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*Full Length Research Paper*

# Optimization of the technology of extracting water-soluble polysaccharides from *Morus alba* L. leaves

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**To optimize the parameters for extracting water-soluble polysaccharides from mulberry leaves using hot water, the extraction process was optimized by the orthogonal test through the single-factor experiment. Experiments were carried out using an $L_9$ ($3^4$) orthogonal design to examine the effects of extraction temperature, extraction duration, concentration of the material and concentration of ethanol on the polysaccharide yield. The optimum extraction conditions determined were as follows: concentration of material was equal to 1:24, extraction temperature was 70°C, extraction duration was 90 min and concentration of ethanol was equal to 80%. Under these conditions, the yield of polysaccharides was 2.64%.**

**Key words:** Polysaccharides, *Morus alba* L., extraction technology, single-factor experiment, orthogonal test.

## INTRODUCTION

Mulberry (*Morus alba* L.) belongs to the family Moraceae and is a perennial deciduous plant. Mulberry leaves have medicinal properties and the tree is found in the list of edible plants declared by the Chinese Ministry of Health. It has a high nutritional and medicinal value, and the active ingredients mainly comprise of polysaccharides, alkaloids, peptides, flavonoids, polyphenols and so on. Various parts of the mulberry are used as medicine in China, Japan and Korea to treat diabetes, paralytic stroke, and beriberi (Kim et al., 2003). However, the total area available for mulberry cultivation is decreasing, and mulberry trees are susceptible to frost damage (Lee et al., 2011). According to Shen Nong's Materia Medica, mulberry leaves are characterized by a sweet-and-bitter taste, are cold in nature, belong to the lung-liver channel, have an antiobesity function, soothe the liver and improve eyesight (Zhao et al., 2008; Nair et al., 2004). They have been applied in traditional medicine for the treatment of diabetes. Modern pharmacological and clinical studies have shown that the active ingredient in mulberry, namely, polysaccharides, lower blood sugar and blood pressure, regulate immunity, and have antibacterial, antiviral and other physical activities (Alamo et al., 2004; Hosseinzadeh et al., 1999; Kodama et al., 2004; Noriko et al., 2005). The Japanese people have studied deeply the effective ingredients of mulberry (Yatsunami et al., 2008). They found that the polysaccharides could lower blood sugar and therefore, they analyzed the structures. In China, the polysaccharides from mulberry leaves have been used as regulators of blood glucose concentration in alloxan-induced diabetes in rats (Fang et al., 1999). China has abundant mulberry resources and proposes to develop functional foods and hypoglycemic drugs with the natural, medicinally effective polysaccharide ingredient extracted from *M. alba* L. leaves (Chen et al., 1996; Yang et al., 1984).

The methods used for the extraction of polysaccharides from *M. alba* L. leaves (hot water extraction and ultrasound extraction) were compared. Hot water extraction is widely used because it is simple, easy to industrialize and involves low cost; nevertheless, the yield

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is less and the process is time-consuming. The yield from ultrasonic extraction is slightly higher than that of hot water extraction, the amount of time spent is short, and the amount of extract needed is less; however, its higher costs are not conducive for industrialization and amplification, and the equipments are costlier. Therefore, it is currently limited to the laboratory. We used hot water extraction to extract water-soluble polysaccharides from mulberry leaves. On the basis of the single-factor experiment, an orthogonal experimental array was adopted to study the effect of several important factors that affect the yield of polysaccharides and the technological conditions were further optimized.

## MATERIALS AND METHODS

Mulberry leaves were collected from the Hunan Institute of Sericulture (Silkworm and Mulberry Improvement Center of China, Changsha subcenters) after the first frost of the year, dried at 50°C, sifted through a 40-mesh sieve and stored in a dryer box.

The trichloroacetic acid, ethanol, sulfuric acid, glucose and anthrone were of analytical grade. An electric constant-temperature water bath, electrically heated hot air oven (Shanghai Jing Hong Experimental Equipment Co., Ltd.), rotary evaporator (Yarong Biochemical Instrument Factory), recycled water pumps (Instrument Factory of Yu Gongyi City, China), high-speed centrifuge and UV-Vis-754 ultraviolet-visible spectrophotometer (Shanghai Precision Instrument Co., Ltd.) were used.

