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A Liquid Chromatography method for the Simultaneous Determination of Safranal and Piperine in Some Marketed Proprietary Chyawanprash Products

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ABSTRACT

A rapid and simple HPLC method has been developed for the simultaneous quantification of safranal and piperine in some marketed proprietary Chyawanprash products. Analysis was performed using C_{18} column (250 x 4.6 mm) by isocratic elution with acetonitrile: water (77:23) and detection at 308 nm using Ultra Violet (UV) detector. The calibration plot was linear over the range studied (safranal: 0.5-10 ppm; piperine: 2.5-50 ppm) with a correlation of 0.999 for safranal and 0.999 for piperine. The method was also validated for the linearity, range, precision, recovery and detection limits. Thus, the method is suitable for routine analysis of safranal and piperine in marketed proprietary Chyawanprash products.

Keywords: Safranal, Piperine, HPLC, Chyawanprash

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INTRODUCTION

Chyawanprash is a traditional Ayurvedic polyherbal semi solid preparation (Avaleha) having multiple health benefits. Chyawanprash is one of the most popular 'Rasayana' groups of formulation which aim to maintaining physique, vigor and vitality, while delaying the aging process[1-3]. Chyawanprash has been used traditionally in Ayurvedic supplements to strengthen digestive system, promote food absorption, prevent hyperacidity and dyspepsia[4], boost memory power[5], promote cardiac fitness and helps in slow down the rate of normal aging and promote longevity. It also eliminates toxins from blood, promotes calcium absorption, promotes muscle toning by protein synthesis and boost the immune system to improve natural well-being, wisdom and glow[3].

Chyawanprash is primarily a generic product prepared by around 50 ingredients by following the method described in Ayurvedic Pharmacopoeia of India[6]. Among the herbal ingredient *Piper longum* (pippali –fruit part) is one of the most important component used for treating general debility, dyspepsia, flatulence and respiratory tract infection[7]. Piperine is a biologically active marker of pippali which is a potent bio-enhancer. Several animal studies on piperine have shown promising results in bioenhancing capacity for various drugs[8]. Hence, the quantification of piperine has direct implication on the quality and efficacy of the product.

Just like many other herbal formulations in the market place today, Chyawanprash ingredients vary widely. Very few manufacturers in India still follow the original formula entirely due to the difficulty in obtaining some of the necessary herbs and the time involved in its preparation. Thus, many top manufacturing companies of India has introduced proprietary Chyawanprash products. The most common ingredient used for these products is saffron (*Crocus sativus*), which is the most costly ingredient but preferred by various manufacturer as it has potent anticarcinogenic, antimutagenic, immunomodulatory and antioxidant like activity. Recent studies have shown the beneficial effects of saffron in depression, premenstrual syndrome (PMS), and Alzheimer's disease[9]. Safranal is an organic compound isolated from saffron. It is the constituent primary responsible for the aroma of saffron.

Safranal is an effective anticonvulsant shown to act as an agonist at $GABA_A$ receptor[10, 11]. Safranal also exhibits high antioxidant and free radical scavenging activity[12], along with cytotoxicity towards cancer cells *in vitro*[13]. It has also been shown to have antidepressant properties. Thus, it is also very important to quantify safranal content in the product[14, 15].

MATERIALS AND METHODS

Chemicals and Reagents

All the solvents were of HPLC grade purchased from MERCK India. Water purified by the Milli-Q water purification system was used. The standards safranal and piperine were purchased from Sigma Aldrich (India).

Chromatographic Conditions

Shimadzu LC-2010CHT quaternary HPLC system was used having auto injector and UV detector. The LC Solutions software was used for integration. Separation was achieved using a Phenomenex Gemini C_{18} column (250 x 4.6 mm, 5µ ID). The solvent system consisted of acetonitrile: water (77:23) was pumped isocratically at a flow rate of 1.0 ml/min. The detection was carried out at 308 nm by UV detector.

