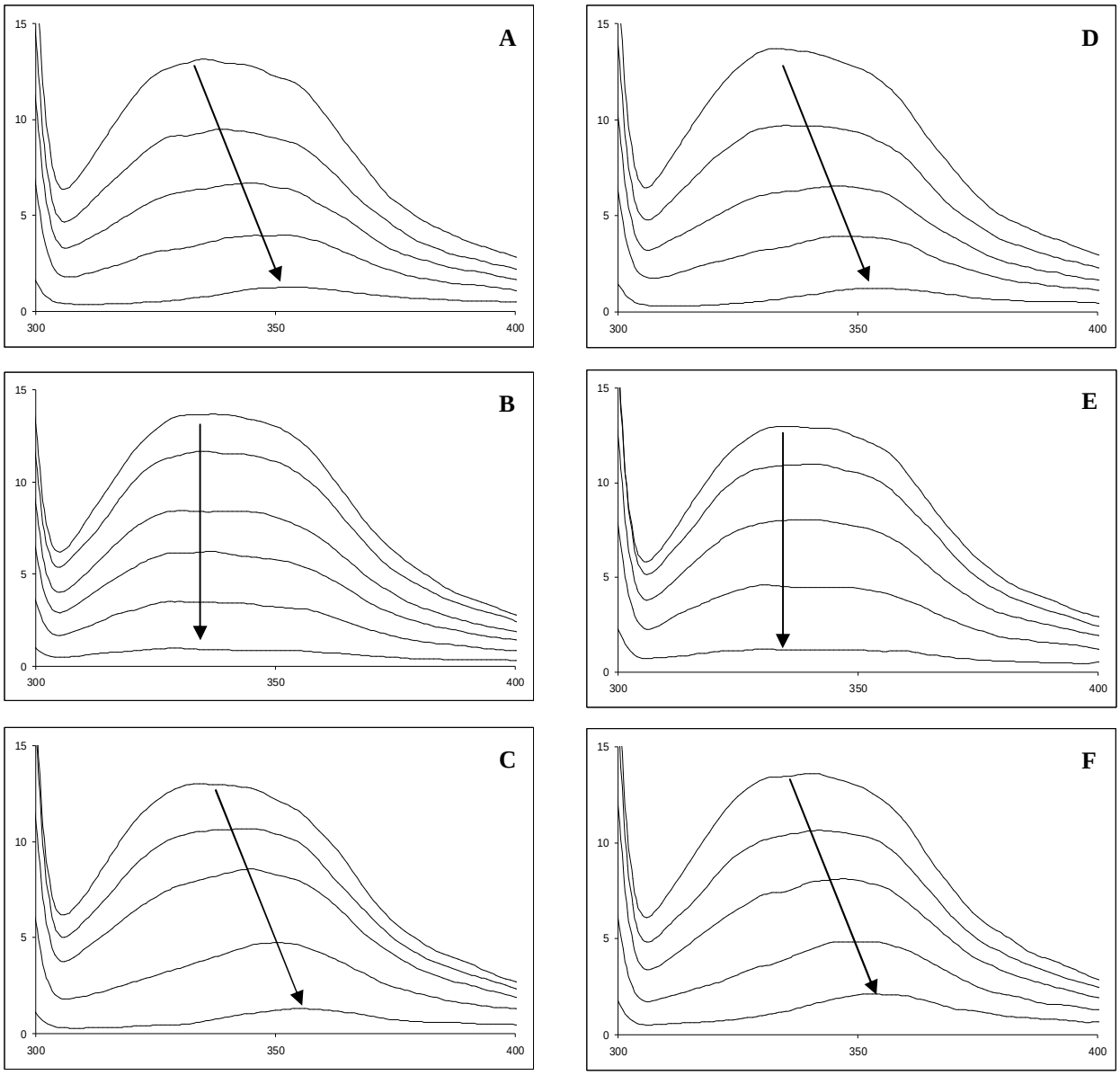

Figure 2: Trp214 fluorescence quenching by Nar (A), N7G (B), N4'G (C), Hesp (D), H7G (E), H3'G (F). Initial HSA–Ligand ratio = 1:0 and final HSA–Ligand ratio = 1:20. Arrows point to the emission λ_{max}.