### The process of extraction

Mulberry leaves were subjected to hot water extraction. The temperature and duration of extraction was investigated in the single-factor study. The extract thus obtained was filtered using gauze filters. The filtrate was concentrated under vacuum, and different volumes of 10% trichloroacetic acid were added to deposit proteins. Subsequently, alcohol precipitation (using different concentrations) was carried out, and the precipitate obtained was redissolved in distilled water, then the polysaccharide content was determined after the solution was further diluted.

### Single-factor experiment

After determining the most appropriate volume of trichloroacetic acid needed to deposit proteins, we used single factor of different raw material concentrations, extraction temperatures, extraction durations, number of extractions and ethanol concentrations, to examine the effect of each factor on the polysaccharide yield.

### Orthogonal experiment

On the basis of the single-factor experiment, an orthogonal array was adopted to study the effects of the various important factors, and then the best technological conditions were obtained.

### The determination of polysaccharide content

The polysaccharide content was determined by the anthrone-sulfuric acid method (Morris 1948). Obtaining the standard curve, different concentrations of glucose (in the same volume) were taken in test tubes, and 0.5 ml of anthrone reagent and 5 ml of concentrated sulfuric acid were added to these tubes. They were placed in a boiling water bath for 15 min; then, they were cooled to room temperature. Distilled water was treated similarly for use as the blank control. The optical density was determined by colorimetry at the wavelength of 620 nm. The extinction-concentration regression equation was $Y = 12.688X + 0.0576$; the correlation coefficient for this regression equation was 0.9991.

Before determination of the polysaccharide content in the sample, 10% trichloroacetic acid was added to the vacuum-concentrated filtrate for precipitation of proteins. Then, 80% ethanol was added, and the tubes were left undisturbed overnight. The tubes were centrifuged, the precipitated pellet was washed with alcohol and distilled water, made up to a fixed volume with distilled water, and analyzed using the anthrone colorimetric method to measure the polysaccharide content in the diluted solution.

## RESULTS AND DISCUSSION

### The single-factor experiment

### Effect of different volumes of trichloroacetic acid on removal of proteins

Mulberry leaf extract (5 ml) was put in six test tubes, and the following volumes of 10% trichloroacetic acid were added to these tubes: 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml, respectively. After mixing, the tubes were stored for 24 h at 4°C; later, they were centrifuged at 4000 rpm for 15 min, the supernatant was removed, and the dry deposits were weighed. The results are shown in Figure 1. Addition of 0.6 ml of 10% trichloroacetic acid for every 5 ml of extract was considered the optimum.

### Effect of different extraction temperatures on the extraction rate

The extraction temperature has an important effect on the extraction rate and the costs of the process. 10 g of the material were taken, and water was added to obtain a solid : liquid ratio of 1:18. The extraction was carried out for 60 min at the following temperatures: 60, 70, 75, 80, 85, 90, and 100°C. The concentration of ethanol used for precipitation was 80%. The results are shown in Figure 2.The results showed that a greater solubility of polysaccharides was obtained as the temperature increased from 70 to 80°C, and the extraction rate decreased when the temperature was above 70°C, with a gradual leveling off after 80°C. Polysaccharide extracts from *M. alba L.* leaves coalesced tightly with the protein, and contain large quantities of inorganic small molecule impurities, and high temperature may cause degradation of the polysaccharides, thus resulting in a decreased extraction rate (Zhang, 2005). Considering that very high temperatures may affect the molecular structure and activity of polysaccharides and easily degrade them, in addition to vaporization of water at high temperatures, which is also not conducive for industrial operations, we

