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High-performance liquid chromatographic method for the quantification of phenolics in 'Chyavanprash' a potent Ayurvedic drug

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Abstract

Quantification of bioactive principles through modern analytical tools is essential for establishing the authenticity, creditability, prescription and usage of Ayurvedic medicines/herbal formulations. 'Chyavanprash' is one of the oldest and most popular Ayurvedic preparations, used widely as a health promotive and disease preventive 'Rasayana' drug in India and elsewhere. The rejuvenating and tonic properties of 'Chavanprash' are considered majorly due to their antioxidant principles, which in turn is due to the presence of phenolic compounds. A simple high-performance liquid chromatography (HPLC) method for the separation and quantitative determination of the major antioxidant compounds from 'Chyavanprash' has been developed. The use of Waters Symmetry® column and an acidic mobile phase enabled the efficient separation of phenolic compounds (catechin, quercetin-3-*O*-rutinoside, syringic acid and gallic acid) within a 35 min analysis. Validation of the method was done with a view to demonstrate its selectivity, linearity, precision, accuracy and robustness. In addition optimization of the complete extraction of phenolic compounds were also studied.

Keywords: 'Chyavanprash'; HPLC; Phenolics; Antioxidants

1. Introduction

'Chyavanprash' is one of the most popular Ayurvedic preparations placed under 'Rasayana' group of drugs, used widely as a health promotive and disease preventive tonic. The word 'Rasayana' literally means the path that 'Rasa' takes ('Rasa': plasma; Ayana: path). 'Rasayana' drugs are very rich in powerful antioxidants, good hepatoprotective and immunomodulating agents [1]. Being a well-known Ayurvedic formulation, 'Chyavanprash' has been the subject of study by several researchers [2–6]. However, the main emphasis has been on correlating the ethnomedicinal uses of some of its ingredients with the medicinal properties attributed to it, and evaluation of its physicochemical values for quality evaluation. Amino acid quantification by HPLC in the 'Chyavanprash' has already been reported [7], but phenolic compounds, as secondary plant metabolites, are most suitable as chemical markers and the presence of which, in the formulation is characteristic and can be used in standardization of 'Chyavanprash' [8,9].

Although there have been different approaches to their analysis, the separation and quantification of the phenolic compounds of a plant extract remains difficult, especially the simultaneous determination of different groups of phenolics in a sole analysis and that too in a poly herbal formulation like 'Chyavanprash' having more than 40 plants ingredients (Table 1). HPLC is the method of choice for the analysis of phenolic compounds because of its versatility, precision and relatively low cost [10–12]. Most frequently, the method is used on reversed phase (RP) C18 columns, a binary solvent system containing acidified water, a polar organic solvent (acetonitrile or methanol), and UV–vis diode array detection (DAD), which constitute a crucial and reliable tool in the routine analysis of plant phenolic compounds [13,14]. Obtaining good resolution is considered the prerequisite for a method targeted for the separation of multiple phenolic groups [11]. According to the most relevant bibliography, the HPLC-DAD chromatographic method seems to be a suitable tool for the separation and quantification of phenolic compounds in plant extracts [13]. On the contrary, spectrophotometric method is ideal for quantitative estimation purposes, but total phenols are indiscriminately measured. There are many difficulties in these quantitative determinations, and their usefulness often questioned. However, some of them, for example, the

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Table 1
The list of ingredient

