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Journal of Chromatography B, 848 (2007) 159–181
JOURNAL OF CHROMATOGRAPHY B
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Review

Methods of analysis and separation of chiral flavonoids

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Received 29 April 2006; accepted 28 October 2006
Available online 20 November 2006

Abstract

Although the analysis of the enantiomers and epimers of chiral flavanones has been carried out for over 20 years, there often remains a deficit within the pharmaceutical, agricultural, and medical sciences to address this issue. Hence, despite increased interest in the potential therapeutic uses, plant physiology roles, and health-benefits of chiral flavanones, the importance of stereoselectivity in agricultural, nutrition, pharmacokinetic, pharmacodynamic, pharmacological activity and disposition has often been ignored. This review presents both the general principles that allow separation of chiral flavanones, and discusses both the advantages and disadvantages of the available chromatographic assay methods and procedures used to separately quantify flavanone enantiomers and epimers in biological matrices.

Keywords: Flavonoid; Flavanone; HPLC; Chiral; Enantiomer; Epimer

Contents

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doi:10.1016/j.jchromb.2006.10.052

1. Introduction

In 1936, Professor Szent-Györgyi reported the isolation of a substance that was a strong reducing agent acting as a cofactor in the reaction between peroxidase and ascorbic acid. This substance was named vitamin P; this substance has been subsequently categorized as the flavonoid rutin. Professor Szent-Györgyi's seminal investigations identified rutin and reported its isolation from both lemons and red pepper [1]. Since then, more than other 4000 flavonoids have being identified and studied. Flavonoids are a group of polyphenolic compounds of low molecular weight [2] that present a common benzo-γ-pyrone structure [3]. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, anthocyanidins, and catechins. The average human diet contains a considerable amount of flavonoids, the major dietary sources of which include fruits (i.e. orange, grapefruit, apple, and strawberry), vegetables (i.e. onion, broccoli, green pepper, and tomato), soybeans and different herbs [4,5]. Among the classes of flavonoids, flavanones have been defined as citrus flavonoids [6–8] due to their almost unique presence in citrus fruits [9–20]. However, flavanones have been also reported in tomatoes [1,21–23], peanuts [24,25] and some herbs, such as mint [26], gaviota tarplant [25,27], yerba santa [25,28], and thyme [25,29].

Flavonoids in general have being studied for more than 70 years *in vivo* and *in vitro* systems. They have been shown to exert potent anti-oxidant capacities [10,22,30–32] in some instances stronger than α-tocopherol [33]. They have been also shown to exhibit beneficial effects on capillary permeability and fragility [3,10,31,34–41], to have anti-platelet [3,10,30,31,34–40], hypolipidemic [30,42–45], anti-hypertensive [14,30,46], anti-microbial [30], anti-viral [3,10,30,31,34–40,47,48], anti-allergenic [49], anti-ulcerogenic [30], cytotoxic [30], anti-neoplastic [9,12,30,50–55], anti-inflammatory [3,10,30,31,34–40], anti-atherogenic [30,56], and anti-hepatotoxic [30] activities.

In addition, within the large family of flavonoids, flavanones present a unique structural feature known as chirality, which distinguishes them from all other classes of flavonoids (Fig. 1). All the flavanones have a chemical structure based on a C_6–C_3–C_6 configuration consisting of two aromatic rings joined by a three-carbon link [57]. Almost all the flavanones have one chiral carbon atom in position 2 (Fig. 1), except for a subclass of flavanones named the 3-hydroxyflavanones or dihydroflavonols that have two chiral carbon atoms in position 2 and 3 (Fig. 2). Some flavanones possess an additional D-configured mono or disaccharide sugar in the C7 position on ring A. These flavanone-7-O-glycosides exist as diastereoisomers or epimers that have the opposite configuration at only one of two or more tetrahedral stereogenic centers present in the respective molecular entities.

The vast majority of chiral flavanones (Figs. 3–5) can be purchased from chemical companies, but they are mainly available only as racemates (equivalent proportions of both enantiomers or epimers). To our knowledge there are only three sterochemically pure flavanones that are currently marketed internationally. Eriodictyol is marketed as the pure *S*-(−)-enantiomer by Fluka (Buchs, Switzerland); however it has been demonstrated by Caccamese et al. that the marketed eriodictyol is indeed a *R*, *S* mixture of eriodictyol enantiomers [58]. Homoeriodictyol is marketed as the pure *S*-(−)-enantiomer by Indofine Chemical Company (Hillsborough, NJ), Extrasynthese (Genay, France), and ITI International Inc. (Miami. FL). Finally, taxifolin is marketed as the pure 2*R*, 3*R*-enantiomer by Alexis Biochemicals (San Diego, CA), Fluka (Buchs, Switzerland), and Extrasynthese (Genay, France).

The importance of stereospecific pomological disposition of racemic flavanones has being recognized and reported in the last 20 years. Most of these investigations report the quantification of a variety of flavanones in citrus fruit juices and herbs [12,25,59–62], or report the separation of flavanones on different stationary phases [63–72]. There is a paucity of investigations detailing the importance of stereospecific pharmacokinetics and pharmacodynamics of chiral flavanones. Of these investigations, only one reports the human urinary excretion of four different flavanones (liquiritigenin, naringenin, dihydrowogonin, and dihydrooroxylin A) after ingestion of different herbal products [62,73,74], unfortunately pharmacokinetics analysis and modeling were not employed.

It is important to consider that it has been reported that some chiral flavanones are stereochemically unstable depending on the substitution pattern of various functional groups around the stereogenic center. When inversion occurs causing the formation of a racemate it is termed racemization, while enantiomerization

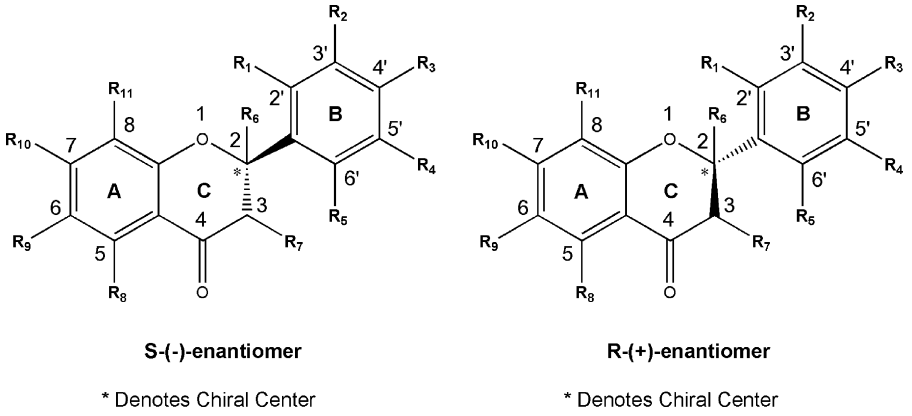


Fig. 1. Spatial disposition of the enantiomers of chiral flavanones.

is the reversible interconversion of enantiomers. For compounds with more than one stereogenic center, a process called epimerization occurs when there is a change of configuration at a single chiral center [75]. The racemization process, which is characterized by a process reaching equilibrium between the two enantiomers is facilitated by temperature, moisture, solvent, pH, among other factors [76]. In addition, flavanones with a free hydroxyl group in the position 4′ (equivalent to R_3 in Fig. 1) (i.e. naringenin and eriodictyol) racemize easier than flavanones with a methoxy group on that position (i.e. hesperetin and isosakuranetin) [77]. Therefore, non-stereospecific assay methods cannot interpret the time-course development of an individual enantiomer and the results of using achiral assays could be misleading in determining concentration dependence of each enantiomer of a racemic flavonoid xenobiotic in terms of efficacy or toxicity.

To our knowledge there are no studies that have examined the pharmacokinetics, anti-cancer, or anti-inflammatory activity of the individual enantiomers of chiral flavanones. However, there is one report where the *S* and *R* enantiomers of naringenin were studied for the inhibition of cyclosporine A oxidase activity in human liver microsomes, which is a cytochrome P450 3A4-dependent activity. Interestingly, no enantioselectivity or significant inhibitory activity were demonstrated for either (*R*)- or (*S*)-naringenin or a mixture of epimers of naringin [58].

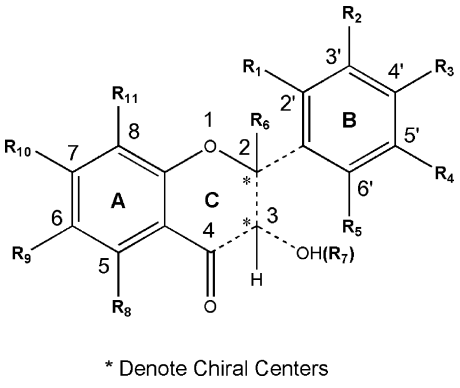


Fig. 2. Chemical structure of the chiral 3-hydroxyflavanones or dihydroflavonols.

Importantly, it should be recognized that other classes of flavonoids including isoflavonoids can also demonstrate chirality in some of their members. Legumes are a rich source of isoflavones that may have pharmacological properties. The isoflavone reductase enzyme reduces achiral isoflavones to chiral isoflavones during the biosynthesis of chiral pterocarpan phytoalexins. Red clover for instance synthesizes (−)-maackiain, garden pea synthesizes predominantly (+)-pisatin, and alfalfa (−)-medicarpin [78,79]. The soy isoflavonoids daidzein and the red clover isoflavonoid formentin are stereospecifically converted to the chroman metabolite *S*-(−)-equol by microbial flora of the gastrointestinal tract [80–82]. In addition, daidzein and genestein are both reduced to racemic (+/−) dihydrodaidzein and (+/−) dihydrogenestein, respectively [83]. In addition, 2-hydroxyformononetin is reduced to *R* and *S* vestitone and subsequently to (+)-medicarpin in peanut and (−)-medicarpin in alfalfa through pterocarpan synthase which can differ between plant varieties [84]. Furthermore, due to their possible therapeutic uses scientists and pharmaceutical companies are now employing flavanones as potential lead compounds and synthesizing a variety of derivatives, such as chiral dihydrofuroflavones [85].

Thus, there is a need for stereospecific assay methods for the quantitation and effective isolation of pure flavonoid enantiomers for their pharmacometric study in *in vivo* and *in vitro* models. This stereospecific analytical methodology would provide valuable information to stereospecifically understand how these xenobiotics are metabolized in plant, human, and animal models and to be able to better understand their disposition, pharmacological activity, as well as therapeutic and toxic effects.

2. Chromatographic methods of separation of enantiomers

The separation, resolution, and analysis of enantiomers have generally been accomplished through the formation of diastereoisomers either transiently or covalently. Diastereoisomers have different physicochemical properties in an achiral environment and thus they can be separated on an achiral