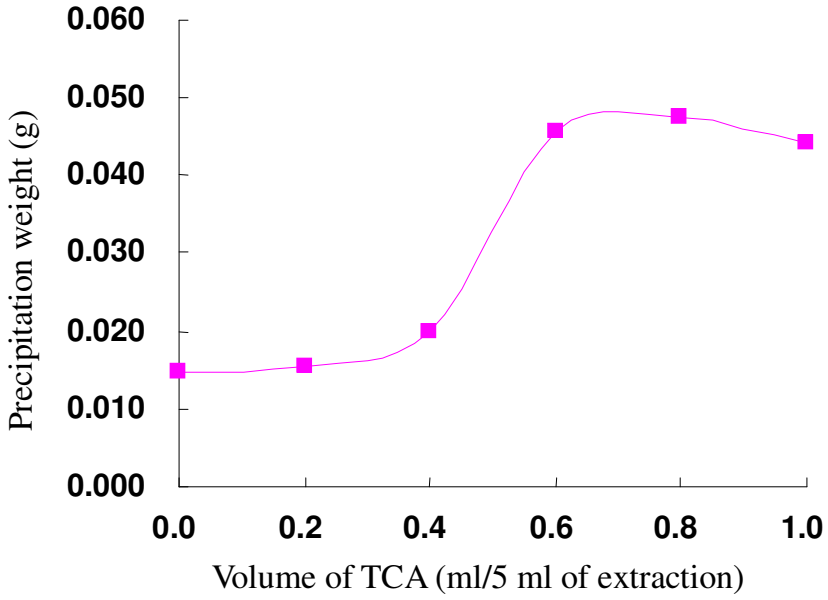


**Figure 1.** Effect of different trichloroacetic acid quantities on protein precipitation.

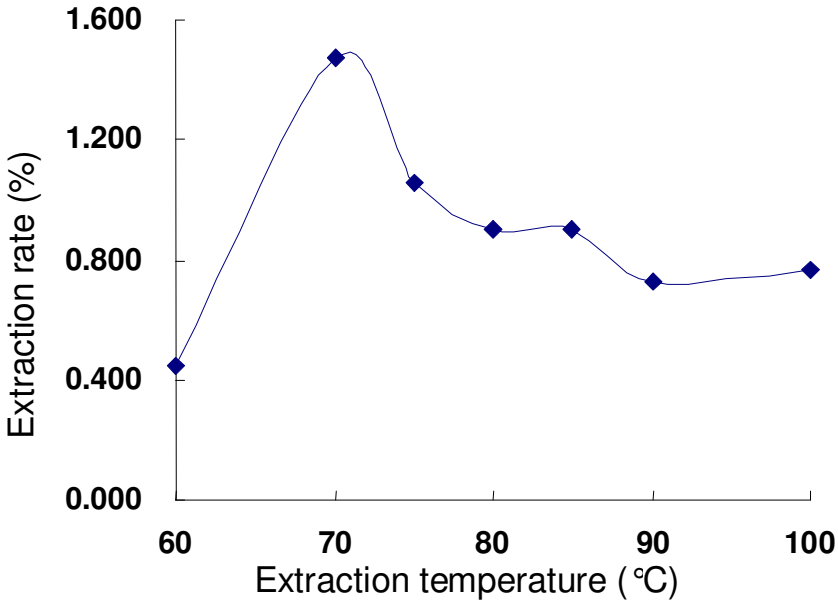


**Figure 2.** Effect of different extraction temperatures on extraction rate.

chose the temperature of 70℃ for the extraction.

**Effect of different extraction durations on extraction rate**

To study the effect of extraction duration on yield, 10 g of raw material were taken, and the solid : liquid ratio was set as 1:80. The extraction temperature was 80℃, and the extraction durations were 30, 45, 60, 75, 90, and 105 min for a single extraction. Further, 80% ethanol was added, and polysaccharide analysis was carried out. The results are shown in Figure 3. Generally, the longer the extraction duration, the more the dissolved polysaccharides and the higher was the extraction rate. There was a significant increase after 75 min, with the highest rate observed at 90 min, which was followed by a slight decrease. Therefore, the appropriate duration of extraction was 90 min.

