DETERMINATION OF HESPERIDIN IN FOOD BY ULTRA PERFROMANCE LIQUID CHROMATOGRAPHY

1.1 Introduction

1.1.1 Ultra Performance Liquid Chromatography, UPLC

For the last 30 years of chemical analysis, High Performance Liquid Chromatography (HPLC) had been considered to be a proven technique which was used in worldwide laboratories. This technique involved the evolution of packing materials of stationary phase to effect separation.

The principles of separation involved the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). One of the variables would be particle size of the packed column and the chromatographic performance can be investigated using established Calibration Plot.

Based on van Deemter equation (Deemter, 1956), when the particle size decreased, a significant gain was observed in chromatographic performance efficiency. By using smaller particles in packed column, speed and peak capacity can be increased. With the application of these principles, Ultra Performance Liquid Chromatography, UPLC is established.

Chromatographic analysis separation can be performed using smaller particles packed columns and higher flow rates in Ultra Performance Liquid Chromatography, UPLC, the effected analysis would had an increased in speed and higher sensitivity.

By using the Instrumentation of Acquity, UPLC, H-Class from Waters (Waters, 2010); this instrument used sub 2 µm particle columns (Acquity UPLC HSS T3, 1.8 micron, 2.1 x 100 mm) in a system that is designed to maximum the advantages of this column.

The UPLC was designed to sustain a pressure tolerant sub 2 µm particle column which was stabled in a wide pH and temperature range. This made the UPLC system more flexible for high efficiency of separations with reduced cycle times and improved resolution and sensitivity.

The UPLC column, Acquity UPLC HSS T3, 1.8 micron, 2.1 x 100 mm featured an eCord Technology that electronically stored all experiments information which include certificate of analysis, dates of installation and usage, number of injections, number of sample sets, maximum temperature and pressure which was very useful for analysis traceability purpose.

The solvent utilization in UPLC application was considered very less compared with HPLC. The solvent consumption for this analysis consumed less 95% than HPLC which was considered more efficient and environment friendly.

The UPLC instrument also equipped with column management with lowest dispersion performance and precise temperature management. This was considered as a critical factor especially for Hesperidin analysis that involved a stable column temperature of 40°C. An active solvent pre-heaters is equipped in this system that ensured effective consistent thermal performance from system to system without additional volume and dispersion that were typical of passive style heat exchangers. These pre-heaters were integrated to the column compartment with robust and pressure-tolerant fittings.

The stacked multi-position Column Manager in this system provided good thermal flexibility, fast heating and cool down times. Column can be switched automatically among multiple columns with a column bypass and waste channel for simple, fast solvent changeover and rapid equilibrium.

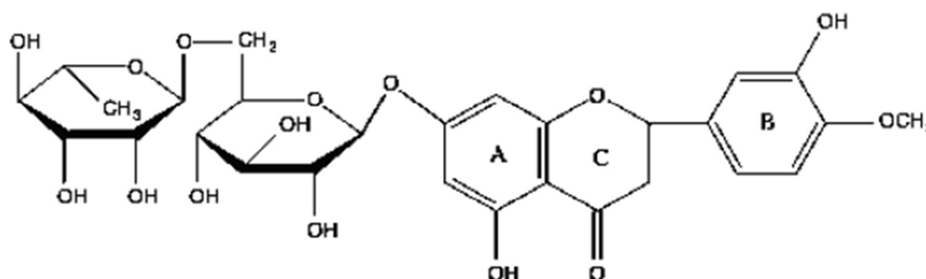
1.1.2 Hesperidin

Hesperidin ($C_{28}H_{34}O_{15}$) is a flavanone glycoside comprised of the flavanone hesperidin and the disaccharide rutinose. The flavonoid hesperidin is primarily found in citrus fruits such as oranges and lemons. Maximum of the hesperidin in these fruits were found in the membranes and peel.

Physical properties of Hesperidin:

CAS Number	520-26-3
Formula	$C_{28}H_{34}O_{15}$
Molecular Weight	610.57 g/mol
IUPAC Name	(2S)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl] oxan-2-yl]oxy-2,3-dihydrochromen-4-one
Classification	Vitamin, Flavanone glycoside, Flavonoid, Bitter compound
Physical State	White to yellow powder
Melting Point	257 - 262°C
Solubility in Water	Soluble
Stability	Stable under ordinary conditions. Hygroscopic, light sensitive.

Molecular structure of Hesperidin



Hesperidin was the most important flavanone of Citrus sp. It had been found that Hesperidin significantly increased high density lipoprotein (HDL) and lowered cholesterol low density lipoprotein (LDL), total lipid and triglyceride levels in normolipidemic rats and in rats with diet- and triton-induced hyperlipidemia (Monforte, *et al.*, 1995).

Hesperidin was evaluated for in its administration and effects on antihypertensive effect on spontaneously hypertensive rats found that Hesperidin improved serum cholesterol composition (Ohtsuki, *et al.*, 2003).

It was found that Hesperidin also featured anti-infection property that it suppressed plasma levels of tumor necrosis factor alpha (TNF-alpha) and high mobility group box chromosomal protein 1 (HMGB-1), decreased the number of apoptotic cells in livers and normalized the activated states of blood coagulation factors such as prothrombin time and platelet numbers caused by infection (Kawaguchi, *et al.*, 2004)

Hesperidin level of 50 and 100 ppm was found to have anti-inflammatory activity and can be a potential therapeutically used as a mild anti-inflammatory agent (Emim, *et al.*, 1994).

Hesperidin was observed with its sedative activity with a possible beneficial use of Hesperidin with benzodiazepines and pain management (Loscalzo, *et al.*, 2008).

Hesperidin displayed nutrigenomic effect on altered leukocyte gene expression to an anti-inflammatory and anti-atherogenic profile after 4 weeks of orange juice consumption. (Deval, *et al.*, 2011).

It is also observed that Hesperidin reduced high glucose-induced intracellular adhesion molecule-1 (HG-ICAM-1) expression via the p38 mitogen-activated protein kinases (MAPK) signaling pathway, contributing to the inhibition of monocyte adhesion to endothelial cells (Kim, *et al.*, 2011).

In the examination of Hesperidin effect in regulating bone metabolism, Hesperidin was found with higher efficacy at a lower plasma concentration (Habauzit, *et al.*, 2011).

It was observed that Hesperidin consumption resulted in a significant increase in bone mineral density (BMD) according to the study of “Hesperidin inhibits ovariectomized-induced osteopenia and shown differential effects on bone mass and strength in young and adult intact rats” (Horcajada, *et al.*, 2008).

