De-Bittering of Kinnow Juice

Peeling

Manual Separation
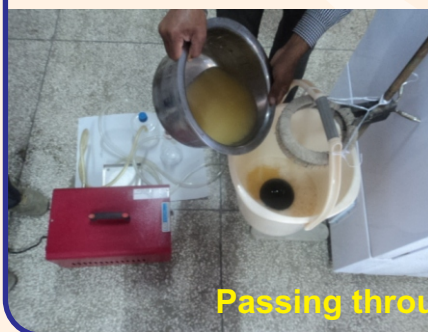
Juice Extraction

Passing through adsorbent

Pasteurized Juice

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PREFACE

Citrus fruits are considered as one of thenutritionallyimportantfruits because these are rich source of β-carotene (Vitamin A source), ascorbic acid (Vitamin C), folic acid and various minerals. Kinnow has emerged as one of the major citrus crops of Punjab and accounts for 45% of its total fruit production. It is known for its superior characters such as heavy bearing, wide adaptability, and excellent fruit quality. Its fresh fruits are available from January to April. Though fresh fruit juice is a common menu in abroad, but in India, fruit juice is still a luxury. During the fruiting season, fresh knnow juice stalls are found in every nook and corner, while abroad commercially manufactured citrus juices like squash, cordial, concentrates etc. are common. The western world does like the citrus juices with slight bitter taste seeing their medicinal effect; however, in countries like India, we do like sweet and tasty juice without bitterness. Kinnow juice tastes sweet immediately after processing but it turns bitter within 6-8 hrs of processing. This limits its consumption as stored juices and their products. This bitter taste is attributed to two major components: naringin (a water soluble flavonoid) present mainly in fruit peel causing initial bitterness and limonin (a water insoluble limonoid) present mainly in citrus seeds causing delayed bitterness due to action of a hydrolytic enzyme, limonoate-D-ring lactone hydrolase. As a result juice industries dealing with kinnow fruit processing are crippling due to lack of suitable low cost process for de-bittering of kinnow juice.

A number of physiochemical and biotechnological approaches have been attempted to reduce bitterness in citrus juices below threshold level for acceptability. These include technologies such as adsorptive de-bittering, chemical methods, treatment with polystyrene divinyl benzene styrene (DVB) resins and β-cyclodextrin etc. Among the physiochemical approaches, various chemicals and adsorptive resins etc. have been employed whereas for biotechnological approaches, use of bacterial cells, immobilized enzymes and cells have been attempted. However, some of these processes are not met with desired success while others are too costly to be adopted by industries. The current technical bulletin entails viable process of de-bittering kinnow juice using hurdle technology.

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Introduction

Kinnow (*Citrus reticulata*) is a first generation hybrid of "King" and "Willow leaf" mandarins (*Citrus nobilis and Citrus deliciosa*) which was evolved by late Dr H.B. Frost at University of California, Regional Fruit Station, USA (Lotha and Khurdiya, 1994; Joshi *et al*., 1997) and introduced in India during early 1940's. It is one of the major citrus fruit crops of northern India with an annual production of over 0.4 million metric tonnes (Khandelwal *et al*., 2006). According to the latest data, the annual production of mandarins (including oranges and kinnows) was about 3.43 million metric tons with an area of cultivation of about 330 thousand hectares for year 2013-14 (National Horticulture Board database, 2015). Its cultivation has assumed great importance among the growers and a large acreage of land is being brought under cultivation particularly in Punjab, Haryana, Rajasthan and Himachal Pradesh. Fully ripe fruits of kinnow have bright and deep attractive color, thin tight and compact skin. The fruits are juicy and freshly extracted juice from the fruit harvested at appropriate stage of maturity has refreshing flavor, characteristic pleasing aroma and thirst quenching properties.

