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Standardization of Cold Solvent Extraction Parameters for Natural Red Pigments

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ABSTRACT

Background: Selecting an appropriate extraction technique and optimization of extraction conditions must be done to improve the efficiency and productivity of natural colorant.

Methods: Flesh and peels of four different raw materials *viz*., beetroot, carrot, pomegranate and grape were extracted independently using 100% ethyl alcohol using cold solvent extraction method adopting 1: 1 and 1: 2 w/v solid to solvent ratio for 24 and 48 hrs, forming 8 treatments replicated thrice in three factorial CRD. The collected extracts were evaporated at 60°C; per cent yield and pigment content were calculated.

Result: Extract yield and pigment content were significantly influenced by raw material, solid to solvent ratio and duration of extraction. Extract yield was higher in flesh of carrot and beetroot as well as in peels of grape and pomegranate. Anthocyanin content in grape and pomegranate and betalain content in beetroot were high in peel where as β-carotene content was higher in carrot flesh extract. Per cent yield and pigment content increased with increased duration of extraction and solid to solvent ratio. Requirement of more solvent and longer extraction time are the drawback of conventional extraction methods and there is a growing demand for non-conventional techniques to enhance yield and maintain pigment stability.

Key words: Anthocyanin, β-carotene, Betalain, Cold solvent extraction, Natural pigments.

INTRODUCTION

Color is considered as an important external quality trait of food products that determines their acceptance. A well textured food, rich in nutrients and flavour, cannot be eaten unless it has the right colour. Hence synthetic colour additives are generally applied to manipulate food colour. However synthetic pigments are hardly nutrient, blamed for toxic to different extents and some are found to be carcinogenic. Colouring of food with natural pigments is of worldwide interest and is gaining importance. Anthocyanin, betalin, carotenoids *etc*. are considered as potential replacement for synthetic colouring agents because of bright attractive colours and water solubility (Kong *et al*., 2003). These pigments are safe to use as a natural food dye in replacement of synthetic ones. The demand for natural source of such compounds is increasing day by day because of the awareness of positive health benefits. It is therefore, essential to explore various natural sources of food grade colourants and their potentials for extraction of pigments using solvents. Conventional extraction methods *viz*., soxhlet extraction, maceration and hydro-distillation, are simple, inexpensive, easy to handle (Veggi *et al*., 2013) and are used to extract natural pigments from a wide array of plant materials. However, extraction efficiency is varied depending on solvents, extraction conditions, extraction techniques and difference in pigment compositions of raw materials (Strati and Oreopoulou, 2011). An experiment was carried out to investigate effect of extraction variables *viz*., raw material, solid to solvent ratio and duration of extraction on naturally occurring red pigments *viz*., carotenoids, anthocyanins and betacyanins at Department of Post-harvest Technology, College of Agriculture, Vellayani, Kerala Agricultural University during 2019-2021.

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MATERIALS AND METHODS

The experiment was carried out at Department of Postharvest Technology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala Agricultural University during 2019-2021. Flesh and peels/ skin of four different raw materials *viz*., beetroot, carrot, pomegranate and grape were utilized for extraction of natural colouring pigments. Good quality fruits/ vegetables were procured, cleaned, peel was separated, flesh and peel were chopped separately into uniform slices and subjected to cold solvent extraction process.

Each 100 gram of peel and flesh was extracted independently with 100% ethyl alcohol in a beaker at ambient temperature (30-35°C), RH (70-83%) and brewed for specific duration without agitation. Conditions of extraction parameters were 2types of raw material (M), flesh (M_1) and peel/ skin (M_2), 2 solid to solvent ratio (R), 1: 1w/v (R_1) and 1: 2w/v (R_2) and 2 extraction time (D) 24 hrs (D_1) and 48 hrs (D_2). Experiment was done with 8 treatment combinations ($M_1R_1D_1$, $M_1R_1D_2$, $M_1R_2D_1$, $M_1R_2D_2$, $M_2R_1D_1$, $M_2R_1D_2$, $M_2R_2D_1$, $M_2R_2D_2$) replicated thrice in a three factorial CRD (Panse and Sukhatme, 1967). The infusion mixture was filtered, evaporated under water bath at 60^0C for complete removal of solvent and yield of the extract (%) and pigment content in extract were calculated.

