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Use of Long-chain Alcohol in Extraction and Purification of Lincomycin from Fermentation Broth

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Abstract–Low selectivity for lincomycin in butanol extraction process leads to relatively higher content of impurities. A novel process for extraction of lincomycin from fermentation broth was studied in this work. Mixture of n-octanol and n-decanol is used as extractant to replace n-butanol in extraction of lincomycin. Optimal operation conditions for the process have been studied. Due to higher extraction selectivity for lincomycin A by long-chain alcohol, content of impurity (lincomycin B) in the final product is much lower than that in product by butanol process. Furthermore, the practicability for combination of long-chain alcohol and butanol in purification of lincomycin was investigated.

Key words: Extraction, Lincomycin, Long-chain Alcohol, Selectivity, Flocculation

INTRODUCTION

Lincomycin is a kind of antibiotics which can be produced by variety of strains of Streptomyces lincolnensis var. lincolnensis, which is active against most of the common gram-positive pathogens [Pratap et al., 1977]. In addition to lincomycin A, several kinds of analogs (lincomycin B, C, D, as shown in Table 1) are also produced in fermentation [Asmus et al., 1983]. Antibiotic activity of lincomycin C is similar to that of lincomycin A, while the other analogs exhibit poorer antibiotic activity and no practical medical value.

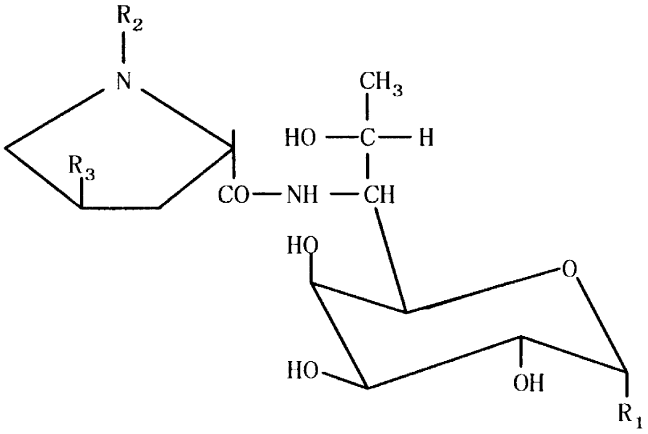

Fig. 1. Chemical structure of lincomycin.

Table 1. Analogs of lincomycin

Analogs	R_1	R_2	R_3
Lincomycin A	$-SCH_3$	$-CH_3$	$-CH_2CH_2CH_3$
Lincomycin B	$-SCH_3$	$-CH_3$	$-CH_2CH_3$
Lincomycin C	$-SC_2H_5$	$-CH_3$	$-CH_2CH_2CH_3$
Lincomycin D	$-SCH_3$	-H	$-CH_2CH_2CH_3$

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For the manufacturers in China, lincomycin in fermentation broth consists mainly of lincomycin A, while about 10% of lincomycin exists in the form of lincomycin B with lower antibiotic activity. Butanol is commonly used as extractant in the extraction of lincomycin from fermentation broth, which results in relatively high content of lincomycin B in final product [Wu et al., 1997, 1998]. With the increase in quantity of lincomycin used as raw material for production of semi-synthetic antibiotics, requirement for final product with lower content of lincomycin B keeps on growing.

Relatively higher selectivity between lincomycin A and B is obtained in long-chain alcohol extraction process than in butanol-extraction process, therefore more pure product can be obtained.

Butanol is partially miscible with water (solubility: 7.4 g of butanol dissolved in 100 g of water). Energy must be consumed in recycling of butanol from the raffinate and strip liquor by distillation. Moreover, relatively low boiling point (390 K) means volatilization loss of butanol. In the traditional butanol-extraction process, loss of solvent and subsequent increase in separation cost is remarkable. Compared with the butanol process, the insolubility and high boiling point of long-chain alcohol will lead to less solvent loss and lower cost in separation process.

Aimed to lower the content of lincomycin B in the end product, a novel process for extraction of lincomycin from fermentation broth was studied in this work. A mixture of long-chain alcohol (n-octanol and n-decanol) is used as extractant. Optimal operation conditions for the process have been studied in details. Furthermore, the practicability for combination of long-chain alcohol and butanol in purification of lincomycin was investigated.