Chiral Flavanone	Other name(s)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}
		2'	3'	4'	5'	6'	2	3	5	6	7	8
Aervanone	Liquiritigenin-8-galactose	H	H	OH	H	H	H	H	H	H	OH	galactose
Alpinetin	7-hydroxy-5-methoxyflavanone, Pinocembrin 5-methylether	H	H	H	H	H	H	H	OCH_3	H	OH	H
Arjunone	5,7,2',4'-tetramethoxyflavanone	OCH_3	H	OCH_3	H	H	H	H	OCH_3	H	OCH_3	H
Artocarpanone	5,2',4'-trihydroxy-7-methoxyflavanone	OH	H	OH	H	H	H	H	OH	H	OCH_3	H
Carthamidin	4',5,6,7-tetrahydoxyflavanone	H	H	OH	H	H	H	H	OH	OH	OH	H
Cryptostrobin	5,7-dihydroxy-8-methylflavanone	H	H	H	H	H	H	H	OH	H	OH	CH_3
Cyrtominetin	5,7,3',4'-tetrahydroxy-6,8-dimethylflavanone	H	OH	OH	H	H	H	H	OH	CH_3	OH	CH_3
Cyrtopterinetin	6,8-dimethyl-4',5,7-trihydroxyflavanone	H	H	OH	H	H	H	H	OH	CH_3	OH	CH_3
Desmethoxymatteucinol	5,7-dihydroxy-6,8-dimethylflavanone	H	H	H	H	H	H	H	OH	CH_3	OCH_3	CH_3
Desmethoxymatteucinol-7-methylether	5-hydroxy-7-methoxy-6,8-dimethylflavanone	H	H	H	H	H	H	H	OH	CH_3	OH	CH_3
Desmethoxymatteucinol-7-O-glucose	5-hydroxy-6,8-dimethylflavanone-7-O-glucose	H	H	H	H	H	H	H	OH	CH_3	O-glucose	CH_3
Didymin	Isosakuranetin-7-O-rutinoside, Neoponcirin	H	H	OCH_3	H	H	H	H	OH	H	O-rutinose	H
Didymocarpin	7-hydroxy-5,6,8-trimethoxyflavanone	H	H	H	H	H	H	H	OCH_3	OCH_3	OH	OCH_3
Dihydrooroxylin A	5,7-dihydroxy-6-methoxyflavanone	H	H	H	H	H	H	H	OH	OCH_3	OH	H
Dihydrotectochrysin	5-hydroxy-7-methoxyflavanone	H	H	H	H	H	H	H	OH	H	OCH_3	H
Dihydrowogonin	5,7-dihydroxy-8-methoxyflavanone	H	H	H	H	H	H	H	OH	H	OH	OCH_3
Dihydroxyflavanone	5,4'-dihydroxy-6,7-dimethoxyflavanone	H	H	OH	H	H	H	H	OH	OCH_3	OCH_3	H
Dihydroxyflavanone	5,4'-dihydroxy-7,8-dimethoxyflavanone	H	H	OH	H	H	H	H	OH	H	OCH_3	OCH_3
Dihydroxyflavanone	5,4'-dihydroxy-7,3'-dimethoxyflavanone	H	OCH_3	OH	H	H	H	H	OH	H	OCH_3	H
Dimethoxyflavanone	7,8-dimethoxyflavanone	H	H	H	H	H	H	H	H	H	OH	OH
Dimethoxyflavanone	5-hydroxy-6,7-dimethoxyflavanone	H	H	H	H	H	H	H	OH	OCH_3	OCH_3	H
Dimethylpinocembrin	5,7-dimethoxyflavanone	H	H	H	H	H	H	H	OCH_3	H	OCH_3	H
Eriocitrin	Eriodictyol-7-O-rutinoside	H	H	OH	OH	H	H	H	OH	H	O-rutinose	H
Eriodictyol	5,7,3',4'-tetrahydroxyflavanone	H	OH	OH	H	H	H	H	OH	H	OH	H
Eriodictyol-3',4'-dimethly ether-5-O-glucose	7-hydroxy-3',4'-dimethoxyflavanone-5-O-glucose	H	OCH_3	OCH_3	H	H	H	H	O-glucose	H	OH	H
Eriodictyol-5-O-rhamnose	7,3',4'-trihydroxyflavanone-5-O-rhamnose	H	OH	OH	H	H	H	H	O-rhamnose	H	OH	H
Eriodictyol-7-O-glucose	5,3',4'-trihydroxyflavanone-7-O-glucose	H	OH	OH	H	H	H	H	OH	H	O-glucose	H
Eriodictyol-7-O-rhamnose	5,3',4'-trihydroxyflavanone-7-O-rhamnose	H	OH	OH	H	H	H	H	OH	H	O-rhamnose	H
Eriodictyol-7-O-rhamnosylglucose	5,3',4'-trihydroxyflavanone-7-O-rhamnosylglucose	H	OH	OH	H	H	H	H	OH	H	O-rhamnosylglucose	H
Eriodictyol-7,3',4'-trimethyl ether	5-hydroxy-7,3',4'-trimethoxyflavanone	H	OCH_3	OCH_3	H	H	H	H	OH	H	OCH_3	H
Eriodictyonone	S-(-)-Homoeriodictyol	H	H	OH	OCH_3	H	H	H	OH	H	OH	H
Farrerol	5,7,4'-trihydroxy-8,8-dimethylflavanone	H	H	H	H	H	H	H	OH	CH_3	OH	CH_3
Farrerol Mono-glucoside	5,4'-dihydroxy-8,8-dimethylflavanone-7-O-glucose	H	H	H	H	H	H	H	OH	CH_3	O-glucose	CH_3
Farrerol Di-glucoside	4'-hydroxy-6,8-dimethylflavanone-5,7-di-O-glucose	H	H	H	H	H	H	H	O-glucose	CH_3	OH	CH_3
Flavanomarein	8,3',4'-trihydroxyflavanone-7-O-glucoside, Isookanin-7-O-glucoside	H	OH	OH	H	H	H	H	H	H	O-glucose	OH
Flavanone	2,3-dihydroflavone	H	H	H	H	H	H	H	H	H	H	H
Fortunellin	Acacetin-7-O-neohesperidose	H	H	OCH_3	H	H	H	H	OH	H	O-neohesperidose	H
Haplanthin	5,2'-dihydroxy-7-methoxyflavanone-6'-O-glucose	OH	H	H	H	O-glucose	H	H	OH	H	OCH_3	H
Hesperetin	5,7,3'-trihydroxy-4'-methoxyflavanone	H	OH	OCH_3	H	H	H	H	OH	H	OH	H
Hesperetin-5-O-glucose	7,3'-trihydroxy-4'-methoxyflavanone-5-O-glucose	H	OH	OCH_3	H	H	H	H	O-glucose	H	OH	H
Hesperetin-7-O-rhamnose	5,3'-trihydroxy-4'-methoxyflavanone-7-O-rhamnose	H	OH	OCH_3	H	H	H	H	OH	H	O-rhamnose	H
Hesperidin	Hesperetin-7-O-rutinose	H	H	OCH_3	OH	H	H	H	OH	H	O-rutinose	H
Homoeriodictyol	5,7,4'-trihydroxy-3'-methoxyflavanone	H	H	OH	OCH_3	H	H	H	OH	H	OH	H
Hydroxyflavanone, 2'	2'-hydroxyflavanone	OH	H	H	H	H	H	H	H	H	H	H
Hydroxyflavanone, 3'	3'-hydroxyflavanone	H	OH	H	H	H	H	H	H	H	H	H
Hydroxyflavanone, 4'	4'-hydroxyflavanone	H	H	OH	H	H	H	H	H	H	H	H
Hydroxyflavanone, 5	5-hydroxyflavanone	H	H	H	H	H	H	H	OH	H	H	H
Hydroxyflavanone, 6	6-hydroxyflavanone	H	H	H	H	H	H	H	H	OH	H	H
Hydroxyflavanone, 7	7-hydroxyflavanone	H	H	H	H	H	H	H	H	H	OH	H
Isokanin	7,8,3',4'-tetrahydroxyflavanone	H	OH	OH	H	H	H	H	H	H	OH	OH
Isokanin-7-O-glucose	8,3',4'-trihydroxyflavanone-7-O-glucose	H	OH	OH	H	H	H	H	H	H	O-glucose	OH
Isosakuranetin	5,7-dihydroxy-4'-methoxyflavanone, Acacetin, Linarigenin, Naringenin-4'-methyl ether	H	H	OCH_3	H	H	H	H	OH	H	OH	H
Isosakuranetin-5-O-glucose	7-hydroxy-4'-methoxyflavanone-5-O-glucose	H	H	OCH_3	H	H	H	H	O-glucose	H	OH	H
Kanakugin	5,6,7,8-tetramethoxyflavanone	H	H	H	H	H	H	H	OCH_3	OCH_3	OCH_3	OCH_3
Lawinal	5,7-dihydroxy-6-formyl-8-methylflavanone, 6-formyl-8-methylpinocembrin	H	H	H	H	H	H	H	OH	CHO	OH	CH_3
Liquiritigenin	7,4'-dihydroxyflavanone	H	H	OH	H	H	H	H	H	H	OH	H
Liquiritin	Liquiritigenin-4'-O-glucoside	H	H	O-glucose	H	H	H	H	H	H	OH	H
Matteucinol	5,7-dihydroxy-6,8-dimethyl-4'-methoxyflavanone, 4'-methylfarrerol	H	H	OCH_3	H	H	H	H	OH	CH_3	OH	CH_3
Matteucinol-7-O-glucose	5-hydroxy-6,8-dimethyl-4'-methoxyflavanone-7-O-glucose, 4'-methylfarrerol-7-O-glucose	H	H	OCH_3	H	H	H	H	OH	CH_3	O-glucose	CH_3
Methoxyflavanone, 4'	4'-methoxyflavanone	H	H	OCH_3	H	H	H	H	H	H	H	H
Methoxyflavanone, 5	5-methoxyflavanone	H	H	H	H	H	H	H	OCH_3	H	H	H
Methoxyflavanone, 6	6-methoxyflavanone	H	H	H	H	H	H	H	H	OCH_3	H	H
Methoxyflavanone, 7	7-methoxyflavanone, 7-hydroxyflavanone methylether	H	H	H	H	H	H	H	H	H	OCH_3	H
Naringenin	5,7,4'-trihydroxyflavanone	H	H	OH	H	H	H	H	OH	H	OH	H
Naringenin-4'-O-galactose	5,7-dihydroxyflavanone-4'-O-galactose	H	H	O-galactose	H	H	H	H	OH	H	OH	H
Naringenin-4'-O-rutinose	5,7-dihydroxyflavanone-4'-O-rutinose	H	H	O-rutinose	H	H	H	H	OH	H	OH	H
Naringenin-4'-O-xylosylglucose	5,7-dihydroxyflavanone-4'-O-xylosylglucose	H	H	O-xylosylglucose	H	H	H	H	OH	H	OH	H
Naringenin-5-O-methyl	7,4'-dihydroxy-5-O-methylflavanone, 5-O-methylnaringenin	H	H	OH	H	H	H	H	OCH_3	H	OH	H
Naringenin-5-O-glucose	7,4'-dihydroxyflavanone-5-O-glucose	H	H	OH	H	H	H	H	O-glucose	H	OH	H
Naringenin-5,7-dimethyl ether	4'-hydroxy-5,7-dimethylflavanone, 5,7-dimethylnaringenin	H	H	OH	H	H	H	H	OCH_3	H	OCH_3	H
Naringenin-6"-galloyl-7-O-glucose	5,4'-dihydroxy-6"-galloylflavanone-7-O-glucose	H	H	OH	H	galloyl	H	H	OH	H	O-glucose	H
Naringenin-6"-O-p-coumaroyl-7-O-glucose	5,4'-dihydroxy-6"-p-coumaroyl-7-O-glucose	H	H	OH	H	O-p-coumaroyl	H	H	OH	H	O-glucose	H
Naringenin-6-glucose	5,4'-dihydroxyflavanone-6-glucose	H	H	OH	H	H	H	H	OH	glucose	OH	H
Naringenin-7-O-fructose	5,4'-dihydroxyflavanone-7-O-fructose	H	H	OH	H	H	H	H	OH	H	O-fructose	H
Naringenin-7-O-glucose	5,4'-dihydroxyflavanone-7-O-glucose	H	H	OH	H	H	H	H	OH	H	O-glucose	H
Naringenin-7-O-rhamnose	5,4'-dihydroxyflavanone-7-O-rhamnose	H	H	OH	H	H	H	H	OH	H	O-rhamnose	H
Naringenin-7,4'-dimethyl ether	5-hydroxy-7,4'-dimethylflavanone, 7,4'-dimethylnaringenin	H	H	OCH_3	H	H	H	H	OH	H	OCH_3	H
Naringenin trimethyl ether	5,7,4'-trimethylflavanone, 5,7,4'-trimethylnaringenin	H	H	OCH_3	H	H	H	H	OCH_3	H	OCH_3	H
Naringin	Naringenin-7-O-neohesperidose	H	H	OH	H	H	H	H	OH	H	O-neohesperidose	H
Narirutin	Naringenin-7-O-rutinoside, Isonaringenin	H	H	OH	H	H	H	H	OH	H	O-rutinose	H
Neoeriocitrin	Eriodictyol-7-O-neohesperidose	H	H	OH	OH	H	H	H	OH	H	O-neohesperidose	H
Neohesperidin	Hesperetin-7-O-neohesperidose	H	OH	OCH_3	H	H	H	H	OH	H	O-neohesperidose	H
Pentamethoxyflavanone	6,7,3',4',5'-pentamethoxyflavanone-5-O-rhamnose	H	OCH_3	OCH_3	OCH_3	H	H	H	O-rhamnose	OCH_3	OCH_3	H
Persicogenin	5,3'-dihydroxy-7,4'-dimethoxyflavanone	H	OH	OCH_3	H	H	H	H	OH	H	OCH_3	H
Persicogenin-5-O-glucose	3'-hydroxy-7,4'-dimethoxyflavanone-5-O-glucose	H	OH	OCH_3	H	H	H	H	O-glucose	H	OCH_3	H
Pinocembrin	5,7-dihydroxyflavanone, Dihydrochrysin	H	H	H	H	H	H	H	OH	H	OH	H
Pinocembrin-7-methylether	5-hydroxy-7-methoxyflavanone	H	H	H	H	H	H	H	OH	H	OCH_3	H
Pinocembrin-7-O-neohesperidose	5-hydroxyflavanone-7-O-neohesperidose	H	H	H	H	H	H	H	OH	H	O-neohesperidose	H
Pinocembrin-7-O-rhamnose	5-hydroxyflavanone-7-O-rhamnose	H	H	H	H	H	H	H	OH	H	O-rhamnose	H
Pinocembrin dimethylester	5-hydroxy-6,7-dimethoxyflavanone	H	H	H	H	H	H	H	OH	OCH_3	OCH_3	H
Pinostrobin	5-hydroxy-7-methoxyflavanone, pinocembrin-7-methylether	H	H	H	H	H	H	H	OH	H	OCH_3	H
Poncirin	Isosakuranetin-7-O-neohesperidose	H	H	OCH_3	H	H	H	H	OH	H	O-neohesperidose	H
Prunin	Naringenin-7-O-glucose	H	H	OH	H	H	H	H	OH	H	O-glucose	H
Sakuranetin	5,4'-dihydroxy-7-methoxyflavanone, Naringenin-7-methyl ether	H	H	OH	H	H	H	H	OH	H	OCH_3	H
Salipurposide	S(-)-Naringenin-5-O-glucose	H	H	OH	H	H	H	H	O-glucose	H	OH	H
Strobopinin	5,7-dihydroxy-6-methylflavanone	H	H	H	H	H	H	H	OH	CH_3	OH	H
Strobopinin-7-methylether	5-hydroxy-6-methyl-7-methoxyflavanone	H	H	H	H	H	H	H	OH	CH_3	OCH_3	H
Tetrahydroxyflavanone	2,7,3',4'-tetrahydroxyflavanone-5-O-glucose	H	OH	OH	H	H	OH	H	H	H	OH	H
Tetramethoxyflavanone	5-hydroxy-6,7,8,4'-tetramethoxyflavanone	H	H	OCH_3	H	H	H	H	OH	OCH_3	OCH_3	OCH_3
Tetramethoxyflavanone	5-hydroxy-7,3',4',5'-tetramethoxyflavanone	H	OCH_3	OCH_3	OCH_3	H	H	H	OH	H	OCH_3	H
Tetrahydroxyflavanone	5,7,2',5'-tetrahydroxyflavanone	H	OH	H	OH	H	H	H	OH	H	OH	H
Trihydroxyflavanone	5,7,2-trihidroxy-6-methyl-8-formylflavanone	H	H	H	H	H	OH	OH	CH_3	CH_3	OH	CHO
Trihydroxyflavanone	7,3',4'-trihidroxyflavanone	H	OH	OH	H	H	H	H	H	H	OH	H
Trimethoxyflavanone	4'-hydroxy-5,6,7-trimethoxyflavanone	H	H	OH	H	H	H	H	OCH_3	OCH_3	OCH_3	H
Trimethoxyflavanone	5-hydroxy-6,7,4'-trimethoxyflavanone	H	H	OCH_3	H	H	H	H	OH	OCH_3	OCH_3	H
Trimethoxyflavanone	5-hydroxy-7,8,4'-trimethoxyflavanone	H	H	OCH_3	H	H	H	H	OH	H	OCH_3	OCH_3
Trimethoxyflavanone	5,4'-dihydroxy-6,7,8-trimethoxyflavanone	H	H	OH	H	H	H	H	OH	OCH_3	OCH_3	OCH_3
Trimethoxyflavanone	5,6-dihydroxy-7,8,4'-trimethoxyflavanone	H	H	OCH_3	H	H	H	H	OH	OH	OCH_3	OCH_3
Trimethoxyflavanone	5,7,8-trimethoxyflavanone	H	H	H	H	H	H	H	OCH_3	H	OCH_3	OCH_3
Trimethoxyflavanone	5,7-dihydroxy-3',4',5'-trimethoxyflavanone	H	OCH3	OCH3	OCH3	H	H	H	OH	H	OH	H
Trimethoxyflavanone	7-hydroxy-5,2',4'-trimethoxyflavanone	OCH_3	H	OCH_3	H	H	H	H	OCH_3	H	OH	H