Presently 95% of kinnow production goes for fresh fruit market. During peak periods, there is glut in the market, prices fall down drastically and due to poor post-harvest infrastructure, wastage of kinnow is around 25-30% and that only 5% of the total production is processed (Khandelwal *et al*., 2006). In order to fully utilize the high production of kinnow, it is necessary to process it into juices and other juice based products. However, processing of kinnow into juice has intimidating problems of bitterness development (Puri *et al*., 2005) due to the presence of two bittering components – limonin (a limonoid) and naringin (a flavanoid). Presence of these compounds in extracted juice adversely affects its consumer acceptability (Hasegawa *et al*., 1989; Ferreira *et al*., 2008). Naringin causes initial bitterness while limonin is responsible for causing delayed bitterness. Naringin is found more in peel while limonin is concentrated mainly in seeds. In order to solve the ever-existing problem of bitterness, it is imperative to think over two types of bitterness i.e. primary bitterness (by naringin) and delayed bitterness (by limonin). Seeing the huge potential of this horticultural crop for juice processing industry, it is imperative to evolve a suitable low cost technology by using physical, chemical and/or enzymatic methods for de-bittering of kinnow juice for a viable juice processing industry for growers and processors.

A number of physiochemical and biotechnological approaches have been attempted to reduce bitterness in citrus juices below threshold level for consumer acceptability. These include technologies such as adsorptive de-bittering, chemical methods, treatment with polystyrene divinyl benzene styrene (DVB) resins and β-cyclodextrin (Thammawat *et al*., 2008). All these approaches have been focused on curative means to attack the problem of bitterness. Some processes are not met with desired success while others are too costly to be adopted by industries. Previously, de-bittering was tried using various resins as adsorbents like polystyrene divinyl benzene and Indion NPA1 at ICAR-CIPHET, Abohar (Singh *et al*., 2008, 2009). However, polystyrene divinylbenzene is being used by all 34 commercial juice de-bittering units operating worldwide (Shaw *et al*., 2000). But the high cost involved in using these resins is major constraint in citrus processing industry.

Causes of bitterness- Role of naringin and limonin in juice bitterness

Kinnow fruit can broadly be divided into peel, juice sacs and seeds. The juice sacs are bonded in partitioned septa (Fig. 1). Kinnow peel can be divided in to two parts: outer colored layer is called flavedo which is having colored pigments and oil cells (from which essential oil can be extracted). Inner white colored rags and layer is called albedo which is
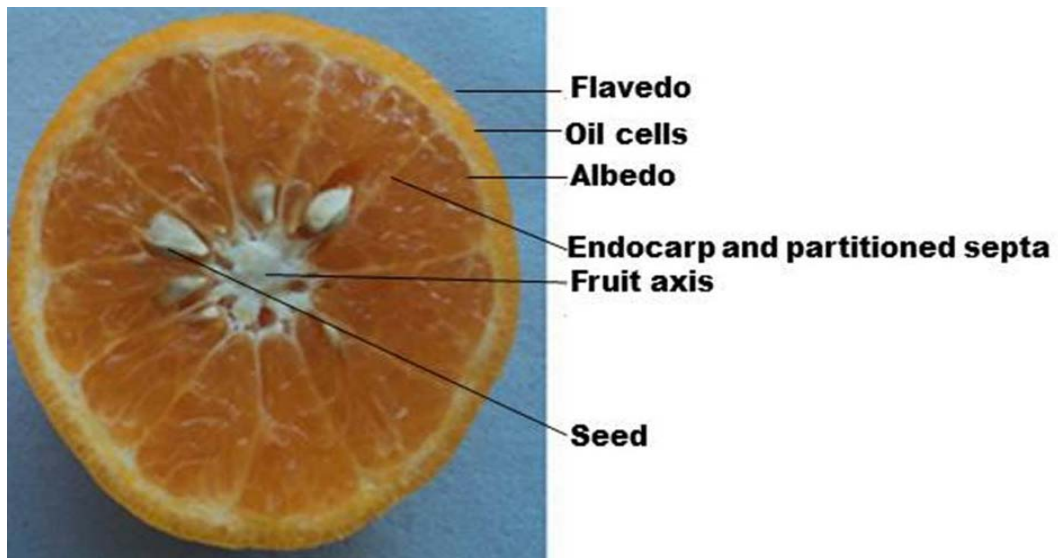
Fig. 1: Cross-section of kinnow showing various fruit portions

