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Original Article

Determination of urea content in urea cream by centrifugal partition chromatography



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ABSTRACT

The objective of this study is to establish a centrifugal partition chromatography (CPC) method for determination of the urea ingredient in urea cream. The mechanism of this method is that urea is determined by UV detector at 430 nm after being extracted from the cream and derivatized on line via Ehrlich reaction in rotor of CPC, where the reaction products dissolve in the mobile phase and the cream matrix retains in the stationary phase. The mixed solvent consisting of *n*-hexane, methanol, hydrochloric acid and *p*-dimethylaminobenzaldehyde with a ratio of 1000 mL:1000 mL:18 mL:2.0 g is used for solvent system of CPC. The CPC method proposed offers good precision and convenience without complex sample pretreatment processes.

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1. Introduction

Cream is one of the common dosage forms for topical applications. The assay of active pharmaceutical ingredients in cream preparations usually requires tedious processes for sample pretreatment, including heating, dissolving, freezing, extracting, and diluting to a constant volume. These procedures may cause systematic errors or the degradation of some heat-labile ingredients during sample pretreatment. Additionally, other factors, such as extraction methods, pH value, time of heating and freezing, or mode and intensity of

flask shaking, may also influence final analysis results. Cream or ointment containing urea is usually used to treat hand and feet chapping, and the urea-content determination in pharmaceutical preparations is mainly carried out by UV-Vis spectrophotometry, infrared spectroscopy, and urease methods [1]. According to Chinese Pharmacopoeia, Japanese Pharmacopoeia Fifteenth Edition, European Pharmacopoeia v7 and US Pharmacopoeia 35, the current methods for urea-content determination in raw material is titrimetry, which is not suitable for urea cream, because the cream matrix usually interferes with the color change of the indicator, causing unstable results with a relative standard deviation (RSD) >2%

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[2,3]. According to British Pharmacopoeia 2009, Volume III, the determination of urea cream is fulfilled by the urease method [4,5].

In our study, a centrifugal partition chromatography (CPC) method for the determination of urea cream was proposed for the first time. In the CPC method, *p*-dimethylamino- benzaldehyde (PDAB) is a derivatization agent that reacts with the urea in the cream inside the CPC partition cells. The derivatization product is separated by CPC and detected by UV detector, thus achieving the combination of chromatography and spectrophotometry.

Counter-current chromatographic (CCC) methods are a form of liquid-liquid partition chromatography invented in the 1940s [6–9]. In modern era of CCC, CPC is equipped with a horizontal rotor consisting of the superposition of disks engraved with small cells that are connected by head/tail ducts on the basis of hydrostatic equilibrium systems. Currently, CPC is widely applied to analyze natural products, pharmaceuticals, and other synthetic organic and inorganic chemicals due to its high stationary phase retention, rapid separation and convenient operation [10–12]. Since CPC does not need any solid support [13], the stable and uniform emulsion samples can be injected into the apparatus without pretreatment, which simplifies sample preparation, minimizes systematical error, and eliminates the influence of cream matrix on assay results. Therefore, developing a simple, rapid, and precise CPC method for the determination of the urea ingredient in urea cream is advantageous and practical.

2. Methods

2.1. Apparatus

CPC 240 (Sanki Engineering, Kyoto, Japan) is equipped with a Waters 2707 autosampler, a Waters 2489 UV detector, and a Waters 1515 pump (Waters; Milford, MA, USA). The rotor of the CPC 240 is made up of 12 disks, with each disk consisting of 178 partition cells. Each partition cell consists of a channel and a duct, and the total volume for 12 disks is ~240 mL, 85% of which is for the channels and 15% for the ducts.

2.2. Materials and reagents

The urea reference substance and PDAB were provided by National Institutes for Food and Drug Control. The urea cream sample (containing urea $0.1 \text{ g}\cdot\text{g}^{-1}$) was purchased from Shanghai General Pharmaceutical Co., Ltd., Shanghai. *n*-Hexane (analytical grade) and hydrochloric acid (analytical reagent: ~36% to 38%) were obtained from Fuyu Fine Chemical Co., Ltd, Tianjin, China. Methanol (high-performance liquid chromatography grade) was purchased from Concord Technology Co., Ltd, Tianjin, China. Ultrapure water was made by the Merck Millipore MILLI-Q system (EMD Millipore; Billerica, MA, USA).

2.3. Preparation of standard solutions and test solutions

The standard stock solution was prepared as follows: 50 mg of urea reference substance was weighed and dissolved in a 10-mL volumetric flask with a moderate amount of methanol under ultrasonic conditions and then diluted to scale.

The preparation of the standard solution was as follows: 5 mL standard stock solution was measured in a 50-mL volumetric flask, 2 mL PDAB solution and 2 mL hydrochloric acid were added, and then the solution was diluted to scale with the mobile phase. After being fully shaken and sitting away from light for 10 min, the standard solution was immediately injected into the CPC.

The preparation of test solution was as follows: 1.0 g urea cream was weighed, added to a 20-mL volumetric flask, and dissolved in methanol under ultrasonic conditions.