Fig. 3. Comprehensive list of naturally occurring chiral flavanones.

chromatographic column through differential interaction and retention. Racemic flavonoid resolution has generally been accomplished by chromatographic enantiospecific resolution through temporary formation of diastereoisomers on a chemically bonded chiral stationary phase (CSP) with an achiral mobile phase.

2.1. Direct methods of analysis: chiral stationary phases (CSP)

A number of different CSPs have been utilized to resolve and separately quantify the enantiomers of chiral flavonoids including: chiral polymer phases. These chiral polymer phases can be further sub-divided into polysaccharide-derived columns, and cyclodextrin and "mixed" cyclodextrin columns

2.1.1. Chiral polymer phases

2.1.1.1. Polysaccharide-derived columns. A variety of chiral columns employing synthetic polysaccharides particularly D-cellulose esters to which a variety of terminal groups are attached have been employed. Resolution of flavanone enantiomers by HPLC utilizing polysaccharide derivatives, such as cellulose trans-tris (4-phenylazaphenylcarbmate) columns was first established in 1980's [72]. This was followed by separation on cellulose tris (3,5-dimethylphenylcarbamate) columns [86,87]. Unsubstituted flavanone can be easily

Chiral 3-Hydroxyflavanone or Dihydroxyflavonol	Other name(s)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}
		2′	3′	4′	5′	6′	2	3	5	6	7	8
Ampelopsin	3,5,7,3′,4′,5′-hexahydroxyflavanone, Dihydromyricetin	H	OH	OH	OH	H	H	OH	OH	H	OH	H
Aromadendrin	3,5,7,4′-tetrahydroxyflavanone, 2,3-dihydrokaempferol	H	H	OH	H	H	H	OH	OH	H	OH	H
Aromadendrin-3-O-glucose	5,7,4′-trihydroxyflavanone-3-O-glucose	H	H	OH	H	H	H	O-glucose	OH	H	OH	H
Aromadendrin-4′-methyl ether	3,5,7,-trihydroxy-4′-methoxyflavanone	H	H	OCH_3	H	H	H	OH	OH	H	OH	H
Aromadendrin-4′-O-xylose	3,5,7-trihydroxyflavanone-4′-O-glucose	H	H	O-glucose	H	H	H	OH	OH	H	OH	H
Aromadendrin-7-O-glucose	3,5,4′-trihydroxyflavanone-7-O-glucose	H	H	OH	H	H	H	OH	OH	H	O-glucose	H
Aromadendrin-7,4′-dimethyl ether	3,5-dihydroxy-7,4′-methoxyflavanone	H	H	OCH_3	H	H	H	OH	OH	H	OCH_3	H
Aromadendrin-7,4′-dimethyl ether-5-O-glucose	3-hydroxy-7,4′-methoxyflavanone-5-O-glucose	H	H	OCH_3	H	H	H	OH	O-glucose	H	OCH_3	H
Astilbin	5,7,3′,4′-tetrahydroxyflavanone-3-O-rhamnose, Taxifolin-3-O-rhamnose	H	OH	OH	H	H	H	O-rhamnose	OH	H	OH	H
Cedeodarin	3,5,7,3′,4′-pentahydroxy-6-methylflavanone	H	OH	OH	H	H	H	OH	OH	CH_3	OH	H
Cedrin	3,5,7,3′,4′,5′-hexahydroxy-6-methylflavanone, 6-methyldihydromyricetin	H	OH	OH	OH	H	H	OH	OH	CH_3	OH	H
Cedrinoside	3,5,7,4′,5′-pentahydroxy-6-methylflavanone-3′-O-glucose, Cedrin-3′-O-glucose	H	O-glucose	OH	OH	H	H	OH	OH	CH_3	OH	H
Dihydrokaempferide	3,5,7-trihydroxy-4′-methoxyflavanone	H	H	OCH_3	H	H	H	OH	OH	H	OH	H
Dihydromyricetin-5′-methyl ether-4′-O-rhamnose	3,5,7,3′-tetrahydroxy-5′-methylflavanone-4′-O-rhamnose	H	OH	O-rhamnose	OCH_3	H	H	OH	OH	H	OH	H
Dihydrorobinetin	3,7,3′,4′,5′-pentahydroxyflavanone	H	OH	OH	OH	H	H	OH	H	H	OH	H
Dihydromorin	3,5,7,2′,4′-pentahydroxyflavanone	OH	H	OH	H	H	H	OH	OH	H	OH	H
Engelitin	5,7,4′-trihydroxyflavanone-3-O-rhamnose	H	H	OH	H	H	H	O-rhamnose	OH	H	OH	H
Fustin	3,7,3′,4′-tetrahydroxyflavanone, 2,3-dihydrofisetin	H	OH	OH	H	H	H	OH	H	H	OH	H
Fustin-3-O-methyl	7,3′,4′-trihydroxy-3-O-methylflavanone	H	OH	OH	H	H	H	OCH_3	H	H	OH	H
Fustin-8-hydroxy	3,7,8,3′,4′-pentahydroxyflavanone, 8-hydroxyfustin	H	OH	OH	H	H	H	OH	H	H	OH	OH
Garbanzol	3,7,4′-dihydroxyflavanone	H	H	OH	H	H	H	OH	H	H	OH	H
Pinobanksin	3,5,7-trihydroxyflavanone	H	H	H	H	H	H	OH	OH	H	OH	H
Pinobanksin dimethyl ether	3-hydroxy-5,7-dimethoxyflavanone	H	H	H	H	H	H	OH	OCH_3	H	OCH_3	H
Sepinol	3,7,3′,5′-tetrahydroxy-4′-methoxyflavanone, Dihydrorobinetin-4′-methyl ether	H	OH	OCH_3	OH	H	H	OH	H	H	OH	H
Taxifolin	3,5,7,3′,4′-pentahydroxyflavanone, 2,3-dihydroquercetin	H	OH	OH	H	H	H	OH	OH	H	OH	H
Taxifolin-3-O-galactose	5,7,3′,4′-tetrahydroxyflavanone-3-O-galactose	H	OH	OH	H	H	H	O-galactose	OH	H	OH	H
Taxifolin-3-O-glucose	5,7,3′,4′-tetrahydroxyflavanone-3-O-glucose	H	OH	OH	H	H	H	O-glucose	OH	H	OH	H
Taxifolin-3-O-methyl	5,7,3′,4′-tetrahydroxy-3-methylflavanone, 3-O-methyltaxifolin	H	OH	OH	H	H	H	OCH_3	OH	H	OH	H
Taxifolin-3,5-di-O-rhamnose	7,3′,4′-trihydroxyflavanone-3,5-di-O-rhamnose	H	OH	OH	H	H	H	O-rhamnose	O-rhamnose	H	OH	H
Taxifolin-3′-O-glucose	3,5,7,4′-tetrahydroxyflavanone-3′-O-glucose	H	O-glucose	OH	H	H	H	OH	OH	H	OH	H
Taxifolin-7-O-galactose	3,5,3′,4′-tetrahydroxyflavanone-7-O-galactose	H	OH	OH	H	H	H	OH	OH	H	O-galactose	H
Taxifoliol	(2R,3R)-taxifolin, Distylin	H	OH	OH	H	H	H	OH	OH	H	OH	H
Tetrahydroxyflavanone	3,5,7,2′-tetrahydroxy-5′-methoxyflavanone	OH	H	H	OCH_3	H	H	OH	OH	H	OH	H
Trihydroxyflavanone	3,5,4′-trihydroxy-6,7-methoxyflavanone	H	H	OH	H	H	H	OH	OH	OCH_3	OCH_3	H

Fig. 4. Comprehensive list of naturally occurring chiral 3-hydroxyflavanones or dihydroflavonols.

separated on cellulose mono and disubstituted carbamates including cellulose-4-substituted triphenylcarbamate derivatives, cellulose chloro-substituted triphenyl carbamate, and cellulose methyl-substituted triphenylcarbamate supported in silica gel [71]. Hesperetin has been successfully separated in a validated reverse phase HPLC method and a commercially available Chiralpak AD-RH tris (3,5-dimethylphenylcarbmate) derivative of amylose column [88].

The chiral recognition of microcrystalline triacetate may involve inclusion complexation. Three commercially available columns of microcrystalline cellulose triacetate were able to resolve several flavanones including naringenin, hesperetin, eriodictyol, homoeriodictyol, pinocembrine, and isosakuranetin [66]. For instance, Chiralcel OA is a commercially available cellulose triacetate column coated on macroporous silica gel [67]. The seminal work on separation of some racemic flavanones was accomplished on microcrystalline cellulose triacetate supported on non-macroporous silica gel diol [67]. This CSP employed in normal (apolar) phase using gradient elution was found to be superior to a commercially available cellulose triacetate columns for separation of polyhydroxylated flavanones particularly the 5,7-dihydroxy substituted on ring A (i.e. pinocembrine, isosakuranetin, naringenin, eriodictyol, homoeriodictyol, and hesperetin). Normal phase chromatography was far superior to reverse (polar) elution to separate flavanone enantiomers. In addition, flavanone glycosides could also be resolved together with their aglycones and this was applied to analysis of naringenin enantiomers in tomato skin [67]. The performance of Chiralcel OA also indicated that 5- and 7-methoxyflavanone could be resolved as well as naringenin [65].

The Chiralcel OD column is a macroporous silica gel coated with cellulose tris (3,5-dimethylphenylcarbamate), which has demonstrated ability to separate a variety of flavanone derivatives including (i.e. flavanone [68,89], 4′- and 6-methoxyflavanone [68,89], 5-methoxyflavanone; 2′- or 6-hydroxyflavanononе; pinostrobin [68]; and 7-methoxyflavanone [65]). A study administered the Chinese herbal medicines Daisiko-to and Shosaiko-to to human subjects and analyzed the urine post-administration, resolving several polysubstituted flavanones including liquiritigenin, naringenin, dihydrowogonin, and dihydrooroxylin A [62,73,74]. Chiralcel OD can also separate and resolve naringin epimers during grapefruit maturation [12]. The Chiralcel OD-RH (tris-3,5-dimethylphenylcarbamate) column has demonstrated the ability to resolve naringenin enantiomers in isocratic reverse phase in a validated assay in biological matrices [90]. Chiralcel OD in normal phase has been utilized for the direct separation of epimers of the glycosides narirutin, hesperidin, neohesperidin, and naringin [91], and the aglycones naringenin, hesperetin, eriodictyol, and pinocembrin [58]. The 2,3,4-tris-*O*-(3,5-dimethylphenylcarbamoyl) CSP demonstrated the ability to resolve flavanone [69]. The Chiralcel OC column (tris-phenylcarbamate) has been demonstrated to resolve flavanone as well as 4′-, 5-, and 6-methoxyflavanone and homoeriodictyol [65]. Furthermore, the Chiralcel OJ column (tris 4-methylphenyl-benzoate ester) can resolve flavanone, 4′-, 5-, and 6-methoxyflavanone, eriodictyol, and hesperetin [65].