Table 1: Binding constants (K_1) of selected ligands to HSA (2 μM) and HSA – DNSS (1:1) complex in a pH 7.4 phosphate buffer at 25 °C. a) Fluorescence monitoring at 340 nm following excitation at 295 nm (HSA single Trp residue), b) fluorescence monitoring at 490 nm following excitation at 370 nm (HSA – DNSS complex).

Ligand	**Trp214 a)** K_1 **(M^{-1})**	**R**	**DNSS b)** K_1 **(M^{-1})**	**R**	ε_L **(M^{-1} cm^{-1})** 295 nm
Nar	77 (± 1) × 10^3	0.9997	36 (±1) × 10^3	0.9985	9910
N7G	47 (± 1) × 10^3	0.9989	29 (±1) × 10^3	0.9988	11390
N4'G	34 (±1) × 10^3	0.9992	25 (±1) × 10^3	0.9994	10020
Hesp	85 (±1) × 10^3	0.9997	29 (±1) × 10^3	0.9991	10570
H7G	60 (±1) × 10^3	0.9988	27 (±1) × 10^3	0.9993	12270
H3'G	37 (±1) × 10^3	0.9996	26 (±1) × 10^3	0.9975	9450

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Publication N° 5

Etude biologique sur les glucuronides

Etude biologique sur les glucuronides

Approche Scientifique et Technique

Plus précisément la première étude Clinique, dans quelle les glucuronides des flavanone ont utilisé, s'est déroulée à Clermont-Ferrand sur 5 volontaires soumis à la prise de 600 mL de jus d'orange, à 600 mL de jus de pamplemousse puis à 600 mL de boisson reconstituée sans phyto-micronutriment (dite boisson contrôle). Chaque prise était distante de 7 jours par rapport à la précédente. Des échantillons de sang ont été prélevés au Tmax des flavanones (5h30) et des prélèvements urinaires fractionnés ont également été collectés. Les deux types d'échantillons ont fait l'objet d'analyses métabolomiques en LC-ESI-MS-MS sur la plateforme de Clermont-Ferrand. La nature des principaux métabolites de l'hespérétine et de la naringénine a été confirmée par synthèse organique des glucuronides et sulfates et des analyses chromatographiques sur colonne chirale réalisées à Bordeaux.

Les travaux sur cellules ont été réalisée à Clermont-Ferrand pour les cellules HUVEC épithéliales et à Bordeaux pour les cellules musculaires lisses (CML) provenant de souris HL-/- et de souris C57Bl6/J. Les principaux métabolites circulants (naringénine-4'-O-glucuronide, l'hespérétine-3'-O-sulfate, l'hespérétine-7-O glucuronide) ont été testés sur les cellules HUVEC à des doses de 2 et 10µM. Les paramètres enregistrés sont la viabilité, l'adhésion des monocytes aux cellules endothéliales, la prolifération et la migration. Une étude en Super-array spécifique des cellules endothéliale est prévue. Sur les CML en revanche, seules l'hespérétine-3'–O-sulfate et l'hespérétine-7–O-glucuronide ont pu être testées à 1 et 10 µM.

