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Separation of Saikosaponins by On-line Sample Stacking CE Method

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ABSTRACT

Saikosaponins a, c, d, b_1 and b_2 which are the trace constituents in the crude extract of Bupleuri Radix can be separated successfully by the on-line sample stacking technique (Sweeping method) of capillary electrophoresis. The method uses aqueous phosphoric acid with a pH lower than 2 to prepare the extracting solvent and buffer solution of the same electric conductance. The former is prepared with methanol and the latter is prepared by adding SDS, methanol and acetonitrile. Bupleuri Radix extract analyzed by CE-sweeping method gives better result than by MEKC method with a higher separation efficiency, lower detection limit and ability to detect five saikosaponins. Applied to the analysis of two Chinese herbal formulas, CE-sweeping method is found to be able to determine the contents of saikosaponins a and b_1 successfully in Minor Bupleurum Combination and Major Bupleurum Combination, both of which contain Bupleuri Radix as the imperial drug. However, the migration times and resolution of the two marker substances, saikosaponins a and b_1, were changed obviously by the complicate constituents in Chinese herbal preparations.

Key words: CE-sweeping method, saikosaponins, Bupleuri Radix and its preparations

INTRODUCTION

Owing to the limited detection capability of UV detectors, which can only reach a level of 10^{-6} M[1], the on-line sample stacking CE method becomes especially significant in the analysis of trace components. The on-line sample stacking technique was developed in Mikker's experiment[2], which was later applied to a series of studies by Lauer[3] and Burgi and coworker[3-6], making it an important technique in CE analyses.

At the present, there has been a plurality of on-line sample stacking methods, of which the field-amplified method[5,6] is the most popular, but it is not suitable for the analysis of neutral substances. Liu[7] and Terabe[8-13] *et al.* recently have found that by utilizing the MEKC mode of stacking technique, neutral compounds can be analyzed effectively. In 1998, Terabe reported a sweeping method[14] which can effect infiltration of analytes into the pseudostationary phase of the sample zone by applying an electric potential. Thereby the molecules of the substance under analysis can be detected and stacked, creating a specific cumulating effect like that of ground sweeping. This sweeping technique can increase the concentrations up to 1 + k times, where k is the capacity factor. The feature with this method is that both sample solution and buffer solution (background solution, BGS) must have the same electric conductance. By using a buffer solution with a pH lower than 2 to make the silanol group on the wall of the capillary tube protonated, the electroosmotic flow will approach nearly zero. The action mechanism is shown in Figure 1. As the sample solution (S, prepared with analyte having the same electric conductance as the BGS) is injected into the capillary tube (Figure 1-A), the micelles from BGS

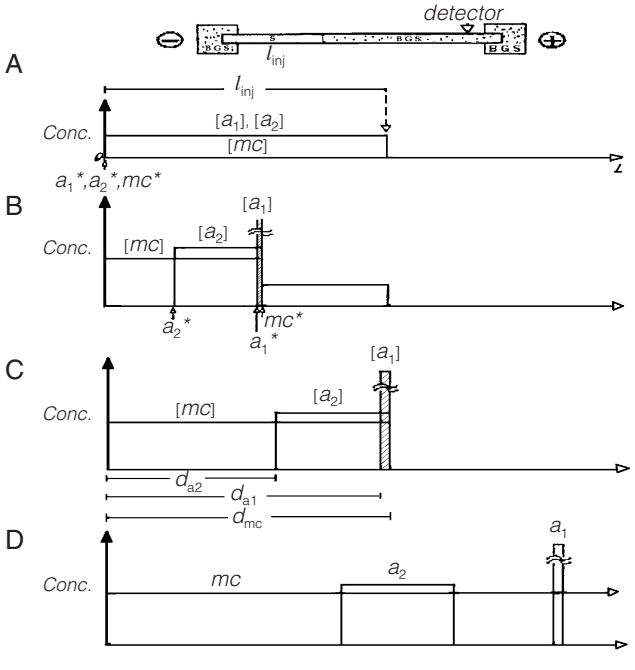

Figure 1. Progression process of the sweeping methods. (A) Initial state, as the capillary tube is filled with BGS, a segment (*l*inj.) of the sample solution (S) containing analytes a_1 and a_2 is injected, with both ends of the capillary tube being placed in BGS. (B) Micelles from the cathode trough migrate into S zone or the capillary tube, making the analytes swept to a narrow zone, where $Ka_1 > Ka_2$. (C) The first herd of micelles (mc*) that migrate to the S zone arrive at the interface of S and BGS zones, where a_1* and a_2* represent the analyte molecules farthest to the interface. (D) MEKC method is used to separate the zones.[14]