In addition, chiral columns employing amylose esters, such as amylose tris (3,5-dimethylphenylcarbamate) and tris (3,5-dichlorophenylcarbamate) supported on silica gel have demonstrated the ability to resolve flavanone [71]. The amylose tris (3,5-dimethylphenylcarbmate) column Chiralpak IA has the advantage of being and immobilized chiral stationary phase instead of a silica gel supported stationary phase allowing to afford a wider range of solvents to be employed as the mobile phase. Furthermore, it has been shown to have the ability to resolve hesperidin, neohesperidin, narirutin, and naringin [91].

Chiralpak OP (+) is based on macroporous silica gel coated with poly(diphenyl-2-pyridylmethylmethacrylate). The separation of flavanone, 5-, 6-, and 4′-methoxyflavanone were achieved on this column [68]. ChiraSpher is a small-pore silica gel chiral polymer (poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester), with this the separation of flavanone, 2′-, 4′-, and 6-hydroxyflavanone, 4′-, 5-, and 6-methoxyflavanone, and pinostrobin have been described although naringenin and naringenin tribenzoate were not separated [68]. The use of a Chiralpak AS-H (tris (*S*)-1-phenylethylcarbamate) to separate naringenin, eriodictyol, hes-

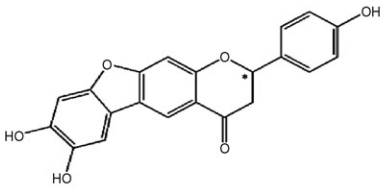
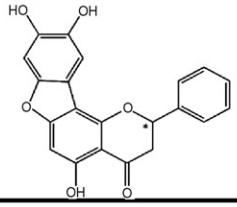
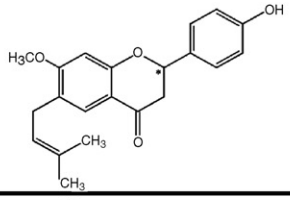
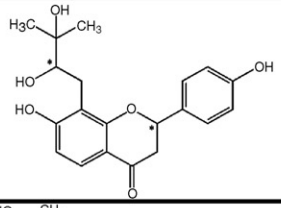
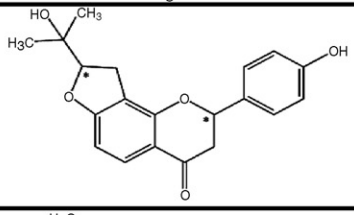
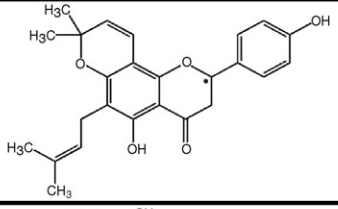
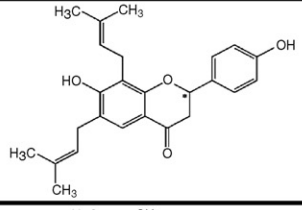
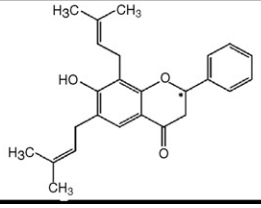
Chiral Flavanone	Other name(s)	Structure
Anastatin-A		
Anastatin-B		
Bavachinin-A	4'-hydroxy-7-methoxy-6-(3-methyl-2-butenyl)-flavanone, Bavachinin	
Brosimacutin-A	7,4'-dihydroxy-8-(2,3-dihydroxy-3-methylbutyl)flavanone	
Brosimacutin-E		
Cajaflavanone	5,4'-dihydroxy-6-prenyl-6',6'-dimethylpyrano-(2',3',7,8)-flavanone	
Diprenylflavanone	7,4'-dihydroxy-6,8-diprenylflavanone	
Diprenylflavanone	7-hydroxy-6,8-diprenylflavanone	

Fig. 5. Comprehensive list of chiral flavanones and 3-hydroxyflavanones having complex substituents.

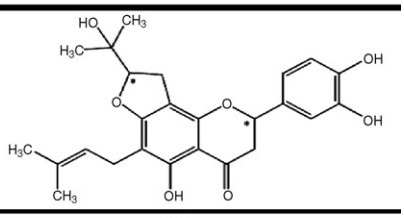
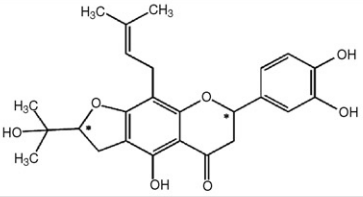
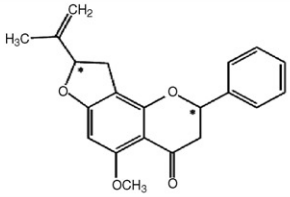
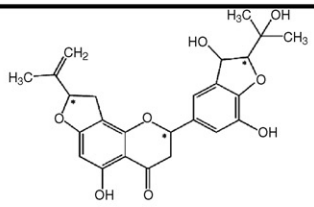
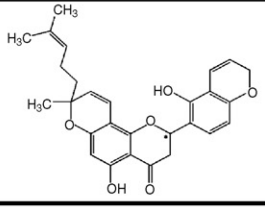
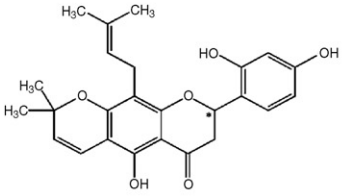
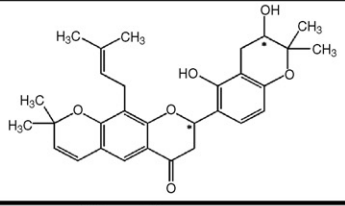
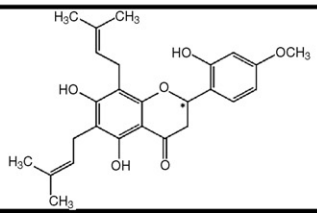
Chiral Flavanone	Other name(s)	Structure
Dorsmanin-F		
Dorsmanin-G		
Emoroidenone		
Epimedokoreanin-A		
Flemichin-A		
Flemichin-D	5,2',4'-trihydroxy-6'',6''-dimethylpyrano(2'',3'',7,6)-8-prenylflavanone	
Flemichin-E		
Flemiflavanone-A	5,7,2'-trihydroxy-4'-methoxy-6,8-dimethylflavanone	

Fig. 5. (*Continued*)

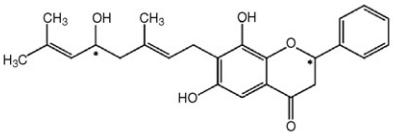
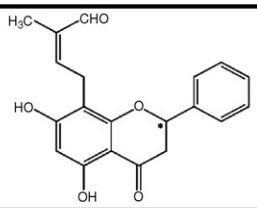
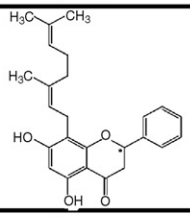
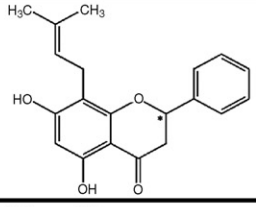
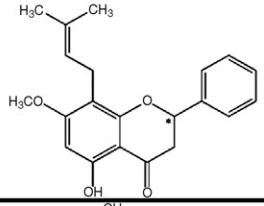
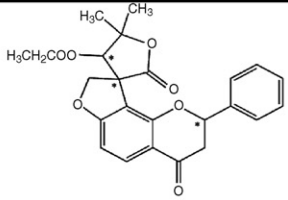
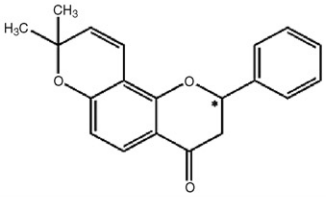
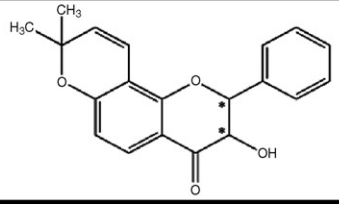
Chiral Flavanone	Other name(s)	Structure
Flemiflavanone-B	6,8-dihydroxy-7-(5-hydroxygeranyl)-flavanone	
Formylallylflavanone	5,7-dihydroxy-8-(β-methyl-β-hydroxy-formylallyl)-flavanone	
Geranylflavanone, 8	5,7-dihydroxy-8-geranylflavanone	
Glabranin	5,7-dihydroxy-8-prenylflavanone	
Glabranin, 7-methyl-	5-hydroxy-7-methoxy-8-prenylflavanone, 7-methyl-glabranin	
Glabatephrin		
Isolonchocarpin	6",6"-dimethyl-(2",3",7,8)-pyranoflavanone	
Isolonchocarpin, 3-hydroxy-	3-hydroxy-6",6"-dimethyl-(2",3",7,8)-pyranoflavanone	

Fig. 5. (*Continued*)

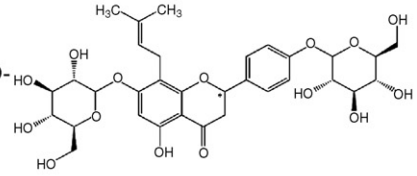
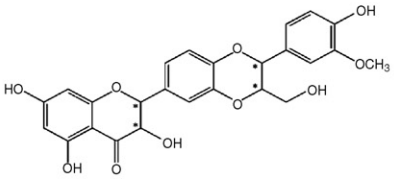
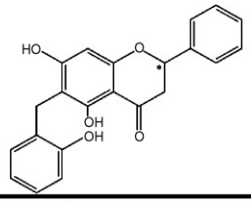
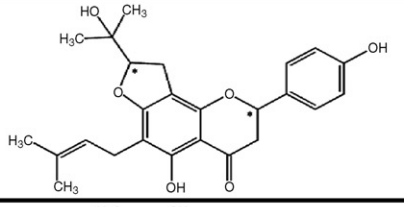
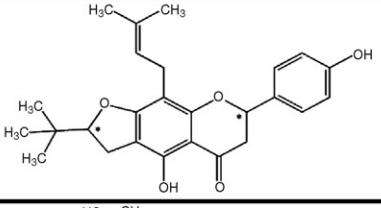
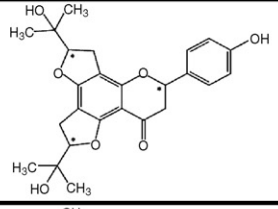
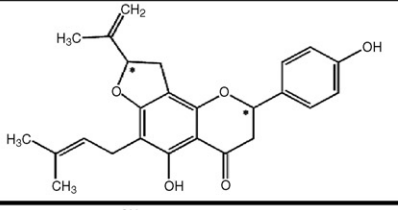
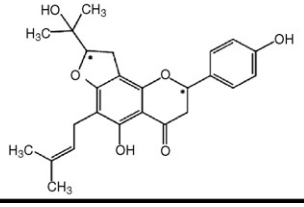
Chiral Flavanone	Other name(s)	Structure
Isopentylnaringenin-7,4'-diglucoside	5-hydroxy-8-C-prenylflavanone-4',7-di-O-glucose	
Isosilybin	2-(2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-1,4-benzodioxin-6-yl)-3,5,7-trihydroxyflavanone	
Isouvaretin	5,7-dihydroxy-6-(2-hydroxybenzyl)-flavanone	
Lonchocarpol-C		
Lonchocarpol-D		
Lonchocarpol-E		
Lupineol		
Lupiniol-A		

Fig. 5. (*Continued*)