rich in pectin and has bitterness due to a flavonoid, naringin. Seeds are wedge shaped present in center at fruit axis in varying numbers from 3-24, depending upon size, variety and growth of fruit. Seeds are intensely bitter and contain a limonoid, limonin. Method of extraction plays an important role on contents of bitter principles in citrus juices (Lotha *et al.*, 1994; Singh *et al.*, 2003). The extraction of juice from citrus fruits (intact or peeled) can be accomplished by hydraulic pressing, expressing, reaming or by squeezing. The juice obtained by squeezing or peeling fruit and extraction of juice in such a way that seeds are not crushed, with a soft press was found to be better than other methods, but the only disadvantage is lesser juice recovery (Premi *et al.*, 1994). Limonin is a highly oxygenated triterpenoiddilactone which occurs naturally in plants from the *Rutaceae* and *Meliaceae* families, and which is particularly abundant in seeds (Roy and Saraf, 2006). It is an intensely bitter triterpenoiddilactone which develops gradually in juices after juice extraction and is responsible for post-processing/delayed bitterness. The intact fruits do not normally contain limonin rather a non-bitter precursor, a limonoate-A-ring lactone (Khandelwal *et al.*, 2006). Once the juice is extracted, this conversion is accelerated by the action of enzyme limonoate-D-ring lactone hydrolase that is present in citrus fruits (Ferreira *et al.*, 2008). Naringin (4,5,7-trihydroxyflavonone-7-rhamnoglucoside), on the other hand, is the primary bittering water-soluble component in fruit membrane and albedo, which gets extracted in fruit juices.

Review of previously used methods: A brief review of previously used methods for de-bittering of citrus juice given in Table 1 below.

Table 1: Previously used methods for de-bittering of citrus juice

S. No.	Approach used	Fruit	Reduction in bittering compounds		Reference
			Naringin	**Limonin**	
1.	β-cyclodextrin	Orange and grapefruit	33-47%	29-59%	Shaw (1990)
2.	α-cyclodextrin	Citrus	50%	50%	Shaw and Wilson (1985)
3.	Ethylene	Citrus		50%	Maier *et al.* (1977)
4.	Naringinase	Kinnow	76%		Puri *et al.* (2005)
5.	Naringinase	Grapefruit	75%		Ferreira *et al.* (2008)
6.	Limonoate dehydrogenase	Kinnow		66%	Puri *et al.* (2002)

S. No.	Approach used	Fruit	Reduction in bittering compounds		Reference
			Naringin	**Limonin**	
7.	Amberlite XAD-16HP	Washington navel orange		97-100%	Kola (2005)
8.	Dowex Optipore L285	Washington navel orange		95-99%	Kola (2005)
9.	Polyvinyl pyrrolidone	Grapefuit	78%	17.5%	Nisperos and Robertson (1982)
10.	Amberlite XAD-2	Citurs		100%	Chandler and Johnson (1977)
11.	Polystyrene divinyl benzene	Citrus	70%	80%	Puri (1984)
12.	Anion exchange styrene polymer	Citrus	97%	100%	Mitchell *et al.* (1985)
13.	Cellulose acetate powder	Orange	8%	70%	Cheng (1990)

Effect of fruit ripening on bittering components of kinnow juice: Kinnow fruits were analyzed at different ripening stages at monthly interval from September, 2014 to April, 2015 for their bittering factors and other biochemical parameters. Both naringin and limonin content decreased with the advancement of fruit ripening which indicated that fruit harvested at later stage has less tendency to turn in to bitter juice as compared to its early harvesting season. Naringin content decreased from an initial value of 396.13 ppm (September month harvested fruits) to 146.2 ppm (April month harvested fruits) while the respective decrease in case of limonin was from 52.37 to 12.7 ppm (Fig. 2). In early stage, it is very difficult to remove peel along with albedo from the fruits. Naringin, a primary bittering water-soluble component in the fruit membrane and albedo, gets extracted more in fruit juices. Its taste threshold is approximately 20–50 ppm (by HPLC) for kinnow (Hasegawa *et al.*, 1996; Mongkolkul *et al.*, 2006). TSS increased from an initial value of 7.43 ºB in September 2014 to 12.63 ºB in April, 2015 while there was a corresponding decrease in % titratable acidity from 1.41 to 0.41 %. Ascorbic acid content showed a bell shaped curve. It increased upto January, 2015 (from an initial value of 23.33 to 45.27 mg/ 100 ml) and decreased thereafter to 16.57 mg/ 100 ml.
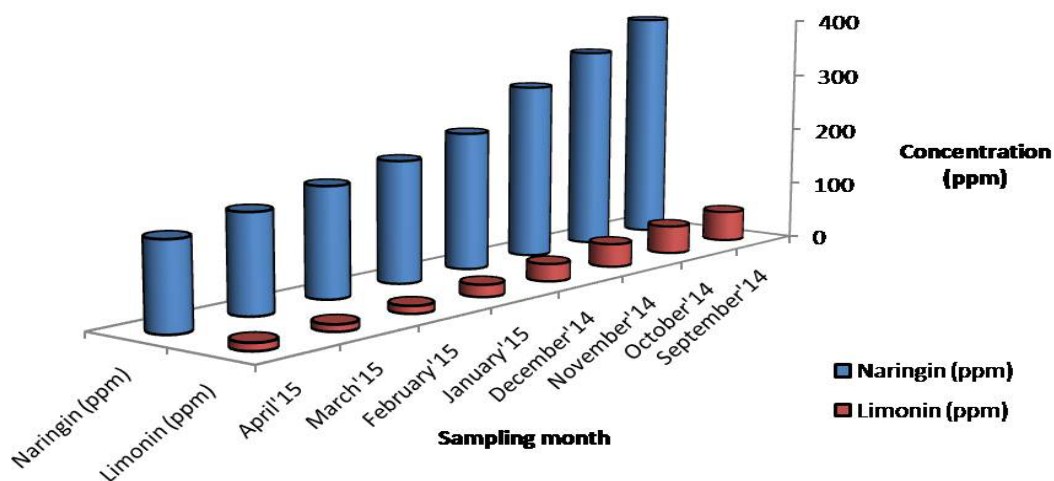
Fig. 2: Bitterness in kinnow juice as affected by fruit ripening

