



## Development and Validation of Ion Exchange Chromatographic Method for the Quality Control of Extract of *Hordeum Vulgare*

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### Abstract

**Introduction:** Yavakshara is an ayurvedic alkaline formulation which is used to treat urinary tract disorders like nephrolithiasis (kidney stone disease), by having an alkalinizing effect which helps in dissolving kidney stones. Though it is a commonly prescribed kshara in ayurveda, but there are no published protocols for establishment/authentication of its quality.

**Methods:** In the present study, physico-chemical properties and content analysis of the powder preparation was done. Energy Dispersive X-ray Scanning Electron Microscopy (EDX-SEM) analysis showed the presence of smooth and spherical particles and also indicated the presence of elements like sodium, chlorine, and potassium. X-ray Powder Diffraction (XRPD) analysis indicated the presence of NaCl crystals in the form of Halite. Further, the counterions present in Yavakshara were determined via Ion-exchange Chromatography (IC).

**Results and Discussions:** Though intense resolved peaks for chloride and sodium were observed at 8.70 min and 4.64 min respectively, potassium was not detected by the IC method. The IC method was also validated as per ICH guidelines for use in regulated environments for the establishment of the safety and efficacy of product.

**Keywords:** Ion-exchange Chromatography; Yavakshara; Sodium; Chloride; X-ray Powder Diffraction; Alkaline Extract

### Abbreviations

CaCO<sub>3</sub>: Calcium Carbonate; Cl: Chlorine; EDS: Energy Dispersive X-ray; ICDD: International Center for Diffraction Data; IC: Ion-exchange Chromatography; JCPDS: Joint Committee on Powder Diffraction Standards; LOD: Limit of Detection; LOQ: Limit of Quantitation; K<sub>2</sub>CO<sub>3</sub>: Potassium Carbonate; KCl: Potassium Chloride; PDF: Powder Diffraction File; RSD: Relative Standard Deviation; SEM: Scanning Electron Microscopy; Na: Sodium; NaCl: Sodium Chloride; SD: Standard Deviation; XRPD: X-ray Powder Diffraction.

### Introduction

Barley, being one of the oldest cultivated medicinal plants, has an important place in Ayurveda, the Indian traditional medicine. It

also finds reference in *Charak Samhita* or *Compendium of Charaka*, one of the foundational Hindu texts on Ayurveda. Known as Yava in Sanskrit and Jau in Hindi, barley is botanically known as *Hordeum Vulgare* and belongs to family Poaceae. Barley is also often used in Chinese herbal medicine and reported to contain highest mineral content, especially sodium [1].

Kshar is a white colored ayurvedic preparation, which is obtained as a residue after evaporation of filtrate of plant ash dissolved in water. It is mainly done to extract alkali rich matter by controlled burning of plant materials [2]. It has medicinal properties for curing many gastric and urinary tract disorders [3]. In ayurveda, yavakshara is given twice to thrice a day with buttermilk

before food for 1 month to treat urolithiasis subsequently which reduces the symptoms like haematuria and dysuria [4].

Yavakshara is a kshara prepared from barley which exhibits alkaline properties and is used for treatment of many gastric, renal and urinary tract disorders [5]. Basically, it exerts alkalinizing effect on kidney environment. Though it is one of the commonly used kshara but pharmaceutically very little is known about it.

Identification and determination of chemical constituents in such multi-component extracts is important to establish quality control parameters, which further have implications on efficacy and safety of the herbal drug. However, due to the complex and variable nature of the plant derived products, it becomes difficult to confine the quality parameters in set or defined ranges. So, determination of chemical markers, especially in unorganized herbal extracts can help in establishment of protocols for establishment of the quality of these products. Modern analytical techniques are being used for standardization of plant-derived products.

There are no available reports on the standardization and content evaluation of Yavakshara. In the present study, we have developed an ion exchange chromatographic method (IC) for the estimation of constituents of Yavakshara. This work also describes the analytical method validation of the method for use in regulated environments [6-9]. Ion exchange chromatography, being a sensitive method compared to other analytical techniques, can be used for detection of a wide variety of organic and inorganic acids [10].