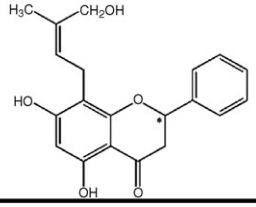
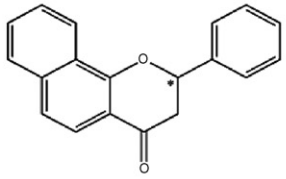
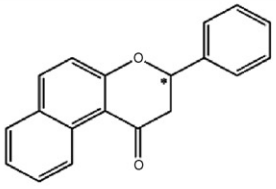
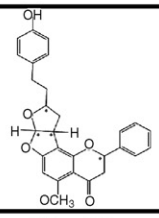
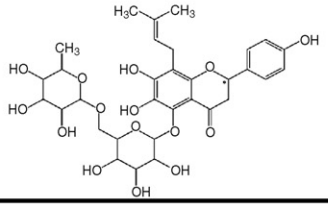
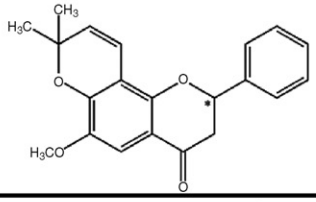
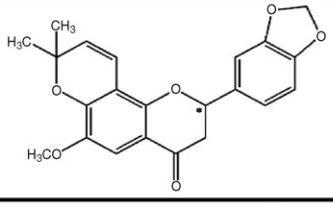
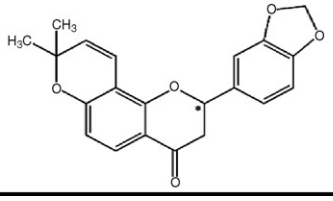
Chiral Flavanone	Other name(s)	Structure
Methylallylflavanone	5,7-dihydroxy-8-(β-methyl-β-hydroxy-methylallyl)-flavanone	
Naphthoflavanone, α	7,8-benzoflavanone, α-Naphthoflavanone	
Naphthoflavanone, β	5,6-benzoflavanone, β-Naphthoflavanone	
Neocalixin		
Nirurin	5,6,7,4'-Tetrahydroxy-8-(3-methyl-2-butenyl)flavanone-5-O-rutinoside	
Ovalichromene	6-methoxy-6",6"-dimethyl-(2",3",7,8)-pyranoflavanone	
Ovalichromene-A	6-methoxy-6",6"-dimethyl-(2",3",7,8)-pyrano-3',4'-methylenedioxyflavanone	
Ovalichromene-B	6",6"-dimethyl-(2",3",7,8)-pyrano-3',4'-methylenedioxyflavanone	

Fig. 5. (*Continued*)

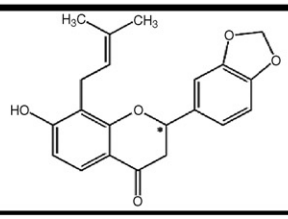
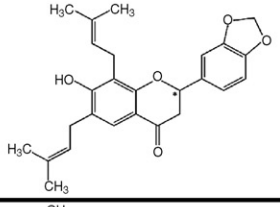
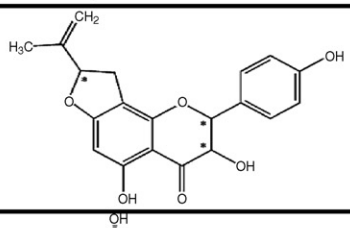
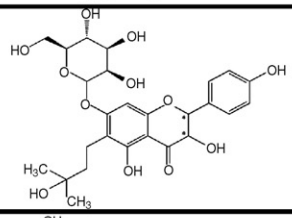
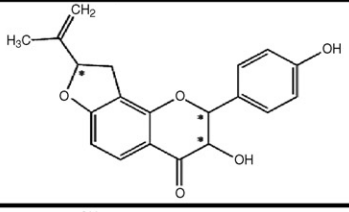
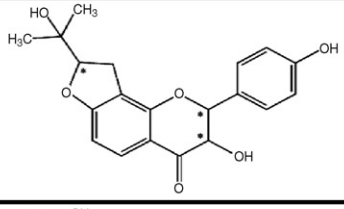
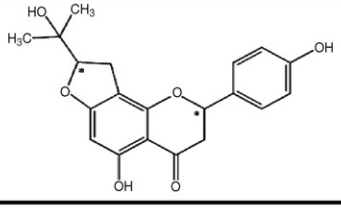
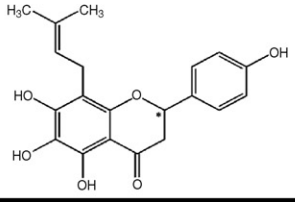
Chiral Flavanone	Other name(s)	Structure
Ovaliflavanone-C	7-hydroxy-8-prenyl-3',4'-methylenedioxyflavanone	
Ovaliflavanone-D	7-hydroxy-6,8-diprenyl-3',4'-methylenedioxyflavanone	
Phellamurin		
Phellavin	3,5,7,4'-tetrahydroxy-6-(3-hydroxy-emethyl-butyl)-flavanone-7-O-glucose	
Phellodensin-A		
Phellodensin-C		
Phellodensin-D		
Prenylnaringenin	5,7,4'-trihydroxy-8-C-prenylflavanone	

Fig. 5. (*Continued*)

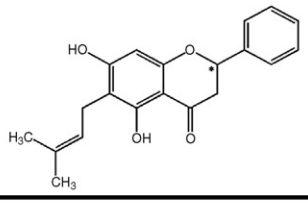
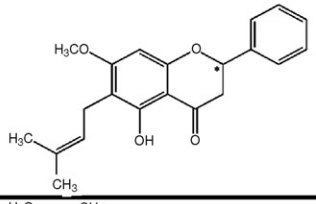
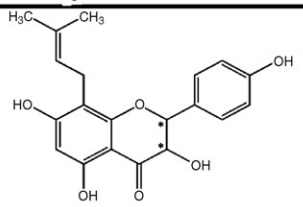
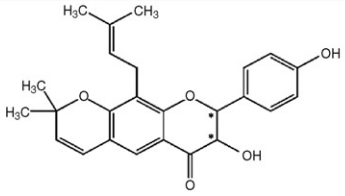
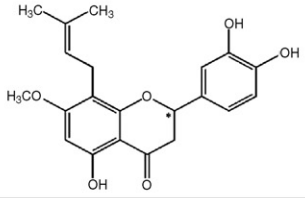
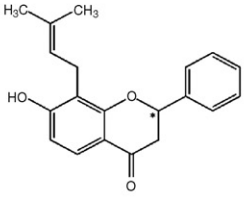
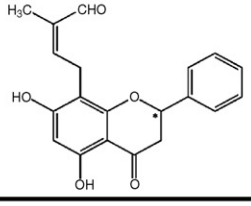
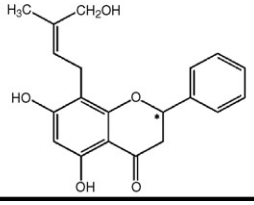
Chiral Flavanone	Other name(s)	Structure
Prenylpinocembrine, 6	5,7-dihydroxy-6-C-prenylflavanone, 6-prenylpinocembrine	
Prenylpinocembrine 7-methylether	5-hydroxy-7-methoxy-6-prenylflavanone, 6-prenylpinocembrine-7-methyl ether	
Prenylflavanone	3,5,7,4'-tetrahydroxy-8-prenylflavanone	
Prenylflavanone	3,5,4'-trihydroxy-6'',6''-dimethyl-(2'',3'',7,8)-pyrano-8-prenylflavanone	
Prenylflavanone	5,3',4'-trihydroxy-7-methoxy-8-prenylflavanone	
Prenylflavanone	7-hydroxy-8-prenylflavanone	
Prenylpinocembrine, 8	5,7-dihydroxy-8-C-(β-Methyl-β-formylallyl)-flavanone, 8-prenylpinocembrine	
Prenylpinocembrine, 8	5,7-dihydroxy-8-C-(β-Methyl-β-hydroxymethylallyl)-flavanone, 8-prenylpinocembrine	

Fig. 5. (*Continued*)

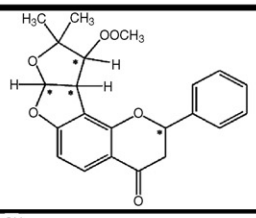
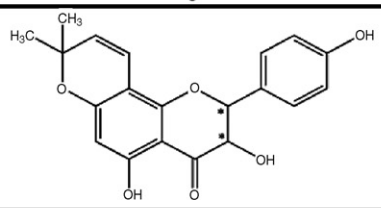
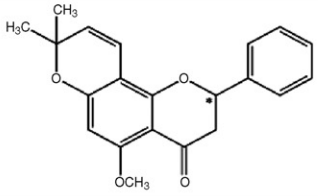
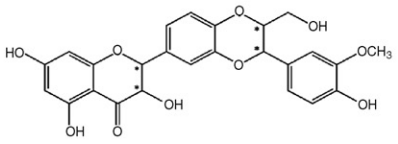
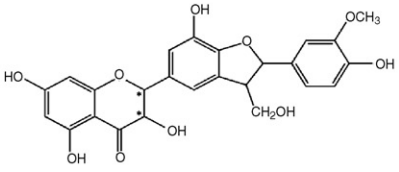
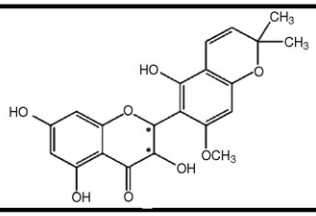
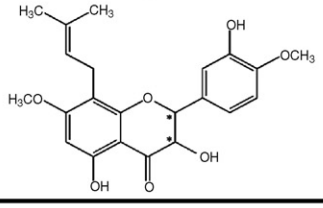
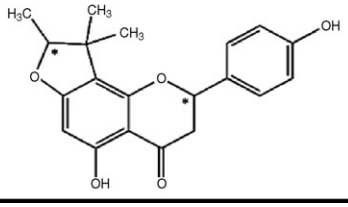
Chiral Flavanone	Other name(s)	Structure
Purpurin		
Pyranoflavanone	3,5,4'-trihydroxy-6'',6''-dimethyl-(2'',3'',7,8)-pyranoflavanone	
Pyranoflavanone	5-methoxy-6'',6''-dimethyl-(2'',3'',7,8)-pyranoflavanone	
Silybin	2-(2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl)-3,5,7-trihydroxyflavanone, Silibinin, Silymarin	
Silychristin		
Sophoronol	3,5,7-trihydroxy-2'-methoxy-6'',6''-dimethyl-(2'',3'',4',3')-pyranoflavanone	
Tirumalin	3,5,3'-trihydroxy-7,4'-dimethoxy-8-prenylflavanone	
Ugonin-D		

Fig. 5. (*Continued*)

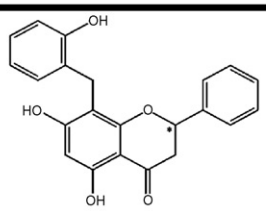
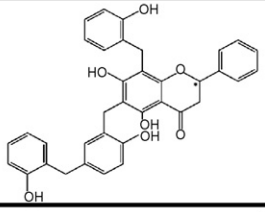
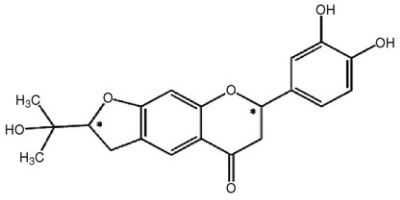
Chiral Flavanone	Other name(s)	Structure
Uvaretin	5,7-dihydroxy-8-(2-hydroxybenzyl)-flavanone	
Uvarinol		
Velloeriodictyol		

Fig. 5. (*Continued*).

peretin, and pinocembrine has recently been reported [58]. Furthermore, a Chiralcel AD column has also been reported to separate naringenin [58].

2.1.1.2. Cyclodextrin and "mixed" cyclodextrin columns. Cyclodextrins are cyclic oligomers of α-D-glucose bonded through α-(1,4) linkages. In this group of CSP columns, there is another group that consists of β-cyclodextrin bonded phase type columns, from which the silica-supported cyclodextrin columns are available. Cyclobond I is a β-cyclodextrin column made up of cyclic glucoamyloses that have been found to separate flavanone, 2′- and 6-hydroxyflavanone as well as the 4′- and 6-methoxyflavanone [68]. Acetylating the 3-hydroxylgroups on the mouth of the cyclodextrin molecule introduces further binding sites and an acetylated Cyclobond I column can resolve several flavanones including: flavanone, 2′- and 6-hydroxyflavanone as well as 6-methoxyflavanone [68]. In addition, Cyclobond I column can resolve several flavanones glycosides including prunin, naringin, narirutin and neohesperidin. The flavanones with 7-*O*-neohesperidose sugars attached were better resolved (i.e. naringin and neohesperidin) [61]. Recently, Cyclobond I 2000 has been utilized to baseline separate naringin, neohesperidin, and separate narirutin and hesperidin [92].

Columns utilizing cyclodextrin bonded silica as well as cellulose-coated silica gel have been successfully employed. Silica coated with a 2-hydroxy-3-methacryloyloxypropyl β-cyclodextrin-co-*N*-vinylpyrrolidone copolymer has been successfully utilized in reverse phase mode to resolve flavanone and monosubstituted flavanones, such as 6- and 7-methoxyflavanones and 6-hydroxyflavanone [93]. Ureido-bonded methylated β-cyclodextrin CSP columns can also separate flavanone; 5-, 6- and 7-methoxyflavanone; hesperetin; naringenin; and taxifolin [70].

New dichloro-, dimethyl- and chloromethylphenylcarbamate derivatives of α, β, γ-cyclodextrin were prepared as CSPs using normal phase liquid chromatography resolved flavanone. In particular 2,5- and 3,4-dichlorophenylcarbamates of β-cyclodextrin as CSPs provided better resolution than dimethylphenylcarbamate derivatives [64]. Enantioseparation of various flavanones on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94]. More recently column coupling with achiral reverse phase chromatography has been utilized to separate the flavanone glycosides. For this, a β-cyclodextrin column is coupled with mass spectrometry operated with negative ion electrospray ionization, which has been utilized to separate and detect eriocitrin, hesperidin, and neohesperidin enantiomers, and applied to their analysis in citrus fruit juices [95].