Ethylene experiment on intact fruits: In order to assess effect of ethylene on removal of limonin mediated bitterness, ethylene (100 ppm) dip treatment on intact fruits was performed for 4 h.The treated fruits were stored for 5 d at ambient and the extracted (control as well as treated) juice was analysed for limonin. Artificial ripening of fruit with the use of ethylene significantly influenced limonin content in treated juice. Application of 100 ppm ethylene reduced limonin from 39.1 ppm to 22.3 ppm. However artificial ripening failed to achieve the tolerance limit of 6 ppm in the treated fruits.

Bitterness index of various portions of kinnow fruit: Different compartments of kinnow fruit contain different concentration of bittering factors. Different portions were processed for extracting limonin and naringin separately and analyzed concentration via reverse phase

Table 2: Bitterness index in different potions of kinnow fruit

Sample	Limonin (ppm)	Naringin (ppm)
Flavedo	56.95	13589.82
Albedo	-	4037.83
Juice	20.33	105.67
Seed	224.37	710.82
Pulp	114.91	131.84

HPLC. The concentrations are elaboarated Table 2. Limonin was found highest in seed and the value corresponded to 224.37 ppm while the lowest limonin was reported in extracted juice having 20.33 ppm. Albedo was found to contain negligible amount of limonin at that dilution level. On the other hand, naringin was found highest (13589.82 ppm) and lowest (105.67 ppm) in flavedo and juice portions of kinnow fruit, respectively. On the basis of these data, it was concluded that if seeds and peel (albedo+ flavedo) are removed (manually) before juice extraction, we can cut down a major portion of bitterness being drifted in kinnow juice while processing due to tissue disruption. Manual separation of peel and seed, followed by juice extraction was found to have no limonin content.

Steps involved in de-bittering of kinnow juice: The de-bittering process comprise of manual peeling of fruit with hand and thereafter carefully removing albedo portion attached to peeled fruit with the help of knife. The juice was extracted in such a way that seeds may not get crushed in to juice. The clarified juice was heated at 90 °C for 5 min and centrifuged at 7000 rpm for 10 min to separate pulp.This juice was used for quantification of bitterness causing compounds in extracted juice using HPLC technique and a process protocol was established accordingly for de-bittering of juice using various hurdle techniques.