Estimation of betalain

The concentrated beetroot extract (1 ml) was diluted in 50 ml of 100 per cent ethanol, agitated for 10 seconds and homogenate was centrifuged at 6000 rpm for 10 min. The supernatant was collected and same step was repeated twice to ensure maximum betalain extraction. The absorbance was read by UV-VIS spectrophotometer at 538 nm and 480 nm and betalain concentration was calculated as mg/g basis (Castellar *et al*., 2003) using the formula:

$$\frac{A \times DF \times MW \times 1000}{\varepsilon \times l}$$

Where,

A is the absorption value, l the path length of cuvette (1 cm) and DF the dilution factor; MW: molecular weight of betalain (550 g/mol), ε: the extinction coefficient for betalain (60000 l/mol).

Estimation of beta carotene

Five gram carrot extract was mixed with 10-15 ml acetone adding a few crystals of anhydrous sodium sulphate crystals. The supernatant was decanted into a beaker, process was repeated twice, transferred the combined supernatant to a separatory funnel, added 10-15 ml petroleum ether and mixed thoroughly, which resulted in formation of two layers. The lower layer was discarded, upper layer was collected in a 100 ml volumetric flask, made up the volume to 100 ml with petroleum ether and optical density was recorded at 452 nm using petroleum ether as blank.

$$\beta\text{-carotene } (\mu g/100g) = \frac{OD_{452} \times 13.9 \times 10^4 \times 100}{\text{wt. of sample} \times 560 \times 1000}$$

Estimation of anthocyanin

One gram grape and pomegranate extracts were independently extracted with ethanolic HCl, filtered through a Buchner funnel using Whatsman No. 1 filter paper. The filtrate was diluted with ethanolic hydrochloric acid to 50 ml and anthocyanin content was calculated (Ranganna, 1997) in mg per 100g of sample.

$$= \frac{[(\text{Absorbance at 535 nm}) \times \text{Volume made up} \times (\text{Total volume}) \times 100]}{\text{Volume (ml) of the extract used} \times \text{Weight of the sample taken}}$$

RESULTS AND DISCUSSION

Parameters influencing extraction method to obtain colouring pigments from natural sources were investigated to determine the optimum conditions for better performance.

Extraction yield (Per cent)

Beetroot (*Beta vulgaris*) vacuoles are store houses of pigments, hence used as the main source for natural red dye, "beetroot red". Yield of beetroot extract was significantly influenced by raw material, solid to solvent ratio and duration of extraction (Fig 1). Extract yield was high from beetroot flesh (5.023%) compared to that from peel (4.328%). Yield was high when the solid to solvent ratio was 1:2 (5.075) compared to 1: 1 ratio (4.275) and was high when duration of extraction was 48 hrs (5.003) compared to 24 hrs (4.348). The interaction effect of raw material, duration and ratio was not significant.

The oldest way to obtain carotenoids is extraction from plant material. Carrot is rich in β-carotene, ascorbic acid and tocopherol (Hashimoto and Nagayama 2004). Extract yield was high from carrot flesh (5.628%) compared to that from peel (5.565) (Fig 2). Extract yield was high when the solid to solvent ratio was 1:2 (5.845) compared to 1: 1 ratio (5.348) and yield was high when duration of extraction was 48 hrs (5.828) compared to 24 hrs (5.365). The interaction effect of raw material, duration and ratio was significant. Yield was highest (6.21) when flesh was extracted by soaking

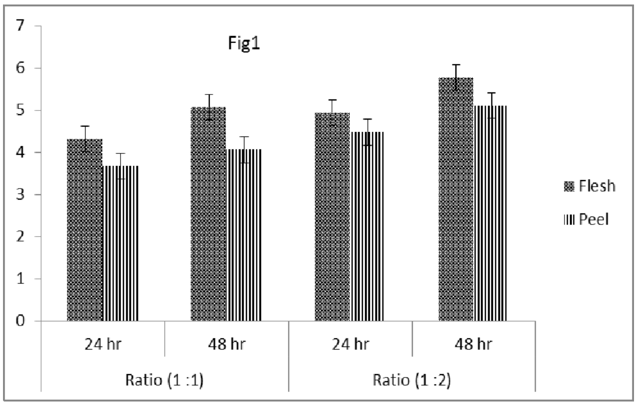

Fig 1: Per cent yield of extract from beetroot.