Preparation of standard stock solution

The stock solutions containing 10 ppm of safranal and 50 ppm piperine were prepared in acetonitrile. These stock solutions were stored in amber colored containers. Aliquots of safranal (0.5-10 ppm) and piperine (2.5-50 ppm) were prepared in the mobile phase.

Preparation of sample solution

The components safranal and piperine from Chyawanprash product were extracted by acetonitrile. Both the constituents were extracted from 10 g sample by adding 25 ml of acetonitrile and sonicated for 15 minutes. The solution was then filtered using a Whatmann filter paper No 41. The process was repeated thrice and the filtrate was pooled together. Then the filtrate was evaporated in rotary evaporator under vacuum and reconstituted in acetonitrile. Then the content was transferred into a 10 ml volumetric flask and final volume was made up by acetonitrile.

METHOD VALIDATION

Linearity

The calibration curve was linear over the concentration range of 0.5-10 ppm for safranal and 2.5-50 ppm for piperine.

Precision

Six replicate injections of standard safranal (3 ppm) and piperine (5 ppm) and six replicate injections of sample were analyzed to examine the precision for analytical system.

Specificity

The percentage RSD of retention time of six replicates of standard safranal and piperine as such

and in sample was determined to check specificity.

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at three levels by adding a known quantity of safranal and piperine to the pre-analyzed samples and the mixtures were analyzed according to the proposed method.

Sensitivity

The sensitivity of measurement of safranal and piperine by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were determined by injecting progressively low concentration of solutions. The limit of detection is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio 3). The limit of quantitation is the smallest concentration of the analyte which gives a response that can be accurately quantified (signal to noise ratio 10).

RESULTS AND DISCUSSION

Method Development

The HPLC condition was developed and optimized using trial and error method. Various proportions of different solvents as mobile phase with varying flow rates were tried to get resolution of both the compounds. The optimized mobile phase was acetonitrile: water (77:23) was pumped isocratically at a flow rate of 1.0 ml/min. The optimized mobile phase could resolve both the compounds apart from each other and the peaks obtained were compact too. The detection was carried out at 308 nm by UV detector.

The optimized chromatographic condition yielded a symmetrical peak for both substances with Retention Time (RT) 4.65 minutes (for piperine) and 6.19 minutes (for safranal). The HPLC chromatogram of piperine and safranal is shown in Figure 1.

The developed method was then validated and successfully applied for quantification of safranal and piperine from the samples. Regression analysis data is shown in Table 1 and Figure 3-4.

Table 1: Linearity of safranal and piperine

Parameters	Results	
	Safranal	**Piperine**
Linearity range (ppm)	0.5-10	2.5-50
Slope	2949.42	4126
S. D of Slope	5.51	5.69
Intercept	701.24	439.8
S. D. Intercept	81.36	48.09
Regression equation	Y= 2949x+701.2	Y= 4126x+439.8
Correlation coefficient	0.999	0.999