Principle of delayed bitterness development- Purification of limonoate-D-ring lactone hydrolase (LDLH): An enzyme limonoate-D-ring lactone hydrolase (LDLH)
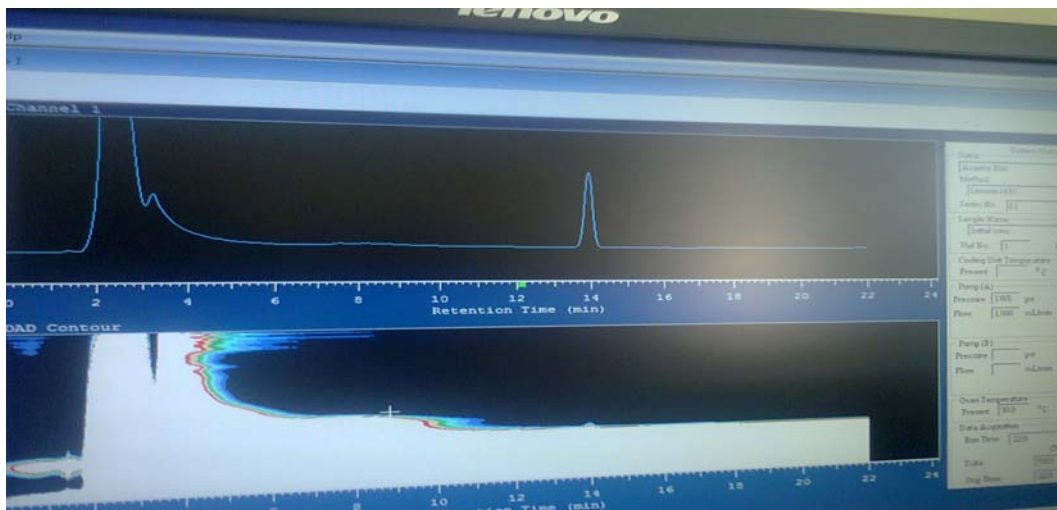

Fig. 3: Estimation of LDLH by HPLC technique

catalyzes reversible conversion of limonoate-A-ring lactone (LARL) to limonin depending upon pH. Under acidic conditions of citrus juices, the LARL precursor (non-bitter) is converted to limonin (bitter), causing delayed bitterness. Limonin is the primary component of limonoid metabolism responsible for causing bitterness in citrus juices (Fayoux *et al.*, 2007). In order to express conversion of LARL to limonin, LDLH enzyme was extracted from kinnow seed and purified using a two stepped sequential process that involved ammonium sulfate precipitation and molecular exclusion chromatography. The enzyme activity was quantified using HPLC (Fig. 3). Molecular exclusion chromatography resulted in 74.57 fold purification of LDLH with 5.02% yield (Table 3). The enzyme reaction depends on pH of reaction mixture. Limonin producing lactonization of D-ring of LARL takes place at pH 6.0 while its reverse (*i.e.* hydrolysis of limonin D-ring to yield LARL) happens at pH 8.0 (Merino *et al.,* 1996).

Table 3: Profile of enzyme limonoate-D-ring lactone hydrolase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold purifi-cation	Yield (%)
Crude extract	7800.0	60440.6	458640.0	7.59	1.00	100.0
Ammonium sulphate saturation (25-85%)	1026.0	4485.7	88236.0	19.67	2.59	19.24
Molecular exclusion chromatography (Seralose CL-6B)	13.0	40.7	23037.2	566.02	74.57	5.02

After ammonium sulphate saturation, LDLH enzyme was dialyzed and concentrated from 1026.0 ml to 60.0 ml using semipermeable membrane and an osmotically active compound, sucrose (Fig. 4). Semipermeable membrane allows water molecules to exit while high molecular weight molecules (including LDLH) to retain inside. During gel filtration chromatography, a column of seralose CL-6B was used for molecular weight base separation of desired enzyme (Fig. 5). Molecular mass as determined by gel filtration was found to be 224 kDa.

Fig. 4: Osmotic dehydration of excess water through semi-permeable membrane (Molecular weight cut-off range: 12 kDa)

Molecular exclusion chromatography
Base material: Seralose CL-6B
Column dimensions: 22 x 1.8 cm
Void volume= 223 ml
Samples collected= 110
Sample volume= 5 ml each
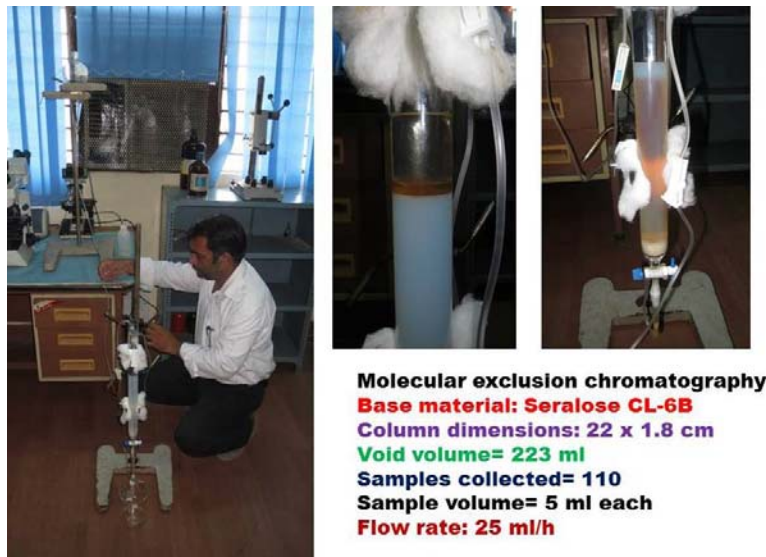**Flow rate: 25 ml/h**

