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Method Development & Validation of High-Performance Liquid Chromatography Method for Organic Acids in Ayurveda Preparation

Bhaskar Chandra Dwivedi, Dr. Rajesh Verma [a], Dr.Gaurav Maheshwari [b]

Quality Research and Analytical Labs, Patparganj New Delhi, India.
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ABSTRACT

Context: Study carried out in Ayurvedic Hajma churna and Psyllium husk. Composition of Hajma churna consisiting Pippali, Maricha, Nimbu saar,Samudra lawana,Sharkara and Sunthi, Citric Acid and Excipient etc.

Objective: In present study,a rapid,simple and cost reduced method has been developed and validated by HPLC for five Organic acids As: Tartaric Acid, Formic Acid, Malic acid, Glacial Acetic Acid and Citric Acid in Hajma churna and Psyllium husk samples were checked through validated HPLC method. The organic acids separated using isocratic ally with the column Phenomenex, Synergi, H20-116711, 5mic, 250 x 4.6 mm and 0.06 %v/v Orthophosphoric Acid in water mobile phase at room temperature with the retention time less than 30 minutes. All organic acid eluted separately and identified and monitored by PDA (Photodiode array detector) at 210 nm. The linearity of the method were magnificent (r2 > 0.999) under the concentration range 50 µg/ml to 626 µg/ml. The statistical evaluation were performed as intra-day and inter-day calibrations and were found method to be satisfactory. Precision results were found with System Precision % CV = 0.21, Method Precision (Repeatability) % CV = 2.0, Intermediate Precision% CV = 1.91. detection limit was found 25 µg/ml. Mean recovery of Citric acid 99.90 %

Keywords: HPLC, PDA, Ayurveda, Validation, Column

Introduction:

An organic compound with acidic properties mostly organic acids are classified based on the number of the carboxylic functions {–COOH} organic acids are support to bind cellular integrity of the gut lining. Its play an important role to improve the digestive process in the human body. Organic acids improve the absorption rate of Amino acids, proteins, minerals etc. method has developed for five organic acids *(Tartaric Acid, Formic Acid, Malic acid, Glacial Acetic Acid and Citric Acid)* in Ayurveda products. Current Validation work done in hajma churna. Hajma churna is one of Ayurveda formulation consisting more than 6 herbs like: Pippali (Botanical name Piper longum), Maricha (Piper Nigrum), Nimbu saar (Citrus limon), Samudra lawana (Sea Salt), Sharkara (White sugar) and Sunthi (Zingiber officinale) including Citric acid. Citric acid is an organic compound which has chemical formula HOC (CO_2H) $(CH_2CO_2H)_2$. It is weak organic acid. Citric acid is naturally occurred in Citrus fruit, it is odourless and colourless compunds.it is added as a flavouring agent and as a preservative in food, herbal & cosmetic products.it keeps food fresh for longer times. Citric acid has protective importance in the human body.it reduced tough stains

Methods and Materials:

The HPLC (Waters, Alliance e2695) consisting of a 100 µl injector (Alliance), waters reciprocating pump, 4 lines in-line alliance degasser and 2998 PDA detector with Empower2 integration software (Waters Corporation) were used for the analysis. The chromatographic separation was achieved using the reverse-phase Phenomenex, Synergi, H20-116711, 5mic, 250 x 4.6 mm under isocratic elution of 0.06 %v/v Orthophosphoric Acid in water with a flow rate of 0.5 mL min^{-1}. The detection wavelength was set at 210 nm, Run time 30 minutes, diluent water and the injection volume was set at 20µl (Indian Pharmacopoeia 2018).

Materials

The Hajma churna samples from the market of India (Delhi) were collected directly from the site and examined to check the concentration of Organic acids As: Tartaric Acid, Formic Acid, Malic acid, Glacial Acetic Acid and Citric Acid by HPLC (PDA). Citric acid standard from Sigma-Aldrich 3050 Spruce Street, Saint Louis, MO 63103 USA with batch no. 0000058903, Placebo without citric acid from the vendor.

