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Chromatographic analysis of botanical based ascorbate

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Abstract
Three different stationary phases of column chromatography viz., neutral alumina (150 mesh), silica gel (230 mesh) and activated charcoal (Darco-G-60) were evaluated for ascorbate recovery efficiency from the methanolic extract of *Emblica officinalis* (Amla) fruit pulp. Screening of efficient mobile phase was carried using fifteen varied proportions of chloroform and methanol *viz*., 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:33, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65 and 30:70. The presence of ascorbate was detected by thin layer chromatography and purity confirmed by high performance liquid chromatography. Results were validated by performing a relative phytoascorbate recovery study. Maximum amount of ascorbate per gram dry weight (2.44%) was recovered when purification was followed by using neutral alumina as stationary phase, followed by silica gel (1.09%). Minimum recovery of ascorbate (0.85%) was observed when charcoal was used as stationary phase. Mobile phase in the ratio of 70:30 (Chloroform: Methanol) resulted in the maximum elution of ascorbate among all the proportions. R_f value of purified ascorbate was calculated as 0.667 against the standard (0.668) during TLC. In the HPLC study, the ascorbate peak in sample eluted within 3.73 minutes as compared to standard which eluted within 3.79 minutes.

Keywords: chromatography, ascorbate, vitamin C, TLC, CC, HPLC

Introduction
Vitamin C, also known as ascorbate or ascorbic acid, is present in millimolar concentrations in plants, where it functions as the major antioxidant and as an enzyme cofactor. Consequently, ascorbate is involved in many different processes within the plant cell, including hormone and cell-wall biosynthesis, stress resistance, photoprotection and cell growth. Isolation of Ascorbate from its natural source like lemon, orange, paprika etc., has well been attempted way back when it was discovered by Szent-Gyorgyi (1928). Subsequently, may researchers succeeded in isolating the ascorbate by a number of processes involving crystallization, derivative formation, decomposition, chromatography etc., (Waugh *et al.*, 1933; Gosh & Guha, 1939; Baumann, 1944 and Hernandez *et al.*, 2005) [18, 4, 3]. Chromatography is a word used to encompass a range of techniques in which mixtures of pure substances are separated into the individual substances by using a mobile phase (usually a liquid) to push the mixture along a stationary phase (usually a solid or liquid coated on a solid). Because the individual substances have different molecular structures, they interact differently with both the stationary and mobile phases, and consequently are "pushed" at different rates by the mobile phase. In the present study ascorbate from the methanolic extract of Amla fruit pulp was purified using Column Chromatography (CC), detected by Thin Layer Chromatography (TLC) and purity confirmed by High Performance Liquid Chromatography (HPLC).

Materials and Methods
a) Column chromatography
- **Preparation of stationary phase:** Three stationary phases were prepared using three different adsorbents for standardization of the purification procedure viz., neutral alumina (150 mesh), silica gel (230 mesh) and activated charcoal (Darco-G-60)
- **Column packing:** A plug of cotton inside the bottom of the column was kept in such a way that the stationary phase would not fall out. The column was fastened tightly to a ring stand. A slurry of stationary phase was prepared by mixing the latter with chloroform in the proportion of 1:2 in a beaker. The slurry was poured into column immediately after stirring to maximize the amount of adsorbent that goes into the column instead of remaining behind in the beaker. The loaded column was allowed to settle down uniformly. To ensure the proper settling, stationary phase was thoroughly washed with chloroform. A Pasteur pipette was used to rinse any stationary phase that was sticking to the sides of the column.
- **Sample preparation:** A known amount of dry soxhleted crude extract of Amla-fruit pulp rich in ascorbate content was taken and mixed with stationary phase by using methanol. The sample was kept in hot air oven below 40°C for evaporation of methanol. The dried material was powdered.
- **Sample loading:** Elutent was fully drained from the column until no solvent above the surface of the stationary phase was left. The powdered sample was layered over the already prepared stationary phase. A layer of cotton was placed over the sample bed to avoid the disturbance while adding the eluting agent.
- **Mobile phase (Eluting agent):** Mobile phases in different ratios of chloroform and methanol were used in succession as follows: 100:0, 95:5, 90:10, 85:15, 80:20, 75: 25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65 and 30:70.
- **Process of column chromatography:** The sample-loaded column was at first eluted with chloroform to remove non-

polar solvent soluble impurities. Different proportions of eluting agent (2-15) were added immediately after the column was washed with chloroform. Care was taken not to allow the stationary phase to dry out as the column progresses which would lead to cracks within the phase. TLC of the each elution was performed to detect the presence of the ascorbate compared with standard. Eluting agent was continuously added until the absence of ascorbate was determined by the TLC.

