ABSTRACT

Citrus fruits are looked upon as one of the healthy eating options. It is attributed to the presence of active components in citrus fruits called as flavonoids which have been identified to be imparting many health benefits. Bioflavonoids are also referred to collectively as vitamin P, although they are not actually vitamins in true sense, but probably due to their health-promoting properties they are being so called. Citrus fruits like Lemons, Oranges, and Sweet lime, etc. are found to contain predominantly flavonoid called hesperidin which is considered to be concentrated in the white, membranous parts and the peel and has been credited with antioxidant, antibacterial, antifungal, antiviral, anticancer and analgesic properties, apart from others.

Flavonoids are naturally occurring polyphenolic compounds. Chemically, hesperidin consists of a flavanone component joined to a sugar group bonded at 7- position to oxygen atom to give the rhamnoglucoside. However, after ingestion, the sugar group is found to be getting removed to leave the parent flavanone, 3',5,7-trihydroxy-4'-methoxyflavanone-7-rhamnoglucoside), also known as hesperetin. Flavonoids are found to exist in two enantimeric forms, R- and S-hesperidin respectively.

There has been a necessity of developing new but more powerful methods for better analysis of hesperidin in biological fluids. Given the chiral nature of hesperidin (HD) which was hitherto not taken into account in evolving at earlier methods, an effort has been made by using Octyl decyl Silane (ODS, non polar) as a stationary phase and further using mobile phase consisting of water/methanol (50:50, v/v) at room temperature containing acidic buffer with pH 4.5, following column conditioning. The detection was done by using on column UV detector at 205 nm and 298 nm. Calibration curves were obtained and were found to be reasonably linear. Sample preparation and processing was done meticulously by using methods like vacuum filtration and ultrasonication etc. before subjecting it to chromatographic analysis. The developed method was successfully applied to the determination of hesperidin enantiomers and it is more of an analytical purification than preparative one.

INTRODUCTION

The genus Citrus belonging to the family of Rutaceae comprises of about 40 species which are distributed over a spectrum of countries namely, India, China, Malaysia, Srilanka, and Australia. Citrus is one of the most important worldwide fruit crops and is consumed either as fresh fruit or in the form of juice because of its nutritional value added upon by special characteristic flavor. Consumption of Citrus juice is found to be beneficial and considered as a healthy food habit as it is instrumental in preventing coronary diseases and chronic asthma [1]. Citrus fruit extracts are found to exhibit a plethora of activities like, anti-oxidant, anti-inflammatory, anti-tumor, anti-fungal, and blood clot inhibition etc.[2][3][4]. The health benefits of Citrus fruit have mainly been attributed to the presence of bioactive compounds such as ferulic acid, hydrocinnamic acid, cyanidine glucoside,
hesperidin, vitamin C, Carotenoid and naringin content[4][5][6]. Further, concern about the safety of the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) have led to the increased interest on natural antioxidants which occur in plants as secondary metabolites. Previous studies on biochemical activities from Citrus were mainly focused on its essential oils which include antimicrobial properties [7], anti-aflatoxigenic activity [8]. Recent accumulative evidences suggest that Citrus contains several possible anti-cancer agents such as flavonoids and limonoids [9][10][11]. Flavonoids are naturally occurring polyphenolic compounds usually present in oranges, other citrus fruits and herbal products. They are conjugated with β-glucosides e.g. in hesperidin the sugar is bonded at position 7 of the flavonone (3’,5,7-trihydroxy-4’-methoxyflavonone-7-rhamnoglucoside.) (Fig.1A). However, after juice ingestion, the rutinose sugar molecule is rapidly cleaved off during its transit through gastrointestinal tract and liver leading to formation of aglycone molecule, hesperitin (±3,5,7-trihydroxy-4’-methoxyflavonone, HT) (Fig. 1B), a Chiral flavonoid which exits in two enantiomeric forms.

![Fig. 1 Structure of Hesperidin [A] and structure of Hesperitin [B]](image)

Table 1: Physical characteristics of different species of Citrus

<table>
<thead>
<tr>
<th>Citrus species</th>
<th>Colour</th>
<th>Size</th>
<th>Shape</th>
<th>Taste</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hystrix</td>
<td>Yello Green</td>
<td>5 – 6 cm</td>
<td>Pear</td>
<td>Sour</td>
<td>Smooth</td>
</tr>
<tr>
<td>C. aurantifolia</td>
<td>Geenish Yellow</td>
<td>5 – 10 cm</td>
<td>Round</td>
<td>Sour</td>
<td>Fibrous</td>
</tr>
<tr>
<td>C. sinensis</td>
<td>Orange</td>
<td>5 – 10 cm</td>
<td>Round</td>
<td>Sweet</td>
<td>Smooth</td>
</tr>
</tbody>
</table>
HPLC analytical methods are currently used either alone or hyphenated with MS can be used for authentication of foodstuffs i.e. the determination of ingredients a given foodstuff should have. This kind of exercise is of vital importance from the economic and quality point of view.