Résultats Obtenu

La première étude métabolomique menée à Clermont Ferrand par Claudine Manach a permis grâce à la collaboration des chimistes de Bordeaux (Svitlana Shinkaruk-Poix) et d'Avignon (Olivier Dangles) d'identifier les principaux métabolites circulants après

absorption de jus d'orange et de pamplemousse. Il s'agit pour le jus d'orange du 7-O-glucuronide d'hespérétine de configuration (2R), du 3'-O-glucuronide d'hespérétine et du 3'-O-sulfate d'hespérétine. Pour le jus de pamplemousse il n'a pas été identifié de composé sulfaté mais seulement les 4'-O-glucuronide de naringénine et 7-O-glucuronide de naringénine. Cette étude a également permis de déterminer de façon extrêmement convaincante, grâce à un contrôle rigoureux de l'alimentation des volontaires, un profil métabolique propre aux consommateurs de jus d'orange et un autre propre aux consommateurs de pamplemousse. A ce jour plusieurs métabolites majeurs ont été identifiés dont certains étaient inattendus et sont potentiellement intéressants du point de vue nutritionnel. Les travaux d'identification des métabolites se poursuivent.

Le travail sur cellules HUVEC est réalisé par Audrey Chanet, Christine Morand et Dragan Milenkovic à Clermont Ferrand en collaboration avec le Professeur Maier (Faculté de Médecine de Milan). Des tests préliminaires ont permis de déterminer les conditions de culture des cellules sous stress modéré (TNF-α à 0,1 ng/mL). A ce jour, les résultats montrent que pour toutes les doses testées une exposition de 48h aux aglycones ou aux métabolites de flavanones est sans effet sur la prolifération des HUVEC ($P>0,05$). Par ailleurs, la migration cellulaire est significativement augmentée en présence d'hespérétine-3'-O-glucuronide aux concentrations de 2 et 10µM ainsi qu'avec l'hespérétine 3'-O-sulfate à la dose de 2µM ($P<0.01$). Enfin l'adhésion des monocytes (U937) aux HUVEC après stimulation au TNF alpha (0.1ng/mL) semble diminuée en présence de naringénine-4'-O glucuronide, d'hespérétine-7-O-glucuronide et d'hespérétine-3'-O-sulfate. A ce titre, la double pré-incubation des HUVEC et des monocytes avec les métabolites de flavanones avant exposition au stress inflammatoire permet d'obtenir une inhibition plus importante de l'adhésion par rapport à la seule pré-incubation des HUVEC. La répétition de ces résultats est en cours. Des expériences à venir permettront de caractériser l'impact des métabolites de flavanones sur la

production de composés vasoactifs (NO) et la migration transendothéliale des monocytes. Une analyse de la modulation de l'expression de gènes ciblés (microfluidiques dédiées à la biologie des cellules endothéliales) est sur le point d'être réalisée pour explorer les mécanismes moléculaires régissant les effets des polyphenols d'agrumes sur l'activité endothéliale. Cette analyse sera également complétée par la mesure de changement d'expression de protéines d'intérêts.

Discussion Générale et Conclusion

In the last two to three decades there has been a growing awareness of the role of diet in the etiology of the chronic diseases, importantly cardiovascular diseases (CVD) and some cancers that are major contributors to morbidity and mortality in industrialised countries such as Canada, Australia, the United States and Europe. A wide range of bioactive substances have already been identified in foods and drinks and it is likely that many more exist. There are many biologically active substances in fruits including both nutrients and non-nutrients for which protective health effects have been postulated. These include vitamins C, folic acid, carotenoids, dietary fibre and a range of phytochemicals. In some instances the beneficial effect of high fruit and vegetable diets has been ascribed to the concomitant reduction in high fat and energy-dense foods in the diet (i.e. a displacement of foods that could be harmful both in terms of chronic disease and obesity). The research frontier has now moved from study of classical vitamin deficiency diseases, to study of the thousands of phytochemicals that may have important physiologic effects.

Citrus fruit and juices have long been considered a valuable part of a healthy and nutritious diet and it is well established that some of the micronutrients in citrus promote health and provide protection against chronic diseases. More recently, the role of bioactive non-nutrient components called phytochemicals / phytomicronutrients has received increasing attention. During the past decade, many classes of phytomicronutrients have been appeared in literature such as carotenoids, polyphenols (flavonoids), and folate.