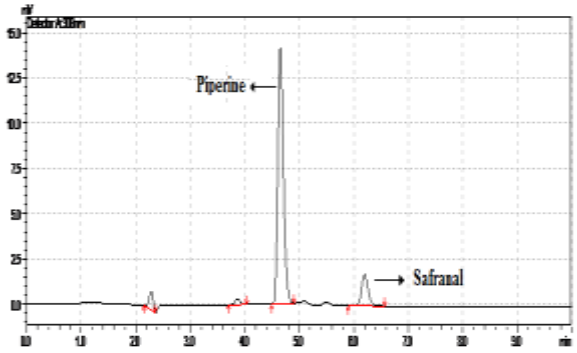

Figure 1: A Typical Chromatogram of safranal and piperine

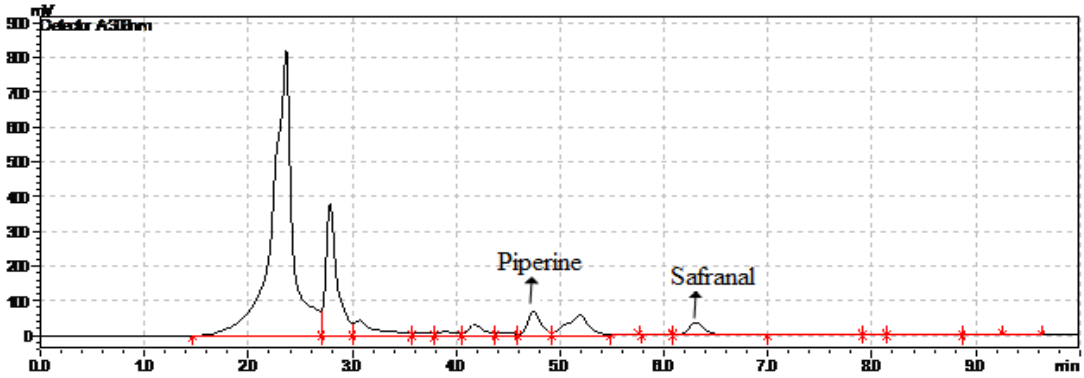

Figure 2: A Typical Chromatogram of chyawanprash

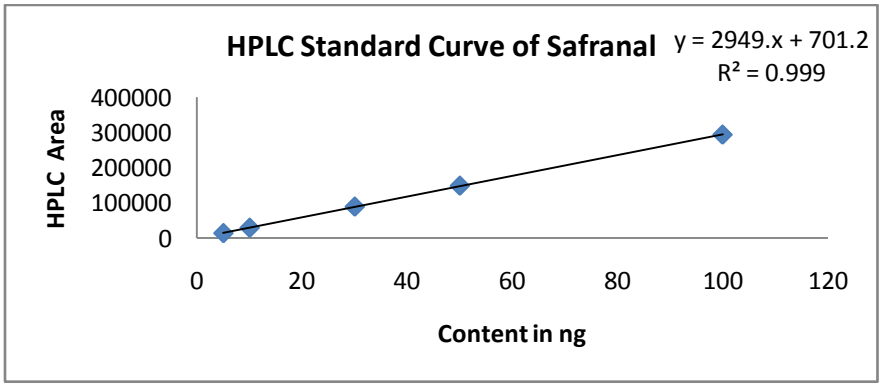

Figure 3: HPLC standard curve of safranal

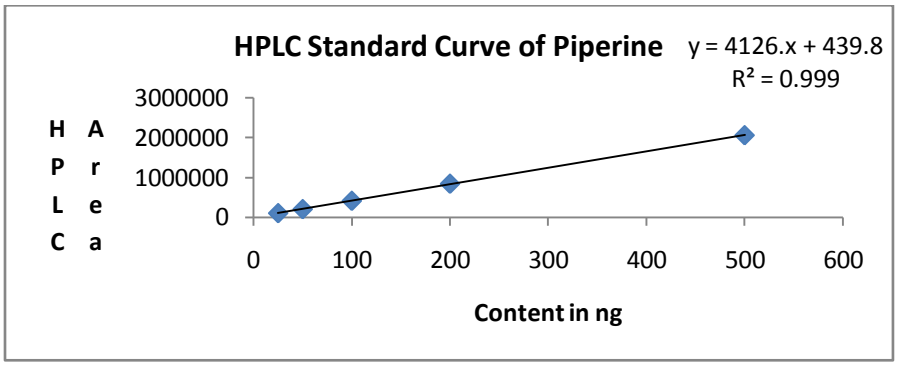

Figure 4: HPLC standard curve of piperine

The calibration curve of safranal and piperine were linear in the range of 0.5 to 10.0 and 2.5 to 50 ppm respectively. Precision, expressed in terms of % RSD of area of safranal and piperine, as standards and in sample of six replicate injections, summarized in Table 2 and 3.