1	Bilva (Rt.)	*Aegle marmelos* Corr. (Rutaceae)
2	Agnimantha (Rt.)	*Premna mucronata* Roxb. (Verbenaceae)
3	Syonaka (Rt.)	*Oroxylum indicum* (Linn.) Vent. (Bignoniaceae)
4	Kasmari (Garnbhari) (Rt.)	*Gmelina arborea* Roxb. (Verbenaceae)
5	Patali (Rt)	*Stereospermum suaveolens* DC. (Bignoniaceae)
6	Bala (Rt.)	*Sida cordifolia* Linn. (Malvaceae)
7	Salaparni (Rt.)	*Desmodium gangeticum* DC. (Fabaceae)
8	Prsniparni (Rt.)	*Uraria picta* (Jacq.) Desv. ex DC. (Fabaceae)
9	Mudgaparni (Rt.)	*Phaseolus trilobus* Ait. (Fabaceae)
10	Masaparni (Rt.)	*Teramnus labialis* Spreng. (Fabaceae)
11	Pippali (Fr.)	*Piper longum* Linn. (Piperaceae)
12	Svadamstra (goksura) (Rt.)	*Tribulus terrestris* Linn. (Zygophyllaceae)
13	Brhati (Rt.)	*Solanum indicum* Linn. (Solanaceae)
14	Kantakari (Rt.)	*Solanum surattense* Burm.f. (Solanaceae)
15	Srngi (Gl.)	*Pistacia kinjuk* Stocks. (Anacardiaceae)
16	Tamalaki (bhumyamalki) (Pl.)	*Phyllanthus amarus* (Euphorbiaceae)
17	Draksa (Dr. Fr.)	*Vitis vinifera* Linn. (Vitaceae)
18	Jivanti (Rt.)	*Leptadenia reticulata* Wt. and Arn. (Asclepiadaceae)
19	Puskara (Rt.)	*Inula racemosa* Hook.f. (Asteraceae)
20	Abhaya (Haritaki) (Fr. P.)	*Terminalia chebula* (Gaertn.) Retz. (Combretaceae)
21	Amrta (guduci) (St.)	*Tinospora cordifolia* (Willd.) Hook.f. (Menispermaceae)
22	Rddhi (Sub. Rt.)	*Fritillaria roylei* Hook. (Liliaceae)
23	Jivaka (Rt.)	*Malaxis acuminata* D.Don (Orchidiaceae)
24	Sati (Rz.)	*Curcuma aromatica* Rosc.
25	Musta (Rz.)	*Cyperus rotundus* Linn. (Cyperaceae)
26	Punarnava (Rt.)	*Boerhavia diffusa* Linn. (Nyctaginaceae)
27	Meda (Sub. Rt.)	*Polygonatum cirrhifolium* Royle (Liliaceae)
28	Ela (Sd.)	*Elettaria cardamomum* (Linn.) Maton (Zingiberaceae)
29	Candana (Ht. Wd.)	*Santalum album* Linn. (Santalaceae)
30	Utpala (Fl.)	*Nymphoea stellata* Willd. (Nymphaeaceae)
31	Vidari (kanda) (Rt. Tr.)	*Pueraria tuberosa* DC. (Fabaceae)
32	Vrsamula (vasamula) (Rt.)	*Adhatoda zeylanica* Medik. (Acanthaceae)
33	Kakoli (Sub. Rt.)	*Lilium polyphyllium*
34	Kakanasika (Fr.)	*Martynia annua* Linn. (Martyniaceae)
35	Amalaka (Fr.)	*Phyllanthus emblica* Linn. (Euphorbiaceae)
36	Pippali (Fr.)	*Piper longum* Linn. (Piperaceae)
37	Tvak (St. Bk.)	*Cinnamomum zeylancium* Blume.
38	Ela (Sd.)	*Elettaria cardamomum* (Linn.) Maton (Zingiberaceae)
39	Patra (Lf.)	*Cinnamomum tamala* (Ham.) Nees and Eberm. (Lauraceae)
40	Kesara (nagakesara) (Fl.)	*Mesua ferrea* Linn. (Clusiaceae)

Fl.-Flower; Fr.-Fruit; Lf.-Leaf; Rt.-Root; Sd.-Seed; St.-Stem; Rz.-Rhizome

Folin–Ciocalteu (FC), are still widely used for a first estimation of the phenolic content in plant extracts [10].

The aim of the present study was to develop an optimized, validated and a simple HPLC method for the analysis of the phenolic compounds present in 'Chyavanprash'. Once the method is developed, extraction parameters optimized to obtain a fast and complete extraction of the phenolic compounds in the compound herbal formulation, method validated on the basis of its selectivity, linearity, precision, accuracy and robustness [15] according to ICH requirements [16], it can successfully be applied for the standardization of 'Chyavanprash'.