EXPERIMENT

1. Material

Fermentation broth was provided by a manufacturer of lincomycin, in the form of acidified filtrate of fermentation broth at the

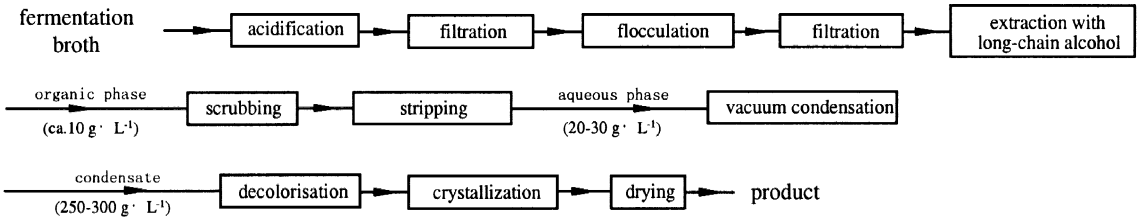

Fig. 2. Process for extraction of lincomycin with long-chain alcohol.

end of fermentation cycle. About 10% of lincomycin was present in the form of lincomycin B.

Lincomycin samples from primary crystallization and secondary crystallization in the butanol-extraction process were used in the experiment, which is intermediate product and final product containing 5.2% and 4.2% of lincomycin B respectively.

Standard sample for lincomycin hydrochloride was purchased from Shanghai Institute of Pharmaceutical Assay and Determination.

2. Method

Experiments for extraction, scrubbing and stripping were conducted in a funnel with a water jacket connected to a thermostat. Over ten minutes of robust shaking was performed to ensure intimate contact of the two phases to attain equilibrium. Multistage counter-current extraction was simulated in funnels connected in series. Samples of aqueous and organic phases were taken after stable state operation in simulation experiment had been achieved.

Lincomycin and palladium chloride react in acidic medium to produce colorful substance, which exhibits absorption peak at wavelength of 380 nm [Lu and Cao, 1986]. This property was utilized in spectrometric determination of lincomycin concentration (including lincomycin A and B). In determination of concentration of lincomycin in fermentation broth, pH value of the broth was firstly adjusted to 11.5±0.5, and then extracted with n-butanol, the extract was diluted to a proper concentration with ethanol and sampled for spectrometric measurement. Organic phase was mixed with ethanol and sampled for spectrometric measurement. Calibration curve was determined by experiments with standard sample of lincomycin hydrochloride, and analytical results were expressed in the form of content of lincomycin A hydrochloride.

Content of lincomycin B in the product was determined by a HPLC method [Asmus et al., 1983]. A C_{18}-bonded silica gel column was used in analysis. Mobile phase consisted of 0.05 mol·L^{-1} sodium tetraborate water solution (pH value adjusted to 6.0 with 85% phosphoric acid) mixed with methanol in a ratio of 4 : 6 (v/v); eluted peaks were detected at a wavelength of 214 nm.

RESULTS AND DISCUSSION

1. Process for Extraction of Lincomycin with Mixed Long-chain Alcohol

Compared with the butanol-extraction process, significant changes will occur in operation conditions in long-chain alcohol extraction process as a result of change in extractant:

(1) severe emulsion problem might occur in extraction;

(2) extraction capacity of lincomycin with long-chain alcohol is obviously lower than with butanol; to achieve high extraction efficiency, changes have to be made in extraction stage, phase ratio, and operation conditions in scrubbing and stripping.

The process for extraction of lincomycin with mixed long-chain alcohol is shown in Fig. 2.

1-1. Flocculation of Filtered Fermentation Broth

Extraction of lincomycin should be conducted in alkaline medium. Emulsion is prone to occur with long-chain alcohol as extractant. Large amounts of impurities exist in the fermentation broth either introduced by the addition of substrates or produced by the metabolic activity of microorganisms [Kim and Ryu, 1993]. To control the emulsion during extraction, acidified filtered fermentation broth should be flocculated to further remove the residual surfactant (such as protein).

In flocculation, chitin and potassium aluminum sulfate were added into the broth, which act as flocculants to precipitate most of the residual proteins. Yield for recovery of lincomycin in flocculation is about 98.9%. The splitting of two phases in following extraction will be facilitated, which aids to decrease loss of lincomycin caused by emulsion in extraction.