Table 2: Precision Data for safranal and piperine

Safranal				Piperine			
Concentration in ppm	**Peak area**	**Mean peak area (n=6) ± SD**	**% RSD**	**Conc. in ppm**	**Peak area**	**Mean peak area (n=6) ± SD**	**% RSD**
3.0	90159 90116 89530 87594 89870 87412	89113.5 ±1268.68	1.42	5.0	201813 202209 201978 198745 198524 194725	199665.7±2932.15	1.47

Table 3: Method Precision Data of safranal and piperine in Sample

S No.	Sample A	
	Safranal (in ppm)	**Piperine (in ppm)**
1	1.421614	3.934432
2	1.423055	3.934822
3	1.425158	3.929413
4	1.427091	3.932737
5	1.420037	3.931041
6	1.428023	3.920953
Mean	1.424163	3.920953
Std dev	0.003137	0.005133
% RSD	0.2203	0.1309

The specificity of the method was assessed evaluating retention times of six injections of safranal and piperine as standards and in sample. The results are summarized in Table 4 and 5.

Table 4: Specificity of safranal and piperine standard

Safranal				Piperine			
Concentration in ppm	**RT in min**	**Mean RT (n=6) ± SD**	**% RSD**	**Concentration in ppm**	**RT in min**	**Mean RT (n=6) ± SD**	**% RSD**
3.0	6.197 6.196 6.196 6.195 6.197 6.195	6.196 ±0.0008	0.014	5.0	4.647 4.647 4.651 4.652 4.648 4.648	4.648±0.002	0.045

Table 5: Specificity of safranal and piperine in Sample

S No.	Sample A	Sample B

	Safranal (RT in min)	Piperine (RT in min)	Safranal (RT in min)	Piperine (RT in min)
1	6.209	4.658	6.204	4.655
2	6.202	4.655	6.202	4.653
3	6.200	4.654	6.203	4.654
4	6.201	4.652	6.201	4.657
5	6.204	4.657	6.205	4.654
6	6.205	4.656	6.206	4.652
Mean	6.2035	4.6553	6.2035	4.654
Std dev	0.0033	0.0022	0.0019	0.0010
% RSD	0.0527	0.0464	0.0302	0.0371

To ensure the accuracy of the method, recovery studies were performed by standard addition method at three different levels, to the pre-analyzed samples and the subsequent solutions were re-analyzed. At each level, three determinations were performed and the results obtained are shown in Table 6.

Table 6: Recovery data for determination of safranal

Safranal					**Piperine**				
Amount present in sample (ppm ± %RSD)	**Amount added [ppm]**	**Total amount [ppm]**	**Total amount recovered [ppm]**	**% Recovery ± %RSD**	**Amount present in sample (ppm ± %RSD)**	**Amount added [ppm]**	**Total amount [ppm]**	**Total amount recovered [ppm]**	**% Recovery ± %RSD**
3.93 ± 0.15	2.7	6.63	2.65	98.02 ± 0.78	6.0 ± 0.001	4.8	10.8	4.91	102.22 ± 1.88
	3.9	7.83	3.86	98.97 ± 5.09		6.0	12.0	6.09	101.66 ± 0.43
	4.68	8.61	4.67	99.71 ± 1.58		7.2	13.2	7.37	102.40 ± 0.98

The sensitivity of measurement of safranal and Piperine by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ are given in Table 7.

Table 7: Results of sensitivity data for safranal and piperine

Parameters	Results	
	Safranal	Piperine
LOD (ppm)	0.01	0.015
LOQ (ppm)	0.03	0.05

The method was applied to the formulation and the chromatogram of the formulation. When the formulation was analysed in HPLC, safranal and piperine gave sharp and well defined peaks at specific RT. The chromatogram is shown in Figure 2.

CONCLUSION

In this proposed method the linearity was observed in the concentration range of 0.5-10 ppm for safranal and 2.5-50 ppm for piperine with co-efficient of correlation, r^2 = 0.999 and r^2 = 0.999 for safranal and piperine respectively at 308 nm. The result of the analysis of combined mixture by the method was found to be highly reproducible and reliable. No interference from Blank was observed at the retention time of safranal and piperine. The matrix and other ingredients present in the product did not interfere with determination of safranal and piperine. So, the developed HPLC method is simple, precise and accurate and can be used for simultaneous determination of safranal and piperine in marketed proprietary Chyawanprash products.

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