2.4. Selection of UV wavelength for CPC detection

A moderate amount of urea reference substance and PDAB were weighed and put into a colorimetric tube, and the solution in the CPC mobile phase was shaken. After 15 min, the UV spectrum of the reaction solution was scanned at 200–500 nm, with the CPC stationary phase used as blank zero.

2.5. Determination of CPC flow rate and dosages of PDAB and acid solution

As the urea derivatization was carried out in the CPC rotor, the reaction time was controlled by changing the CPC flow rate. At 430 nm, the flow rate was set at 1, 2, 3, 4, 5, 6, 7, and $8 \text{ mL}\cdot\text{min}^{-1}$ to determine the peak area of derivatized product with urea concentration of $1.0 \text{ mg}\cdot\text{mL}^{-1}$.

The dosages of PDAB and acid solution were determined as follows: 1.0, 2.0, 4.0, 8.0, and 12.0 g PDAB were transferred into a solution consisting of 20 mL hydrochloric acid and 1000 mL methanol, which acted as the CPC mobile phase. At a flow rate of $5.0 \text{ mL}\cdot\text{min}^{-1}$, the peak areas of derivatized products were determined by the urea concentration of $1.0 \text{ mg}\cdot\text{mL}^{-1}$.

Different concentrations of hydrochloric acid (~ 0.2 – $1.2 \text{ mol}\cdot\text{L}^{-1}$) with $1.0 \text{ mg}\cdot\text{mL}^{-1}$ urea were used and the relevant peak areas of derivatized product were measured.

2.6. CPC analysis procedures

The biphasic solvent system, consisting of *n*-hexane, methanol, hydrochloric acid, and PDAB in a ratio of 1000 mL:1000 mL:18 mL:2.0 g, was mixed and shaken in a 3-L separating funnel for 1 min, and the mixed liquid was separated into two layers within 20 s and allowed to stand for 10 min. The lower layer was used as the mobile phase and the upper layer as the stationary phase. The stationary phase was pumped into the rotor in descending mode at a flow rate of $10 \text{ mL}\cdot\text{min}^{-1}$ and a rotation speed of 100 rpm. The CPC continued to run for 25 min to ensure that the rotor was completely filled with the stationary phase, then the mobile phase was pumped into the rotor in descending mode at a flow rate of $3 \text{ mL}\cdot\text{min}^{-1}$ and a rotation speed of 1200 rpm. The apparatus was kept in motion for ~20 min to equilibrate the

hydrodynamics of the biphasic system in the rotor until the stationary phase ceased outflowing. The standard solution or test solution (500 μL) was injected, and the CPC was kept running under the following conditions: the retention value of the stationary phase was $\sim 80\%$, the maximum pressure of the pump was 750 psi, the apparatus running temperature was $\sim 25^\circ\text{C}$, the flow rate of the mobile phase was $5.0\text{ mL}\cdot\text{min}^{-1}$, and the UV detection wavelength was 430 nm.

The original CPC chromatogram of the standard solution is shown in Fig. 1A. In order to reduce the baseline noise and improve signal-to-noise ratio, the original chromatogram was smoothed by 13 points (Fig. 1B).

2.7. Methodology validation

Linearity determination was undertaken as follows: $0.50\text{ mg}\cdot\text{mL}^{-1}$ standard solutions were scaled at 2, 4, 6, 8, and 10 mL and transferred to 100 mL volumetric flasks. The

calibration curve was drawn under the CPC conditions as described in Section 2.6.

Precision determination was as follows: six replicated injections of test solution with concentrations of 0.10, 0.20, and $0.40\text{ mg}\cdot\text{mL}^{-1}$ were prepared with injection volumes of 500 μL . The average peak area was determined and the precision was calculated.

Recovery determination was as follows: The urea standard solution was added to the methanol solution of three urea cream samples in different proportions (80%, 100%, and 120%), and the addition recovery of urea standard substance was determined.

2.8. Comparison of two modes of urea derivatization

Two modes of urea derivatization were compared to confirm that the derivatization of urea could be carried out inside the CPC rotor: (1) The urea standard solution or the urea cream sample solution reacted with the mobile phase in a test tube