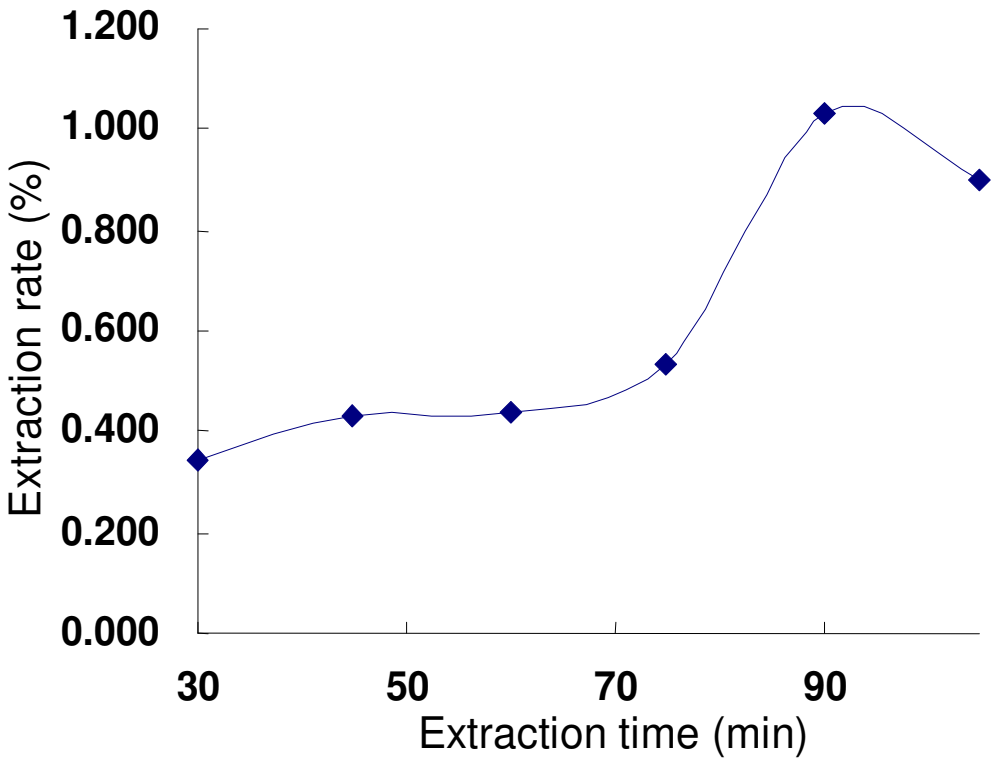


**Figure 3.** Effect of different times on extraction rate.

**Effect of different concentrations of the material on extraction rate**

The solid-to-liquid ratio has a major effect on the extraction rate of polysaccharides. The more the quantity of water, the more conducive the conditions are for the spread of the mass of polysaccharides; however, problems may develop due to the longer time needed for evaporation of the large quantities of water.

10 g of the material were again taken for determination of this parameter. The extraction temperature was set at 80℃ and extraction was carried out for 60 min. The solid-to-liquid ratios used were 1:12, 1:15, 1:18, 1:21, 1:24 and 1:27 for a single extraction. Subsequently, 80% ethanol was added, and polysaccharide analysis was carried out, as described earlier. The results are shown in Figure 4.

The results showed that for the solid-to-liquid ratios in the range of 1:12 to 1:27, the polysaccharide extraction rate increased with increase in volume of the solvent. Between the ratios 1:18 and 1:24, the increase was more pronounced, with the highest been at the ratio of 1:24, followed by slow increases. It is certain that in actual production, too much liquid will not only consume much more solvent, but also reduce the concentration of polysaccharides in the follow-up operation and consume more energy. Therefore, a solid-to-liquid ratio of 1:24 was considered suitable for the extraction.

**Effect of number of extraction times on extraction rate**

10 g of material were taken, with solid-to-liquid ratio of 1:80 and extraction was carried out for 60 min at 80℃. The extract was obtained by repeating the process 1, 2, 3 and 4 times. Later, 80% ethanol was added, and the extract was analyzed for polysaccharide content. The results are shown in Figure 5.

The results showed that the polysaccharide content decreased obviously after a single extraction, whereas, it decreased to zero at the third extraction. Therefore, to save more energy and shorten the production period, extraction of the leaves twice was considered to give better yields.

**Effect of different concentrations of ethanol on extraction rate**

In the process of ethanol precipitation of polysaccharides, the concentration of ethanol had a great effect on the polysaccharide yield. According to Figure 6, for precipitation of polysaccharides, 80% ethanol was the best.

The result of the single-factor experiment indicated that the optimum conditions for extraction were a solid-to-