2.1.2. Chiral mobile phase additives

The addition of an optically active molecule to the mobile phase can facilitate separation of enantiomers on conventional stationary phases. Separation of flavonoids through the addition of cyclodextrins to the mobile phase is a rational approach given the effectiveness of CSP cyclodextrin columns. The interaction of the chiral additive with the enantiomers facilitated the formation of transient diastereomers. These diastereomeric pairs have different physicochemical properties and this may distribute differentially between the adsorbing achiral stationary phase and the organic mobile phase. Capillary electrophoresis can be operated in various modes and the separation of several flavanone-7-*O*-glycosides (naringin, prunin, narirutin, hesperidin, neohesperidin, and eriocitrin) by chiral capillary electrophoresis was accomplished by a variety of cyclodextrin mobile phase additives in borate buffer at a pH range of 8–10

[59]. There is no generally applicable cyclodextrin for flavonoid glycosides separation and assays must be developed individually; however, naturally occurring β and γ-cyclodextrin and neutral cyclodextrin derivatives, such as DM-β-cyclodextrin, HP-β-cyclodextrin, and charged derivatives CM-β-cyclodextrin and CE-β-cyclodextrin were all successful as chiral selectors [59]. These methods were subsequently applied to examine flavanone-7-*O*-glycosides in citrus fruit [60].

A recent publication [75] demonstrated the stereospecific separation of many flavanones and flavanone-7-*O*-glycosides with capillary electrophoresis by adding cyclodextrins or cyclodextrin derivatives as chiral selectors to the background electrolyte. The ionizability of flavanones at high pH requires an anionic cyclodextrin derivative, such as carboxymethyl-cyclodextrin, sulfatocyclodextrin as buffer selectors. While a buffer system at pH 7 containing neutral cyclodextrins does not appear to possess enantiomeric discrimination [75]. It appears that the pH strongly influences the stereospecific separation and that methyl-carboxymethyl and hydroxypropyl-γ-cyclodextrin leads to a better resolution than the corresponding β-cyclodextrin, while sulfato-γ-cyclodextrin provided no separation of the examined flavanones [75].

Separation of some chiral flavanones by micellar electrokinetic chromatography has also been accomplished [63]. γ-cyclodextrin and sodium cholate were used as chiral mobile phase additives. Sodium cholate when used above at critical micelle point concentration forms chiral micelles and was effective at separating flavanone glycosides due to a sugar micelle interaction, while the use of cyclodextrin was more effective in separating flavanone aglycones.

The glycoside neohesperidin was baseline separated while naringin was not. For the aglycones examined, the best resolution was for hesperetin although again baseline resolution was not achieved [63]. A more recent investigation demonstrates that micellar electrokinetic chromatography with (a) sodium cholate or (b) sodium cholate plus cyclodextrins or cyclodextrin derivatives or (c) sodium dodecyl sulfate (SDS) plus cyclodextrin or cyclodextrin derivatives as a chiral surfactants/selectors can be employed for the epimeric separation of flavanone 7-*O*-glycosides [75]. Flavanone aglycones are not separable into their respective enantiomers with sodium cholate alone; however, by adding SDS to a buffer system containing certain γ-cyclodextrins enantioseparation can be obtained. No stereospecific separation was demonstrated for other bile salts, such as sodium deoxycholate and sodium taurocholate; however, baseline separation for neohesperidin and naringin was achieved and this separation was dependent on concentration of sodium cholate and pH of the mobile phase [75].

Separation of several flavanone glycosides and aglycones including eriocitrin, hesperidin, hesperetin, naringin, naringenin, narirutin, neohesperidin, flavanone, 2′- and 6′-hydroxyflavanone, and 6-methoxyflavanone in citrus fruit juices was accomplished by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the chiral selector [96].

Cyclosophoraoses are unbranched cyclic (1 → 2)-β-D-glucans oligosaccharides. Highly sulfated cyclosophoraoses or neutral cyclosophoraoses were applied as chiral additives with SDS for the separation of isosakuranetin and neohesperidin in micellar electrokinetic chromatography [97].

2.2. Indirect method of analysis: chiral derivatization techniques

One of the first reports of HPLC separation of flavanone glycosides was in 1980 [98]. It was suggested that both naringin and narirutin could be acetylated with equal portions of pyridine and acetic anhydride and resolved at low temperatures 0–5 °C [98]. In the mid-1980's, there was some initial separation of prunin (naringenin-7-*O*-glucoside) using benzoylated derivatives to separate the epimers in *Prunus callus* (sweet cherries) [99], oranges and grapefruit [100]. The separation of prunin benzoate and naringin benzoate on Cyclobond I columns has also been reported [61]. There is also mention in the literature of derivatization of naringenin to naringenin tribenzoate and separation on a Chiralcel OD column. However, naringenin enantiomers were not resolved suggesting that the hydroxyl groups present in naringenin hinder chiral recognition on this stationary phase [68].

2.3. Racemization, enantiomerization and epimerization

A feature that exists with chiral xenobiotics is a lack of configurational stability. Some chiral flavanoids undergo non-enzymatic interconversion of one stereoisomeric form into another. When isomerization occurs causing the formation of a racemate it is termed racemization, racemization is the process of an enantioenriched substance becoming a mixture of enantiomeric forms and thus the formation of a racemate from a pure enantiomer. Alternatively stated, racemization is the conversion of one enantiomer into a 50:50 mixture of the two enantiomers of a substance. Racemization is normally associated with the loss of optical activity over a period of time since 50:50 mixtures of enantiomers are optically inactive, while enantiomerization is the reversible interconversion of enantiomers. In epimers when diastereoisomerization occurs by the change of configuration at a single chiral center, the process is called epimerization [75].

For example, *S*-(−)-naringenin racemizes within 3 h in a water/methanol solvent [25]. The importance of temperature and pH dependent epimerization or enantiomerization barriers of many flavanone-7-*O*-glycosides (i.e. naringin, narirutin, neohesperidin and prunin) as well as flavanones (homoeriodictyol and naringenin) have been recently examined [75].

The importance of enantiomerization and epimerization in stereospecific chromatography is that when this occurs during separation on a chiral stationary phase there are some characteristics of the eluting peaks, such as peak broadening, peak coalescence, and plateau formation that suggest interconversion of the enantiomers or epimers under those conditions [75]. For instance, it has been demonstrated for some flavanones (i.e. naringenin and homoeriodictyol) as well as some flavanone-7-*O*-glycosides (i.e. narirutin, naringin, neohesperidin, and prunin) under basic conditions of high pH (9–11) a visually evident tem-

perature dependent plateau is apparent between the peaks of the respective enantiomers and epimers [75].

Non-enzymatic inversion of xenobiotics is important in the pharmaceutical manufacturing process and has implications for the shelf-life of a drug and the economic feasibility of the stereoresolution. Non-enzymatic inversion can also occur during the stereospecific chromatographic procedures. Racemization may also occur in physiological fluids, such as the acidic environment of the stomach.

The biogenic mechanism of epimerization during the maturation of the fruit has been studied by several investigators [101,102]. Naringin is present at very high quantities in young grapefruit and as the fruit increases in size there is a decrease in naringin content as ripening occurs following a characteristic sigmoid pattern [102]. Naringin is essentially in the 2*S*-epimeric form in immature fruits and it is believed that it is produced by enzymatic cyclization of its precursor chalcone glycoside. However, the findings of Wistuba et al. [75] suggests that chalcones were not detected in dynamic electrophoretic studies of the interconversion of flavanones. Further, studies are required to clarify intermediates involved in enantiomerization and epimerization.

During fruit enlargement 2*S* naringin is stored in fruit vesicles and undergoes non-enzymatic racemization at the C-2 position leading to the production of 2*S* and 2*R* naringin. This phenomena appears to be independent of plant habitat and may also affect taste perception [101,102].

Demonstration of racemization or epimerization may have profound consequences for the development of stereochemically pure flavanones as a pharmaceutical/nutraceutical entity. A better understanding of the factors facilitating such interconversions may greatly aid their development by identifying this feature at an early stage and thereby reducing pharmacological and bioanalytical workload. Regulatory agencies are increasingly asking for evidence regarding this phenomena following administration of racemates or single enantiomer drug candidates [103]. Racemization could lead to variability in both the pharmacokinetics and pharmacodynamics of chiral xenobiotics and have implications for preclinical screening and for safety evaluation and be a source of variability in response. As racemization may occur for some stereoisomeric flavanones, an examination of pharmacokinetics and pharmacodynamics of both *in vitro* and *in vivo* after administration of the racemates and the enantiomers or epimers is necessary. For instance, it has been observed that after oral administration of traditional

Chinese medicines Daisaiko-to and Shosaiko-to to healthy volunteers, dihydrowogonin and dihydrooroxylin were predominantly excreted as *S*-enantiomers while naringenin was excreted as *R*, *S* mixture in urine [62]. Therefore, the need to have stereospecific methods of analysis is warranted to study their biological activity and monitor drug development [104,105].

Furthermore, it is prudent for the analyst to avoid any environment that may epimerize or racemize the chiral center of the flavanone. This would be for example the use of extreme alkaline or acidic conditions or elevated temperatures. For instance naringenin plateau formation can be observed at pH 9–11 but not under neutral (pH 7.0) or acidic (pH 2.5) conditions [75]. A hydroxyl group at position 4′ of ring B is a common structural feature of flavanones that undergo enantiomerization or epimerization except for neohesperidin [75]. Naringin, prunin and narirutin all undergo epimerization with the type of saccharide attached on ring A having minimal influence on the interconversion; however, flavanone 7-*O*-glucosides appear to be more prone to inversion than their respective aglycones. In this recent investigation, only naringenin and homoeriodictyol were demonstrated to enantiomerize under the conditions examined [75]. A recent investigation using stopped-flow HPLC, dynamic HPLC and enantioselective HPLC determined that the rate constants of diastereomerization were about eight times higher for naringin and narirutin than for hesperidin and neohesperidin [92]. The rate of diastereomerization between neohesperidoses and corresponding rutinosides were not significantly different. Interestingly, the rate of diastereomerization of naringin was ∼10 times faster using the dynamic HPLC than using a stop-flow method At present the intermediates involved in this enantiomerization and epimerization process have not been clearly delineated and the reasons for differences in the rate and extent of these process within and between each flavonoid require further detailed study.

2.4. Advantages and disadvantages of current methods

All of these methods of analysis may have certain advantages and disadvantages. Some disadvantages might include long run times that make routine analysis of large volumes of samples impractical. In addition, many columns and methods that have shown stereospecific separation are not yet commercially available. The choice of columns are increasing, however, the costs of the columns can also be prohibitive and the mobile phase composition can be rather limited with CSPs In the case of some CSP HPLC columns, they can only be used with non-aqueous solvents and this requires judicial removal of water from biological samples to retain optimal column efficiency.

On the other hand, the ultimate advantage of chiral separation methods over achiral methods include a more thorough understanding of the pharmacokinetics of flavanones and the determination of safe and effective dosing regimens. In the case of racemic flavanones or stereochemically pure flavanones this requires knowledge of the *in vivo* behavior of the enantiomers and epimers. Awareness and appreciation in the drug development process of conformational stability of chiral compounds may have significant bearing on the pharmaceutical, pharmacokinetic, and pharmacodynamic data. Stereospecific analysis methods can enable the study of enantiomerization/epimerization and racemization. Putative differences in therapeutic or adverse effects of the enantiomers would be abolished by rapid interconversion *in vivo* and render the development of stereochemically pure enantiomers ineffective. In the development of stereochemical pure compounds and racemates, chirality must be taken into account *ab initio* in the development process. Many publications report the applicability of CSPs to resolve different chiral flavanones; however, there are still comparatively few published and validated assays in biological matrices.

Finally, the lack of availability of optically pure enantiomers and epimers renders evaluation of configurational stability of chromatographic methods complicated. Regardless of the method of resolution the possibility of non-enzymatic inversion during the assay and biological extraction must be recognized early on in the development and validation process for any new stereospecific assay. The commercial availability of pure enantiomers and epimers from chemical companies to facilitate assay validation and examination of configurational stability would be beneficial to the analyst. A more thorough understanding of fruit regulation and growth also may allow extraction of enantioenriched epimers and enantiomers. Further chemical characterization and synthesis of pure enantiomers to serve as standards would greatly assist the analyst in the development of stereospecific analytical methodology [101,106].

3. Flavanones

3.1. Dihydrowogonin

The enantiomers of dihydrowogonin were resolved on a Chiralcel OD column in normal phase and its presence was detected in post-administrative urine predominantly in the *S* form in patients administered some Asian herbal medicines [62,73,74].

3.2. Dihydrooroxylin A

Dihydrooroxylin A was resolved into its respective enantiomers on a Chiralcel OD column under normal phase conditions and its presence predominantly in the *S*-enantiomer was detected in post-administrative urine of patients administered with some Asian herbal medicines [62,73,74].