Fig. 5: Molecular exclusion chromatography for enzyme purification

LDLH inhibition using food grade chemicals: The purified LDLH was tried to be inhibited using food grade inhibitors at the de-facto pH of juice, the details of which are presented in Table 4. The inhibitors were prepared by dissolving the amount given below in distilled water and adding 10 microliter of each directly in reaction mixture (Table 4). The use of food grade inhibitors like EDTA, CDTA, L-glutamate and sodium hexmetaphosphate was able to inhibit LDLH activity to some extent under lab conditions and therefore may be used to inhibit LDLH mediated conversion of LARL to limonin in kinnow juice (Table 4).

Table 4: Relative inhibition (%) of purified LDLH activity by various inhibitors

Inhibitor	Concentration	Relative inhibition (%)
EDTA	745 mg/L	58.13
Histidine	310 mg/L	N.D.
CDTA	693 mg/L	41.42
L-glutamate	294 mg/L	40.60
Sodium hexmetaphosphate	1223 mg/L	40.10
DL-malic acid	268 mg/L	N.D.

N.D. = Not detected (any inhibition)

Effect of pH and storage temperature on bittering of kinnow juice: The healthy kinnows were harvested directly from 20 years old orchard, washed, wiped (to remove surface moisture) and stored at 10°C for their intended use. The fruit's albedo and flavedo were removed manually and rags were fed into a screw press for juice extraction. The juice was filtered through a sieve to remove fibrous portion. The kinnow juice so obtained was analyzed for its initial biochemical and physico-chemical properties (Table 5). Juice yield was found between 46.0-52.0% for batches of 25 kg fruits taken at a time for juice extraction. Total soluble solids (%), titratable acidity (%) and pH varied, respectively, in the range 11.6-12.5, 0.78-1.0 and 3.6 to 3.9 (Table 5). The organoleptic score for freshly extracted juice was 8.8 (Table 5). In order to determine the conceivable bitterness with time, sensory evaluation of freshly extracted juice was carried out at hourly interval under ambient conditions (13-18°C). It was found that the conceivable bitterness developed after 6 h of juice extraction. The juice was highly acceptable up to 3 h due to its non-bitter taste but thereafter sensory score gets affected due to initiation of bitterness in freshly extracted juice.

Table 5: Initial physico-chemical composition of kinnow juice

S. No.	Parameter	Values
1	Juice yield (%)	46.0-52.0
2	TSS (%)	11.6-12.5
3	Titratable acidity (%)	0.78-1.0
4	pH	3.6-3.9
5	Total soluble protein (μg/ml)	0.402
6	Organoleptic score (fresh juice)	8.8
7	Color	
	'L'	22.13
	'a'	13.57
	'b'	17.05

Kinnow juice was extracted by the process described earlier. The extracted juice was heated, centrifuged and adjusted to pH 3, 4 and 5 using citric acid/NaOH. The juice with different pH values (3, 4 and 5) was filled in aseptic glass bottles, crown corked, pasteurized and stored at low temperature (5±1°C) and ambient condition. The limonin and naringin content decreased with increase in pH of the juice and vice-versa. Storage period increased the concentration of bittering factors under both the conditions. However, magnitude of change was much more under low pH and high temperature storage. There were fewer rises in bitterness at low temperature storage while bitterness increased comparatively faster during ambient storage. This may be due to activation of various bitterness causing enzymes at ambient storage while less activity of these enzymes during low temperature storage. It might be due to reduced activity of LDLH enzyme at higher pH in converting LARL to limonin.Though higher pH reduced the bitterness but it adversely compromised the sensory quality of juice.