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(cathode) will migrate into the S zone under the action of the electric potential, and sweep the neutral analytes to be cumulated at a narrow concentration zone (Figures 1-B, 1-C). Finally, under the MEKC mode, the analytes with different capacity factors in the zone will get separated. (Figure 1-D).

Bupleuri Radix is a commonly used Chinese herbal drug, which possesses diaphoretic, antipyretic, liver soothing, melancholia relieving and yang-chi elevating effects[15] and contains a number of saikosaponins as its major bioactive components[15-17]. Saikosaponins are the herb's specific trace components composed chiefly of saikosaponins a, c, d, b_1 and b_2 as shown in Figure 2. Hsieh *et al.*[18] used MEKC method to analyze the five compounds, but the herb extract had to be condensed to some ten times higher concentration before the components could be detected. In this study, we used the CE-sweeping method to analyze the crude extracts of Bupleuri Radix and two of its preparations, Major Bupleurum Combination and Minor Bupleurum Combination. In addition, a comparative comparison of the strength and weakness of the method against the MEKC method will also be made.

MATERIALS AND METHODS

I. *Reagents and Materials*

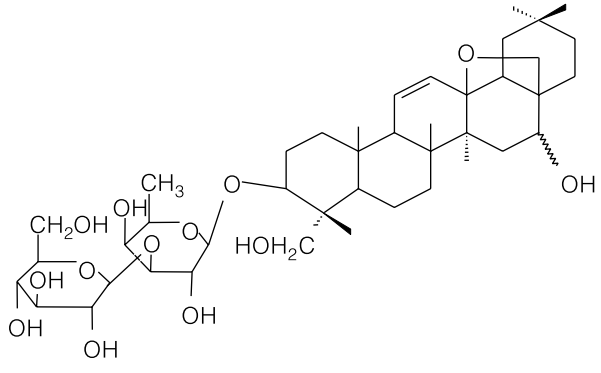
Saikosaponins a, c, and d were purchased from Nacalai Tesque (Kyoto, Japan) and b_1 and b_2 were prepared from a and d respectively[18]. Sodium dodecylsulfate (SDS) was purchased from Sigma (St. Louis, MO, USA) and H_3PO_4 from Aldrich (Milwaukee, WIS, USA). Methanol was of far-UV grade (Mallinchrodt, Paris, KY, USA). Deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Bupleuri Radix and its herbal preparations (Major Bupleurum Combination and Minor Bupleurum Combination) were purchased from a Chinese herbal market in Taipei, Taiwan. Major Bupleurum Combination is composed of Bupleuri Radix, Pinelliae Tuber, Zingiberis Rhizoma Recens, Scutellariae Radix, Paeoniae Radix, Zizyphi Fructus, Aurantii Fructus Immaturus and Rhei Rhizoma; and Minor Bupleurum Combination is composed of Bupleuri Radix, Scutellariae Radix, Pinelliae Tuber, Ginseng Radix, Zizyphi Fructus, Glycyrrhizae Radix and Zingiberis Rhizoma Recens.