3.3. Eriocitrin and eriodictyol

Eriocitrin [(+/−)-5,7,3′,4′-tetrahydroxyflavanone 7-*O*-rutinoside] is a chiral flavanone-7-*O*-glycoside present in lemons, tamarinds and other citrus fruits, as well as in mint, oregano, fennel, thyme, and rose hip. Eriocitrin was successfully resolved into its epimers using a variety of cyclodextrin mobile phase additives and capillary electrophoresis although baseline resolution was not obtained [59]. It has been suggested that eriocitrin is found equally as 2*R* and 2*S* in lemons [60]. Multi-dimensional liquid chromatography through the use of carboxylated β-cyclodextrin columns coupled to mass spectrometry demonstrated that lemon juices contain eriocitrin epimers in approximately equal amounts [58]. Separation of eriocitrin by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector demonstrated that in citrus fruit juices ∼50:50 2*S*/2*R* epimers was evident [96].

After consumption, the sugar moiety is rapidly cleaved off the parent flavanone glycoside eriocitrin in the gastrointestinal tract and liver to leave the aglycone bioflavonoid eriodictyol [(+/−)-5,7,3′,4′-tetrahydroxyflavanone]. Three commercially available columns of microcrystalline cellulose triacetate (MCCTA) were able to resolve eriodictyol isomers [66]. Eriodictyol could be resolved under reverse and normal phase conditions on modified MCCTA [25,67]. Eriodictyol was determined in peanut hull (*Arachis hypogaea*), gaviota tarplant (*Hemizonia increscens*) and thyme (*Thymus vulgaris*) to be predominantly in the *S*-(−) configuration [25]. A recent study that employed the commercially available Chiralcel OD and Chiralpak AS-H separated eriodictyol enantiomers under normal phase HPLC. The authors obtained baseline resolution with the Chiralpak-AS-H, but not with Chiralcel OD, however, the method was not validated in biological matrices [58]. The Chiralcel OJ column (tris 4-methylphenyl-benzoate ester) can resolve eriodictyol [65]. Our laboratory has recently validated a method for the separation of eriodictyol enantiomers under reversed-phase HPLC utilizing the Chiralcel OJ-RH, a cellulose tris (4-methylbenzoate) column [107]. This method is a stereoselective, isocratic, reversed-phase high-performance liquid chromatography (HPLC) method that has been successfully applied for the determination of the enantiomers of eriodictyol and its application to *in vivo* kinetic studies, determine enantiomers in lemons, limes, and lemonade, peanut hulls and thyme and to separately isolate enantiomers for further pharmacological testing [108]. The enantiomeric separation of eriodictyol by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of *R*s = 1.61 with carboxymethyl-β-cyclodextrin [75]. The combined use of the surfactant SDS to a buffer system containing γ-cyclodextrin or hydroxypropyl-γ-cyclodextrin using micellar electrokinetic chromatography demonstrated separation for both and baseline separation for the later [75].

3.4. Flavanone

Resolution of flavanone enantiomers by HPLC was first established utilizing the polysaccharide derivatives cellulose trans-tris(4-phenylazaphenylcarbamate) columns [72]. This was followed by separation on cellulose tris(3,5-dimethylphenylcarbmate columns [86,87]. The Chiralcel OD column is a macroporous silica gel coated with cellulose tris (3,5-dimethylphenylcarbamate) has demonstrated ability to separate flavanone [65,68,89]. While Chiralcel OC, OA and OJ columns can also resolve flavanone [65]. In addition, the Chiralpak AD-RH can effectively baseline resolve flavanone enantiomers [Davies *et al.* unpublished observations].

Unsubstituted flavanone can be easily separated on cellulose mono and disubstituted carbamates including cellulose-4-substituted triphenylcarbamate derivatives, cellulose chloro-substituted triphenyl carbamate, and cellulose methyl-substituted triphenylcarbamate supported in silica gel [71]. Also the 2,3,4-tris-*O*-(3,5-dimethylphenylcarbamoyl) CSP demonstrated the ability to resolve flavanone [69].

The resolution of flavanone has been demonstrated on silica coated with a (2-hydroxy-3-methacryloyloxypropyl β-cyclodextrin-co-*N* vinylpyrrolidone) copolymer that has been successfully employed in reverse phase mode [93]. In addition, reasonable enantioseparation of flavanone (*R*s = 1.31) on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94]. Cyclobond I is a β-cyclodextrin column made up of cyclic glucoamyloses that have been

demonstrated to separate flavanone enantiomers [68]. The acetylated Cyclobond I column [68], and the ureido-bonded methylated β-cyclodextrin column [70] can also effectively resolve flavanone.

In addition chiral columns employing amylose esters, such as amylose tris (3,5-dimethylphenylcarbmate) and tris (3,5-dichlorophenylcarbmate) supported on silica gel have shown ability to resolve flavanone [71]. Chiralpak OP (+) is based on macroporous silica gel coated with poly(diphenyl-2-pyridylmethylmethacrylate) and has been reported to separate flavanone enantiomers [68]. ChiraSpher is a small-pore silica gel chiral polymer (poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester) that has demonstrated the separation of flavanone [68].

Separation of flavanone by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector was also accomplished although baseline resolution was not obtained [96].

3.5. Hesperidin and hesperetin

Hesperidin [+/−-3,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglucoside] is a chiral flavanone-7-*O*-glycoside consumed in oranges, grapefruit, and other citrus fruits and herbal products. Recently, the use of chiral mobile phase additives of β-cyclodextrin, hydroxypropyl β-cyclodextrin and capillary electrophoresis were found to separate hesperidin epimers although baseline resolution was not observed [59]. Hesperidin has been suggested to be in an epimeric ratio between 90:10 and 97:3 with the 2*S* epimer predominating in lemons and 95:5 ratio in sweet orange and mandarin juice [60]. Multi-dimensional liquid chromatography through the use of β-cyclodextrin columns coupled to mass spectrometry demonstrated that fruit juices contain hesperidin epimers predominantly in the 2*S* epimer [95]. In orange/sour orange cross freshly squeezed juice, hesperidin was almost exclusively in the 2*S* epimer (92%) and in lemon juices (96%) [95]. In a recent study, hesperidin was separated using normal phase HPLC in commercial hesperidin and herbal medicine samples and although the 2*S* epimer predominated there was significant 2*R* hesperidin present in some samples [91]. Finally, baseline separation of hesperidin and hesperetin by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector was accomplished and 2*S* hesperidin predominated in lemon and orange juice [96]. Furthermore, the baseline separation of hesperidin by capillary electrophoresis using carboxymethyl-β-cyclodextrin as the selector has also been accomplished [75].

The rutinose sugar moiety is rapidly cleaved off the parent flavanone glycoside hesperidin to leave the aglycone bioflavonoid hesperetin [+/−-3,5,7-trihydroxy-4′-methoxyflavanone], also a chiral flavonoid. There are just a few reports where hesperetin enantiomers were separated although baseline resolution and separation were poor and validation was not undertaken. Ng *et al*. employed multipleureido-covalent bonded methylated β-cyclodextrin columns supported on silica gel [70], while Krause and Galensa used multiple microcrystalline cross-linked acetylcellulose (MCCTA) columns [66]. Hesperetin could also be resolved under reverse and normal phase conditions on modified MCCTA [67]. Unfortunately, these columns are not commercially available, and separation was poor with no baseline resolution and quantification was not validated in biological matrices or applied to pharmacokinetics studies. Hesperetin could be separated using γ-cyclodextrin as a mobile phase additive and micellar electrokinetic chromatography; however, baseline resolution was not obtained [63]. Baseline enantioseparation of hesperetin (Rs = 1.88) on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94]. Nevertheless, there is a recent study that employed the commercially available Chiralcel OD and Chiralpak AS-H, for the separated of hesperetin enantiomers under normal phase HPLC, the authors obtained baseline resolution with the Chiralcel AS-H column only, but the method was not validated in biological matrices [58]. The Chiralcel OJ column (tris 4-methylphenyl-benzoate ester) can resolve hesperetin [65]. We have developed the only validated method for the separation of hesperetin enantiomers under reversed-phase HPLC on a Chiralpak AD-RH column [95] and successfully applied to *in vivo* pharmacokinetic studies and citrus fruit analysis [109]. The enantiomeric separation of hesperetin by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 3.65 with methyl-γ-cyclodextrin and baseline resolution with sulfato-β-cyclodextrin [75]. The combined use of the surfactant SDS to a buffer system containing γ-cyclodextrin or hydroxypropyl-γ-cyclodextrin using micellar electrokinetic chromatography demonstrated separation for both and baseline separation for the former [75].A recent study, [92] demonstrated separation of hesperidin on Cyclobond 1 2000 column in reverse phase and its application to assessment of freshly squeezed and commercial orange juice. The ratio of 2*S*/2*R* hesperidin is much higher in fresh (17.9) than in processed juice (3.2–4.6) [92].

3.6. Homoeriodictyol

Three commercially available columns of microcrystalline cellulose triacetate (CTA I, CTA II, and CTA III available from Merck, Darmstadt, Germany) were able to resolve homoeriodictyol [66]. It could be resolved under reverse and normal phase conditions on modified MCCTA [67]. In yerba santa (*Eriodictyon glutinosum*), homoeriodictyol was determined to be predominantly in the *S*-(−) configuration [25]. Furthermore, it can be resolved on a Chiralcel OC column under normal phase conditions [65]. Finally, separation was achieved using micellar electrokinetic chromatography with γ-cyclodextrin as a mobile phase additive although baseline resolution was not obtained [63]. In addition, the enantiomeric separation of homoeriodictyol by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 6.47 with methyl-γ-cyclodextrin and suitable baseline resolution with hydroxypropyl-γ-cyclodextrin (Rs = 2.22) and sulfato-β-cyclodextrin (Rs = 1.82) [75]. The combined use of the surfactant SDS to a buffer system containing γ-cyclodextrin or hydroxypropyl-γ-cyclodextrin using micellar electrokinetic chromatography demonstrated separation for both and baseline separation for the later [75].

3.7. Hydroxyflavanone, 2′-

Different columns have being reported to separate 2′-hydroxyflavanone including: Chiralcel OD, ChiraSpher, Cyclobond I, and acetylated Cyclobond I [68]. However, baseline resolution was not obtained by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector [96].

3.8. Hydroxyflavanone, 4′-

The separation of 4′-hydroxyflavanone has been described using ChiraSpher, a small-pore silica gel chiral polymer made of poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester [68]. In addition, reasonable enantioseparation of 4′-hydroxyflavanone (Rs = 0.93) on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94]. The enantiomeric separation of 4′-hydroxyflavanone by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 1.39 with methyl-sulfato-β-cyclodextrin [75].

3.9. Hydroxyflavanone, 6-

Different columns have being reported to separate 6-hydroxyflavanone including: Chiralcel OD, ChiraSpher, Cyclobond I, and acetylated Cyclobond I [68]. In addition, near baseline enantioseparation of 6′hydroxy flavanone (Rs = 1.45) on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94]. However, baseline resolution was not obtained by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector [96].

3.10. Isosakuranetin

One commercially available column made of microcrystalline cellulose triacetate (CTA II available from Merck, Darmstadt, Germany) was able to resolve isosakuranetin [66]. It could also be resolved under reverse and normal phase conditions on modified MCCTA [67]. Isosakuranetin could be separated using γ-cyclodextrin as a mobile phase additive and micellar electrokinetic chromatography; however, baseline resolution was not obtained [63]. More recently using highly sulphated cyclosophoraoses as chiral mobile phase additives with SDS using micellar electrokinetic chromatography allowed the resolution of isosakuranetin enantiomers [97]. A follow-up study of the enantiomeric separation of isosakuranetin by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 3.43 with sulfato-β-cyclodextrin and baseline resolution with carboxymethyl-γ-cyclodextrin (Rs = 2.11) and methyl-γ-cyclodextrin (Rs = 2.05) [75]. The combined use of the surfactant SDS to a buffer system containing γ-cyclodextrin (Rs = 1.78) or hydroxypropyl-γ-cyclodextrin (Rs = 1.49) using micellar electrokinetic chromatography demonstrated good separation for both [75].

3.11. Liquiritigenin

Liquiritigenin was resolved into its respective enantiomers on a Chiralcel OD column in normal phase and its presence was detected in post-administrative urine of patients administered herbal medicines predominantly in the *S*-enantiomer [62,73,74].

3.12. Methoxyflavanone, 4′-

Different columns have being reported to separate 4′-methoxyflavanone including: Chiralcel OD [68,89], Chiralpak OP (+), ChiraSpher, Cyclobond I [68], Chiralcel OC and Chiralcel OJ [65].

3.13. Methoxyflavanone, 5-

5-methoxyflavanone has being separated using different columns, such as: Chiralcel OD [65,68], Chiralpak OP (+), ChiraSpher, acetylated Cyclobond I [68], ureido-bonded methylated β-cyclodextrin [70], Chiralcel OC, Chiralcel OJ, and Chiralcel OA [65].