With the amount of 50 and 100 ppm of Hesperidin treatment, it significantly attenuated histopathological alterations on the ischemic reperfusion cerebral injury-induced memory dysfunction (Gaur & Kumar, 2010).

Study had been conducted by using Hesperidin 150 mg twice or three times daily for 30 days found that bleeding bladder varicose veins that bled the haematuria disappeared (Loarca, 2003).

Hesperidin had been confirmed to have hypoglycemic and hypolipidemic effects by altering the activities of glucose-regulating enzymes and reducing the levels of lipids in the serum and liver of the GK rats, according to “Hypoglycemic and hypolipidemic effects of hesperidin and cyclodextrin-clathrated hesperidin in Goto-Kakizaki rats with type 2 diabetes” (Akiyama, *et al.*, 2009).

Based on the published studies, it was found that Hesperidin is essential to aide in human health maintenance. These benefits included cholesterol lowering, anti-hypertension, anti-infection, anti-inflammatory activity, sedative and antinociceptive effects, nutrigenomic effect, endothelial cells effect, plasma concentration efficacy, bone mineral density increased and treatment cure of memory dysfunction, bleeding bladder varices and diabetes.

1.1.3 Introduction to samples subject

1.1.3.1 Calamansi

Scientific Classification

Kingdom	: Plantae
Order	: Sapindales
Family	: *Rutaceae*
Genus	: *Citrofortunella*
Species	: *C. mitis*

Citrofortunella mitis is a shrub or small tree growing to 3 – 6 metres. The fruit of the Calamansi was resembled by a small, round lime, usually 25-35 mm in diameter. The center pulp and juice was the orange color of a tangerine with a very thin green (unripe form) or orange (fully ripe) colored peel.

Calamansi, was under fruit tree in the family *Rutaceae* native to the Philippine Islands. Calamansi was originated from China and speeded throughout Southeast Asia, India, Hawaii, the West Indies, Central and North America (Morton, 1987).

The plant is characterized by wing-like appendages on the leaf stalks and white or purplish flowers. Its fruit had a spongy or leathery rind with juicy pulp that is divided into sections.

The fruit was indigenous and widely cultivated in the Philippines. Calamansi was available year round in the Philippines and usually appeared in unripened state as a dark green fruit and it turned into tangerine orange color when it ripen.

1.1.3.2 Honey Murcott Tangerine/Thai Honey Tangerine

Scientific Classification

Kingdom : Plantae

Order : Sapindales

Family : *Rutaceae*

Genus : *Citrus*

Species : *C. tangerine*

Tangerine (*Citrus tangerina*) appeared as an orange-colored citrus fruit which was closely related to mandarin orange (*Citrus reticulata*). Tangerines were smaller than common oranges, and were usually easier to peel and split into segments. The taste was considered less sour, but sweeter and stronger, than that of an orange (Pittman & Davis, 1999).

Honey tangerines (murcotts as it is called in the industry) were the most widely grown tangerine (Stephen & Larry, 2012). The Ponkan or Chinese honey tangerine was among the very popular fruit around Melrose, Florida, where it was introduced from China.

1.1.3.3 Kaffir Lime

Scientific Classification

Kingdom : Plantae

Order : Sapindales

Family : *Rutaceae*

Genus : *Citrus*

Species : *C. hystrix*

Citrus hystrix appeared as a thorny bush, 5 - 10m tall, with aromatic and distinctively shaped "double" leaves. The kaffir lime featured a rough, bumpy green fruit. The green lime fruit is distinguished by its bumpy exterior and its small size (approx. 4 cm wide).

Citrus hystrix, commonly known in English as kaffir lime, is a fruit native to Indochinese and Malesian ecoregions in India, Philippines, Indonesia, Malaysia and Thailand, and adjacent countries.

1.1.3.4 Key Lime

Scientific Classification

Kingdom	: Plantae
Order	: Sapindales
Family	: *Rutaceae*
Genus	: *Citrus*
Species	: *C. aurantiifolia*

C. aurantiifolia appeared as a shrubby tree, to 5 m, with many thorns. Its trunk rarely grew straight, with many branches, often originated far down on the trunk. The leaves were ovate in shape, 2.5 – 9 cm long, resembled orange leaves.

The flowers were 2.5 cm in diameter and appeared as yellowish white with a light purple tinge on the margins. Flowers and fruits were appeared throughout the year and particularly abundant from May to September in the Northern Hemisphere (Golob, 1999) (Morton, 1987).

1.1.3.5 Korea Valencia/ South Africa Orange

Scientific Classification

Kingdom	: Plantae
Order	: Sapindales
Family	: *Rutaceae*
Genus	: *Citrus*
Species	: *C. sinensis*

The orange was the fruit of the citrus species *Citrus sinensis* in the family *Rutaceae* [United States Department of Agriculture. (2013). *Citrus sinesis* (L) *Osbeck* (*pro sp.*) (*maxima* and *reticulata*) sweet orange . Retrieved June 4, 2013 from http://plants.usda.gov/java/profile?symbol=CISI3]. The fruit of the *Citrus sinensis* is called sweet orange. The orange was a hybrid between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*), cultivated since ancient times (Nicolosi, *et al.*, 2000).

The orange was originated in Southeast Asia. Orange trees were widely grown in tropical and subtropical climates for its sweet fruit, which can be eaten fresh or processed to obtain juice, and for its fragrant peel [United States Department of Agriculture. (2006). Citrus sinensis information from NPGS/GRIN. Retrieved June 4, 2013 from http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?10782].

There were a few varieties for orange; the Valencia orange is considered a late-season fruit, and it was a popular variety when navel oranges were out of season.

1.1.3.6 Lemon

Scientific Classification

Kingdom	: Plantae
Order	: Sapindales
Family	: *Rutaceae*
Genus	: *Citru*s
Species	: *C. limon*

The lemon (*Citrus limon*) appeared as a small evergreen tree native to Asia, and the tree's ellipsoidal yellow fruit. The juice of the lemon contained about 5% to 6% of citric acid, which gave lemons a sour taste.

The lemon was a hybrid between sour orange and citron based on a study conducted on the genetic origin of the lemon (Gulsen & Roose, 2001).

1.1.3.7 Peel Fresh Orange Juice/ Tropicana Twister Orange Juice

Commercial orange juices that were purchased from the market

1.1.3.8 Red Capsicum

Scientific Classification

Kingdom	: Plantae
Order	: Solanales
Family	: *Solanales*
Genus	: *Capsicum*
Species	: *Capsicum annuum*

Capsicum annuum, (Bell pepper) was known as sweet pepper or a pepper (in the United Kingdom and Ireland) and capsicum (in India, Australia and New Zealand. The cultivars of the plant produced fruits in different colors, including red, yellow, orange, green, chocolate/brown, vanilla/white, and purple.