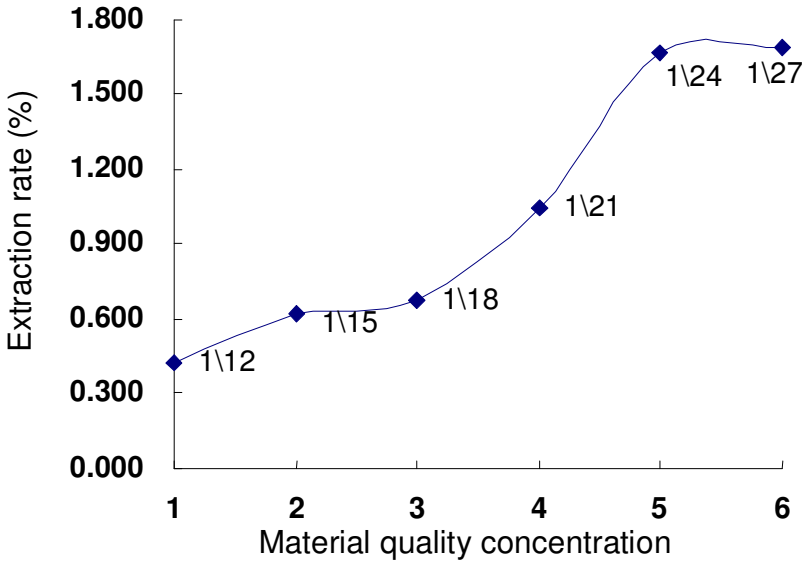


**Figure 4.** Effect of different material quality concentrations on extraction rate.

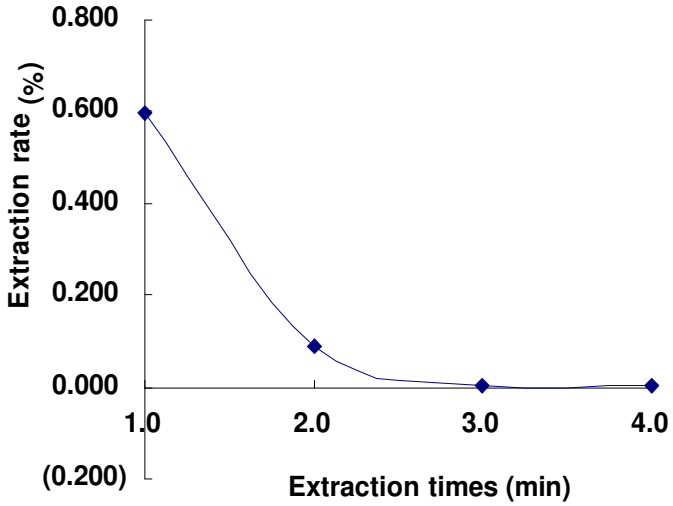


**Figure 5.** Effect of different extraction times on extraction rate.

liquid ratio of 1:24 for a period of 90 min at 80℃ with an ethyl alcohol concentration of 80%.

## The design of the orthogonal test

Integrating the results from the single-factor test, four factors influencing the polysaccharide yield greatly were selected: extraction duration, solid-to-liquid ratio, extraction temperature and ethyl alcohol concentration. Subsequently, a four-factor and three-level orthogonal test (Table 1) was designed according to the $L_9(3^4)$ table. The results are shown in Table 2.

From Table 2 which shows the range-analysis results, the effects of the various factors on polysaccharide yield are in the following descending order: C > A > D > B, that is, extraction temperature > extraction duration > ethanol concentration > solid-to-liquid ratio. According to the