Effect of various other resins and adsorbents on kinnow juice bitterness: XAD7, XAD16 and florisil were used as adsorbent for removing bitterness. The juice was processed and passed through columns of XAD7, XAD16 and florisil. Where XAD was able to reduce bittering compounds from 60-90%, florisil could adsorb bittering compounds nearly 50% (Table 6). Our results are in conformity to as observed by previous researchers (Chandler and Johnson, 1977; Kola, 2005).

Table 6: De-bittering of kinow juice using adsorbent resins

S. No.	Approach used	Fruit	Reduction in bittering compounds	
			Naringin	**Limonin**
1.	XAD7	Kinnow	81%	90%
2.	XAD16	Kinnow	63%	90%
3.	Florisil	Kinnow	46%	50%

Effect of ultrafiltration on de-bittering of kinnow juice: An experiment was conducted to get rid of bittering factors of juice using ultrafiltration. The kinnow juice was heated, centrifuged, passed though Grade-4 filter paper before feeding to ultrafiltartion machine. Ultrafiltarion of kinnow juice was carried out using hollow fiber membrane having molecular weight cut-off 30 kDa at a temperature of 18-20ºC. The inlet peristaltic pressure was 15-18 psi and the flow rate was 110 ml/min. The resultant permeate and retentate were analyzed for limonin and naringin. Naringin content in control sample was 206.93 ppm which got fractionated into permeate (164.2 ppm) and retentate (44.07 ppm) after ultrafiltration (Table 7; Fig. 6). Similarly, limonin content in permeate and retentate was 6.0 and 2.2 ppm, respectively. Since, the concentration of limonin and naringin are considerably reduced by ultrafiltration in retentate which can be used as de-bitteredkinnow juice.

Effect of thermal processing on naringin content: Simple blanching of fruit juice to 90 ºC for 5 min resulted in an increase in naringin content. Fresh juice contained 158.81 ppm naringin and it was increased to 344.93 ppm when the juice was blanched to inactivate enzymes and initial macrobial load. Therefore, to eliminate this inceased naringin amount, there was mandatory to find out some way before pasteurization and packing of juice.

Table 7: Effect of ultrafiltration on kinnow juice quality

Parameter	Control	Permeate	Retentate
Naringin (ppm)	206.93	164.20	44.07
Limonin (ppm)	10.47	6.00	2.20
TSS (ºB)	11.47	10.00	10.93
Titratable acidity (%)	1.03	0.53	0.66
Ascorbic acid (mg/100 ml)	45.27	21.03	27.70

Fig. 6: Ultrafiltration process with permeate and retentate

Role of adsorbent filtration step: The juice was extracted as previously described method. After this, an adsorbent filtration step was introduced where an activated charcoal filter was fitted in a vessel (buccet), the outlet of which was connected to a vacuum pump. The juice was pouredin the vessel fitted with activated charcoal and allowed adsorption (of bittering factors) by giving a contact time of 2 h (between juice and activated charcoal) with intermittent shaking. After this contact time, the juice was filtered through adsorbent using vacuum pump (Fig. 7). Naringin content was reduced to 62.69 ppm from its initial value 344.93 ppm due to adsorbent filtration. Limonin content was reduced to negligible amount as detected by HPLC. TSS changed from 12.73 to 12.20°B. Titratable acidity got reduced to 0.49 % compared to control (0.56%) while ascorbic acid receded from 16.57 to 3.76 mg/100 ml.

Fig. 7: Set-up of carbon filter and vacuum filtration of kinnow juice

Process protocol for de-bittered kinnow juice: The final process protocol for de-bittering of kinnow juice involves following sequential steps (Fig. 8):

- Select kinnow fruits having TSS 12.0^oB or more particularly in February to March.

- Remove manually peel containing upper colored flavedo and lower white papery segemtn called albedo.

- Either remove seeds or extract juice using mechanically without damagind seeds.

- Do blanching at 90^oC for 5 min to inactivate enzymes and initial microbial load.

- Cetnrifuge at 7000 rpm for 10 min to separate pulp.

- Treat centrifuged juice with activated charcoal for 2 hours with intermittent shaking.

- Add EDTA (745 mg/L) or L-glutamic acid (294 mg/L) to juice and re-add centrifuged pulp @ 1% to juice.

- Adjust TSS to 14.5^{o}B and titratable acidity to 0.75%.