Fig 1: Transfer of adsorbent to column

Fig 2: Column loaded with sample (close up)

b) Thin layer chromatography (TLC)

- **Preparation of solvent (mobile phase):** Mobile phase for identification of ascorbate was prepared by mixing Ethyl acetate, Acetic acid Glacial, Formic acid (85%), Distilled water in the proportion of 100:11:11:27 v/v (Ponder, 2004).
- **Preparation of standard solution:** The standard ascorbic acid was prepared by dissolving 1gm of ascorbic acid in 100ml of methanol.
- **Preparation of sample solution:** Sample collected from column chromatography was as such used for determining the ascorbic acid by TLC by comparing with standard.

Process of Thin Layer Chromatography

TLC analysis consists of a number of steps: Preparation of developing container, Preparation of TLC plate; activation of TLC plate, spotting the TLC plate, developing the TLC plate, drying the plate; visualizing the substance spots and measuring the Rf values.

- **Preparation of developing container:** A 100 ml beaker with a watch glass on the top was used (Plate 24 A). Mobile phase was poured into the beaker to a depth of just less than 0.5 cm (Plate 24 B).The beaker was covered with a watch glass, swirled gently and allowed to stand while preparation of TLC plate. A part of the beaker was lined with filter paper to aid in the saturation of the TLC chamber with solvent vapors.
- **Preparation of TLC plate:** The glass plate was thoroughly washed and dried before preparation of TLC plate. Silica gel with binder and florescent indicator was mixed with water (1:2 ratio) to make slurry. The slurry was applied to a plate as a uniform thin layer (250µ thickness) by means of plate spreader.
- **Activation of TLC plate:** TLC plate was placed in an oven at 50-60oC for 15-20 minutes for "activation". Activation involves driving off water molecules that bond to the polar sites on the plate.
- **Spotting the TLC plate:** Sample and standard was spotted on the TLC plate with the help of small capillary tubes. TLC spotting guide was used to ensure a uniform base line. Solvent was allowed to completely evaporate from the spot. Spotting was done in such a way that the spots should not touch the solvent in the chamber.
- **Developing the TLC plate:** TLC plates containing the sample and standard were placed carefully in the developing chamber containing mobile phase. Care was taken that sample and standard spots are above the level of mobile phase. It was noted that the TLC plates was not too much tilted. If the plate is excessively tilted, solvent will not advance uniformly along the plate and development will not take place properly. Similarly, if the bottom of the plate is against the wall of the glass container, solvent will advance more rapidly up the edges of the plate than in the middle, causing the substances to be pushed toward the center of the slide as they move up. Left the plate in the chamber until solvent had advanced to the top of the TLC plate and not cross the edge of the glass plate.
- **Drying and visualization of the TLC plate:** The plate was placed in an oven at a temperature of 50-60 ^{o}C for drying. When the plate is completely dry, it was visualized under UV illuminator (Fig. 3).
- **Measurement of Retention Factor (Rf):** The retention factor or R_f, is defined as the distance traveled by the compound divided by the distance traveled by the solvent. The R_f for a compound remains same from one experiment to the next only if the solvent system, adsorbent thickness of the adsorbent, amount of material spotted and temperature is kept constant.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

c) High performance liquid chromatography (HPLC)

- **Equipment and operational conditions:** HPLC was performed with a SHIMADZU SYSTEM consisting of a column monitor (SCL 6A) and column printer (CR 6A), A UV-Visible diode array detector (SPD 6AV) and a liquid chromatography pump (LC 6A). Shimadzu software was used to calculate peak areas. The column used was a partition type Shim-pack CLC NH_2 column (6.0mm i.d. x 15cm).