II. *Preparation of Sample Solutions*

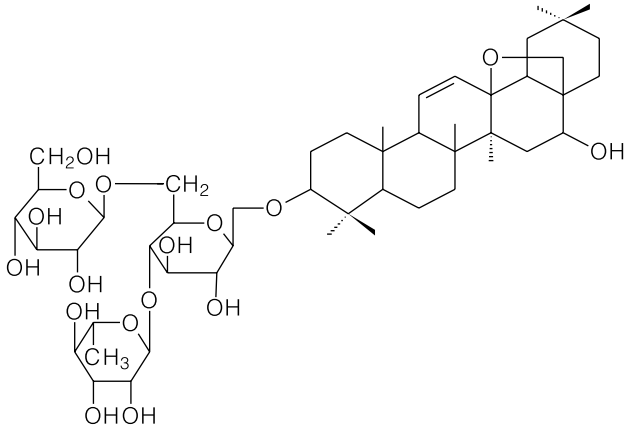
An extracting solvent made of 0.1M H_3PO_4 with suitable amount of CH_3OH was adjusted to have an electric conductance approaching that of the buffer solutions. A 0.2 g sample of Bupleuri Radix was extracted by ultrasonic vibration in the extracting solvent (6 mL) for 30 min, then centrifuged at 1500 ×g (Universal, Hettich Zentrifugen) for 5 min. After filtering through a 0.45 μm filter, the filtrate was used as a test solution of the herb drug. For the preparation of the test solutions of the herb preparations, a 0.5-g sample, each from the herb preparations, was extracted with 10 mL of the extracting solvent according to the above-mentioned operation.

III. *Apparatus and Conditions*

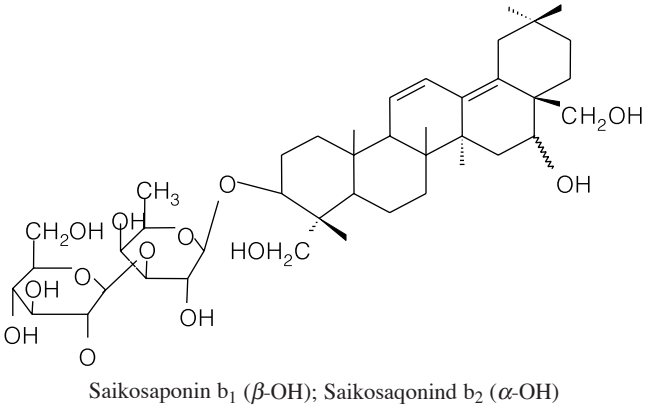
Electrophoresis were performed on a Spectra Phoresis 1000 capillary electrophoresis system equipped with an UV detector set at 200 nm and 255 nm and a 70 cm x 75 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) with the detection window placed at



Saikosaponin a (β-OH); Saikosaqonind d (α-OH)



Saikosaponin c



Saikosaponin b_1 (β-OH); Saikosaqonind b_2 (α-OH)

Figure 2. Structures of the saikosaponins

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62.5 cm. The conditions were as follows:

injection mode, 30 s x 1 psi (hydrodynamic); cartridge temperature, 20˚C; run time, 50 min.; applied voltage, -15 kV. The electrolyte was a buffer solution comprising 88% of 0.1 M H_3PO_4 and 100 mM SDS, 20% of CH_3CN and 2% of CH_3OH (v/v). Pre-buffer consisted of 88% of 0.1 M H_3PO_4, 20% of CH_3CN and 2% of CH_3OH (v/v).

Daily start up: H_2O 5 min (30˚C) → MeOH 5 min (30˚C) → H_2O 10 min (60˚C) → 0.1 M H_3PO_4 5 min (60˚C) → H_2O 10 min (30˚C)

Pre-wash for each run: 0.1 M H_3PO_4 3 min (30˚C) → H_2O 3 min (20˚C) → pre-buffer 2 min (20˚C) → buffer 2 min (20˚C)

Post-wash for each run: H_2O 3 min (30˚C) → MeOH 3 min (30˚C) → H_2O 3 min (30˚C)

RESULTS AND DISCUSSION

Saikosaponins a, c and d are trace components of Bupleuri Radix that exist in higher amounts and are usually used as markers for estimating the drug quality, whose λ_{max} of UV absorption is 194 nm. When saikosaponins a and d are treated with acid, they are easily converted into b_1 and b_2 that possess conjugated double bonds whose λ_{max} also shifts to 252 nm. Saikosaponins a, d, b_1 and b_2 all have the same molecular weight of 780 and are two pairs of stereoisomers. In order to obtain the maximal absorbance, the detecting wavelengths are set at 200 nm and 255 nm respectively.