## Experimentals

### Chemicals and reagents

Yavakshara was purchased from Dindayal Industries Ltd., India. High-purity water was collected from a Millipore Milli-Q water purification system (Millipore, Milford, MA, USA). Multi-element ion chromatography standard solutions of cations and anions were purchased from Sigma-Aldrich.

### Physico-chemical characterization (Composition Analysis) of Yavakshara: An alkaline herbal preparation

#### X-ray powder diffraction

X-ray Powder Diffraction (XRPD) analysis is widely used analytical technique for the study of crystal structures and atomic spacing. X-rays scattered by sample at different angles are recorded at each displacement along the course of rotational movements of the detector and sample. The intensity of the reflected X-rays is recorded and processed by the detector in the form of a signal peak.

MiniFlex X-Ray Diffractometer equipped with D/teX Ultra detector and MiniFlex 300/600 Goniometer was used for analysis of Yavakshara. Finely powdered sample was placed evenly on a quartz glass slide sample plate and pressed. The sample plate was fixed upon the standard sample holder of the X-ray instrument and analysed at 40kV, 15 mA in continuous scan mode. The sample was scanned at a speed of 10°/min in the range of 5°-80°. Interpretation of spectra was done using Philips X'PERT Highscore Plus software v 3.0 [11].

### Scanning electron microscopy coupled with energy dispersive X-ray analyzer

Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-ray (EDS) is a technique used for elemental analysis and inorganic compound/substance characterization [11]. This technique is also known as Energy-dispersive X-ray Spectroscopy, occasionally termed as Energy Dispersive X-ray Microanalysis or Energy Dispersive X-ray Analysis. It works on a fundamental principle that each element has its unique atomic structure giving a unique set of peaks on electromagnetic emission spectrum. Emitted X-rays strike an EDS detector or solid-state detector (lithium-drifted silicon or Si (Li) detector) producing a transient electrical signal whose energy is proportional to the energy of the X-ray. These signals are sorted according to their energy and counted in a multi-channel analyser [12]. EVO MA 15 Scanning Electron Microscope coupled with 51N1000 EDS System (Zeiss, Germany) was used for the analysis of Yavakshara. The sample was made conductive by coating with gold using QUORUM (Q150RES) sputter coater. The sample was kept in sputter coater for 90 seconds at 20 mA current to form a uniform coat of about 7-8 nm thickness. The coated sample was dried via thermal evaporation inside the coater and then kept in the sample holder and analysed. Each sample was analysed thrice (number of iterations = 3). Corresponding reference standards used in the study were CaCO<sub>2</sub> for Carbon, Albite for Sodium, KCl for Chloride and MAD-10 Feldspar for Potassium.

### Ion-exchange chromatography (IC)

Ion-exchange chromatography is one of most effective methods for simultaneous determination of ions with precision, high sensitivity and rapidity. Ion-exchange chromatography was performed to supplement the findings of x-ray diffraction and SEM-EDS studies.

### Instrumentation and chromatographic conditions

Dual channel 930 Compact IC Flex Ion exchange chromato-

graphic system (Metrohm, Switzerland) equipped with 919 IC auto-sampler, a cation suppressor, and a conductivity detector were used for the study. The quantitation of cations was performed on Metrohm C4 and MetrosepA Suppressor 5 (250 × 4.0 mm, 5.0 μm particle size) was used for anions. HPLC grade water was used as mobile phase and the analysis was done at a temperature of 25°C maintaining a flow rate of 0.7 mL/min and pump pressure of 13.1 MPa in anionic compartment. Similarly, in the cationic compartment the flow rate and pump pressure were maintained at 0.9 mL/min and 7.9 MPa respectively. The data obtained was processed using the MagIC Net ver. 2.1 (Metrohm, Switzerland).