- **Mobile phase preparation:** Sodium phosphate buffer (10mM) of 2.6 pH was prepared by mixing 1.380 g of dibasic sodium phosphate into 1 liter of HPLC grade water. pH of the solution was adjusted to 2.6 by adding HCL (5ml in 45 ml HPLC water). Acetonitrile (HPLC grade) and Phosphate buffer (10mM) in the ratio of 3:1 v/v were prepared by mixing the 500 ml of Phosphate buffer into 1500 ml of Acetonitrile.
- **Preparation of standard solution:** Stock standard was prepared by dissolving 10mg in 10ml of the mobile phase. Working standard was prepared by taking 1ml from the stock and made up to 10ml with the mobile phase.
- **Sample preparation:** The plant extract eluted by column chromatography was filtered through "Millex syringe driven filter unit" and diluted with the solvent system used in HPLC.
- **Procedure:** The column was run with mobile phase for one hour for stabilization. Column temperature was maintained at 40 °C. HPLC machine was programmed for detection of the compound. The unit was set, by monitoring the sample run (1.5ml/min) and the detector was adjusted to UV range of 240nm. The standard solution was serially diluted with the mobile phase and injected using syringe (20µl) until the desired peak was repeatedly obtained at a constant time and peak area of known concentration. Then the serial dilutions of sample (purified ascorbate) were prepared and injected into the unit until the desired peak was obtained. Identification of compounds was achieved by comparing their retention times as detected by UV spectra with that of standard. Concentration of purified ascorbate was calculated as per the standard peak area.

Results and Discussion

a) Column chromatography

TLC was performed at a constant time interval with the 15 proportions of chloroform and methanol *viz*., 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:33, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65 and 30:70 and presence of ascorbate was confirmed by comparing with standard ascorbate. Till 75:25 mobile phase proportion, no presence of ascorbate was detected through TLC. Ascorbate started eluting as soon as 70:30 mobile phase was used. With continuous TLC observation all the elutent was pooled and concentrated.

Maximum amount of ascorbate per gram dry weight (2.44%) was recovered when purification was followed by using neutral alumina as stationary phase, followed by silica gel (1.09%). Minimum recovery of ascorbate (0.85%) was observed when charcoal was used as stationary phase (Table 1).

Table 1: Comparative recovery of purified ascorbate under different stationary phases of column chromatography

Column	VC/ml of sample (mg)	VC/g dry wt. (mg)	Recovery %
Neutral Alumina	2.406	24.439	2.44
Silica gel	1.832	10.992	1.09
Charcoal	1.426	8.559	0.85

b) Thin layer chromatography (TLC)

Ascorbate in Amla fruit extract, purified by column, was detected by TLC with mobile phase, Ethyl acetate: Glacial Acetic acid: Formic acid: Distilled water (100:11:11:27). R_f value of purified ascorbate was calculated as 0.667 against the standard (0.668) (Fig. 4)

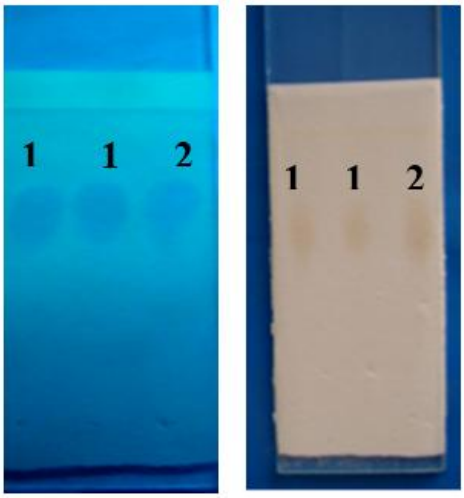

Fig 3: TLC chromatogram of purified vitamin C against standard under, A: visible light, B: UV Illumination
1: Purified ascorbate sample, 2: Standard ascorbate