2. Materials and methods

2.1. Standards and chemicals

Caffeic acid, chlorogenic acid, catechin, ferulic acid, gallic acid, protocatechuic acid, quercitrin, rutin, syringic acid and vanilic acid were obtained from Sigma Co. Ltd. HPLC grade acetonitrile, water and phosphoric acid were obtained from Merck (Darmstadt, Germany).

2.2. Samples

'Chyavanprash' was procured from the market (Lucknow) and 25 g was defatted with hexane and chloroform and then subsequently extracted with methanol (3× 100 ml). The extract was then filtered, concentrated using a rotary evaporator (Buchi, USA) and dried using lyophiliser (Labconco, USA). Simple mobile phase was used as the control to see the blank peaks.

2.3. Solid-phase extraction columns

SAMPREP RP18 SPE non-endcapped columns (Ranbaxy Fine Chemicals Ltd., India) of size 500 mg sorbent mass/6 ml reservoir volume.

2.4. Purification of phenolic extract by SPE column

About 1.5 g of extract was subjected to extraction as previously described. The methanolic extract was taken to dryness under reduced pressure (40 °C), and re-dissolved in 50 ml of methanol. The methanolic solution was then passed through SAMPREP RP18 column, previously conditioned with 5 ml of methanol and 5 ml of water. The loaded cartridge was passed with 30% methanol water to get the phenolics. The injection volume for HPLC analysis was 10 μl.

2.5. HPLC-DAD system for qualitative and quantitative analysis of phenolic compounds

Analyses were performed in a liquid chromatograph with Waters (Milford, MA, USA) pumps (Waters 515) equipped with an online degaser, a Waters PCM, Rheodyne 7725 injection valve furnished with a 20-μl loop, a Waters 2996 photodiode array detector and Waters Empower software. Separation was carried out using a Waters Symmetry® (150 mm × 3.6 mm, i.d., 5 μm pore size), guard column of same chemistry.

HPLC finger print profile was established for phenolic fraction. Elution was carried out at a flow rate of 1 ml/min with

water:phosphoric acid (99.7:0.3, v/v) as solvent A and acetonitrile:water:phosphoric acid (79.7:20:0.3, v/v) as solvent B using a gradient elution in 0–5 min with 88–85% A, 5–10 min with 85–75% of A, 10–20 min with 75–70% of A, 20–25 min with 70–50% of A, 25–30 min with 50–30% A and 30–35 min with 30–88% of A.

2.6. Calibration

The content of the active phenolic compounds was determined using a calibration curve established with seven dilutions of each standard, at concentrations ranging from 2.5 to 200 μg/ml. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the phenolic compound injected. Peak identification was achieved by comparison of both the retention time and UV absorption spectrum with those obtained for standards. The used reference substances were chlorogenic acid, caffeic acid, protocatechuic acid, ferulic acid, catechin, rutin, quercitrin, vanilic acid, syringic acid and gallic acid.

2.7. Validation parameters

2.7.1. Selectivity and peak purity

Selectivity was checked by using an extract of 'Chyavanprash' and a mixture of available standards optimizing separation and detection. The purity of the peaks was checked by DAD (λ = 200–400 nm) by multivariate analysis. The three spectra corresponding to upslope, apex and downslope of each peak were computer normalized and superimposed. Peaks were considered pure when there was a coincidence between the three spectra (match factor was ≥98%).

2.7.2. Linearity, limits of detection and quantification

The linearity of the detector response for the prepared standards was assessed by means of linear regression regarding the amounts of each standard, measured in μg, and the area of the corresponding peak on the chromatogram. Linearity was also confirmed for 'Chyavanprash' extract. After chromatographic separation, the peak areas obtained were plotted against the extract concentrations by linear regression. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method.

2.7.3. Precision

The repeatability of the injection integration was determined for both standard phenolics and the 'Chyavanprash' constituents. A standard solution containing the 10 reference compounds was injected 10 times, Chyavanprash was also extracted 10 times to evaluate the repeatability of the extraction process. The mean amount and R.S.D. values of each constituent were calculated. The precision was calculated at two different concentrations high and low tested in the concentration range. For standardization the sample was injected at five different concentrations and linearity was noted.