Standard preparation

Weigh accurately 25.0 mg of each Tartaric Acid, Formic Acid, Malic acid, Glacial Acetic Acid and Citric Acid reference standard & transfer it into 100 ml volumetric flask. Add about 50 ml water & sonicate for 5-10 minutes to dissolve and makeup to the mark with water. Shake well to homogenize. Filter 2 mL of this solution through 0.45µ Nylon filter into auto injector vial.

Sample preparation

Weigh and transfer the sample approx. 0.125 g into a 50 mL volumetric flask. Add around 10-15 mL of water, sonicate for 10 minutes and makeup to the mark with water. Shake well to homogenize. Filter 2 mL of this solution through 0.45µm Nylon filter into auto injector vial and was injected into the HPLC.

Procedure

Inject combined reference standard of organic acid, placebo of sample without organic acid & sample of hajma churna including citric acid into the HPLC and chromatogram recorded as shown in figure:1, figure:2, figure:3

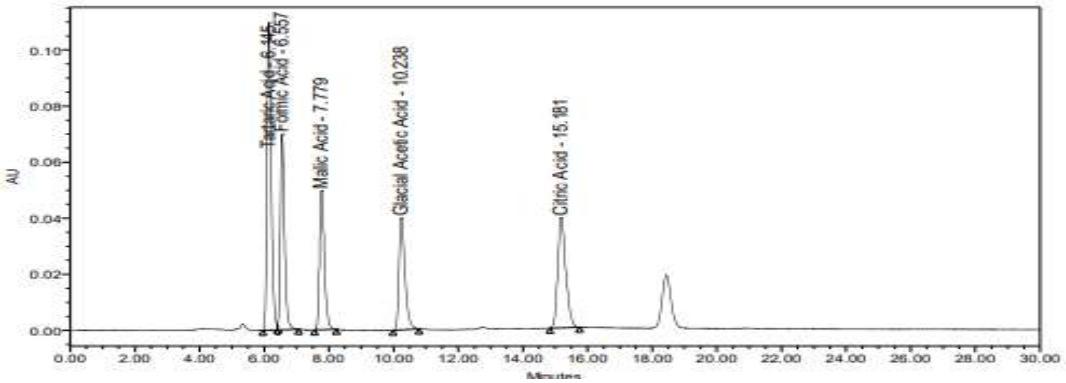

Figure 1: Chromatogram of a standard mixture solution

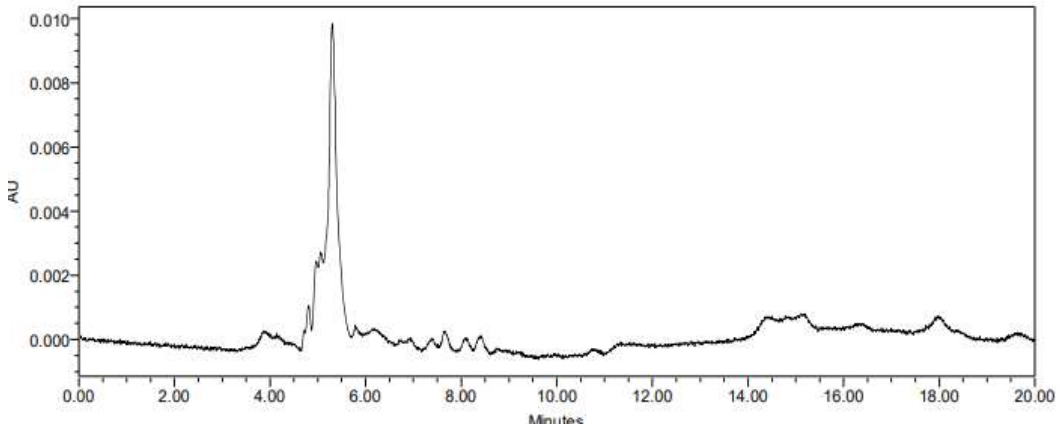

Figure 2: Chromatogram of a placebo solution

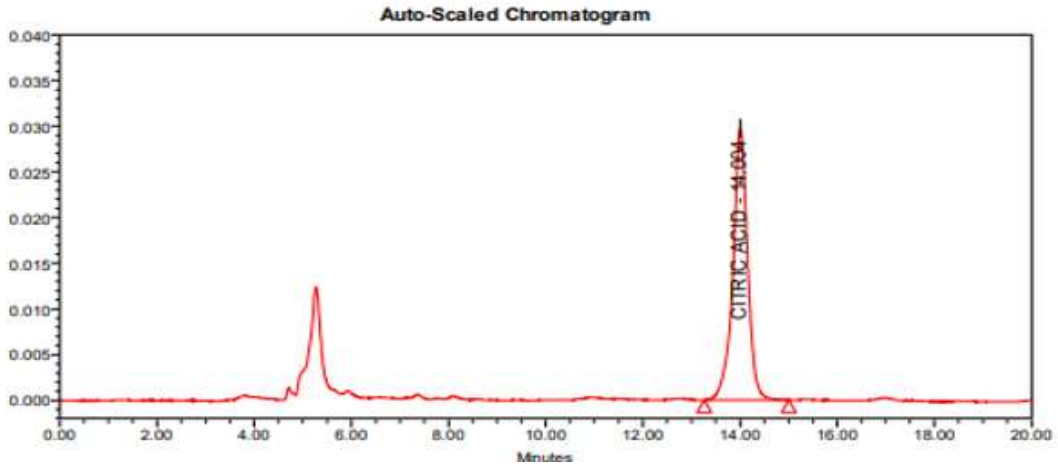

Figure 3: Chromatogram of a sample (Hajma churna) solution

Results and discussion

A) Chromatographic condition

Different trial method applied to get peak separation during development process following chromatographic parameter was set to achieve better separation, peak shape and resolution. Mobile phase 0.06 % orthophosphoric acid, flow rate-0.5 ml/min, wavelength set at 210nm by scanning of reference standard solution of organic acid. Organic acids are less chromophoric on HPLC (PDA) detector.

B) Optimization of method