Polyphenols are a wide and complex group of secondary plant metabolites. So far, over 8000 compounds have been identified. Structures of the compounds range from simple molecules such as phenolic acids, to highly polymerized compounds like proanthocyanidins. Polyphenols are essential for the physiology of plants, having functions in growth, structure, pigmentation, pollination, allelopathy, and resistance for pathogens, UV radiation and predators. The mechanism(s) through which flavonoids may decrease the risk of chronic diseases is not currently authenticated. Flavonoids have e.g. antioxidant, vasodilatory, antithrombotic and anti-inflammatory properties, which may account for their protective effects.

This current doctoral study was a part of AGRUVASC program (2007 – 2009) whose purpose was to establish the effects of phytomicronutrients consumption through orange juice on vascular risk factors. The goal was reached through an interventional clinical study. This

study was completed by additional experiments leading to better understand mechanisms of action of citrus fruit specific phytomicronutrients: (1) *in vivo* studies on atherosclerotic mouse models; (2) *in vitro* studies on vascular cells implicated into atherosclerosis process, using the circulating forms of phytomicronutrients, alone or in combination. FLAVANONES are one of the major phytomicronutrients and the dominant polyphenolic compounds found in citrus fruits.

Citrus production (oranges, tangerines/mandarins, grapefruit, lemons and limes) is continuously increasing with an estimated world production of 82 million metric tons in the session 2009-10, among which the major commercially important orange fruits accounts for about 50 million metric tons (USDA, 2010). The domestic and industrial use of these large quantities of citrus fruits, especially for the production of juice, results in the accumulation of high amounts of by-products such as peel, seed, cell and membrane residues which account for about half of the fruit weight. The extraction of phytochemicals (flavanone glycosides) from these by-products has been proposed as an alternative. To get higher productions of phytochemicals, ultrasound application was recognised as potential breakthrough in comparison to conventional method. The extracts obtained from these by-products can be used as food grade antioxidants to replace some synthetic ones.

After the consumption of citrus fruits and juices, the flavanones are passed through gastrointestinal tract and converted into their metabolic forms which include the glucuronide and sulphate forms. This report also details the chemical synthesis of flavanone glucuronides (circulating forms) that were used as standards by nutrition biologists for *in vivo* and *in vitro* studies. The glucuronides of naringenin (4′- and 7-*O*-*β*-D-glucuronides) and hesperetin (3′- and 7-*O*-*β*-D-glucuronides), the major flavanone aglycones in grapefruit and orange respectively, have been chemically synthesized by careful protection and deprotection of flavanone and glucuronic acid moieties. The benzoyl protecting groups (used for flavanones) were found more stable and higher yielding in comparison to acetate groups during the whole synthesis pathway. Final products were completely characterised by UV-visible spectroscopy, UPLC-MS spectrometry, NMR (mono- and bi-dimensional) spectroscopy and HR-MS. Moreover, NMR gave an estimation of two enantiomers based on the peaks in NMR spectra.

The synthetic study was further extended to prepare the chalcone forms of flavanone aglycones. Chalcones were synthesised under optimised highly alkaline conditions. In mild basic conditions, the chalcone forms are highly susceptible to isomerise back to their aglycone

forms. But the addition of human serum albumin (HSA) slows down this process, possibly due to the strong association between the chalcones and HSA. The mechanism can be interpreted in the presence of chalcone metabolites in blood circulation and their possible bioactivities.

The interaction of polyphenol metabolites with HSA is an important factor in their transport to biological sites. The synthesised flavanone glucuronides and their parent aglycones have shown a moderate affinity for HSA. Moreover, the weak displacement of probe (dansyl sarcosine) in sub-domain IIIA suggests the sub-domain IIA as the primary binding site of flavanone aglycones and its glucuronides. In conclusion, based on the circulating concentrations of HSA (ca. 0.6 mM) and flavanone metabolites (<1 mM) in blood plasma and on the estimated binding constants, it can be suggested that the flavanone metabolites are transported by HSA in the blood circulation.