2.7.4. Accuracy

The accuracy of the method was determined by analyzing the percentage of recovery of the main constituents in the 'Chyavanprash' extract. The samples were spiked with three different amounts of standard compounds before extraction. The spiked samples were extracted by triplicate and analysed under the previously established optimal conditions. The obtained average contents of the target compounds were used as the "real values" to calculate the spike recoveries.

2.7.5. Robustness

For the determination of the method's robustness a number of chromatographic parameters, such as both column package and size, mobile phase composition and gradient ratio, flow rate and detection wavelength, were varied to determine their influence in the quantitative analysis. Interday and intraday variability was studied for the sample, by injecting the same concentration of the sample on three different days and the standard error mean was calculated.

2.8. Statistics

When applicable, one-way or two-way analyses of variance (SPSS11.0 for window) were used to assess the observed differences in the phenolic content. Differences were considered to be statistically significant when the P-value was <0.05.

3. Results and discussion

Four phenolics in 'Chyavanprash' were identified, viz., gallic acid (RT: 2.377, 0.36%, w/w), catechin (RT: 9.495, 0.12%, w/w), syringic acid (RT: 11.559, 0.16%, w/w) and rutin (RT: 18.193, 0.47%, w/w) (Fig. 1). The chromatograms also shows many other peaks apart from the 10 standards studied (Fig. 2), work is in progress to identify them also. The sample were injected at five different concentrations and the linearity was observed with the regression coefficient being 0.93.

The HPLC method was validated by defining the linearity, peak purity, limits of quantification and detection, precision, accuracy, specificity and robustness. For qualitative purposes, the method was evaluated by taking into account the precision in the retention time, peak purity, and selectivity of phenolic compounds eluted. A high repeatability in the retention time was obtained with (R.S.D.) values lower than 1.5% for both standards and extracts even at high concentration. The peak purity was studied in the major peaks. In no case were impurities or co-elutions were observed (match factor ≥ 96%). Linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated for quantitative purposes (Table 2). Thus, LOD and LOQ values ranged from 0.6 to 2.94 μg/ml, and from 3.8 to 7.8 μg/ml, respectively, which suggested full capacity for the quantification of each phenolic compound investigated. R^2 values of the phenolic compounds were higher than 0.98 thus confirming the linearity of the method. The high recovery values (close to 95%) and a high repeatability indicated a satisfactory accuracy in the proposed method. Likewise, the accuracy was independent of both the