1-2. Extraction of Lincomycin

1-2-1. Influence of Composition of Mixed Alcohol

Experimental results for extraction of lincomycin with different concoction of mixed alcohol are shown in Table 2. Original aqueous phase is a solution of secondary crystallization of lincomycin from butanol-extraction process with a concentration of 4 g·L^{-1} and adjusted to pH 9.7. Organic phase consists of n-octanol(C_8) and n-decanol(C_{10}) in different concentration ratio.

As is shown in Table 2, increase in content of n-octanol leads to increase of the extraction capacity of mixed alcohol. Severe emulsion will occur if only n-octanol is used as extractant. Decanol is added into the extractant mixture to facilitate phase splitting after extraction. The molecule of n-decanol has stronger selectivity for lincomycin A than n-octanol [Wu et al., 1998]. Therefore, higher content of n-decanol in the mixture will lead to slight increase in

Table 2. Effect of concoction of mixed alcohol on extraction and splitting of phases*

Concoction (v/v, %)	D	Phase splitting speed
70%octanol-30%decanol	2.99	Relatively fast phase splitting
75%octanol-25%decanol	3.12	Medium phase splitting
80%octanol-20%decanol	3.24	Medium phase splitting
85%octanol-15%decanol	3.33	Relatively severe emulsification
90%octanol-10%decanol	3.51	Severe emulsification

*T=298.2 K, V_O/V_A=1/3, $C_{A,o}$=4 g·L^{-1}

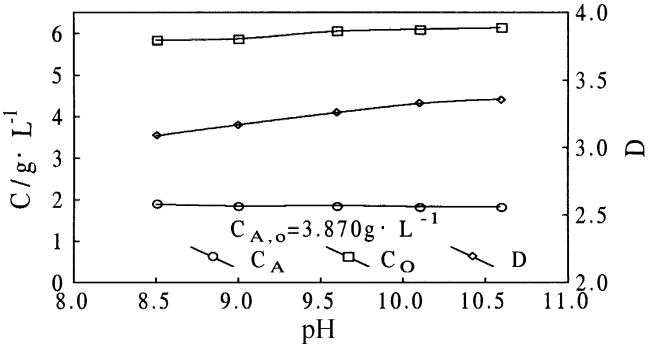

Fig. 3. Extraction of lincomycin at various pH value.

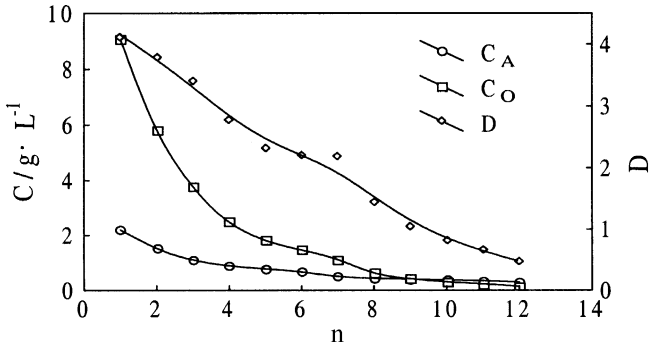

Fig. 4. Multistage counter-current extraction of lincomycin from fermentation broth.

selectivity but lower distribution ratio. To guarantee a high level of extraction yield, small sacrifice of extraction selectivity has to be made by lowering the content of n-decanol if too many stages of extraction are not desired. In consideration of phase splitting speed, extraction capacity and selectivity between lincomycin A and B, a mixture of 80% n-octanol and 20% n-decanol was used in the following experiments.

1-2-2. Influence of pH Value

Lincomycin is easy to be extracted in alkalinous medium. Aqueous solutions of secondary crystallization of lincomycin from butanol-extraction process were extracted with mixed alcohol (80% n-octanol and 20% n-decanol) under different pH values.

As is shown in Fig. 3, there is a very slight increase of C_O and distribution ratio (D) with the increase of pH value, while there is a slight decrease of C_A in the meantime. Just a little higher extraction yield is available at relatively higher pH value (near to 10.0).