The suppressors used during anion exchange play an important role in detection by reducing the background conductivity of the eluent. This in turn, increases the conductivity of the analytes and is done by replacing the counterions (cations) in the sample with H<sup>+</sup>. This results in overall increase in the sensitivity of analysis. The suppressor needs to be rotated by 120° before each analysis, so that it is regenerated. The suppressor is usually regenerated using diluted sulphuric acid, followed by rinsing with HPLC grade water or eluate. The detector interface was set with a detector range of 100 μS/cm and scale of 20 μS/cm for a run time of 27 min.

### Preparation of solutions

Certified multi-element ion chromatographic standard solutions of cations and anions (Sigma-Aldrich) of concentrations 1, 3, 5, 7 and 10 ppm were used as reference standards to calibrate the instrument before the sample run. All dilutions were made in Milli-Q water. The test solutions of Yavakshara were prepared at concentration of 20, ppm. The concentration of anions and cations present in the test solutions was estimated using validated ion-exchange chromatographic method in triplicate (n=3).

### Method development and validation

During the process of development of an analytical method, conditions required for production of reproducible results are selected through multiple trials. These parameters are then validated to prove that they are suitable for their intended use. The ion-exchange method developed for the estimation of ions present in Yavakshara was validated as per ICH guidelines to achieve the conditions as mentioned in Section 3.3.1. "Chromatographic Conditions".

- **Specificity:** Specificity is the ability of the analytical method to analyze with certainty the given analyte in presence of substances which might be degradants, impurities, matrix com-

ponents, etc. The specificity of the developed ion-exchange chromatographic method was established for the presence of anions such as chloride, nitrate, bromide, nitrite and sulfate; and cations such as sodium, potassium, magnesium and calcium. The specificity of the method was determined based on the detection of individual anionic or cationic peak through the chromatographic procedure.

- **Precision:** In an analytical procedure, precision expresses degree of scatter (concurrency) between a series of measurements of multiple samples used from the same homogeneous bulk under the prescribed chromatographic conditions. Precision may be measured by estimating the repeatability and ruggedness of the method. Repeatability or intra-assay precision was checked over the concentration range with six determinations at each concentration and percentage relative standard deviation was calculated. Ruggedness (intermediate precision) which determines within-laboratory variations was also estimated by carrying out the method on different days, different analytes, etc.
- **Accuracy:** It is a measure of how close the experimental values are to the true value. Accuracy was determined by incorporating known amount of drug (Yavakshara) by weight in the medium. Three individually prepared replicates (n = 3) were analysed and accuracy was calculated using formula: %Recovery = (Recovered concentration/Added concentration) × 100.
- **Linearity and Range:** This parameter defines the confidence of the analytical method to obtain results within a range which is directly proportional to the concentration of the analyte in the sample. In order to establish the linearity of the method, concentration of individual (cations/anions) in the range of 10 μg/mL to 50 μg/mL were prepared. The peak areas of individual ions (cations/anions) were plotted against the corresponding concentrations to obtain the calibration curve and correlation coefficients for the regression lines.
- The range is estimated via linearity studies. It can be defined as the determination of highest and lowest limit of the analyte present in the sample that has been indicated to be measurable using the method along with the applicable degree of accuracy, linearity and precision within the stated range of the analytical procedure. Results collected after the accuracy, precision and linearity analysis were utilised to estimate/establish the range of the developed method or analytical procedure.

- Limit of detection (LOD) and limit of Quantitation (LOQ):** LOD and LOQ both are two vital performance parameters of method validation. LOD is the lowest quantity or concentration of an analyte in a sample which can be reliably detected but not inevitably quantitated. LOQ is the lowest concentration of an analyte in a sample which can be reliably quantified along with suitable accuracy and precision via the analytical procedure. LOQ and LOD were determined as per the ICH guidelines. There are various methods to determine them, however, in the present method determinations were made using formula:  $LOQ=10\sigma/S$  and  $LOD=3.3\sigma/S$  where,  $\sigma$  is the Standard Deviation (SD) of the response and S is the slope of the calibration curve.  $\sigma$  can be determined from SD of y-intercepts (of regression lines). Other approaches are based upon Calibration curve, Signal to Noise ratio, SD of blank and visual evaluation.