The Peppers was originated in Mexico, Central America and northern South America.

1.1.3.9 Red Chili

Scientific Classification

Kingdom	: Plantae
Order	: Solanales
Family	: *Solanaceae*
Genus	: *Capsicum*
Species	: *Capsicum frutescens*

Capsicum frutescens was a species of chili pepper that is considered to be part of the species *Capsicum annuum* [Missouri Botanical Garden. (2010). The Plant List. Retrieved June 4, 2013 from http://www.theplantlist.org/tpl/record/kew-2698415]. *Capsicum frutescens* can be cultivated annual or short-lived perennial plants.

Its flowers were white with a greenish white or greenish yellow corolla, and insect- or self-pollinated. The plants' berries typically grew erectly; ellipsoid-conical to lanceoloid shaped.

Capsicum frutescens usually were very small and pungent, 10 – 20 mm long and 3–7 mm in diameter (Solanaceae, 2011). Its fruit typically grew a pale yellow and matured to a bright red. The *Capsicum frutescens* species were originated in South or Central America.

1.1.3.10 Red Onion

Scientific Classification

Kingdom	: Plantae
Order	: Asparagales
Family	: *Amaryllidaceae*
Genus	: *Allium*
Species	: *Allium cepa*

Red onions (purple onions) were cultivars of the onion with purplish red skin and white flesh tinged with red. Red onions appeared to be medium to large in size and have a mild to sweet flavor.

Red onions were available throughout the year. The red color of the red onion came from anthocyanidins such as cyanidin and it had high flavonoids (Gennaro, *et al.*, 2002). The onion plant typically grew to a height of 15 to 45 cm. The leaves were blueish-green and grew alternately in a flattened, fan-shaped swathe. They were fleshy, hollow and cylindrical, with one flattened side. They were at their broadest about a quarter of the way up beyond which they tapered towards a blunt tip. The base of each leaf was a flattened and white sheath that grew out of a basal disc. From the underside of the disc, a bundle of fibrous roots extended for a short way into the soil. As the

onion matured, food reserves began to accumulate in the leaf bases and the bulb of the onion swells (Brickell & Christopher, 1992).

The red onion flower-head appeared as the form of a globular umbel of white flowers with parts in sixes. The seeds were glossy black and triangular in cross section (Brickell & Christopher, 1992).

1.1.4 Objective

The objectives of this study are as follows:

(a) To validate the methods used in term of linearity, accuracy/recovery studies, precision/repeatability, precision/ intermediate precision, limit of detection and quantitation before applying for Hesperidin study.

(b) To evaluate Hesperidin level in citrus fruits, vegetables and commercial fruit juices using Ultra Performance Liquid Chromatography with UV detector at 285 nm

2.1 Literature Review

2.1.1 Review of analytical method for "DETERMINATION OF NARINGIN AND HESPERIDIN IN CITRUS FRUIT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY. THE ANTIOXIDANT POTENTIAL OF CITRUS FRUIT"

2.1.1.1 Sample preparation and extraction technique

The analytical method employed a sample preparation and extraction technique that the fruit was peeled and the pulp was blended to fine slurry. The juice was obtained by mechanical juice maker. Then, the juice was divided into both juice and pulp and stored frozen at −80°C for future analysis. 7.1 g of slurry was weighed and centrifuged at 3000 rpm in a refrigerated Eppendorf centrifuge.

2.1.1.2 Solvent extraction procedures

The supernatant from the extract was separated and stored. The residue was washed with 3 x 5 ml deionized water and the aqueous solutions were combined. The concentration of the solution of the total weight of edible fruit was 286 g/L. The residues were extracted 3 times with dimethyl sulfoxide. The aqueous and DMSO fractions were subjected to HPLC analysis for determination of naringin (sweeties and grapefruits) and hesperidin (oranges).

2.1.1.3 HPLC Instrumentation

The Hesperidin was analyzed using HPLC Shimadzu (Kyoto, Japan) DGU-14A system equipped with a model LC-10AT-VP liquid chromatography pump, an autoinjector, and a diode-array detector. The peak areas calculation was performed using Shimadzu software. Compounds were separated on a Spherisorb ODS1 column from Waters Instruments (MA, USA).

The analysis was using a gradient prepared from 2% aqueous acetic acid, pH 2.58 (component A) and acetonitrile (component B). The composition of the gradient, with 100% component A at 0 – 15 minutes. At time 15 minutes, the composition changed to 70% of component A and 30% of component B. The analysis continued until 50 minutes. At time 50 minutes, 100% of component B is utilized for another 5 minutes. At time 60 minutes, a 100% switch of component B to component A was performed and the analysis ended at 90 minutes.

The injection volume utilized for this analysis was 20 μL (Hamilton syringe; Reno, NV, USA) with the mobile phase flow rate 1 mL/min, the oven temperature was set at 40°C, and the detection wavelength at 285 nm.

Commercial hesperidin samples were also measured under the same conditions.

2.1.1.4 Sample Preparation for Analysis of Polyphenols and Determination of Antioxidant Potential

The extraction and hydrolysis of total polyphenols was performed using 50 mg of lyophilizate and were extracted with 5 ml of 1.2 M of hydrochloric acid in 50:50 % v/v of methanol and water. The samples were vortex mixed for one minute and heated at 90°C for 3 hours. The vortex mixing was repeated every 30 minutes. The samples were cooled, diluted to 10 mL with methanol, and centrifuged for 5 minutes at 4000 g with a bench-top centrifuge, to remove solids (Vinson, *et al.*, 2001). Extraction of polyphenols for determination of antioxidant potential was also performed with water, acetone, methanol, and DMSO.

Total polyphenols were analyzed using Folin–Ciocalteu method and measured at 765 nm with gallic acid as standard (Singleton, *et al.*, 1999). The utilization of Trolox-equivalent antioxidant capacity (TEAC) is based on the ability of antioxidants to scavenge the blue-green $ABTS^{+}$ radical cation (2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) compared with the scavenging ability of the water-soluble vitamin E analogue trolox.

The $ABTS^{\cdot+}$ radical cation was generated by reaction of 250 μM ABTS with 40 μM $K_2S_2O_8$. The absorbance was monitored exactly 1 and 6 minutes after addition of 990 μL $ABTS^{\cdot+}$ solution to 10 μL of fruit extracts or trolox standards with final concentration of 0 – 20 μM in methanol or phosphate-buffered saline pH 7.4. The decrease in absorbance at wavelength 734 nm was calculated and plotted as a function of extract concentration, or of trolox for standard reference data.