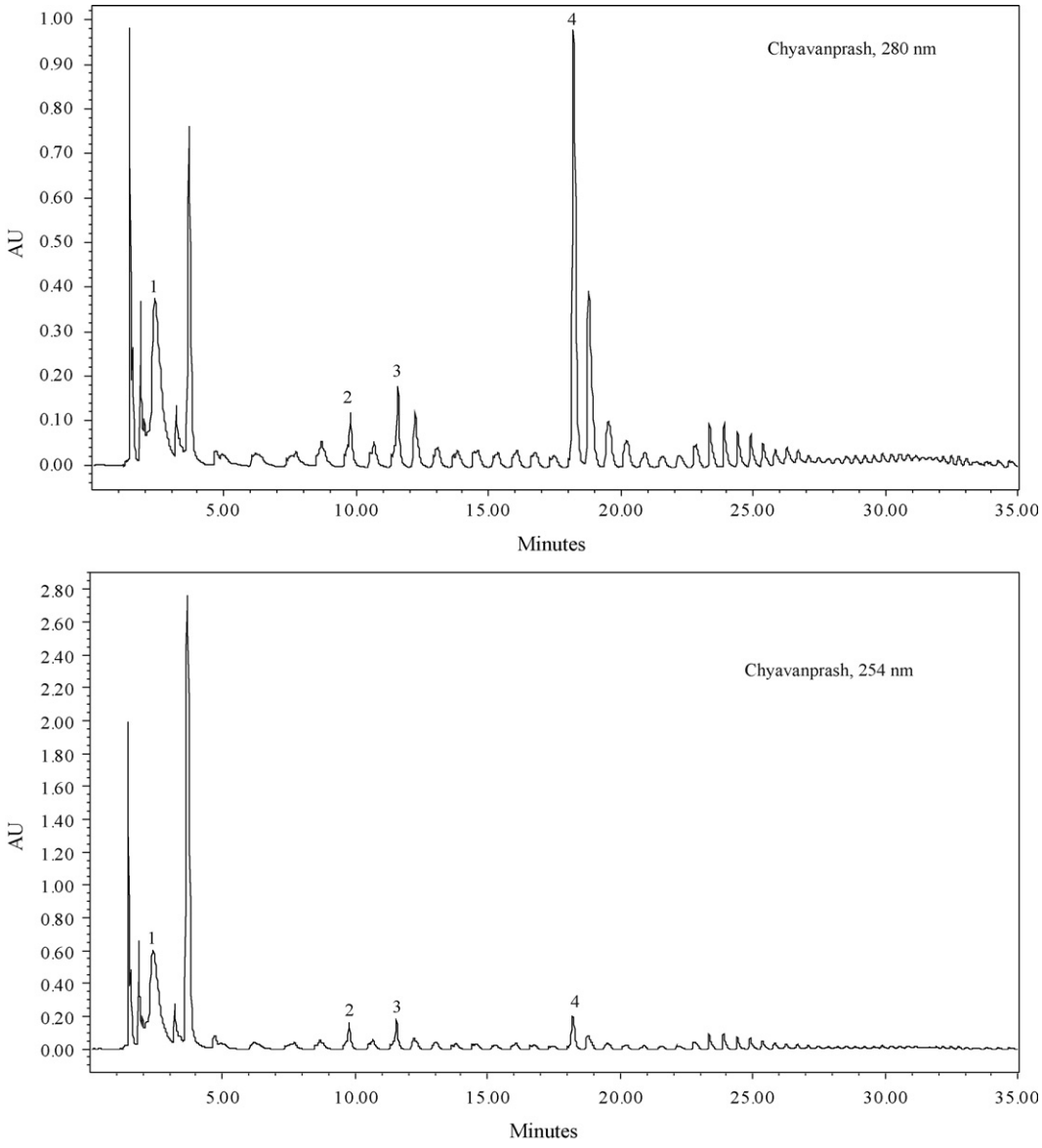

Fig. 1. Chromatograms registered for Chyavanprash at 280 and 254 nm, showing the phenolics: (1) gallic acid; (2) catechin; (3) syringic acid; (4) rutin.

compound concentration and the chemical structure. Finally, the robustness of the method was also assessed. Minor modifications of the initial mobile phase gradient (from 7 to 18% solvent B instead of 12%) had no effect on the peak resolution of the compounds. Therefore, this HPLC method can be regarded as selective, accurate, precise and robust. The precision in the RT and in the concentration was maintained with very little variations viz. R.S.D. around 3 and 0.9% in RT and concentration respectively at two different concentrations. The method is very adaptable because of the precision and repeatability for the compound herbal formulations like 'Chyavanprash' which is a major advantage of the current over other methods available. There was not much variation in the interday and intraday injections performed with the R.S.D. being 2.74 and 1.4%, respectively.

'Chyavanprash' is an important compound formulation of Ayurveda claimed to be best the Rasayana (immunomodulator

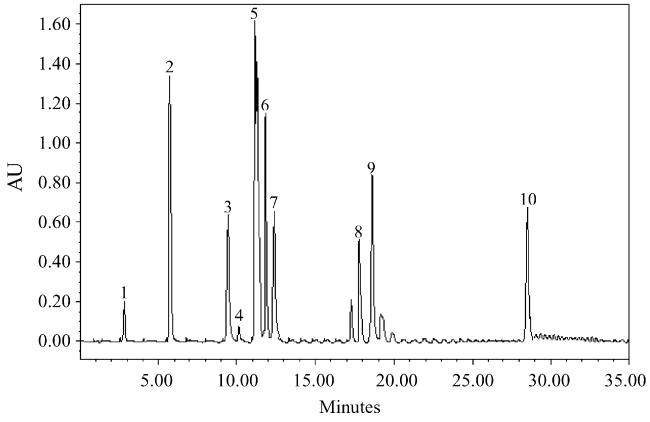

Fig. 2. Chromatograms registered for standard at 280 nm, showing the phenolics: (1) gallic acid; (2) protocatechuic acid; (3) catechin; (4) caffeic acid; (5) vanillic acid; (6) chlorogenic acid; (7) syringic acid; (8) rutin; (9) ferulic acid; (10) quercitrin.