MATERIALS & METHODS

a) Preparation of sample:
Fresh fruits of Citrus, C. hystrix, C. aurantifolia, C. microcarpa, C. sinensis at the commercial mature stage were made available from local market in the months of March-April from Pune, India. Healthy fruits were selected randomly for uniformity of shape and colour. Physical characteristics of each species of Citrus fruits are described and given in table 1.

The fruits were washed thoroughly in potable water followed by Millipore water. The extract was collected by crushing the peel and membranous part of each of fruit. The extract was filtered using vacuum filtration technique using 0.2 µ filter paper and filtrate was stored at -20°C until further analysis.

b) Chemicals and reagents:
All chemicals and solvents used in this study were of analytical grade. Water used is Millipore.

c) Identification of Hesperidin by HPLC:
High Performance Liquid Chromatography coupled to UV –Vis detector was used to analyze hesperidin content of Citrus samples according to method of (Ortuna et al., 1997) using separation module equipped with a C18 column (inertsil, phenomex, 250 X 4.6 mm, 5 µm particle size). The sample were eluted using isocratic system of water:methanol (30 : 70 v/v) as the mobile phase at the flow rate of 1.0 ml/min.

The temperature of the column was ambient and the injection volume was 20 µL. The peaks of std. hesperidin were monitored at 205 nm and 298 nm for identification and quantification of hesperidin was accomplished by comparing the retention times of peaks in sample to those of standard. Calculation of hesperidin concentration was carried out by an external std. method using calibration curves of standard hesperidin.

d) Precision and accuracy:
The precision and accuracy studies were performed with replicate assays (n=6) in the same day (within-run precision) and over 3 consecutive days (between-run precision) at different level of concentrations of Hesperidin. The precision was evaluated calculating the relative standard deviation (RSD) of the enantiomer concentrations. While accuracy was estimated based on the mean percentage error of measured and actual concentration.
INSTRUMENTATION:

Spectrophotometer conditions:
System: UV – 1650PC (Shimadzu Make)
Software: UV Probe
Source: 50 W Deuterium Lamp
Wavelength Range: 190 – 1100 nm
Detector: Silicon Photodiode

Chromatography Conditions
System: LC–10AT vp (Shimadzu Make)
Software: Spinchrome

Column: Inertsil C-18 (ODS) (250 x 4.60 mm, 5 µ particle size)
Flow rate: 1 ml/min
λ max: 280 nm.
Mobile phase: Water : Methanol (1:1, v/v)
(isocratic elution)
Column Temperature: Ambient
Run time: 5 min.
Injection Volume: 20 µL of the prepared standard and sample solution

Figure 1. Chromatogram showing analysis of Orange fruit peel & membranous part extract at 298 nm

Figure 2. Chromatogram showing analysis of Sweet Lime fruit peel & membranous part extract at 298 nm
Figure 3. Chromatogram showing analysis of Lemon fruit peel & membranous part extract at 298 nm

Figure 4. Chromatogram showing analysis of Ext. Std. Hesperidin at 298 nm

Figure 5. Chromatogram showing analysis of Lemon fruit peel & membranous part extract at 205 nm
Figure 6. Chromatogram showing analysis of Orange fruit peel & membranous part extract at 205 nm

Figure 7. Chromatogram showing analysis of Sweet Lime fruit peel & membranous part extract at 205 nm

Figure 8. Chromatogram showing analysis of Ext. Std. Hesperidin at 205 nm
RESULTS AND DISCUSSION

The table.1 shows the physical characteristics of different species of citrus fruits under investigation. The fig.1 shows a chromatogram for Orange at 298 nm which results into separation of one of the enantiomers similar to one witnessed in chromatogram for Hesperidin at same wavelength and with comparable retention time. The fig.2 shows a chromatogram for Sweet lime at 298 nm which results into separation of one of the enantiomers similar to one witnessed in chromatogram for Hesperidin at same wavelength and with comparable retention time. The fig.3 shows a chromatogram for Lemon at 298 nm which results into separation of one of the enantiomers similar to one witnessed in chromatogram for Hesperidin at same wavelength and with comparable retention time. The fig.4 shows a chromatogram for external standard, Hesperidin at 298 nm which results into separation of one of the enantiomers similar to one witnessed in citrus fruits and with comparable retention time. It is interesting to note that the retention characteristics for different citrus fruits vary slightly and this variation may be attributed to differential interaction between a range of compounds in citrus fruits apart from flavonoids.

The present study describes a simple stereoselective, isocratic, reversed-phase high performance liquid chromatography (HPLC) method for the determination of the enantiomers of hesperedin.

CONCLUSIONS

In the present study, the extraction, analysis and separation of enantiomers of flavonoids from three species, namely, lemon, sweet lime and orange was undertaken at variable wavelengths from the peel and membranous part of the citrus fruits successfully. Two enantiomers R- & S- are found to absorb at different wavelengths and the advantage of these absorption characteristics was taken for their separation purpose.
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REFERENCES