Terabe *et al*. used a buffer solution comprising 88% of 0.1 M H_3PO_4 and 50 mM SDS, 20% of CH_3CN and 2% of CH_3OH, which successfully separate and condense their samples[14]. In this study, we applied the condition to analyze the five maker substances of Bupleuri Radix and found that only b_1 and b_2 had good resolution (resolution value, Rs = 2.75) and peaks of a and c and peaks of a and d had partial overlap. The Rs values for the two pairs of peaks were 0.80 and 0.67 respectively. Therefore, two buffer solutions containing 80 mM and 100 mM SDS were prepared and tested, respectively. The results showed that as SDS increased gradually in concentration, the resolutions of a/c and a/d pairs gradually improved, whereas the b_1/b_2 pair showed sign of slight approach to each other. At 80 mM SDS, a/c pair had two separated peaks. The Rs value of a/d pair was 1.5, and that of b_1/b_2 pair was 1.0. At 100 mM SDS, a and c pair of peaks were 2 min separate from each other in migration time. The Rs value of a/d was 1.87; and b_1/b_2, 1.45, all reaching the baseline separation (Figure 3-A). Electropherogram from MEKC was shown in Figure 3-B.[18]

As the electrolyte containing 100 mM SDS was used in analyzing the extract of Bupleuri Radix, it was found that the absorption bands of saikosaponins were too minute to be detectable. Thus, we used saikosaponin c as a marker and gradually increased the injection time starting from 3 sec. As a result, the injection amount was found to have a

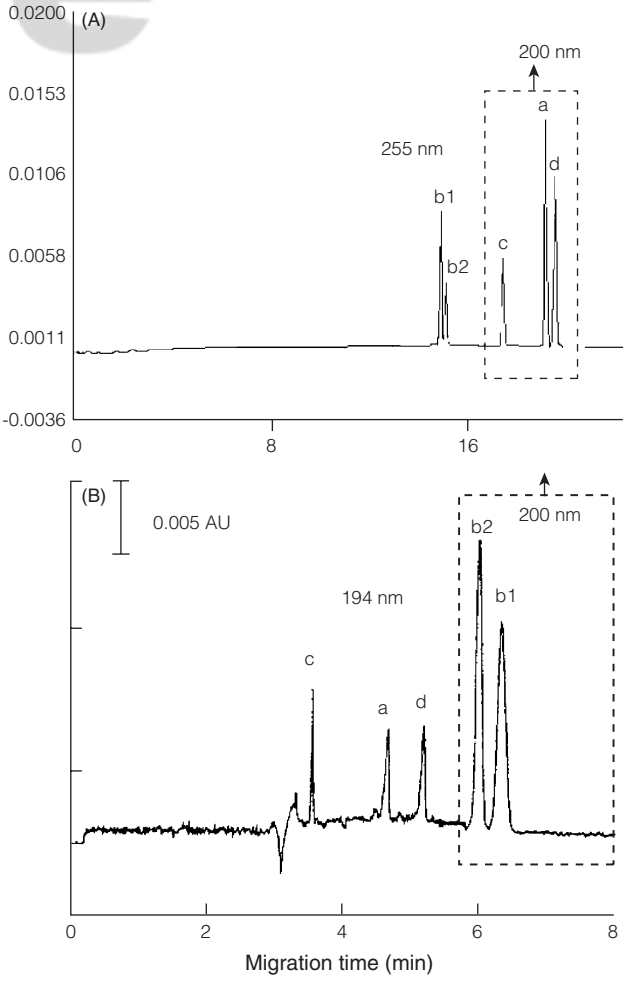

Figure 3. Capillary electropherograms of a mixture of the five saikosaponins performed by (A) sweeping method (B) MEKC.[18]