## Results and Discussion

### X-Ray powder diffraction analysis

The x-ray diffractogram of Yavakshara showed peaks at  $d=2.81176\text{\AA}$  ( $2\theta=31.8$ ),  $d=1.99074\text{\AA}$  ( $2\theta=45.52$ ),  $d=3.13517\text{\AA}$  ( $2\theta=28.44$ ) confirming the presence of crystalline components in it (Figure 1). For most materials, the peaks and their corresponding intensity have been documented in the form of Powder Diffraction File (PDF). The PDF is a database of X-ray powder diffraction patterns maintained by the International Center for Diffraction Data (ICDD) and JCPDS (Joint Committee on Powder Diffraction Standards). These PDF reference patterns (Figure a) are retrieved by using software's like PANalytical X'Pert HighScore Plus v3.0 and used to compare with the XRD pattern of unknown samples and predict their characteristics. In this study, PDF patterns were retrieved using the PDF reference code/number and search-match procedure using stick patterns (Figure 2-3) along with other details like name, formula, crystallographic parameters and references of the database. From the stick pattern, it was inferred that NaCl crystals in the form of Halite and  $K_2CO_3$  crystals are present in Yavakshara.

### Scanning electron microscopy coupled with energy dispersive X-ray analyzer

The characteristic x-ray energies vary from element to element with limited spectral overlapping. The micrograph of Yavakshara showed smooth surface morphology with a spherical shape with particle size being in the micrometer range (Figure 4,5). SEM-EDS data showed the presence of sodium, chloride, potassium and carbon in the kshara sample. The highest intensity peak was recorded for chloride indicating its abundance in sample followed by that of sodium. The percentage weight of elements and atoms present in Yavakshara has been shown in table 1.

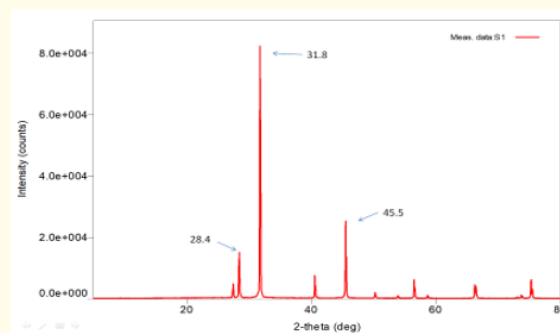


Figure 1: XRD Spectra of Yavakshara.

Pattern List	Scan List	Peak List	Anchor Scan Data			
No.	Pos. [2Th.]	Height [cts]	FWHM [°2 $\theta$ ]	d-spacing [Å]	Rel. Int. [%]	Tip width [°2 $\theta$ ]
1	24.4925	83.55	0.2362	3.63455	0.10	0.2400
2	27.4720	4572.96	0.1181	3.24675	5.65	0.1200
3	28.4507	14810.60	0.1378	3.13726	18.29	0.1400
4	31.8008	80985.41	0.1181	2.81399	100.00	0.1200
5	40.6050	7220.16	0.0787	2.22187	8.92	0.0800
6	45.5312	25140.18	0.0960	1.99063	31.04	0.0800
7	45.6699	12316.34	0.0480	1.98984	15.21	0.0400
8	50.2686	1839.55	0.1200	1.81357	2.27	0.1000
9	50.4128	992.77	0.0720	1.81322	1.23	0.0600
10	53.9435	864.36	0.0960	1.69837	1.07	0.0800
11	54.0961	441.80	0.0720	1.69815	0.55	0.0600
12	56.5464	6152.94	0.0960	1.62621	7.60	0.0800
13	56.7109	3079.58	0.0960	1.62592	3.80	0.0800
14	58.7174	919.68	0.0960	1.57115	1.14	0.0800
15	58.8818	463.91	0.0720	1.57105	0.57	0.0600
16	66.2947	4445.93	0.0960	1.40876	5.49	0.0800
17	66.4743	3944.94	0.0960	1.40538	4.87	0.0800
18	66.6531	1110.62	0.0720	1.40553	1.37	0.0600
19	73.1312	365.51	0.0960	1.29301	0.45	0.0800
20	73.7644	1062.12	0.1200	1.28346	1.31	0.1000
21	73.9815	592.93	0.0720	1.28342	0.73	0.0600
22	75.3488	5977.58	0.1200	1.26036	7.38	0.1000
23	75.5701	2978.59	0.0960	1.26034	3.68	0.0800