For the modified assay, ABTS was dissolved in 20 mM acetate buffer of pH 4.5 and prepared with potassium persulfate. The absorbance was monitored exactly 1 and 6 minutes after addition of 990 μL $ABTS^{\cdot+}$ solution to 10 μL of fruit extracts or trolox standards with final concentration of 0 – 20 μM in methanol or phosphate-buffered

saline pH 7.4. The decrease in absorbance at wavelength 734 nm was calculated and plotted as a function of extract concentration, or of trolox for standard reference data.

The ability of the antioxidants in the fruit samples to reduce ferric tripyridyl-triazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) which absorbed light at 593 nm was measured using the ferric-reducing antioxidant power (FRAP) assay. The complexes of the ferrous and ferric forms of iron with TPTZ were the main products of this reaction. FRAP was calculated by plotting a standard curve of absorbance against concentration of Fe^{2+} standard solution or trolox (Benzie & Strain, 1996)(Szeto, *et al.*, 2002).

In the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay the volume of fruit extracts in different test tubes was adjusted to 100 μL by addition of Methanol and a methanolic solution of 5 μL of 0.1 mM 1,1-diphenyl-2-picrylhydrazyl radical was added to the tubes. The control was prepared in the same way but without extract, and Methanol was used for baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Butylated hydroxyanisole was used for comparison (Singh RP, *et al.*, 2002)(Ozgen, *et al.*, 2006).

Three antioxidant assays (DPPH, ABTS and FRAP) were compared after the same periods of time at 10, 30, 60, and 120 minutes using methanolic extracts of the fruit of the same concentration. For each antioxidant assay trolox was used to establish a standard curve. All data were then were expressed as trolox equivalents (TE).

50 g of pulp was weighed and extracted with water, methanol, acetone, and dimethyl sulfoxide for the oxygen radical absorbance capacity (ORAC) assay. The solutions were combined and subjected to ORAC assay (Huang, *et al.*, 2002) with minor modifications on a fluorescent plate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). The results were expressed as μmol trolox equivalent (TE) per 100 g fresh weight (FW).

3.1 **Sampling, transport, processing and storage of samples**

3.1.1 Sampling

Food samples were taken from the food market randomly in regardless of Origin

3.1.2 Sample Transportation

Samples were packed in original containers or Polythene bags during transportation to prevent degradation

3.1.3 Sample preparation and processing prior to analysis

The sample subject was identified by product type. Samples from perishable such as fruits or vegetables were prepared fresh prior analysis. Commercial fruit juices was stored at refrigerator at temperature below $4^{o}C$ prior analysis

3.2 Glassware

All glassware were soaked overnight in 2% Decon 90 (Potassium Hydroxide) diluted with water. Then it was cleaned thoroughly with brush and rinsed 3 times with distilled water. The glassware was oven dried at $105^{o}C$ a day prior analysis.

3.3 Standards, calibration solution, etc.

3.3.1 <u>Identity, purity, and storage of standards</u>

Pure standard of Hesperidin is received from Sigma on 19 November 2012. The Hesperidin came with a product number of H5254, part code 1001131563 and lot number 011M1865V.

Hesperidin standard appeared to be a light beige color powder. Hesperidin appeared as slightly hazy when solubility in 50 mg/L of pyridine was conducted.

It was found that the pure standard was extracted from the peel of *Citrus lemon* and *Citrus sinesis* originated from Spain.

The assayed purity of this Hesperidin standard was 95% with recommended storage temperature of 2 – 8 ^{o}C.

Figure 3.1 illustrated the FTIR spectrum of Hesperidin pure standard. It is observed that the maximum IR absorption was strong at 1260 cm^{-1} (methyl group), 750–800 cm^{-1} (aromatic group), 3000-2900 cm^{-1} (O-H group), 1680–1690 cm^{-1} (C=O group).

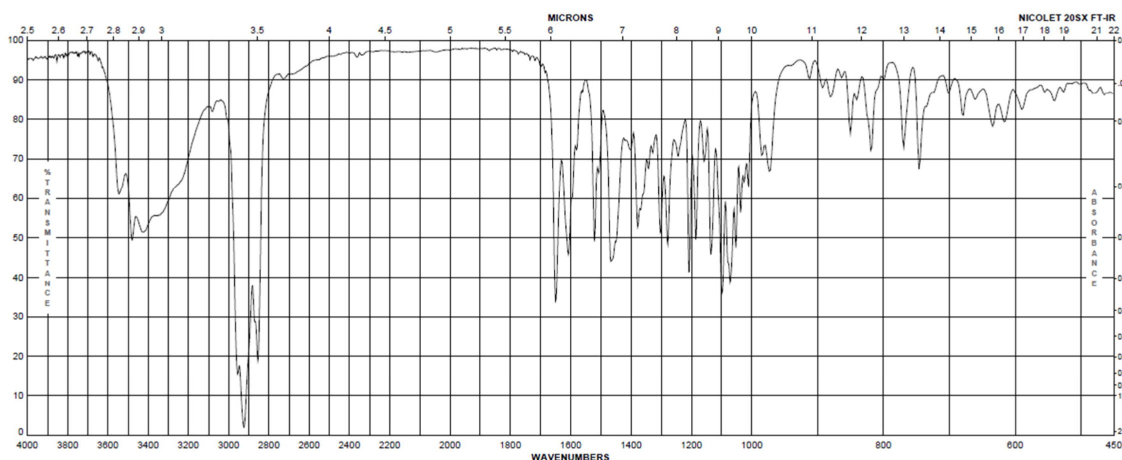


Figure 3.1 FTIR Spectrum of Hesperidin Pure Standard

3.3.2 Preparation and storage of stock standards

Hesperidin Pure Standard was obtained from Sigma (Spain, Purity 95%). A low standard stock was prepared by weighing 58.2 mg of Hesperidin Pure Standard and diluted to 50 ml of 72:28% v/v with 5mM ammonium acetate, pH 4.45 and acetonitrile. The final concentration of this low standard Hesperidin stock would be 1105.8 ppm.

Another set of high standard stock was prepared by weighing 2637.7 mg of Hesperidin Pure Standard and diluted to 50 ml of 72:28% v/v with 5mM ammonium acetate, pH 4.45 and acetonitrile. The final concentration of this high standard Hesperidin stock would be 50116.3 ppm.

Both low and high Hesperidin stock was kept in an amber glass bottle and stored at 2 – 8 ^{o}C.