To increase the pH value of acidified filtrate of fermentation broth, alkali (e.g., solution of sodium hydroxide) should be added. When the pH value of fermentation broth is adjusted to the range of 8-11, relatively less alkali is consumed. However, when the pH value of fermentation broth is adjusted to over 11, the quantity of consumed alkali will increase sharply. In consideration of more alkali consumed to achieve higher pH value, pH 10 is deemed to be suitable for extraction of lincomycin from fermentation broth.

1-2-3. Multistage Counter-current Extraction of Lincomycin from Fermentation Broth

In the butanol-process, only two stages is needed to achieve thorough extraction of lincomycin from fermentation broth. However, the extraction capacity of lincomycin with long-chain alcohol is obviously much lower than with butanol, more stages will be needed in the multistage counter-current extraction with long-chain alcohol as extractant.

Acidified filtered broth was flocculated and adjusted to pH value of 10.0. Experimental simulation of 12-stage of counter-current extraction of the broth with mixed alcohol (80% n-octanol and 20% n-decanol) was conducted at 298.2 K and volumetric flow-ratio V_O/V_A of 1/3. The equilibrium concentrations in organic and aqueous phase of each stage after stable state of the simulation had been achieved are shown in Fig. 4. Calculation on the exit concentration of organic phase exhibits an extraction yield of 92.8%. Therefore, sufficiently high extraction yield can be obtained in 12 stages of counter-current extraction. Higher extraction yield can be obtained if more stages are used, however, more impurities including lincomycin B will enter the extract phase.

As is shown in Fig. 4, distribution ratio D decreases rapidly in the range of low concentration of lincomycin. Effect of lincomycin concentration on distribution ratio is shown in Fig. 6. A simple extraction experiment with diluted fermentation broth (lincomycin concentration 0.307 $g \cdot L^{-1}$) at pH 10.0 exhibit distribution ratio D of 2.23 which is much higher than that in 12th stage as shown in Fig. 4. It was experimentally shown that simple decrease in concentration is not responsible for the decrease in distribution ratio.

Determination of distribution of lincomycin A and B in organic phase and aqueous phase shows that better selectivity is obtained in extraction by mixed long-chain alcohol. Selectivity value is 4-6 in the concentration range in the experiments (Fig. 4). Lincomycin B exhibits relatively stronger polarity than lincomycin A. In the multistage counter-current extraction, lincomycin B in the fermentation broth was enriched in the dilute section, which resulted in the decrease of general distribution ratio of lincomycin A and B as shown in Fig. 4.

1-3. Scrubbing of Extract

Scrubbing aids to remove impurities in organic phase such as pigments, inorganic salts and lincomycin B and upgrade the purity of final product. Effluent aqueous phase in scrubbing will be mixed with fermentation broth for feed in extraction, and therefore no loss in total yield of lincomycin is generated. Results in simulation experiment of two stages of counter-current scrubbing are shown in Table 3. Buffer solution of pH 10.0 was used as the inlet aqueous phase in scrubbing under 298.2 K.

Based on experimental results as shown in Table 3, the following operation conditions for scrubbing is recommended: a buffer solution of pH 10 used to scrub extract at 298.2 K in two-stage counter-current operation with a volumetric flow-ratio of 2-3.

1-4. Stripping

Solute in the form of acid or alkali in the extract phase is easy to be stripped by alkali or acid [Han et al., 2000]. Complete stripping

Table 3. Two-stage scrubbing of extract under various flow-ratio

Flow-ratio V_O/V_A	Yield for lincomycin in organic phase after scrubbing/%
1.0	77.3
2.0	90.8
3.0	91.7

Table 4. Multistage counter-current stripping

Stage number	Aqueous phase		Organic phase	D
	pH	C_A/g·L^{-1}	C_O/g·L^{-1}	
1	2.09	0.0210	0.0433	2.06
2	2.10	0.665	0.0531	0.0798
3	2.29	24.60	0.114	0.00463

of lincomycin from the scrubbed extract phase can be obtained by the addition of hydrochloric acid in stoichiometric ratio. Concentrated aqueous solution of lincomycin is obtained and organic phase is recycled for extractant. As the mutual solubility of long-chain alcohol and water is much lower than that of butanol and water, fewer stages are required for stripping compared to that of former butanol-extraction process.