- Fill hot, pasteurize, cool and store juice in glass bottles at low temperature.

Selection of kinnow fruit

↓

Manual removal of kinnow peel (flavedo and albedo)

↓

Juice extraction using screw press without damaging seeds or manually separating seeds

↓

Blanching (90°C; 5 min)

↓

Centrifugation (10 min; 7000 rpm) to separate sticky pulp

↓

Treatment of centrifuged juice with activated charcoal for 2 h with intermittent shaking

↓

Addition of LDLH inhibitor and re-addition of centrifuged pulp

↓

Adjusting TSS (14.5 ^{o}B) and acidity (0.75%)

↓

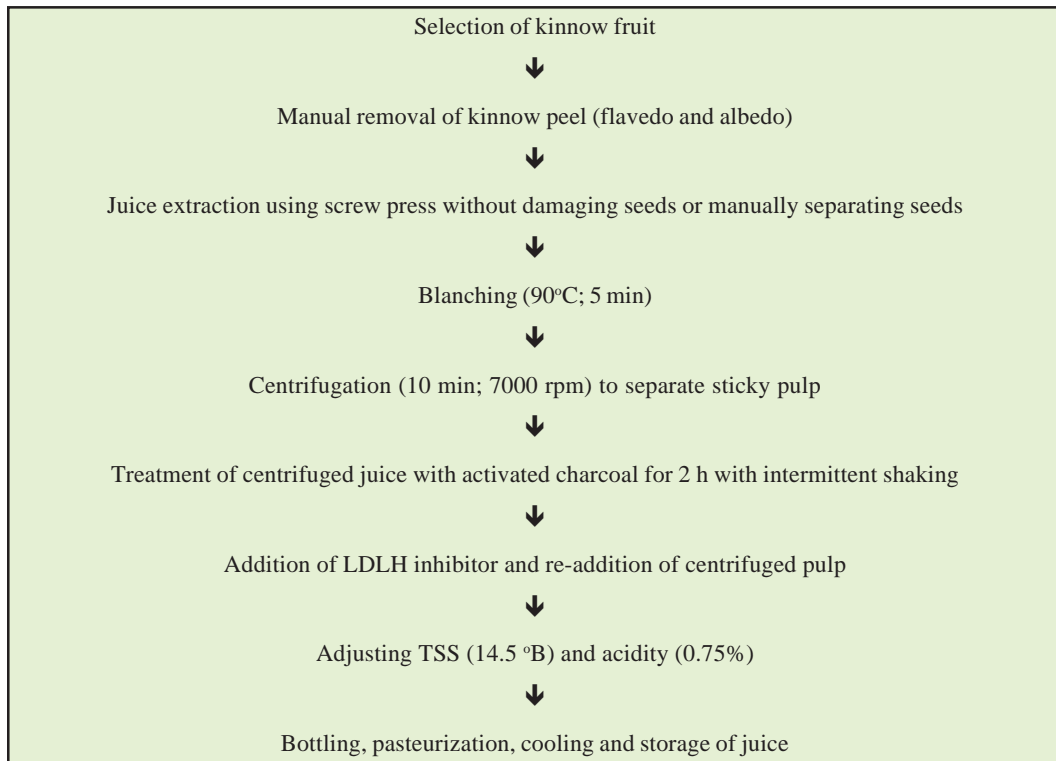Bottling, pasteurization, cooling and storage of juice

Fig. 8: Process flow chart for production of de-bittered kinnow juice

The present intervention is easy and economical addressing this complex problem of bitterness by reducing bittering factors below their threshold levels. The developed process protocol offers a comparatively cheap alternative of kinnow juice de-bittering. Currently the de-bittering technology involves costly equipment set-up along with use of costly resins like

polystyrene divinyl benzene and Indion NPA1 for selective adsorption of limonin and naringin. The developed process protocol tested at laboratory level involves selective removal of physical parts of fruit bearing major concentration of bitterness causing components. Also, it takes care of LDLH enzyme which becomes active after tissue disruption at acidic pH while juice extraction process using food grade inhibitors to some extent. Quite interestingly, the bittering factor naringin bolsters due to heating which can be overcome by using non-selective adsorbent like activated charcoal. However, development of bitterness is a complex process mediated by two different types of metabolites (limonin and naringin) whose localization and *modus operandi* are different. If we try to curtail one, the concentration of other becomes sizeable.

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NOTES

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