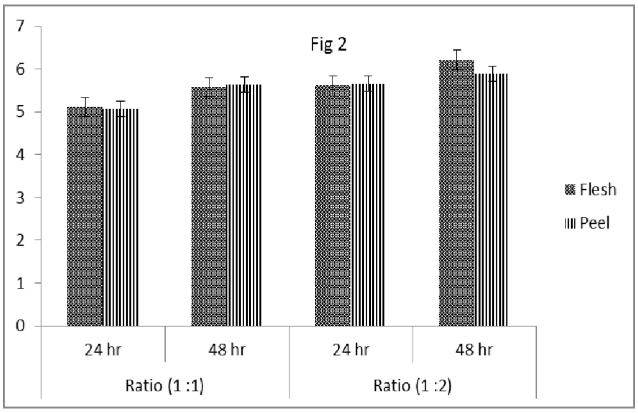

Fig 2: Per cent yield of extract from carrot.

in ethanol in 1:2 ratio for 48 hours and lowest (5.07) when extraction of peel in ethanol in 1: ratio for 24 hours. Among the different extraction conditions, time, temperature and amount of solvent have been reported as the important parameters affecting extraction yield of carotenoids from plant materials (Wang and Liu, 2009).

Grapes are rich sources of phenolic compounds including flavonoids and non-flavonoids and one of the most abundant classes of flavonoids include anthocyanins (Crecelius *et al*., 2000). Colour yield was high from grape peel (18.703%) compared to that from flesh (12.145) (Fig 3). The yield was high when solid to solvent ratio was 1:2 (18.633) compared to 1: 1 ratio (12.215) and when the duration of extraction was 48 hrs (16.503) compared to 24 hrs (14.345). Azoubel *et al*., (2019) could observe better anthocyanin extraction efficiency for longer solvent exposure times. When optimization of simultaneous flavanol, phenolic acid and anthocyanin extraction from grape skin, seed and pulp was performed (Mane *et al*., 2007), optimum extraction times and solid-to-liquid ratios varied according to the part.

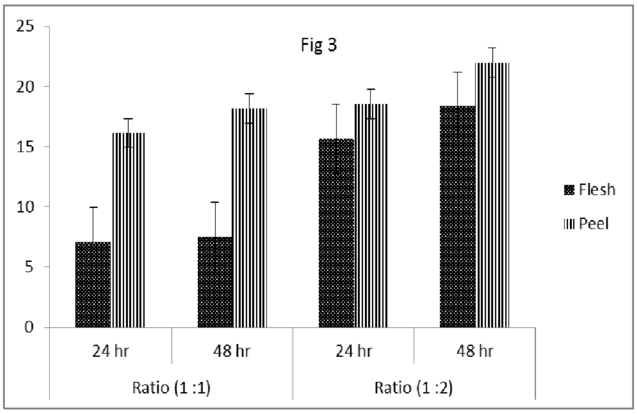

Fig 3: Per cent yield of extract from grape.

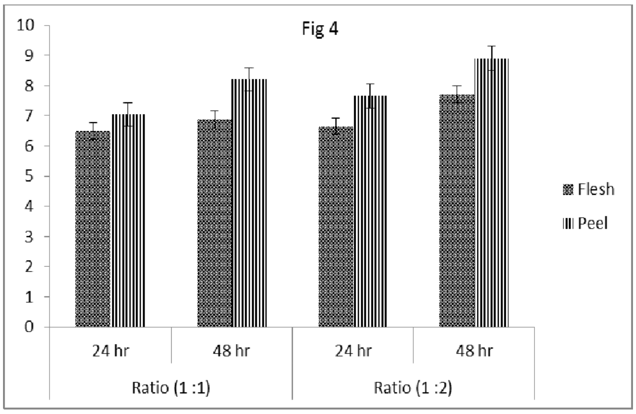

Fig 4: Per cent yield of extract from pomegranate.