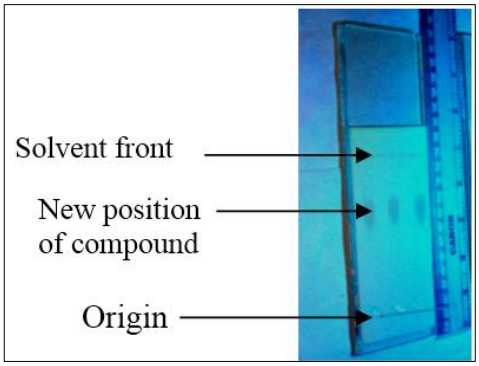

Fig 4: Calculation of R_f Value

c) High performance liquid chromatography (HPLC)

Purity of ascorbate in Amla fruit extract, purified by column, was tested in HPLC. Ascorbate peak in sample eluted within 3.73 minutes (table 2) as compared to standard which eluted within 3.79 minutes (table 3). In standard, injection of 2µg ascorbate showed the peak area of 62350. Purified ascorbate at 1.5 times dilution showed area of 39878 which confirmed the same concentration as standard.

Fig 5: HPLC Chromatograph of purified vitamin C

Table 2: Specification of HPLC Chromatograph for purified ascorbate

S, No.	Time	Area	Concentration
1	0.050	2437	5.412
2	0.100	2006	4.454
3	0.350	1135	2.520
4	2.210	109	0.242
5	3.735	39878	86.763
6	4.763	274	0.609
Total		45839	100

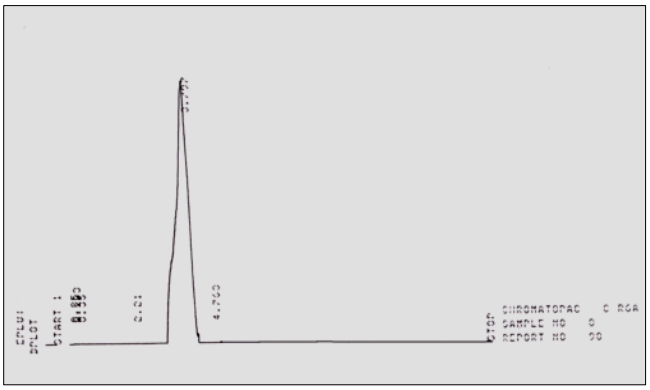

Fig 6: HPLC Chromatograph of standard vitamin C

Table 3: Specification of HPLC chromatograph for standard ascorbate

S. No	Time	Area	Concentration
1	0.053	153	0.217
2	3.797	62350	88.043
3	5.063	8314	11.741
Total		70817	100

In recent years HPLC has become the preferred method of determination, but this method has the disadvantages of being relatively labour intensive and costly on chemicals for analysis of sample. The quantification of ascorbate in column-purified sample was therefore carried out by following the spectrophotometric method for regular use. The HPLC was used only for the purity check.

In the current investigation purification of ascorbate was done by using the methanolic extract of *E. officinalis*, as this botanical extract was found to have the maximum content of ascorbate among all the botanicals short-listed. Purification was carried out using column chromatography. Standardization of mobile phase and stationary phase was done on the basis of the efficiency of the two phases to recover maximum ascorbate. With regard to the mobile phase among fifteen different proportions of chloroform and methanol, 30: 70 proportion was observed to be the effective elutent for ascorbate purification as detected by thin layer chromatography (TLC). Further, with respect to stationary phase, among the three phases used, maximum ascorbate per gram dry weight (2.44%) was recovered when purification was followed by using neutral alumina as stationary phase, followed by silica gel (1.09%) and charcoal (0.85%). Information is scanty regarding the purification of ascorbate from plants by following column chromatography. The present results are in agreement with Gosh and Guha (1939) [4] who purified the ascorbate from plant tissue material and pressed juice by column chromatography after elution with a mixture of 30% chloroform and 70% absolute alcohol. Similarly, Guha and Sengupta, (1938) [5] attempted to isolate ascorbate from cabbage juice by adsorption on charcoal and subsequent elution of the adsorbate with chloroform-alcohol mixture. Further purification of the mixture was effected by them by precipitation of the inactive substances by sodium tungstate and sulphuric acid. The filtrate was subjected to vacuum evaporation which yielded brownish color mass containing reducing substance (ascorbate). Different workers have put forth different method for the purification of ascorbate from plants using different materials and methods (Waugh *et al.*, 1933; Baumann, 1944 and Hernandez *et al.*, 2006) [18, 3, 6].