3.3.3 Preparation, use and storage of working standards

Hesperidin working standard was prepared by weighing accurate amount of stock standard and diluted to corresponding concentration level with 72:28% v/v with 5 mM ammonium acetate, pH 4.45 and acetonitrile as illustrated in Table 3.1

The low working Hesperidin standard consisted of 4.97 ppm, 62.79 ppm, 108.68 ppm, 190.65 ppm, 326.73 ppm and 1105.80 ppm. The high working Hesperidin standard consisted of 5610.83 ppm, 10140.08 ppm and 50116.30 ppm.

Table 3.1 Low & High Working Standard

No.	Working Standard	Concentration (ppm)
1	Low	4.97
2	Low	62.79
3	Low	108.68
4	Low	190.65
5	Low	326.73
6	Low	1105.80
7	High	5610.83
8	High	10140.08
9	High	50116.30

All low and high working Hesperidin standards was kept in an amber glass bottle and stored at 2 – 8 ^{o}C.

3.4 Extraction and concentration

3.4.1 Extraction conditions

This study employed a sample preparation and extraction technique that the whole fruit was extracted using Fruit Juice Extractor at ambient temperature and the obtained juice was filtered with 0.22 micron filter and stored at 2 – 8 ^{o}C prior analysis as illustrated in Table 3.2.

The Hesperidin evaluation was performed at 3 different group such as fruits (Citrus origin), vegetables and commercial fruit juice. The fruits (Citrus origin) under analysis were Calamansi, Honey Murcott Tangerine, Kaffir Lime,

Key Lime, Korea Valencia, Lemon, South Africa Orange and Thai Honey Tangerine.

The vegetables under analysis were Red Capsicum, Red Chili and Red Onion. As for the analysis of commercial fruit juices, it was randomly picked from the available market.

Table 3.2 Extraction Condition & Temperature for Sample Subject

No.	Product	Type	Extraction Condition	Extraction Temperature
1	Calamansi	Fruit	Whole fruit was extracted using Fruit Juice Extractor and filter through 0.22 micron filter	Ambient
2	Honey Murcott Tangerine	Fruit		
3	Kaffir Lime	Fruit		
4	Key Lime	Fruit		
5	Korea Valencia	Fruit		
6	Lemon	Fruit		
7	Peel Fresh Orange Juice	Commercial fruit juice	Directly filter through 0.22 micron filter	Ambient
8	Red Capsicum	Vegetable	Whole vegetable was extracted using Fruit Juice Extractor and filter through 0.22 micron filter	Ambient
9	Red Chili	Vegetable		
10	Red Onion	Vegetable		
11	South Africa Orange	Fruit	Whole fruit was extracted using Fruit Juice Extractor and filter through 0.22 micron filter	Ambient
12	Thai Honey Tangerine	Fruit		
13	Tropicana Twister Orange Juice	Commercial fruit juice	Directly filter through 0.22 micron filter	Ambient

3.4.2 Extract concentration and dilution to volume

The determination of sample subject dilution was solely based on the whole fruit extraction condition. In this analysis, it was found that Honey Murcott Tangerine and South Africa Orange required a 1:1 dilution with distilled water due to the thick slurry obtained during extraction. However, Calamansi, Kaffir Lime, Key Lime, Korea Valencia, Lemon and Thai Honey Tangerine did not require further dilution as the extracted juice able to be filtered through 0.22 micron filter. As for Red Capsicum, Red Chili Red Onion and commercial fruit juices, these samples also did not require further dilution. The requirement of sample subject dilution was illustrated in Table 3.3.

Table 3.3 Sample Subject Dilution

<table>
<tr><th>No.</th><th>Product</th><th>Dilution</th></tr>
<tr><td>1</td><td>Calamansi</td><td>Nil</td></tr>
<tr><td>2</td><td>Honey Murcott Tangerine</td><td>1 : 1</td></tr>
<tr><td>3</td><td>Kaffir Lime</td><td rowspan="8">Nil</td></tr>
<tr><td>4</td><td>Key Lime</td></tr>
<tr><td>5</td><td>Korea Valencia</td></tr>
<tr><td>6</td><td>Lemon</td></tr>
<tr><td>7</td><td>Peel Fresh Orange Juice</td></tr>
<tr><td>8</td><td>Red Capsicum</td></tr>
<tr><td>9</td><td>Red Chili</td></tr>
<tr><td>10</td><td>Red Onion</td></tr>
<tr><td>11</td><td>South Africa Orange</td><td>1 : 1</td></tr>
<tr><td>12</td><td>Thai Honey Tangerine</td><td>Nil</td></tr>
</table>

13	Tropicana Twister Orange Juice	Nil

3.5 Experimental

3.5.1 Chemicals and reagents

The use of high purity reagents and solvents help to minimize analysis interference. HPLC-grade acetonitrile was purchased from Tedia with part code AS1122-001 and lot number 1006236. AR Grade of Ammonium acetate was purchased from QRec with part code A 5034-1-1000 and lot number 114834-1214.

3.5.2 Instrumentation

The Hesperidin was analyzed using Acquity, UPLC, H-Class (MA, USA), an auto-injector, and a photo diode-array detector (PDA). The peak areas calculation was performed using Empower software. Compounds were separated on a Acquity UPLC HSS T3, 1.8 micron, 2.1 x 100 mm column, part number 186003539 from Waters Instruments (MA, USA).

The analysis was using an isocratic mobile phase prepared from 5mM ammonium acetate, pH 4.45 (component A) and acetonitrile (component B). The composition of the isocratic mobile phase with 72% component A and 28% of component B at all time. The run time set for this analysis was 5 minutes.

The injection volume utilized for this analysis was 1 µL (Waters syringe; MA, USA) with the mobile phase flow rate 0.4 mL/min, the oven temperature was set at 40°C, and the detection wavelength at 285 nm. The observed pressure for this analysis is about 7698 psi.

Hesperidin samples of fruits, vegetables and commercial fruit juice origin were also measured under the same conditions.

3.5.3 Preparation of mobile phase

5 mM ammonium acetate, pH 4.45 was prepared by weighing an accurate amount of 0.3854 g ammonium acetate and diluted to 1L with distilled water. The buffer was adjusted to pH 4.45 with acetic acid.

The mobile phase that used for this analysis was prepared by adding 280 ml of HPLC-grade acetonitrile in 1000 ml of 5 mM ammonium acetate, pH 4.45 (28:72, %v/v). Then, the mobile phase was filtered under a vacuum through 0.22 micron nylon filters and degassed before use.