Table 2
Regression curves, linearity, limit of quantification (LOQ), limit of detection (LOD) and recovery

Compound	t_R (min)	R^2	Concentration range[a]	LOD[a]	LOQ[a]	Recovery (%)
Caffeic acid	11.88 ± 0.07	0.998	10–100	1.17	4.6	91.12 ± 1.8
Catechin	9.76 ± 0.04	0.994	10–100	0.6	3.8	96.4 ± 1.6
Chlorogenic acid	10.3 ± 0.02	0.985	10–200	2.94	5.1	95.6 ± 0.86
Ferulic acid	18.50 ± 0.16	0.992	5–100	1.72	6.5	93.54 ± 1.28
Gallic acid	2.37 ± 0.09	0.990	10–200	0.66	4.2	97.86 ± 0.78
Protocatechuic acid	4.77 ± 0.11	0.989	10–100	1.16	5.8	94.6 ± 1.66
Quercitrin	28.04 ± 0.11	0.997	5–100	2.6	7.8	97.8 ± 0.83
Rutin	18.19 ± 0.03	0.996	2.5–100	1.4	3.8	96.0 ± 1.93
Syringic acid	11.56 ± 0.06	0.998	5–100	2.92	4.9	89.79 ± 2.48
Vanilic acid	10.8 ± 0.15	0.996	5–100	1.55	4.4	97.66 ± 2.12

The retention times (t_R) are the mean of 10 replicates ±S.D.
[a] Values expressed in μg/ml.

and rejuvenator) [5]. Certain lymphocyte functions are potentiated by H_2O_2 or other reactive oxygen intermediates [17]. The intact immune system thus appears to require a delicate balance between peroxidant and antioxidant conditions. It is interesting to note that several of ingredients of 'Chyavanprash' are used ethnomedicinally [2]. It has been successfully used as a preventive and a curative tonic and also useful during chronic constipation and urinary infections [22] it revitalises the metabolic functions. Some evidence has been provided to show that oxygen radicals and released enzymes such as myeloperoxidases and hydrolases play an important role in the auto-immune diseases and that therapy directed against them have been proven clinically useful. A few individual case reports have appeared as to the benefits of oxygen radical scavengers in the treatment of human autoimmune disease although proper control trials have not been done. It is also used as a food supplement for strength and energy, maintains youthfulness by renewing tissues and counterattacking degeneration. Free radical theory of ageing proposes that normal ageing results from random deleterious damage to tissues by free radicals produced during normal aerobic metabolism. Depressed immunocompetence associated with ageing, various diseases and poor nutrition may result from an excess generation of ROS due to the down regulation of the enzymes, viz., lipoxygenase and cyclooxygenase. Various antioxidants may prevent and/or correct immune dysfunction. These also include dietary or oral supplements in the form of Vitamins C and E, β-carotene, zinc and selenium [23,24]. 'Chyavanprash' has also been reported to contain *Emblica officinalis* as one of the major ingredient, which is reported to contain antioxidant tannoid principles [25].

During the past decade, the polyphenols have been subjected to a number of investigations due to their biological properties and benefit effects on health. Phenolic compounds seem to protect against cardiovascular disease and have certain potential anticarcinogenic properties due to their antioxidant activity and their function as free radical scavengers [18–21].

The results indicate that 'Chyavanprash' contain a number of phenolics which inturn may be responsible for their therapeutic activity. The HPLC method developed here thus helps in standardization of the 'Chyavanprash' using biologically active chemical markers (phenolics). Since some of plants used in the 'Chyavanprash' (Table 1) also contain these phenolics for example *Aegle marmelos* is reported to contain rutin, while *Phyllanthus amarus* contains gallic acid and many more, which can also be standardized by using this method. With the growing demand of herbal drugs in the herbal drug market and with the increased belief in the usage of green medicine (herbal drugs), this standardization tool will help in maintaining the quality and batch to batch consistency of this important Ayurvedic preparation.

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