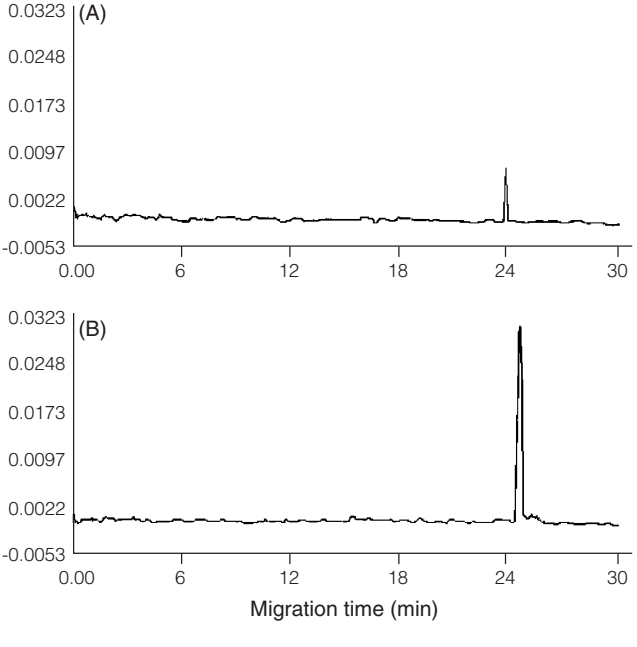

Figure 4. Electropherograms of saikosaponin C in different injection times (A) 3 sec (B) 30 sec.

normal proportional relation with the peak area. As the injection time was increased to 30 sec, the peak area increased 10 times, and the theoretical plate number remains approximately the same value (Figure 4). With the injection time set at 30 sec, the electropherogram is as shown in Figures 5-A and 5-B. In the figure, the marker peaks (made by addition of authentic standards to serve as controls) showed that saikosaponins a, c, d, b_1 and b_2 could be successfully detected, and the migration times did not differ much from those of the pure compounds. By combining Figure 5-A and Figure 5-B into Figure 5-C which is compared with the electropherogram from Hsieh *et al.*[18], the sweeping method used in this study possesses the advantages of skipping the sample concentration step, higher sensitivity, higher theoretical plate number, ability to detect more components and lower detection limits (about 1/300)(Table 1), albeit the analysis time is about three times that of the MEKC method, which nevertheless still falls within the acceptable range. Hence, the sweeping method can be applied to the analysis of trace components contained in Chinese herbal drugs.

As the method was used in analyzing Minor Bupleurum Combination and Major Bupleurum Combination, both of which contain Bupleuri Radix as their imperial ingredient, the results were as shown in Figure 6 and Figure 7. Both electropherograms showed that in the matrices of complicate herb preparations, the migration times of the various components varied considerably. As saikosaponin authentic standards were added to the test solutions, the marker substances were found to be masked by the complicate and abundant components of the herb preparations and only saikosaponins a and b1 were detectable. The absorption peaks of the two components in the extract of Major Bupleurum Combination had achieved good baseline separation result.

This work has successfully demonstrated that by optimizing parameters such as pH, injection time, surfactant concentration of the electrophoretic media and sample extracting solvent, high-resolution separations of a complicated mixture can easily be achieved. It is believed that this technique can be extended to the determination of the

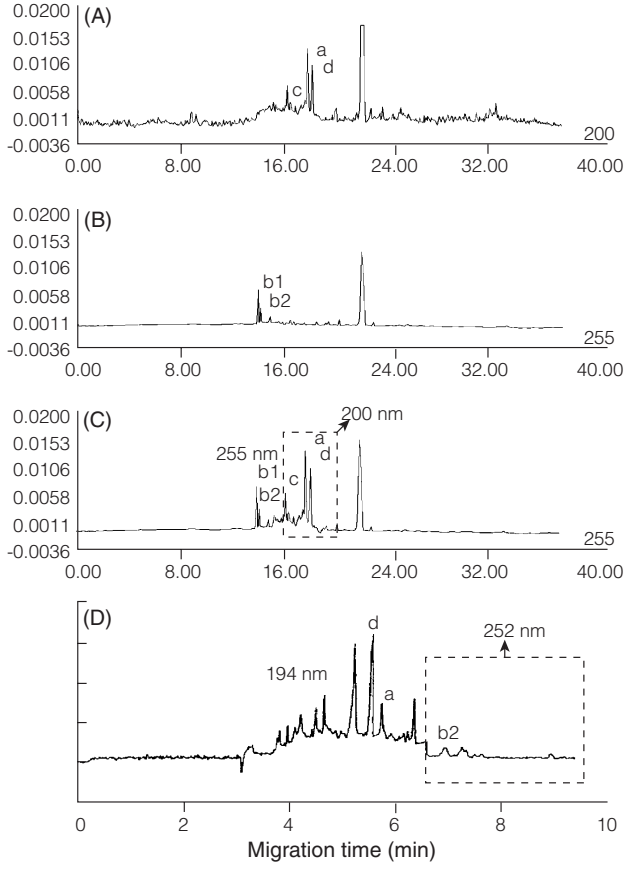

Figure 5. Electropherograms of the extract of Bupleuri Radix. (A) 200nm (B) 255nm (C) combining electropherogram of A and B (D) combining electropherogram performed by MEKC.[18]