3.14. Methoxyflavanone, 6-

6-methoxyflavanone has being separated using different columns, such as: Chiralcel OD [68,89], Chiralcel OC, Chiralcel OJ [65], Chiralpak OP (+), ChiraSpher, Cyclobond I [68], and ureido-bonded methylated β-cyclodextrin [70]. Separation of 6-methoxyflavanone by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector was accomplished although baseline resolution was not obtained [96]. It has also been resolved using a silica coated with a (2-hydroxy-3-methacryloyloxypropyl β-cyclodextrin-co-*N* vinylpyrrolidone) copolymer in reverse phase mode [93]. In addition, reasonable enantioseparation of 6-methoxy flavanone (Rs = 1.31) on mono (6^A-*N*-allylamino-6^A-deoxy) permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94].

3.15. Methoxyflavanone, 7-

7-methoxyflavanone has being separated using different columns, such as: Chiralcel OA, Chiralcel OD [65], and ureido-bonded methylated β-cyclodextrin [70]. It has also been resolved using a silica coated with a (2-hydroxy-3-methacryloyloxypropyl β-cyclodextrin-co-N vinylpyrrolidone) copolymer in reverse phase mode [93]. In addition, baseline enantioseparation of 7-methoxyflavanone (Rs = 2.28) on mono (6^A-*N*-allylamino-6^A-deoxy)permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported [94].

3.16. Naringin and naringenin

Naringin [(+/−)-4′,5,7-trihydroxyflavanone 7-rhamnoglucoside] is a chiral flavanone-7-*O*-glycoside present in citrus fruits, tomatoes, cherries, oregano, beans, and cocoa [110–115].

After consumption, the neohesperidose sugar moiety is rapidly cleaved off the parent compound in the gastrointestinal tract and liver to leave the aglycone bioflavonoid naringenin. The ratio between the amount of naringenin and naringin varies among different food products. For instance, citrus fruits contain higher amounts of the glycoside naringin, while tomatoes have higher amounts of the aglycone naringenin [116]. Naringin was acetylated and separated on an achiral column [98]. A Cyclobond I column can also resolve naringin epimers [61]. Only about 2% of naringin is in the 2*R* configuration in immature freshly squeezed grapefruit, while ripe grapefruit contained 66% 2*S* and 34% 2*R* naringin, and grapefruit from a commercial source 60% 2*S* and 40% 2*R* [61]. A CSP using MCCTA in normal phase provided resolution in a tomato ketchup sample [67]. The Chiralcel OD column can separate naringin in albedo grapefruit and examine epimer changes during grapefruit maturation using normal phase isocratic HPLC [12]. The stereochemistry of naringin changes with the diameter of fruit with greater concentrations of *S*-naringin in the smallest diameter of grapefruit [12]. A recent report using β-cyclodextrin, dimethyl-β-cyclodextrin, and hydroxypropyl β-cyclodextrin as mobile phase additives in capillary electrophoresis resolved the epimers of naringin although baseline resolution was not obtained [59]. Naringin could be separated using sodium cholate as a mobile phase additive under micellar electrokinetic chromatography; however, baseline resolution was not obtained [63] although a follow-up study demonstrated pH dependent baseline resolution [75]. The *S*:*R* ratio in sour oranges and marmalade made from sour oranges was 60:40, while in immature grapefruits both naringenin enantiomers were detected, and the *S*-enantiomer clearly predominated and decreased as the fruit matured [60]. The use of carboxylated β-cyclodextrin columns in reverse phase demonstrated that Jaffa grapefruit juices contain naringin epimers mainly in the 2*S* form [95]. In freshly squeezed red grapefruit juice, 56% of the naringin was in the 2*S* form whereas lower percentage of the 2*S* epimer was found in commercial white and red grapefruit juice [95]. A Chiralpak IA column was also able to separate naringin directly under normal phase isocratic conditions although baseline resolution was not obtained [91]. Finally, separation of naringin and naringenin by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector was accomplished and grapefruit juice was determined to be essentially 50:50 in naringin epimers [96]. A more recent study reported the enantiomeric separation of naringenin by capillary electrophoresis using the various cyclodextrins as selectors and demonstrated separation with the best resolution of Rs = 4.85 with hydroxypropyl-γ-cyclodextrin and baseline resolution with methyl-γ-cyclodextrin (Rs = 3.81), carboxymethyl-γ-cyclodextrin (Rs = 2.26), and sulfato-β-cyclodextrin (Rs = 3.63) [75]. The combined use of the surfactant SDS to a buffer system containing γ-cyclodextrin or hydroxypropyl-γ-cyclodextrin or sulfato-β-cyclodextrin using micellar electrokinetic chromatography demonstrated separation for all and baseline separation (Rs = 1.72) for γ-cyclodextrin [75]. Finally, the Cyclobond I 2000 column has been shown to demonstrate baseline resolution of naringin in reverse phase.

Three commercially available columns of microcrystalline cellulose triacetate (CTA I, CTA II, and CTA III available from Merck, Darmstadt, Germany) were able to resolve naringenin [66]. A commercially available cellulose triacetate column coated on macroporous silica gel (Chiralcel OA, Daicel) separated naringenin enantiomers in normal phase although baseline resolution was not obtained [65,67]. A microcrystalline cellulose triacetate (MCCTA) coated on 7 μm Nucleosil diol, and depolymerized MCCTA using normal and reverse phase provided baseline resolution. A CSP using MCCTA in normal phase provided resolution in a tomato sample demonstrating the presence of both enantiomers [67]. A CSP using cellulose triacetate in normal phase in thyme samples demonstrated stereospecific disposition of the *S*-(−)-enantiomer and both enantiomers in a tomato ketchup sample [67]. Naringenin was also resolved into respective enantiomers on a Chiralcel OD column in normal phase and its presence was detected in post-administrative urine of patients administered herbal medicines predominantly in the *S*-enantiomer [62,73,74]. The utility of the Chiralcel OD column was also demonstrated by others [58].

There are, however, a couple of reports demonstrating that micellar electrokinetic chromatography with chiral γ-cyclodextrin as a mobile phase additive [63], and multidimensional liquid chromatography coupled with mass spectroscopy [95] can separate naringenin enantiomers. However, baseline resolution and separation was not evident [63], and quantification was not validated in biological matrices [63,95]. Ureido-bonded methylated β-cyclodextrin CSP columns can also separate naringenin [70].

There was a report by Geiser *et al.* at Pittcon 2000 reporting the use of supercritical fluid chromatography (SFC) with the analytical column Chiralpak AD-RH to separate the enantiomers of naringenin. In our laboratory using a Chiralpak AD-RH column with HPLC we failed to demonstrate baseline resolution for the analysis of naringenin in biological matrices. However, we were successful in naringenin separation with the commercially available Chiralcel OD-RH column, and to our knowledge this is the only validated direct assay method for stereospecific analysis of naringenin enantiomers in the literature [90]. There is also a recent study that employed the commercially available Chiralpak AS-H (an amylose-derived column) for the separation of naringenin under normal phase HPLC, the authors obtained baseline resolution but the method was not validated in biological matrices [58]. Our method is a stereoselective, isocratic, reversed-phase high-performance liquid chromatography (HPLC) method that has been successfully validated and applied to the determination of the enantiomers of naringenin and its application to disposition in tomato fruit and *in vivo* kinetic studies [116–118]. Furthermore, naringenin stereospecific disposition in pears, strawberries, sweet cherries, apples, and apple products has recently been determined [Davies *et al.* unpublished observations].

3.17. Narirutin

Narirutin was first separated indirectly by derivatization through acetylation and separation on an achiral column [98].

In addition, Cyclobond I column can resolve narirutin directly and has shown a higher concentration of 2*S* narirutin than 2*R* narirutin in grapefruit juice but equal 2*R* and 2*S* concentrations in sweet orange juice [61]. The use of the mobile phase additives γ-cyclodextrin and dimethyl-β-cyclodextrin demonstrated ability to separate the epimers of narirutin, although baseline resolution was not obtained [59]. Narirutin was approximately 50:50 in sweet orange juice [60]. The use of carboxylated β-cyclodextrin columns in reverse phase demonstrated that grapefruit and orange juice contain narirutin epimers in approximately equal amounts [95]. Separation of narirutin by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the selector was accomplished and suggested that 2*S* was slightly higher in grapefruit but equal to 2*R* in oranges [96]. The pH dependent separation of sulfobutyl ether β-cyclodextrin has been verified by a more recent publication [75]. Narirutin was separated using normal phase HPLC in commercial herbal medicine samples using a Chiralpak IA column with the 2*S* epimer predominating ∼60–80% [91].A recent investigation, failed to achieve baseline resolution of narirutin on a Cyclobond I 2000 column [92].

3.18. Neoeriocitrin

The resolution of neoeriocitrin epimers using hydroxypropyl β-cyclodextrin as a chiral mobile phase additive in borate buffer with capillary electrophoresis was reported [60]. Neoeriocitrin was further determined to be ∼50:50 ratio in sour orange juice [60]. A recent investigation using β-cyclodextrin as a chiral selector in capillary electrophoresis demonstrated poor resolution ability (Rs = 0.35) [75].

3.19. Neohesperidin

The Cyclobond I column can resolve neohesperidin directly and it has been demonstrated that the presence of 2*S* neohesperidin predominates in marmalade processed from bitter oranges (*Citrus aurantium*) [61]. A recent investigation, also achieve baseline resolution of neohesperidin on a Cyclobond I 2000 column [92]. The resolution of neohesperidin was accomplished using natural, neutral and charged cyclodextrins as mobile phase additives using capillary electrophoresis [59,75]. The baseline resolution using hydroxypropyl-β-cyclodextrin and dimethyl-β-cyclodextrin and β-cyclodextrin was reported [59]. The separation on neohesperidin on carboxylated β-cyclodextrin, permethylated β-cyclodextrin and acetylated β-cyclodextrin columns in reverse phase was also suggested [95]. In addition, carboxymethyl-β-cyclodextrin can baseline resolve neohesperidin [75]. Neohesperidin could be separated using sodium cholate as a mobile phase additive in micellar electrokinetic chromatography with baseline resolution obtained [63,75]. Neohesperidin was predominant in sour orange juice in the 2*S* form [60], and it could be separated using normal phase HPLC in commercial herbal medicine samples using a Chiralpak IA column [91]. More recently the separation of neohesperidin has being demonstrated using highly sulphated cyclosophoraoses as chiral mobile phase additives with SDS under micellar electrokinetic chromatography [97].

3.20. Pinocembrine

Three commercially available columns of microcrystalline cellulose triacetate were able to resolve pinocembrine [66]. It could also be resolved under reverse and normal phase conditions on modified MCCTA [67]. Pinocembrine could be separated on both Chiralcel OD and Chiralpak AS-H columns, although baseline resolution was not obtained [58].

3.21. Pinostrobin

It could be resolved utilizing the Chiralcel OD and Chira-Spher column [68]. Pinostrobin could also be separated using γ-cyclodextrin as a mobile phase additive and micellar electrokinetic chromatography; however, baseline resolution was not obtained [63]. The enantiomeric separation of pinostrobin by capillary electrophoresis using the various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 1.44 with methyl-γ-cyclodextrin [75].

3.22. Prunin

Separation of prunin using benzoylated derivatives and reverse phase HPLC demonstrate stereospecific disposition in sweet cherries [99], oranges and grapefruit [100]. In addition, the Cyclobond I column can resolve prunin and has demonstrated its presence almost exclusively in the 2*S*-epimer in immature grapefruit [60,61]. Finally, the use of mobile phase additives containing β-cyclodextrin and dimethyl-β-cyclodextrin demonstrated their ability to separate prunin epimers, although baseline resolution was not obtained [59]. Recent investigations using sulfato-β-cyclodextrin as a chiral selector in capillary electrophoresis demonstrated excellent baseline separation of prunin epimers [75].

3.23. Taxifolin

Ureido-bonded methylated β-cyclodextrin CSP columns can separate taxifolin but not to baseline resolution [70]. However, our laboratory has recently being able to separate taxifolin utilizing the Chiralcel OJ-RH with baseline resolution [Davies *et al.* unpublished observations].

4. Conclusions

Over the last several decades a number of methods and techniques have been developed for the analysis of chiral flavanones by scientific researchers from a number of disciplines. The direct chromatographic approach has dominated this field of investigation with resolution being achieved through chiral polymer phases of oligosaccharides and their derivatives. Indirect derivatization methods have been very limited and mostly observational. There has been an increase use of chiral mobile phase additives in recent years often coupled to capillary electrophoresis. Since the seminal work in this field of Krause and Galensa in the 1980's there has been increased awareness and interest in developing the techniques to separately analyze chiral

flavanoids. It is apparent that the importance of enantiomerization and epimerization needs to be examined when developing assays for chiral flavanones. There remains a lack of stereospecific assays published in the literature for a plethora of chiral flavanones. There also remains very few validated stereospecific assays in biological matrices for the majority of compounds in this class, however, ongoing investigations in laboratories throughout the world are in progress and are rapidly advancing our stereospecific knowledge of this important class of compounds and applying this knowledge to biological applications.

Acknowledgment

The authors would like to thank the grants from the Washington State Tree Fruit Commission and the Organic Center.

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