3.6 Results & Discussion

3.6.1 Validation of the chromatographic method

3.6.1.1 Linearity and range

Linearity was determined by using low and high Hesperidin working standard at nine different concentrations between 4.97 and 50116.30 ppm. The slope and intercept values together with relative standard deviations were determined using the regression analyses. The responses of Hesperidin were linear in the range of study with regression coefficients of 0.9903. The results obtained were illustrated in Table 3.4 and Figure 3.2.

Table 3.4 Results of assessment of the linearity of the UPLC method for the assay of Hesperidin employing the analytical working standard dissolved in mobile phase

Concentration (ppm)	Peak area as mean of 6 injections (n=6)	Peak area RSD (%)
4.97	3553.4	0.7
62.79	10171.2	0.5
108.68	14043.6	0.2
190.65	20293.6	0.7
326.73	29120.4	0.2
1105.80	124377.5	0.2
5610.83	117918.6	0.7
10140.08	188332.5	0.2
50116.30	1179517.5	0.4
Correlation coefficient: $r^2 = 0.9903$; Equation for regression line: $y = 23.392x + 13089$ (n = 6)		

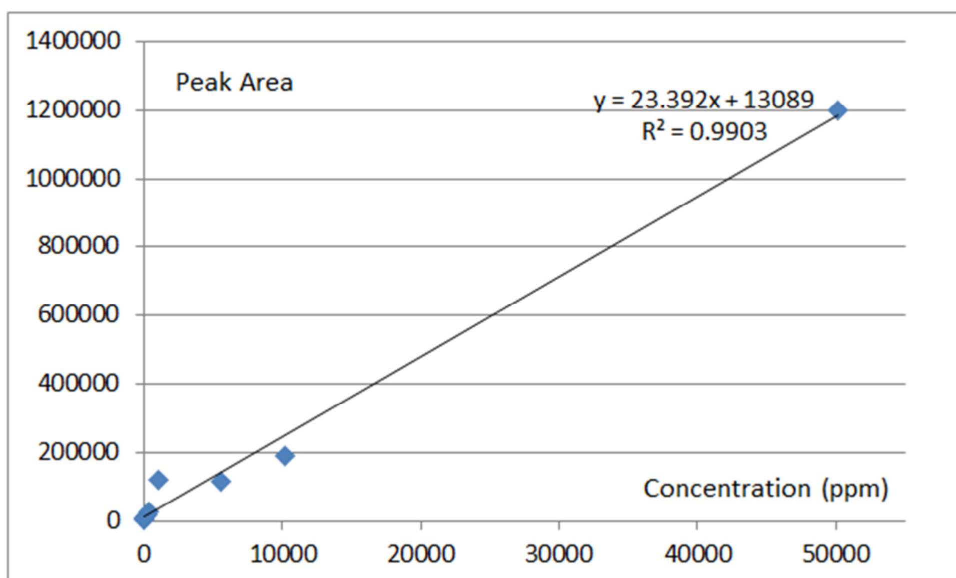


Figure 3.2 Graph measured peak area versus Hesperidin concentration demonstrating linearity

Acceptability of linearity data was judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r^2) is > 0.99 of 9 different concentrations was generally considered as evidence of acceptable fit of the data to the regression line.

In this study, linearity was studied in the concentration range 4.97-50116 ppm (n = 6) and the following regression equation was found by plotting the peak area (y) versus the Hesperidin concentration (x) expressed in ppm: $y = 23.392x + 13089$ ($r^2 = 0.9903$). The demonstration coefficient (r^2) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of Hesperidin (Figure 3.2). The data obtained from linearity experiments are presented in Table 3.4.

The range was derived from linearity studies, and the assay procedure confirmed providing an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within, or at the extremes of the specified range, of the test method. In this case, the concentration range was from 4.97 to 50116 ppm.

3.6.1.2 Accuracy/recovery studies

Accuracy/recovery studies were determined by comparing obtained concentration from samples and expected concentrations. In this study, QC samples of known concentration of 1000.85 ppm were performed for 6 replicates. The recoveries were calculated versus expected concentration as illustrated in Table 3.5.

Table 3.5 Accuracy/recovery of Hesperidin from QC Samples with known concentration

Sample Number (n=6)	**Nominal (ppm)**	**Recovery Amount (ppm)**	**Recovery (%)**
1	1000.85	1001.359	100.05%
2	1000.85	1002.134	100.13%
3	1000.85	1003.720	100.29%
4	1000.85	1000.034	99.92%
5	1000.85	1002.161	100.13%
6	1000.85	1002.810	100.20%
Mean recovery: 100.12%; RSD 0.1%			

The accuracy of an analytical method was the closeness of test results obtained by that method to the true value. Accuracy can be assessed by analyzing a sample of known concentration (reference materials), and comparing the measured value to the true value. Accuracy criteria were that the mean recovery would be 100 ± 2% of measured value to the true value.

In this study, known added amounts of Hesperidin QC Samples were injected and percent recoveries of response factor (concentration) were calculated. The results

of accuracy studies are shown in Table 3.5 indicated the mean recovery was 100 ± 2%, and it was evident that the analytical method was accurate.

3.6.1.3 <u>*Precision/Repeatability*</u>

Precision/Repeatability measured the degree of repeatability of an analytical method under normal operation and expressed as percent relative standard deviation (RSD%). Repeatability (intra-day) is the ability of an analytical method when repeated several times in a single day by single analyst to give the same results. Repeatability of analytical method was calculated by analyzing 0.09992 ppm samples in 10 replicates in intra-day precision study as illustrated in Table 3.6.

Table 3.6 Demonstration of the repeatability of the UPLC assay for Hesperidin as shown by the results of 10 replicate injections of one solution at 0.09992 ppm concentration

Injection Number (n=10)	**RT (min)**	**Peak Area**
1	1.175	118
2	1.177	120
3	1.174	121
4	1.173	118
5	1.178	122
6	1.171	118
7	1.173	120
8	1.170	120
9	1.177	119
10	1.173	121

Mean	1.174	120
RSD (%)	0.23%	1.18%

A precision criterion at limit of quantitation in instrument precision (repeatability) RSD would be ≤ 5%. In this study, precision of the method was evaluated through the repeatability of the method (intra-assay precision) by assaying ten replicate injections of Hesperidin at the same concentration (0.09992 ppm), during the same day, under the same experimental conditions. The RSD values of the retention time and area of Hesperidin peak were found to be 1.18%, as presented in Table 3.6 and it was evident that the analytical method was precise.