The experiment done with all five organic acid as mentioned above. All five-organic acid (Tartaric Acid, Formic Acid, Malic acid, Glacial Acetic Acid and Citric Acid) were eluted simultaneously with the better resolution as shown in figure: 1.in the placebo no interferences occurs corresponds to the standards retention time as shown in figure:2. In the sample there is only Citric acid component was found in the sample as shown in figure: 3 during analysis. There is only citric acid was added in the sample. Thus validation activity was processed further only for Citric acid in the sample.

C) Method Validation

1) System suitability parameter

Inject standard reference solution and record the chromatogram for system suitability if SST (System Suitability Test) limits are under acceptance criteria that must be met prior to sample analysis. the following parameter than go forward for further experiment.

Acceptance Criteria

Theoretical plates : NLT 5000

Tailing factor : NMT 2

% CV (Citric acid RT) : NMT 1 % for six replicate injections of standard

% CV (Citric acid Peak Area) : NMT 2 % for six replicate injections of standard

Resolution : NLT 2 (between the analyte and closest eluting peak)

The test samples were injected after the system suitability acceptance criteria were met.

Demonstrating the Citric acid standard and sample wavelength spectra and Peak purity spectra showing in figure: 4, figure: 5 and peak purity angle and purity threshold are given below table-1

Table-1: Purity Angle & Purity Threshold

Sample	**Analyte**	**Retention Time**	**Purity Angle**	**Purity Threshold**	**Purity1 Flag**
Citric Acid Reference Standard	Citric Acid	14.145	0.804	1.024	NA
Sample	Citric Acid	14.165	0.371	0.567	NA
Reagent Blank	Citric Acid	14.193	*	*	*