Dilute hydrochloric acid (0.01 mol·L^{-1}) was used to strip the scrubbed extract (C_O=9.159 g·L^{-1}) in 3-stage stripping with a volumetric flow-ratio V_O/V_A of 3 at 298.2 K. The final stage of stripping was conducted under agitation, pH value of aqueous phase was adjusted to the range of 2.0-2.5 with hydrochloric acid (1.0 mol·L^{-1}). As shown in Table 4, thorough stripping can be obtained in two or three stages of counter-current operation when pH value is adjusted to 2.0-2.5 and with a volumetric flow-ratio of 3-5.

1-5. Condensation, Crystallization and Drying

Strip liquor was vacuum concentrated and decolorized, and then acetone was added into the strip liquor. The mixture was then kept at 277.2 K to precipitate lincomycin. Spectrometric measurement showed a specific activity of 849 μg/mg in the final product. Analysis by HPLC showed that content of lincomycin B in the final product (1.3%) is much lower than that in final product by butanol-extraction process (4.2%).

As is shown from structural formula, lincomycin A exhibits relatively weaker polarity than lincomycin B. Long-chain alcohol (with lower polarity) exhibit higher extraction selectivity for lincomycin A, therefore content of lincomycin B will decrease in a counter-current multistage extraction process with long-chain alcohol. Butanol exhibits rather high extraction capacity for lincomycin and almost no selectivity between lincomycin A and B [Jariwala, 1978], so relatively high content of lincomycin B remains in the final product of butanol-extraction process.

2. Process for Purification of Lincomycin with Butanol and Mixed Long-chain Alcohol

Demand for lincomycin with higher purity (e.g., content of lincomycin B less than 2%) as raw material in pharmaceutical preparation keeps on increasing in recent years. Price increases sharply with decrease in content of lincomycin B. The price for lincomycin

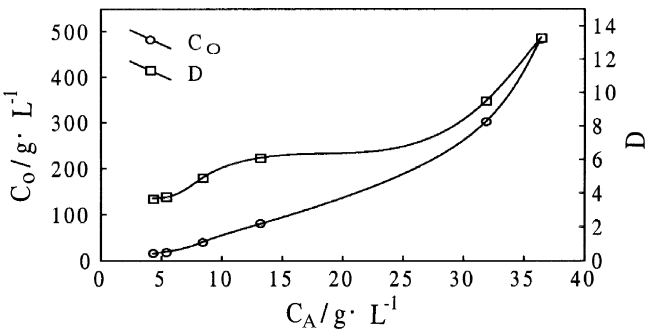

Fig. 6. Extraction of primary crystal from butanol-process by mixed long-chain alcohol.

with content of lincomycin B less 1.5% can be twice as much as that of crude lincomycin with 4% lincomycin B.

On the premise of retaining the original butanol-extraction process, the lincomycin product can be further purified with mixed long-chain alcohol to lower the content of lincomycin B. Therefore, the process for extraction in combination of lincomycin with butanol and long-chain alcohol was studied, the flow chart of which is shown in Fig. 5.

2-1. Extraction

Aqueous solution of product of primary crystallization in butanol-extraction process was adjusted to pH 10.0 with sodium hydroxide, and extracted with 80% n-octanol and 20% n-decanol at 298.2 K. As is shown in Fig. 6, when extraction is operated in the range of high concentration of lincomycin (C_A>10 g·L^{-1}), distribution ratio is much higher than that in the normal range of concentration (C_A= 1-5 g·L^{-1}). High load of lincomycin in organic phase (C_O≥300 g·L^{-1}) leads to high viscosity and causes much inconvenience to process operation and control. Moreover, high distribution ratio at high concentration will hinder the separation of lincomycin A and lincomycin B.

Aqueous solution of lincomycin of proper concentration was extracted with mixed alcohol. As is shown in Table 5, two-stage counter-current extraction in a volumetric flow-ratio V_O/V_A of 0.5-1 can guarantee an extraction yield over 90%. If only a small part of primary crystal from butanol-process needs to be further purified, then just one stage of extraction can be performed with extraction yield of 82%.

Lincomycin left in the raffinate can be further processed by stripping and crystallization, the final product containing more lincomycin B can be mixed with secondary crystal from butanol-process and used in situations where high grade purity of lincomycin is not required.