Figure a: Sample peak List of XRD Spectra of Yavakshara.

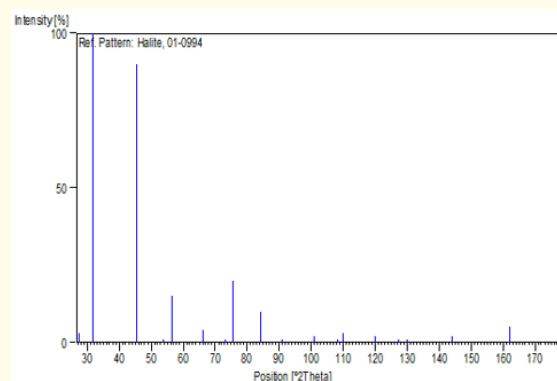
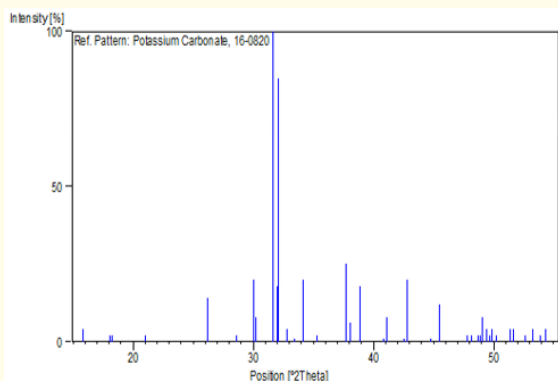
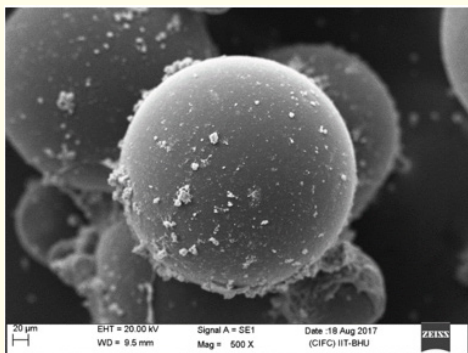


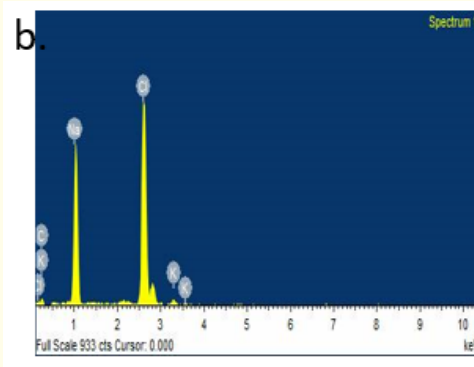
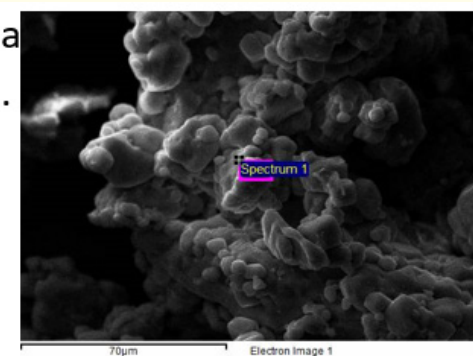
Figure 2: Calculated XRD Stick Pattern of Halite (Rock Salt - Sodium Chloride) from Powder Diffraction File (PDF) database. The data as obtained from the database- Reference code:01-0994; Mineral name: Halite; PDF index name: Sodium Chloride; Empirical formula: ClNa; Chemical formula: NaCl. References-Primary reference: Davey., Phys. Rev., 21, 143, (1923); Optical data: Data on Chem. for Cer. Use, Natl. Res. Council Bull. 107; Unit cell: The Structure of Crystals, 1st Ed.