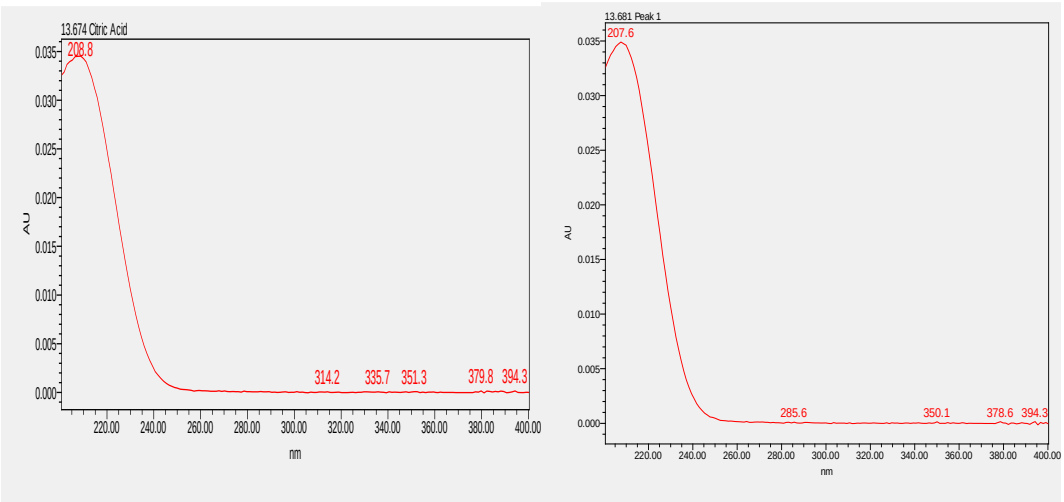

Figure: 4 Spectrum of Reference Standard and Sample

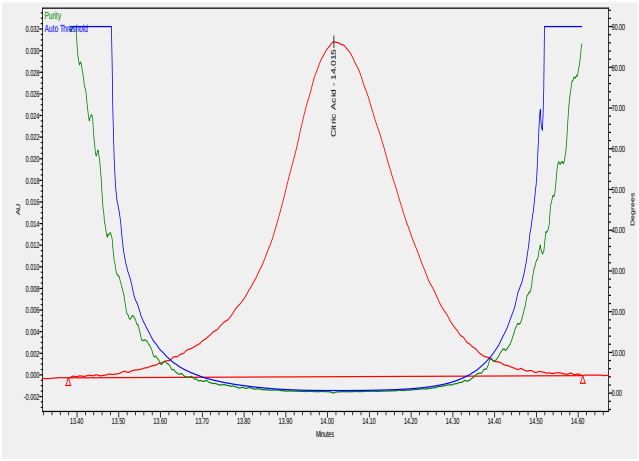

Figure: 5 Spectrum of Peak Purity

2) Calibration Curve (Linearity)

Calibration curve prepared using Citric acid reference standard solution concentration from 182 µg/mL,210 µg/mL,248 µg/mL,328 µg/mL,382 µg/mL,448 µg/mL to 626 µg/mL by taking seven concentration levels, injecting lower level and higher level solutions in six replicates and other levels in triplicate. Plotted a graph with concentration on X-axis against the peak area responses on Y-axis and calculated the slope of the regression line, the correlation coefficient, y-intercept, residual sum of squares and % CV for lower level and higher level peak area responses. The results are summarized in the Tables Linearity of the calibration curve was excellent (r2 > 0.999) Linearity chart given below in figure: 6.and Plot of "Residuals" Versus "Concentration (µg/mL)" (for Calibration Curve prepared using Reference Standard Stock Solution diluted with Diluent) shown in figure:7