3.6.1.4 Precision/ Intermediate Precision

Precision/ Intermediate Precision measured the degree of reproducibility (inter-day) of an analytical method when repeated several times in different day and if possible different analysts and laboratories to give the same results.

Reproducibility of analytical method was calculated by analyzing 190.65 ppm samples in 3 replicates in inter-day precision study as illustrated in Table 3.7.

Table 3.7 Demonstration of the intermediate precision of the HPLC assay for Hesperidin results in QC Samples 190.65 ppm recoveries

Operator 1 (n=3)	**Recovery Amount (ppm)**	**Recovery (%)**
Day 1	190.973	100.17%
Day 2	190.465	99.90%
Mean	190.719	100.04%
RSD (%)	0.19%	0.19%

Intermediate precision (inter-day variation) was the results obtained from within lab variations, due to random events, such as different days. A precision criterion for an assay method was that the intra-assay precision will be ≤ 2%.

In this study, intermediate precision (within-laboratory variation) was demonstrated by different days using same UPLC system and evaluating the relative percent purity data across the two days at same concentration level 190.65 ppm. The mean and RSD across the different days were calculated from the individual recovery. The RSD values presented in Table 3.7 were 0.19% for different days and illustrated the good precision of the analytical method.

3.6.1.5 Limit of Detection and Quantitation

The instrument limit of detection was determined from 10 replicates injection of standard solution with low concentration that gave peak area detection at the signal-to-noise of 3 as illustrated in Table 3.8.

Table 3.8 Demonstration of Limit of Detection of the UPLC assay for Hesperidin as shown by the results of 10 replicate injections of one solution at 0.09992 ppm concentration

Injection Number (n=10)	**RT (min)**	**Peak Area**
1	1.175	118
2	1.177	120
3	1.174	121
4	1.173	118
5	1.178	122
6	1.171	118
7	1.173	120
8	1.170	120
9	1.177	119
10	1.173	121
Mean	1.174	120
RSD (%)	0.23%	1.18%

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyte.

LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. In this study, LOD for a 1 µL injection of Hesperidin standard was 0.09992 ppm.

LOQ is defined as the lowest amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N = 10 which equivalent to 3.333 LOD. In this study, LOQ for a 1 µL injection of Hesperidin standard was 0.333 ppm.

3.6.1.6 Stability of Analytical Solutions

The stability of Analytical Solution was assessed using Samples and standards that were tested over 24 hours period (for Hesperidin assay) and quantitation of QC samples was determined by comparison to freshly prepared standards. The stability of analytical solution was determined from 3 replicates injection of QC Samples 190.65 ppm as illustrated in Table 3.9.

A stability criterion for assay methods was that sample and standard solutions and the mobile phase were stabled for 24 hours under storage at 2 – 8 ^{o}C. Stability is considered to be acceptable when the change in the standard or QC sample recovery is within 2% relative to freshly prepared standards.

Table 3.9 Stability of Hesperidin in QC Samples 190.65 ppm

Operator 1 (n=3)	**Recovery Amount (ppm)**	**Recovery (%)**
Day 1	190.973	100.17%
Day 2	190.465	99.90%
Difference	0.508	0.270%

In this study, the stability of Hesperidin solutions was investigated. Hesperidin was chromatographed at the beginning, and after 24 hours. The stability of Hesperidin and the mobile phase were calculated by comparing recovery at 190.65 ppm over time. QC solution was stored in a capped volumetric flask under storage at 2 – 8 ^{o}C for 24 hours, and was shown to be stabled with no significant change in Hesperidin concentrations over this period (Table 3.9). This was indicated by 0.270% changes in recovery between T = 0 hours and T = 24 hours. Based on analyzed data, the data shown quantitative recovery through 24 hours, Hesperidin solutions can be assayed within 24 hours of preparation.

3.6.1.7 System Suitability

System suitability tests were an integral part of HPLC methods, and used to verify that the accuracy and precision of the system were adequate for the analysis to be performed. Although there was no defined USP guideline for System Suitability of UPLC methods, parameters, such as repeatability (RSD of retention time and area for six repetitions) were determined and compared against the specifications set for the method. The system suitability of analytical solution was determined from 6 replicates injection of QC Samples 1000.85 ppm as illustrated in Table 3.10.

Table 3.10 Demonstration of the system suitability of the UPLC assay in QC Samples 1000.85 ppm for Hesperidin

Injection Number (n=6)	**RT (min)**	**Peak Area**
1	1.167	74947
2	1.175	74996
3	1.175	75096
4	1.171	74863
5	1.172	74998
6	1.172	75039
Mean	1.172	74989.83
RSD (%)	0.25%	0.11%
System Suitability Parameter	**Acceptance Criteria**	**Results**
Injection precision for area (n = 6)	RSD ≤ 1%	0.11%
Injection precision for retention time (min)	RSD ≤ 1%	0.25%

In this study, the system suitability test was demonstrated with the accuracy and precision of the system, by injecting six injections of a solution containing 1000.85 ppm of Hesperidin. The obtained RSD for peak area and retention time was 0.11% and 0.25% respectively as illustrated in Table 3.10 and it was evident that the analytical method met system suitability criteria.

3.6.2 Application of analytical method to samples

The proposed analytical method using UPLC was applied for the determination of Hesperidin in three different group of samples subject such as fruit, vegetables and commercial fruit juices. The sample Hesperidin level was determined from 2 replicates injection of sample solution as illustrated in Table 3.11.

Table 3.11 Hesperidin level in Food

No.	Product (n=2)	Type	Dilution	Hesperidin from analysis (ppm)	Hesperidin Actual (ppm)
1	Calamansi	Fruit	Nil	7003	7003
2	Honey Murcott Tangerine	Fruit	1 : 1	31208	62416
3	Kaffir Lime	Fruit	Nil	71875	71875
4	Key Lime	Fruit	Nil	19454	19454
5	Korea Valencia	Fruit	Nil	29361	29361
6	Lemon	Fruit	Nil	73053	73053
7	Peel Fresh Orange Juice	Commercial fruit juice	Nil	9331	9331
8	Red Capsicum	Vegetable	Nil	9850	9850
9	Red Chili	Vegetable	Nil	883	883
10	Red Onion	Vegetable	Nil	5346	5346
11	South Africa Orange	Fruit	1 : 1	292632	585264
12	Thai Honey Tangerine	Fruit	Nil	17773	17773
13	Tropicana Twister Orange Juice	Commercial fruit juice	Nil	9670	9670

Figure 3.3 illustrated the Hesperidin level in the analyzed food. It was found that the top three Hesperidin contribution in food were originated from citrus fruits