Colour yield was high from pomegranate peel (7.955%) compared to that from flesh (6.933) (Fig 4). The percent yield was high when the solid to solvent ratio was 1:2 (7.730) compared to 1: 1 ratio (7.158) and high when the duration of extraction was 48 hrs (7.925) compared to 24 hrs (6.963). Ali *et al*., (2016) reported that pomegranate colour yield slightly increased by increasing the extraction time from 30 to 90 minutes and the effects were not statistically significant. The interaction effect of raw material, duration and ratio was significant. Yield was highest (8.90) when peel was extracted by soaking in ethanol in 1:2 ratio for 48 hours and least (6.49) when extraction was done using flesh soaked in solvent in 1: 1 ratio for 24 hrs.

In general colour yield was higher in flesh of carrot and beetroot and in peels of grape and pomegranate. Percent yield was increased with increase in duration of extraction and solid to solvent ratio. As raw material to solvent ratio and duration time increased, diffusion rate, solubility and mass transfer of the compounds increases, improves contact of raw material with solvents, thereby resulting in improved extraction efficiency (Richter *et al*. 1996).

Pigment content

The extraction methods and the choice of solvent affected noticeably the content of individual compounds in the extract (Kujala *et al*, 2001).

The main component of beetroot extract is betalains, which are widely used as a food colorant in yogurts, ice creams, sweets and other products. Betalain of the beetroot extract was significantly influenced by raw material, solid to solvent ratio and duration of extraction (Table 1). Betalain was higher in beetroot peel extract (14.665 mg100 $^{g-1}$) compared to flesh extract (12.79 mg100 $^{g-1}$). This is in accordance with Kujala *et al*., (2001) who had reported that beetroot pomace extract had a high content of bioactive compounds, namely phenolics and betalains. Betalain was higher when solid to solvent ratio was 1:2 (14.625 mg100 $^{g-1}$) than 1:1 ratio (12.83 mg100^{g-1}). The content was more when extraction was done for 48 hrs (15.19 mg100 $^{g-1}$) compared

Table 1: Betalain content as influenced by raw material, solid to solvent ratio and extraction time.

Beetroot (M)	Betalain content (mg 100 $^{g-1}$)				
	Ratio (1 :1) (R_1)		Ratio (1 :2) (R_2)		Mean (M)
	24 hr (D_1)	48 hr (D_2)	24 hr (D_1)	48 hr (D_2)	
Flesh (M_1)	10.11	13.55	13.17	14.33	12.79
Peel (M_2)	12.11	15.55	13.67	17.33	14.665
	Mean (R)			Mean (D)	
	R_1- 12.83, R_2- 14.625			D_1- 12.265, D_2- 15.19	
	CD- 0.333 (M, R, D)				
	Interaction M xR xD - 0.665				

to 24 hrs(12.265 mg100 $^{g-1}$). The interaction effect of raw material, solid to solvent ratio and duration of extraction was significant. Betalain content was highest (17.33 mg100 $^{g-1}$) when beetroot peel was extracted using ethanol in 1:2 ratio for 48 hrs and lowest (10.11 mg100 $^{g-1}$) when beetroot flesh was extracted using ethanol in 1:1 ratio for 24 hrs.

β-carotene is a valuable food additive used as an orange-red pigment in many food product. β-carotene of carrot extract was significantly influenced by raw material, solid to solvent ratio and duration of extraction (Table 2). β carotene was higher in carrot flesh (3.988 mg100^{g-1}) compared to that in peel extract (3.418 mg100^{g-1}). β carotene was higher when solid to solvent ratio was 1:2 (3.818 mg100 $^{g-1}$) than in 1:1 (3.588 mg100^{g-1}). The content was more when extraction was done for 48 hrs (3.813 mg100^{g-1}) compared to extraction for 24 hrs (3.593 mg100^{g-1}). The extraction yield of β-carotene was reported to be strongly influenced by time and temperature treatment given to particular food (Fikselova *et al*., 2008).

Anthocyanin of the grape and pomegranate extract was significantly influenced by raw material, solid to solvent ratio and duration of extraction (Table 3 and 4.).