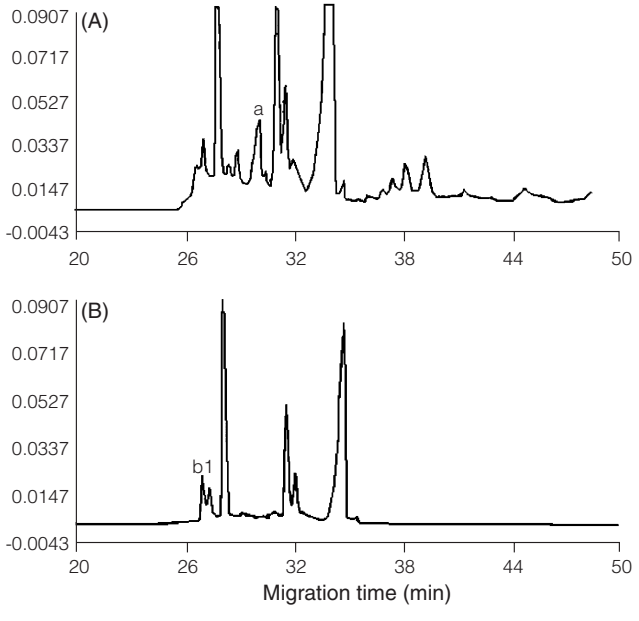

Figure 6. Electropherograms of the extract of Minor Bupleurum Combination (A) 200nm (B) 255nm.

Table 1. Comparison of sweeping and MEKC methods in the analysis of Bupleuri Radix

Item method	Sweeping method	MEKC methods[18]
Theoretical plate number (N)	$1.30 \times 10^6 \sim 2.11 \times 10^6$	$1.48 \times 10^5 \sim 9.12 \times 10^5$
Tailing factor	0.67 ~ 1.03	0.83 ~ 1.11
Analysis time	About 25 min	About 7.5 min
Detectable component	a, c, d, b_1, b_2	a, d, b_2
Detection limit	0.02 ~ 0.11 μg/ mL	7 ~ 33 μg/ mL
Concentration step	No	Yes

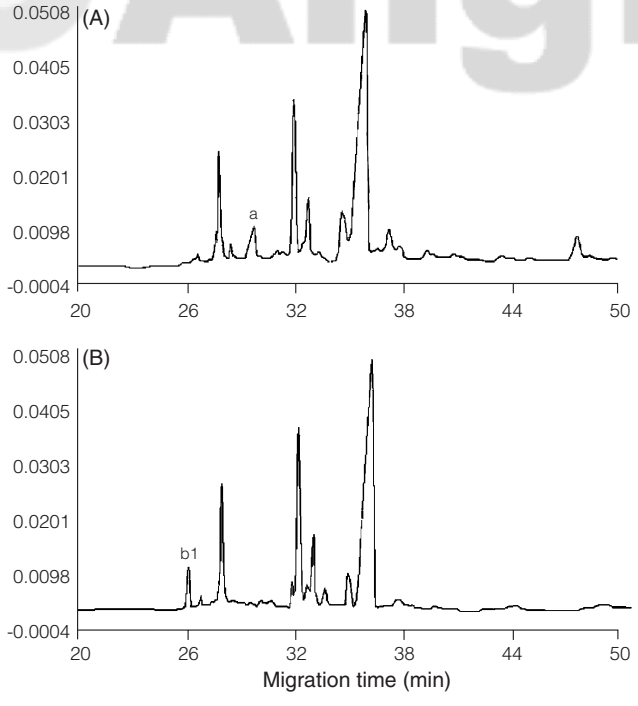

Figure 7. Electropherograms of the extract of Major Bupleurum Combination (A) 200nm (B) 255nm.

trace components of other Chinese herbal drugs and the samples with very dilute concentration.

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