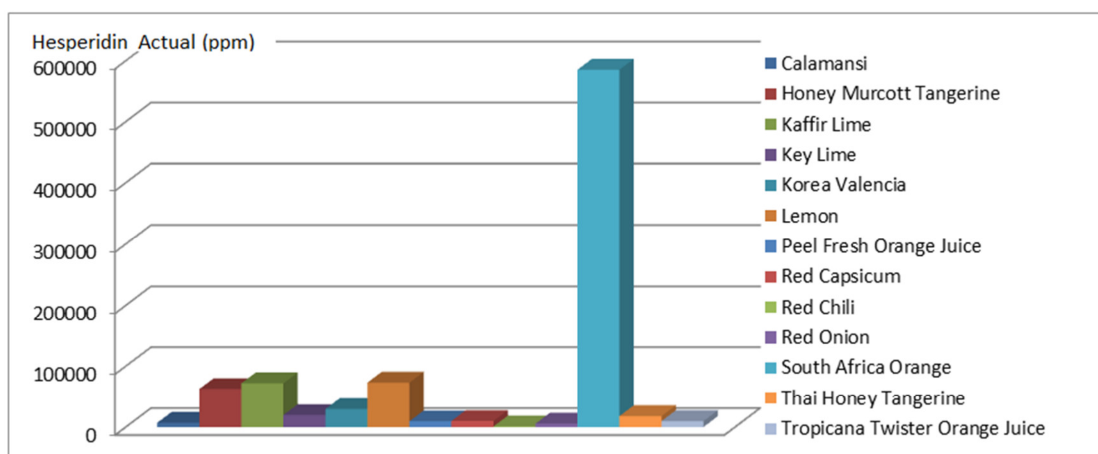


Figure 3.3 Comparison graph of Hesperidin level in Food

Hesperidin level in citrus fruits, vegetables and commercial fruit juices using Ultra Performance Liquid Chromatography with UV detector at 285 nm was evaluated. The Hesperidin level in citrus fruits ranged from 7003 – 585264 ppm. The Hesperidin level with concentration of 7003 ppm was present in Calamansi sample. A concentration of 62416 ppm Hesperidin was detected for Honey Murcott Tangerine. As for Kaffir Lime, 71875 ppm of Hesperidin was found.

Key Lime exhibited Hesperidin level of 19454 ppm and Korea Valencia was found with 29361 ppm of Hesperidin. The Hesperidin level with concentration of 73053 ppm was present in Lemon sample. The Hesperidin level of South Africa Orange and Thai Honey Tangerine were 585264 ppm and 17773 ppm respectively.

As for the vegetables group, Hesperidin with concentration of 9850 ppm was present in Red Capsicum sample. The Hesperidin level of Red Chili and Red Onion were 883 ppm and 5346 ppm respectively. The Hesperidin level in commercial fruit juices ranged from 9331- 9670 ppm.

It has been shown that citrus fruit is a major Hesperidin contributor in food. Based on the analysis as per Figure 3.3, the Hesperidin contribution was in the order South Africa Orange >>> Lemon = Kaffir Lime > Honey Murcott Tangerine > Korea Valencia > Key Lime > Thai Honey Tangerine.

As for vegetables, the amount of Hesperidin level was quite low compared with citrus fruits. The Hesperidin contribution was in the order Red Capsicum > Red Onion > Red Chili. Commercial fruit juices exhibited higher Hesperidin level compared with vegetables but less with citrus fruits which was in the range of 9000 – 10000 ppm.

UPLC chromatograms obtained from the sample subject extraction shown that Hesperidin was detected at UV 285 nm and at retention times (RT) of 1.17 minutes. The linear regression line of the calibration plot was extrapolated for Hesperidin level determination in Kaffir Lime, Lemon and South Africa Orange due to its high Hesperidin concentrations.

Based on the food subject under study, it was observed that South Africa Orange exhibited the highest Hesperidin level in the citrus fruits grouping. Under the analyzed vegetables groups, it was found that Red Capsicum had the highest Hesperidin level. Commercial fruit juices Hesperidin levels were in between the citrus fruits and vegetables group.

4.1 Conclusion

Hesperidin analysis in food is a simple application involving chromatographic based technique. The challenges would be maintaining the stability of Hesperidin during the duration of the study. This is accomplished by keeping the analyte under controlled storage condition and faster analysis cycle time to overcome the degradation of this compound.

The objectives of this study was the method development and validation of the fast and reliable Ultra Performance Liquid Chromatography technique for the determination of Hesperidin belong to different group of food including citrus fruits, vegetables and commercial fruit juices.

In the sample treatment methodology, a direct extraction technique was selected as the more suitable method for routine analysis of Hesperidin in food sample. This technique reduced extraction losses and achieved short cycle time to minimize sample degradation.

This method gave satisfactory analytical performance parameters for most targeted Hesperidin and analysis of food samples proved its feasibility for the intended purpose. A satisfactory of achieved linearity was observed based on the calibration curve plotted in a nine different concentrations with 6 replicates.

The accuracy of the analytical method was proven with achieved mean recovery of 100 ± 2%, and this method is considered precise as documented in the analysis data above. The establishment of limit of detection at 0.09992 ppm and limit of quantitation at 0.333 ppm is applicable to low level of Hesperidin study.

Based on all analyzed data gathered, this analytical method using Ultra Performance Liquid Chromatography was validated and found to be suitable for the application of Hesperidin study.

In the application of Hesperidin study, it was found that by using chromatographic technique of Ultra Performance Liquid Chromatography with UV detector at 285 nm, the obtained Hesperidin level of citrus fruits were among the highest Hesperidin contributor of the group of fruits, vegetables and commercial fruit juices that were evaluated.

In the fruit category, the highest contributor of Hesperidin level was found to be originated from the South Africa Orange. However, with the similar plant species i.e. Korea Valencia had a lower Hesperidin level compared with South Africa Orange. As both oranges were planted in different locations e.g. South Africa and Korea, thus it was deduced that different locations of the plantation give rise to different Hesperidin level in the fruit. This is further confirmed with different Hesperidin level found in tangerine species of Honey Murcott Tangerine and Thai Honey Tangerine.

In the vegetable category, Red Capsicum was among the highest Hesperidin contributor in this group. However, the Hesperidin level in this group had a much lower Hesperidin level compared with the citrus fruit group. Commercial fruit juices also shown a lower Hesperidin level compared with the citrus group. Thus, it was deduced that Hesperidin level is predominant in citrus fruits family.

5.1 Supplementary

5.1.1 Appendix A

Refer to Appendix A for data sources and raw data.

5.1.2 Bibliography

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