Anthocyanin was higher in grape peel extract (104.238 mg100 $^{g-1}$) compared to flesh extract (13.388mg100^{g-1}) (Table 3). Rice-Evans *et al*., (1996) had reported large amounts of phenolic compounds, mostly flavonoids in grape skins. Anthocyanin was higher when solid to solvent ratio was 1:2 (60.655 mg100^{g-1}) than in 1:1 (56.97mg100^{g-1}). Spagna *et al*. (2003) reported that type of solvent and concentration were significant factors in the extraction operation. The content was more when extraction was done for 48 hrs (62.988 mg100^{g-1}) compared to extraction for 24 hrs (54.638mg100^{g-1}). The interaction effect of raw material, solid to solvent ratio and duration of extraction was significant. Anthocyanin was highest (115.11mg100 $^{g-1}$) when grape peel was extracted using ethanol in 1:2 ratio for 48 hrs and lowest (11.23 mg 100 $^{g-1}$) when grape flesh was extracted using ethanol in 1:1 ratio for 24 hrs.

Table 2: β-carotene content as influenced by raw material, solid to solvent ratio and extraction time.

Carrot	β-carotene (mg 100g^{-1})				
	Ratio (1 :1)		Ratio (1 :2)		Mean (M)
	24 hr	48 hr	24 hr	48 hr	
Flesh	3.86	4.03	3.94	4.12	3.988
Peel	3.12	3.34	3.45	3.76	3.418

Mean (Ratio): R_1- 3.588, R_2- 3.818
Mean (Duration): D_1- 3.593, D_2- 3.813
CD- 0.078 (Raw material, Ratio, Duration)
CD Interaction M xR xD- NS

Table 3: Anthocyanin content of grape as influenced by raw material, solid to solvent ratio and extraction time.

Grape	Anthocyanin content(mg100 $^{g-1}$)				
	Ratio (1:1)		Ratio (1:2)		Mean (M)
	24 hr	48 hr	24 hr	48 hr	
Flesh	11.23	14.57	12.43	15.32	13.388
Peel	95.13	106.95	99.76	115.11	104.238

Mean (Ratio): R_1- 56.97, R_2- 60.655
Mean (Duration): D_1- 54.638, D_2- 62.988
CD- 0.646 (Raw material, Ratio, Duration)
Interaction M xR xD - 1.292

Table 4: Anthocyanin content of pomegranate as influenced by raw material, solid to solvent ratio and extraction time.

Pomegranate	Anthocyanin content (mg100 $^{g-1}$)				
	Ratio (1 :1)		Ratio (1 :2)		Mean (M)
	24 hr	48 hr	24 hr	48 hr	
Flesh	216.21	225.76	220.98	234.54	224.373
Peel	315.12	365.21	323.50	381.76	346.398

Mean (Ratio): R_1-280.575, R_2- 290.195
Mean (Duration): D_1-268.953, D_2- 301.818
CD-0.999 (Raw material, Ratio, Duration)
Interaction M xR xD - 1.997

Anthocyanin was higher in pomegranate peel extract (346.398 mg100^{g-1}) compared to that in flesh extract (224.373mg100^{g-1}) (Table 4). Zhao *et al*. (2013) reported that the anthocyanin pigment was more complex in fruit peel compared to pomegranate juices. Waste generated from processed fruits and vegetables can be used as a source of carotenoids and can be used as functional food ingredients to replace their synthetic equivalents. Anthocyanin was higher when solid to solvent ratio was 1:2 (290.195 mg100^{g-1}) than in 1:1 (280.575mg100^{g-1}). The content was more when extraction was done for 48 hrs (301.818 mg100^{g-1}) compared to extraction for 24 hrs (268.953 mg100^{g-1}).

The efficiency of conventional extraction methods directly depends on the solubility of a solute from a plant material into an extraction solvent (Cowan, 1999). With increase in raw material to solvent ratio and duration time, contact of raw material with the solvents increased, improving diffusion rate, solubility and mass transfer of the compounds thereby resulting in higher colour yield and pigment content. Devi and Joshi (2012) reported enhanced anthocyanin yield with enhanced solid to solvent ratio. But requirement of large amount of solvent and long extraction time are considered as drawbacks of conventional extraction methods (Cheok *et al*., 2014).

CONCLUSION

Pigments of plant origin are gaining importance as a potential source of natural food colours for their versatility and to avoid health hazards caused by synthetic colours. Selecting an appropriate extraction technique for each type of natural pigment must be done to improve efficiency and productivity of natural colorant. Optimization of extraction conditions is very important in order to obtain maximum coloring component in a solvent. However, traditionally used method *viz*., cold solvent extraction requires relatively high amounts of solvent and there is a growing demand for non-conventional extraction techniques as a means to enhance yield and maintain pigmentstability.

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