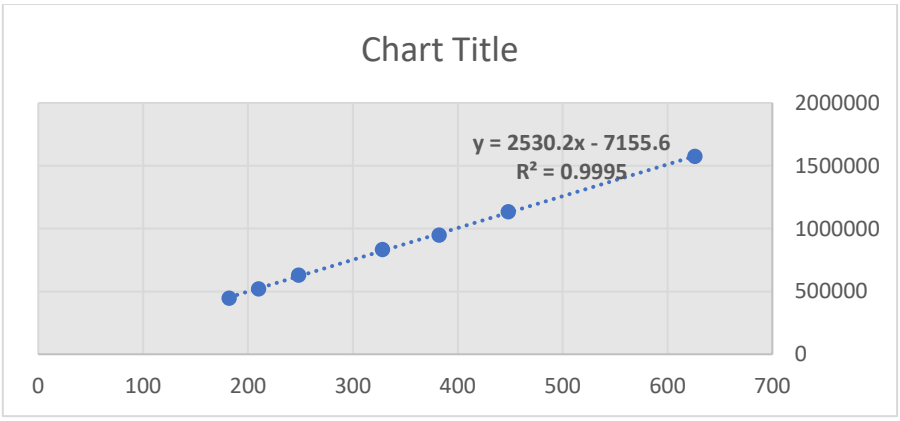

Figure: 6.Linearity plot of "Peak Area" Versus "Concentration (µg/mL)".

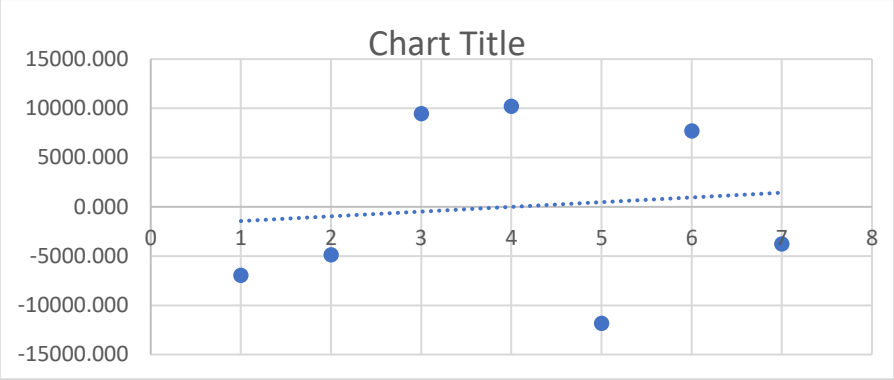

Figure: 7 Plot of "Residuals" Versus "Concentration (µg/mL)".

3) Recovery

The recovery study determined by taking the peak area obtained from the added Citric acid reference standard spiked into the placebo of the sample at 50 µg/mL (LOQ) level to separate portions of Placebo at 80% level, 100% level and 120% level of the test concentration of Citric acid in triplicate at each level and inject each in duplicate onto the chromatographic system.described in table-2

% Spike Level			**Peak Areas**	**Avg. Peak area**	**Amount recovered (µg/mL)**	**Average**	**SD±**	**%CV**
80	**Rep-1**	**Inj 1**	139624	137208	52.38	98.93	0.67	1.30
		Inj 2	134791					
	Rep-2	**Inj 1**	133241	133823	51.09			
		Inj 2	134405					
	Rep-3	**Inj 1**	134878	134706	51.43			
		Inj 2	134534					
100	**Rep-1**	**Inj 1**	155018	153763	58.70	99.83	0.32	0.54
		Inj 2	152508					
	Rep-2	**Inj 1**	154804	154514	58.99			
		Inj 2	154223					
	Rep-3	**Inj 1**	154052	152860	58.36			
		Inj 2	151668					
120	**Rep-1**	**Inj 1**	186605	186187	71.08	100.93	0.56	0.79
		Inj 2	185768					
	Rep-2	**Inj 1**	184054	183396	70.01			
		Inj 2	182737					
	Rep-3	**Inj 1**	184063	184024	70.25			
		Inj 2	183985					

Table-2; Recovery chart

4) Precision

Present method is very precise and accurate. System precision as determined given below in table-3.method precision activity was followed by repeatability and intermediate precision (between different day and different time precision) for Citric acid.