Discussion on biological work

In conclusion, it is now beginning to be appreciated that the biologically active, non-nutrient compounds found in citrus and other plants (phytochemicals) can be helpful to reduce the risk of many chronic diseases. In our diets, citrus consumption has a considerable potential to expand as part of the overall recommended increase in fruit and vegetable consumption. There is a well known phrase

"An apple a day keeps the doctor away"

After this whole literature and experimental study, I would like to emphasis on the recommendations by French Nutrition Health program and will say that

"*Five fruits or vegetables a day keep the chronic diseases away"*

Résume

Un groupe d'études épidémiologiques fournit une bonne preuve de la relation inverse associé à la consommation de fruits et légumes et les maladies chroniques important comme maladies cardiovasculaires et certains types de cancers. Après les longues années d'études sur phytomacronutrients, le rôle de phytomicronutrients tels que les polyphénols est désormais très étudiée et appréciée dans le contrôle de ces maladies dégénératives. La présente étude combine les études d'extraction, de synthèse et d'analyse sur les principaux polyphénols des fruits d'agrumes, FLAVANONES.

Connaissance de nutritionnels et de santé a augmenté la production d'agrumes en provenance des dernières décennies. Ces productions plus générer des bye-produits. Pour leur utilisation alternative à des antioxydants extraits riches, l'extraction assistée par ultrasons (UAE) des polyphénols en particulier flavanones de l'orange (*Citrus sinensis* L.) par son peau en utilisant l'éthanol comme solvant de qualité alimentaire a été prouvé son efficacité en comparaison avec la méthode conventionnelle . Un plan composite central (CCD) a révélé que l'approche des conditions optimisées pour UAE ont une température de 40 ° C, une puissance de 150W sonication et un 4:1 (v / v) d'éthanol: ratio de l'eau. En outre, l'activité antioxydante déterminée par les tests DPPH et ORAC a confirmé la pertinence des UAE pour la préparation d'extraits de plantes riches en antioxydants.

Les glucuronides de flavanone sont les principaux métabolites phénoliques détectés dans le plasma humain après la consommation d'agrumes. Jusqu'à maintenant, toutes les études sur les cellules liées au cancer ou les maladies cardiovasculaires ont été réalisées soit sur les aglycones ou sur leurs glycosides. Par conséquent, il ya grand besoin de glucuronides flavanone pure pour démontrer le potentiel réel de flavanones dans la prévention de ces maladies. Dans ce travail, glucuronides de naringénine (4'- et 7-*O*-*β*-D-glucuronides) et de hespérétine (3'- et 7-*O*-*β*-D-glucuronides), les aglycones flavanone majeur dans le pamplemousse et d'orange, respectivement, ont été synthétisés chimiquement par une protection et la déprotection sélective des groupements d'acide glucuronique et de flavanone. La caractérisation structurale complète de composés purifiés a été réalisée par résonance magnétique nucléaire et spectrométrie de masse.

L'affinité des quatre glucuronides pour l'albumine sérum d'humaine (HSA) a été testée par leur capacité à éteindre la fluorescence intrinsèque de HSA (*Trp*, seul résidu de sous-domaine IIA). Leurs constantes de fixation (*K*) ont été estimées de l'ordre de 30 à 60 × 10^3 M^{-1} et comparées à celles de l'aglycones (70 à 90 × 10^3 M^{-1}). Les enquêtes de la liaison compétitive ou non compétitive de la glucuronides dans la présence de sondes fluorescentes (sarcosine dansyl) nous a permis d'obtenir un aperçu dans les sites de liaison. L'étude a également été étendue aux chalcones hespérétine et naringénine (synthétisés en utilisant des conditions alcalines optimisée), qui sont les précurseurs de biosynthèse des flavanones.

Mots-clés: agrumes, les polyphénols, flavanone, l'extraction assistée par ultrasons, la synthèse, l'albumine sérique humaine.