2-2. Scrubbing

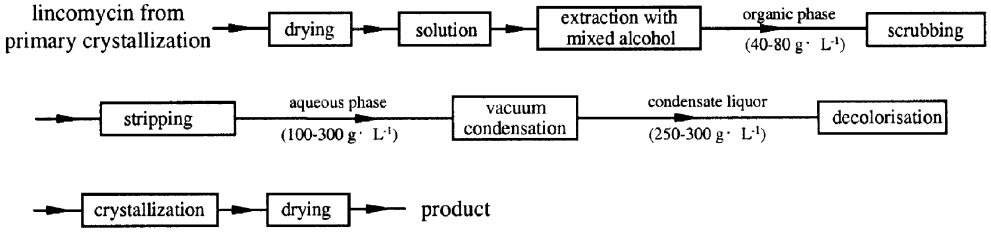

Fig. 5. Process for extraction of lincomycin with butanol and long-chain alcohol.

Table 5. Extraction of solution of crude lincomycin crystal

Extraction stage number	Concentration in feed $C_{A,o}$/g·L^{-1}	Volumetric ratio V_O/V_A	Extraction yield/%
2	106.95	1.0	97.4
2	53.39	0.5	91.1
2	26.66	0.5	89.2
1	53.24	1.0	82.4

Small volumetric ratio should be adopted in scrubbing to ensure the low content of lincomycin B in scrubbed organic phase. A buffer of pH 10.0 was used as aqueous phase, organic phase with lincomycin concentration of 40-80 g·L^{-1} was scrubbed with a volumetric flow ratio V_O/V_A of 1. Single stage scrubbing at 298.2 K gives rise to a yield of 82.7-86.2% of lincomycin remained in organic phase.

2-3. Stripping and Crystallization

According to the process shown in Fig. 5, lincomycin from primary crystallization was further purified by 2-stage counter-current extraction ($C_{A,o}$=5-10 g·L^{-1}, V_O/V_A=0.5-1) and single stage scrubbing (V_O/V_A=1). Lincomycin in the scrubbed organic phase was stripped with 1 mol·L^{-1} hydrochloric acid. Strip liquor was vacuum concentrated and decolorized, then acetone was added to precipitate lincomycin. After filtration and washing with acetone, lincomycin crystals were vacuum dried. Content of lincomycin B analyzed by HPLC in final purified product is 1.29-1.78%, which is remarkably lower than that in primary crystallization (5.2%) and secondary crystallization (4.2%) in the butanol-extraction process.

With the growing requirement for lincomycin with higher purity, the great gap on prices for lincomycin of different purity promotes production of lincomycin with lower content of lincomycin B. Therefore, based on the original extraction process with butanol as solvent, crude product of lincomycin produced by butanol-extraction process can be further purified by extraction with mixed long-chain alcohol, content of lincomycin B in the final product will be remarkably decreased. Small expenditure is needed for installing apparatus for further purification, such as single stage extractor and crystallizer, and great improvement on product purity and more economic benefit is obtained.

For deep purification of primary crystal of lincomycin from butanol-extraction process with mixed long-chain alcohol, more than one stage of extraction and scrubbing are needed. Through adjustment on operation conditions such as stage number and volumetric flow-ratio of extraction, scrubbing and stripping, different requirements on purity of final product can be met.

CONCLUSIONS

1. Under optimal operation conditions, extraction of lincomycin from fermentation broth with mixed long-chain alcohol can lead to remarkable enhance of product purity compared with butanol-extraction process.

2. Based on the original extraction process with butanol as extractant, mixed long-chain alcohol can be used in further purification of crude product to sharply decrease the content of lincomycin B, while no great improvement on original production apparatuses is required.

3. Flocculation treatment with potassium aluminum sulfate aids to precipitation of residual proteins in filtered fermentation broth. And henceforth, coalescence of dispersion and clarification after extraction can be facilitated.

ACKNOWLEDGEMENT

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NOMENCLATURE

C_A : concentration of lincomycin in aqueous phase [g·L^{-1}]
$C_{A,o}$: concentration of lincomycin in aqueous phase before extraction [g·L^{-1}]
C_O : concentration of lincomycin in organic phase [g·L^{-1}]
D : distribution ratio, (=C_O/C_A)
n : stage number for continuous extraction
T : temperature for extraction [K]
V_A : volume of aqueous phase [L]
V_O : volume of organic phase [L]

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