**Figure 3:** Calculated XRD Stick Pattern of Potassium Carbonate from Powder Diffraction File (PDF) database. The data as obtained from the database-Reference code: 16-0820; PDF index name: Potassium Carbonate; Empirical formula:  $CK_2O_3$ ; Chemical formula:  $K_2CO_3$ . References-Primary reference: de Wolff, P, TechnischPhysischeDienst, Delft, The Netherlands., ICDD Grant-in-Aid.



**Figure 4:** SEM image of Yavakshara.



**Figure 5:** a. EDS inspection field selected for elemental analysis and; b. EDS Spectrum showing the presence of elements in Yavakshara.

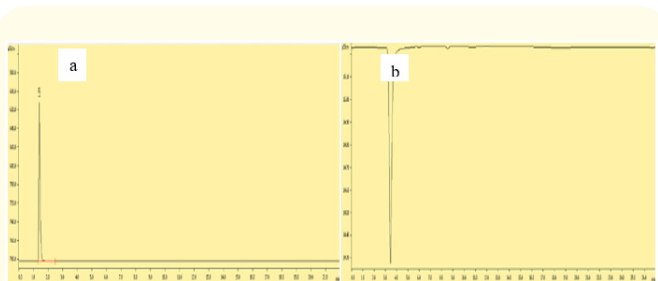
Element	C	Na	Cl	K	Total
Weight %	12.33	33.17	52.65	1.86	100.01
Atomic %	25.65	36.05	37.11	1.19	100.00

**Table 1:** Weight % and Atomic % of elements present in Yavakshara.

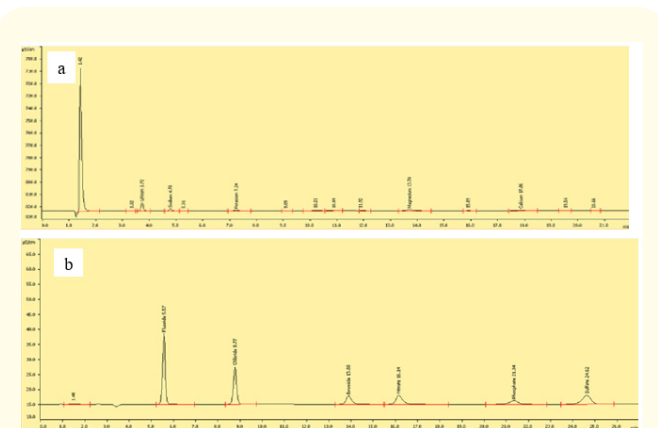
### Ion exchange chromatography

Validation helps in establishing a documented proof that a particular method can be used with high level of confidence for detection of analytes of interest. In the present study, a simple and precise ion-exchange chromatographic method was developed for determination of the quality of Yavakshara. Currently, there is no analytical method available for analysis of Yavakshara and the proposed method will help in establishing the quality of Yavakshara. Satisfactorily resolved chromatographic peak shapes and consistent retention times were achieved with accurately and scientifically selected mobile phase. The chromatogram of blank consisting of ultra pure water (cations) and ultrapure water (anions) have been shown in figure 6 (a-b).

The retention time of cations lithium, sodium, potassium, magnesium and calcium in standard solution was observed at 3.79, 4.78, 7.17, 13.07 and 17.21 min respectively (Figure 7a) and that of anions fluoride, chloride, bromide, nitrate, phosphate and sulphate were recorded at 5.57, 8.77, 13.88, 16.14, 21.34, and 24.62 min respectively (Figure 7b).