System Precision	% CV for six replicate injections of standard preparation should not be more than 2.0	**% CV = 0.21**
Method Precision (Repeatability)	% CV for % Assay of six sample preparations should not be more than 10.0	**% CV = 2.0**
Intermediate Precision	1) %CV for % Assay of six sample preparations should not be more than 10.0	**1) % CV = 1.91 % CV (Cumulative) =1.95**

	The overall % CV of % Assay from twelve determinations (six from method precision data and six from intermediate precision data) should not be more than 10.0	

Table-3; Precision chart

5) Detection limit & Quantification limit

The Limit of Detection and Limit of Quantitation was determined on the basis of signal-to-noise (S/N) ratio for Tartaric Acid, Formic Acid, Malic Acid, and Glacial Acetic Acid & Citric Acid the results are summarized in the Tables. LOD level 25 µg/mL and LOQ level 50 µg/mL. Signal-to-noise ratio limit for LOD & LOQ are shown in below table-4.

Conc. of Analyte (ppm)	**Peak Areas for Tartaric acid**				**Signal-to-Noise Ratio**			
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**
25 (LOD)	98566	97212	97973	97917	73.67	77.77	79.89	77.11
50 (LOQ)	193747	194438	195023	194402.67	160.08	126.03	147.9	144.67
Conc. of Analyte (ppm)	**Peak Areas for Formic acid**				**Signal-to-Noise Ratio**			
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**
25 (LOD)	61892	60569	62341	61600.67	47.05	49.53	51.35	49.31
50 (LOQ)	123424	122677	123728	123276.33	102.39	80.86	94.65	92.63
Conc. of Analyte (ppm)	**Peak Areas for Malic acid**				**Signal-to-Noise Ratio**			
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**
25 (LOD)	51225	52707	51833	51921.67	32.35	35.87	36.08	34.77
50 (LOQ)	102561	102284	103429	102758.00	72.49	57.24	65.6	65.11
Conc. of Analyte (ppm)	**Peak Areas for Glacial Acetic acid**				**Signal-to-Noise Ratio**			
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**
25 (LOD)	51818	51352	51156	51442.00	27.21	29.08	29.62	28.64
50 (LOQ)	101649	101905	101599	101717.67	59.73	47	54.03	53.59
Conc. of Analyte (ppm)	**Peak Areas for Citric Acid acid**				**Signal-to-Noise Ratio**			
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**
25 (LOD)	65987	66851	68620	67152.67	26.54	28.52	29	28.02
50 (LOQ)	134352	134915	132864	134043.67	58.92	46.45	53.36	52.91

Table-4; Signal-to-Noise Ration

6) Specificity test

Specificity test determined in placebo of the sample and No Significant Peaks were observed at the retention time of the analyte as shown in the above figure: 2 under Procedure.

7) Stability of analytical solution

The reference standard solution was stable up to 48 hours at ambient temperature and for 48 hours when kept at 2-8°C.The sample solution was stable up to 48 hours at ambient temperature and for 48 hours when kept at 2-8°C.

Discussion

There are so many methods for analysing Organic acids quantification However we have selected pH buffering method typically an acidic mobile phase which used in the ion exclusion mode. This method used widely for the acid analysis by HPLC. it means dissociation is inhibited for weak acids as electroconductivity detector under such conditions will not provide adequate sensitivity. So in this pH buffering, a pH is continuously added to the column effluent to adjust the pH closed to neutral to promote dissociation of organic acids in the column and reduce noise during analyte isolation. it shows high sensitivity and selectivity for all compounds presents in the sample.

Conclusion

The separation of organic acid all peak has been investigated and confirmed at different retention time As: Tartaric Acid, (6.14minutes) Formic Acid, (6.55minutes), Malic acid (7.77minutes), Glacial Acetic Acid (10.23minutes), and Citric Acid (15.11minutes) peak separation achieved using HPLC column; Phenomenex, Synergi, H20-116711, 5mic, 250 x 4.6 mm.this method is easy to use for the organic acid analysis in food and Ayurveda categories. The method can be considered proportionally rapid, simple and economically cheapest for the day to day routine analysis. In (alliance 2695 PDA at λ max 210 nm wavelength excellent area was observed of every component with suitable mobile phase 0.06 %v/v Orthophosphoric acid in water. The isocratic separation used in the process avoids the complications of the gradient separation.

Thus, validation criteria which have been set and discussed, that shows the method is suitable and reliable for the intended purpose and industrial use.

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