Thin layer chromatography (TLC) or high performance thin layer chromatography (HPTLC) is primarily used as an inexpensive method for separation, for qualitative identification or for the semi-quantitative visual analysis of samples. TLC is thus often described as a pilot method for HPLC (Rozylo and Janicka, 1991 & 1996) [12, 13]. However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis (Weins & Hauck 1996 and Kalasz & Bathori, 1997) [19, 7]. In the present study detection of ascorbate in sample obtained from column chromatography was performed regularly by thin layer chromatography (TLC) by comparing with the standard ascorbate. The ascorbate purified by column, was detected by TLC (Fig. 25 & 26) with mobile phase, Ethyl acetate: Glacial Acetic acid: Formic acid: Distilled water (100:11:11:27). Thin layer chromatography has been widely used to determine ascorbate concentration in foodstuffs, pharmaceutical preparations and biological materials. Aburjal *et al.*, (2000) [1] performed the TLC which involved direct application of methanolic solutions of tested samples on silica gel TLC plates using water: methanol (95: 5 v/v) as developing system. The developed plates were then directly scanned at 260 nm using a TLC scanner. Roomi and Tsao (1998) [11] carried out a thin-layer chromatography (TLC) for isomers of ascorbate and its oxidation product, dehydroascorbic acid (DHA) on sodium borate impregnated silica gel and cellulose plates. This procedure has been adopted to separate and identify the ascorbic acid and dehydroascorbic acid in fresh orange and lemon juices, pharmaceutical preparations and guinea pig tissues (liver, kidney and eye lens) and fluids (plasma and urine). Suntornsuk *et al.*, (2002) [14] detected the ascorbate by TLC in freeze-dried samples of guava (*Psidium guajava* Linn.) amla (E. officinalis), lemon (*Citrus aurantifollia* Swing), sweet pepper (*Capsicum annuum* Linn.) and passion fruit (*Passiflora laurifoia* Linn.) after 8 weeks. Navon (1978) measured the daily activity loss of ascorbate and its analogs at equimolar concentrations in the diet of Egyptian Cotton Leaf worm, *Spodoptera littoralis* by 2, 4-dinitrophenylhydrazine-thin layer chromatography method.

In the present study, column- purified ascorbate of amla fruit extract was analyzed for purity using high performance liquid chromatography (HPLC). Acetonitrile (HPLC grade) and Phosphate buffer (3:1 v/v) were used as mobile using CLC NH_2 column. With this mobile phase 2µg purified ascorbate showed the peak within 3.73 minutes of elution whereas that of standard ascorbate gave the peak within 3.79 minutes

(Table 47 & 48 and Fig. 27 & 28). The results of purified as against standard ascorbate confirmed purity of ascorbate isolated by column chromatography. Detection and quantification of ascorbate in plants has been reported by many researchers at different operational conditions. Khopde *et al.*, (2001) [8] determined ascorbate content of amla extract by HPLC (Spectra Series, P 100) with UV detector at 265 nm, C18 column and 5% v/v methanol in 0.01 M KH_2PO_4 as the mobile phase, at a flow rate of 1 ml/min. Sample solution (0.2%) was prepared in the mobile phase. They were able to get the peak for ascorbate at 2.60 min retention time as standard solution ascorbate which gave a peak at 2.64 min. The difference in the results observed by Khopde *et al.*, (2001) [8] and in present study could be attributed to the difference in column used, flow rate and more importantly the mobile phase. Similarly, Walker *et al.*, (2006) [17], Tedone (2004) [16], Asami *et al.*, (2003) [2], Yildrim and Tokusoglu (2005) [20], Burini (2007) and Hernandez *et al.*, (2005) have used the HPLC for qualitative and quantitative analysis of plant based ascorbate using different columns, mobile phases, stationary phases, retention times *etc*., and different reports have been put forth.

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