**Figure 6:** Ion Exchange chromatogram of a) Ultrapure water cations and; b) Ultrapure water anions.



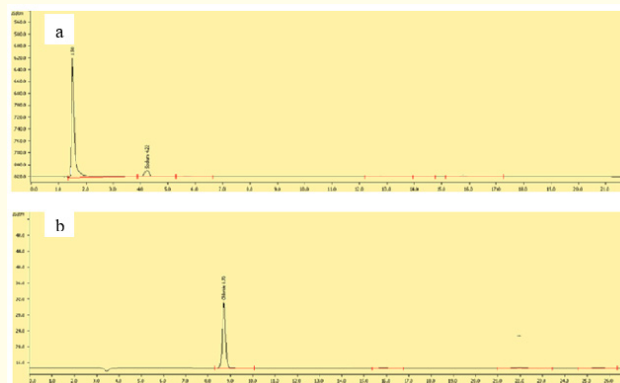
**Figure 7:** Ion Exchange chromatogram of standard solutions of (a) cations and; b) anion at a concentration of 10 ppm.

### Test sample analysis

A sample of 20 ppm of Yavakshara was prepared and analyzed to find the concentration of cations and anions present in the sample. As can be observed in Figure 8a, majorly sodium along with Cl were detected in the Yavakshara sample (Figure 8b). It can be thus inferred that Yavakshara is majorly composed of Cl, and Na. Potassium, however could not be detected in the sample using the IC method.

Table 2 shows the retention times, linearity and range, calibration curves, detection limits (LOD), quantification limits (LOQ) and precision data for the chromatographic analysis. In the present study, well resolved peaks of the ions were observed in the chromatograms for the yavakshara sample (Figure 8 a-b). Intense resolved peaks of chloride and sodium were observed at 8.70 min and 4.64 min respectively in the sample. Typically, relative standard deviation (RSD) should be less than or equal to 2% for bulk drugs

and for minor components it should be less than or equal to 5%. Less than 2% relative standard deviation was observed for intra-day (repeatability) and inter-day precision (ruggedness) studies.



**Figure 8:** Ion Exchange chromatogram of (a) cations and; b) anions present in Yavakshara.

Element	Chloride	Sodium
Parameters		
Retention time (min)	8.78	4.26
Y <sup>α</sup>	0.1599x	0.1043x
Rsd	0.8078	0.745
Linearity range (µg/mL)	0-50	0-50
DL <sup>β</sup> (µg/mL)	2.044	1.746
QL <sup>∞</sup> (µg/mL)	6.208	5.207
Precision	0.56-1.1	0.11-1.77
Accuracy	95%	96.5%

**Table 2:** Validation data for the ion chromatographic analysis of ions present in yavakshara.

<sup>α</sup>Intercept; <sup>β</sup>Detection limit; <sup>∞</sup>Quantitation limit

### Conclusion

The present method was developed and validated with an objective to devise a technique for the standardization of yavakshara. Currently, there are no quality control protocols in place for evaluation of safety and efficacy of this alkaline khara, but is being used commonly for the treatment of nephrolithiasis and other urinary tract infections. There are a number of methods available for the detection and quantification of anions/cations via both non-chromatographic methods like potentiometry [13], electrophoresis us-

ing ion-selective electrodes [14] or capillary method with indirect UV detection [15] and chromatographic methods based on light scattering [13], suppressed [16] or non-suppressed conductivity detection [17], or indirect UV detection [18]. However, we developed and validated a novel ion chromatographic method with suppressed detection and high sensitivity as per ICH guidelines which can be used in industrial set up for the establishment of the safety and efficacy of yavakshara. The stick pattern analysis showed the presence of NaCl crystals in halite form which was in concurrence with SEM-EDS data which showed presence of chloride and sodium in yavakshara. The determination of concentration of anions/cations in the drug substance is a pre-requisite to establishment of the quality in terms of mass, stoichiometry, presence of functional groups, etc. which also are required for drug authenticity studies.

### Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

### Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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