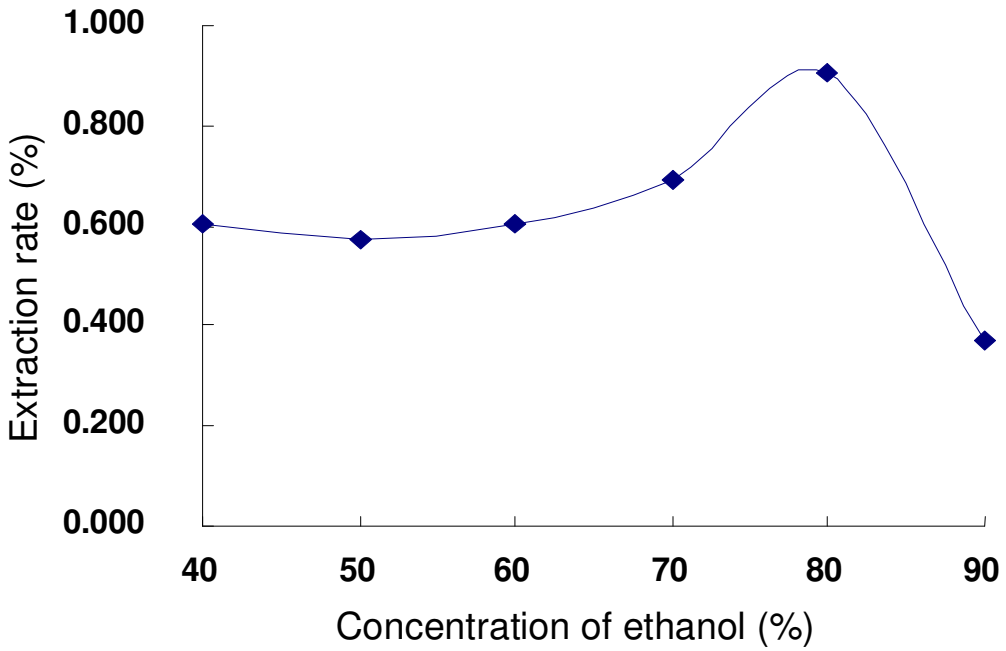


**Figure 6.** Effect of different concentration of ethanol on extraction rate.

**Table 1.** Factors and levels of orthogonal test.

| Factor level | Extraction time (min) (A) | Solid to liquid ratio (B) | Extraction temperature (C) | Ethanol concentration (%) (D) |
|---|---|---|---|---|
| 1 | 75 | 1:21 | 60 | 70 |
| 2 | 90 | 1:24 | 70 | 80 |
| 3 | 105 | 1:27 | 80 | 90 |

orthogonal experimental results, the optimum conditions for extraction of polysaccharides from *M. alba* L. leaves showed $A_2B_2C_2D_3$ as the following: a solid-to-liquid liquid ratio of 1:24, extract for 90 min at 70°C using an ethyl alcohol concentration of 80%. The highest extraction rate was 2.64% under optimum conditions.

In comparison with the published papers, there are some methods for the extraction of polysaccharides. In the Zhao's report, material was stirred in 1.0 M NaOH and the supernatant was obtained by filtration, then the protein in the supernatant was removed using the Sevag method (Zhao et al., 2008; Whistler, 1965). Polysaccharides had been extracted by circumfluence with methanol or ethyl acetate from sample (Liu et al., 2007). The defatted figs powder was extracted with water under ultrasound assistant (Yang et al., 2009). Currently, some reports have stated that it is difficult to purify polysaccharides, mainly because the structure of polysaccharides is complex; further, not enough basic research has been carried out on polysaccharide extraction in depth (Yao et al., 2002). In the last century, some researchers have purified polysaccharides using natural clarifying agents, including type II ZTCl+I and chitosan (Yokoyama, 1992). The use of a clarifying agent is superior to the traditional method which involves water extraction and alcohol precipitation in the context of removing impurities such as protein, wax, tannin and resin, and retaining the effective elements such as polysaccharides and soluble solids. It has the merits of high efficiency, low cost, simple operation and good stability. Of course, the use of clarifying agents affects the quality and stability of the products to a certain extent. Therefore, it is suggested that further works should be performed on the isolation and identification of the key components from water-soluble polysaccharides of *M. alba L*. leaves.

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**Table 2.** $L_9(3^4)$ try scheme and test result.

| Test number | Factor | | | | Extraction rate (%) |
|---|---|---|---|---|---|
| | (A) | (B) | (C) | (D) | |
| 1 | 1 | 1 | 1 | 1 | 0.504 |
| 2 | 1 | 2 | 2 | 2 | 1.144 |
| 3 | 1 | 3 | 3 | 3 | 1.095 |
| 4 | 2 | 1 | 2 | 3 | 1.524 |
| 5 | 2 | 2 | 3 | 1 | 1.499 |
| 6 | 2 | 3 | 1 | 2 | 0.623 |
| 7 | 3 | 1 | 3 | 2 | 0.407 |
| 8 | 3 | 2 | 1 | 3 | 0.875 |
| 9 | 3 | 3 | 2 | 1 | 0.998 |
| K1 | 2.744 | 2.435 | 2.002 | 3.002 | ∑xi=8.670 |
| K2 | 3.646 | 3.518 | 3.666 | 2.174 | n=9 |
| K3 | 2.280 | 2.717 | 3.002 | 3.494 | |
| X1 | 0.915 | 0.812 | 0.667 | 1.001 | |
| X2 | 1.215 | 1.173 | 1.222 | 0.725 | |
| X3 | 0.760 | 0.906 | 1.001 | 1.165 | |
| R | 1.366 | 1.084 | 1.664 | 1.320 | |
| $S=R^2/9$ | 0.207 | 0.130 | 0.308 | 0.194 | |

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