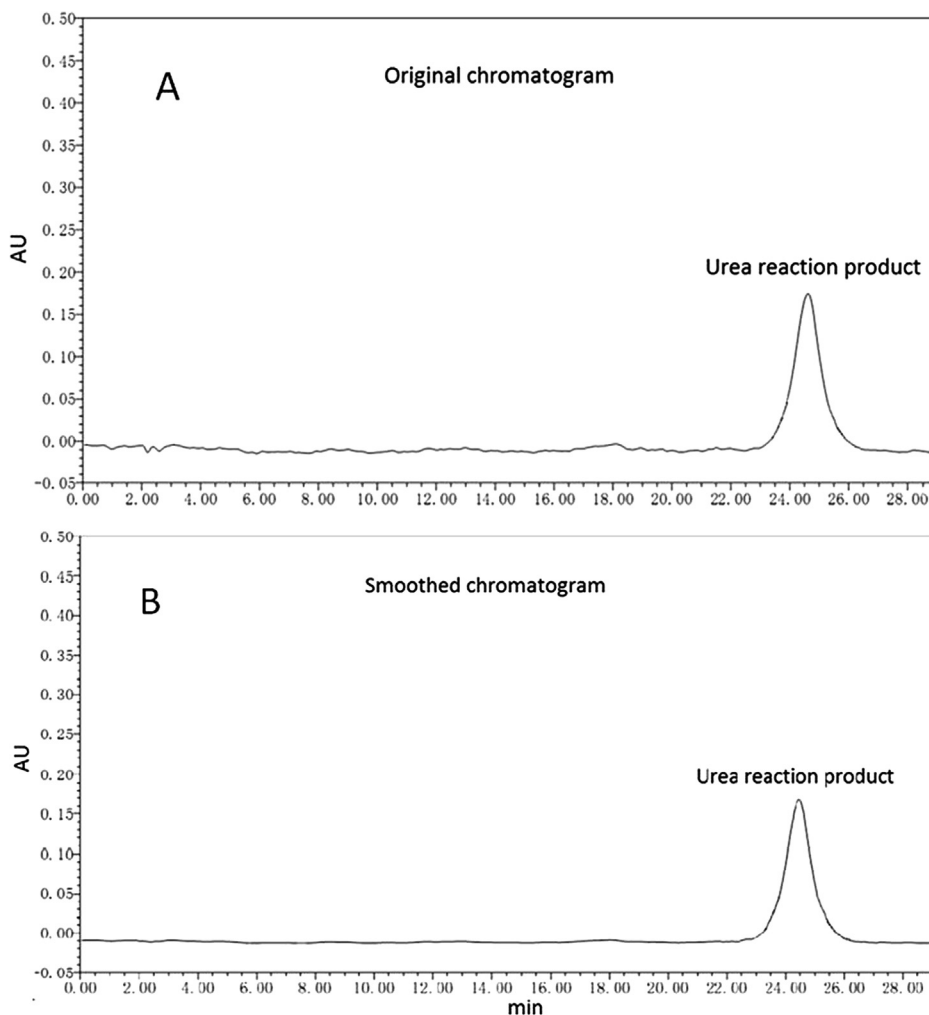


Fig. 1 – The CPC chromatogram at 430 nm of the urea standard solution (urea-PDAB-HCl in the mobile phase). (A) Original chromatogram; (B) smoothed chromatogram. CPC = centrifugal partition chromatography; HCl = hydrochloric acid; PDAB = *p*-dimethylaminobenzaldehyde.

Table 1 – Influence of different CPC solvent systems on retention of the stationary phase, baseline noise, and retention time.^a

Solvent systems ^b	Retention of the stationary phase (%)	Baseline noise (mv)	Retention time (min)
n-hexane-methanol (containing hydrochloric acid)	85	0.015	25
n-hexane-methanol (containing sulfuric acid)	85	0.030	25
n-hexane-ethanol (containing hydrochloric acid)	80	0.055	28
n-hexane-ethanol (containing sulfuric acid)	78	0.055	28

CPC = centrifugal partition chromatography.
^a Flow-rate was 5 mL·min⁻¹.
^b Upper phase volume: lower phase volume ($V_u:V_l$) = 1:1. The lower phase containing hydrochloric acid or sulfuric acid and PDAB worked as the mobile phase, and the upper phase worked as the stationary phase.

for 15 min, then the product was injected into the CPC to determine the peak area of outflow opponent; (2) The urea standard solution or the urea cream sample solution reacted with the mobile phase in the CPC rotor for 15 min, then the derivatization product was eluted and measured.

3. Results

3.1. Development and optimization of the CPC method

3.1.1. Selection of the solvent system and wavelength

The CPC solvent system was optimized by measuring the retention value of the stationary phase, the UV baseline noise, and the retention time of the active ingredient. In order to keep the lipophilic matrix of the urea cream in the stationary phase, n-hexane (upper phase) was chosen as the stationary phase. The methanol solution containing hydrochloric acid and PDAB (lower phase) was used as the mobile phase. The low viscosity of this solvent system contributed to the

formation of biphasic solvent without emulsification. Thus, the selected solvent system produced low loss of stationary phases, a stable baseline, and a high signal-to-noise ratio (Table 1).

Fig. 2 shows the UV-visible spectra of the mobile phase (containing 100 mg·L⁻¹ PDAB) and the reaction solution of urea and PDAB in the mobile phase. At 430 nm in the spectra, the mobile phase indicated almost no absorption, while the reaction solution showed good absorbency. Therefore, 430 nm was chosen as the wavelength for the detection of the urea derivatization product.

3.1.2. Flow rate and PDAB dosage

As the CPC mobile and stationary phases are both liquid, a small amount of stationary phase can be washed by the mobile phase, which makes the baseline unstable. Fig. 3A illustrates the relationship between the peak area of the urea derivatization product and CPC flow rate, and indicates that the peak area remains steady with a flow rate of 1–5 mL·min⁻¹, and declines with the increase in flow rate.

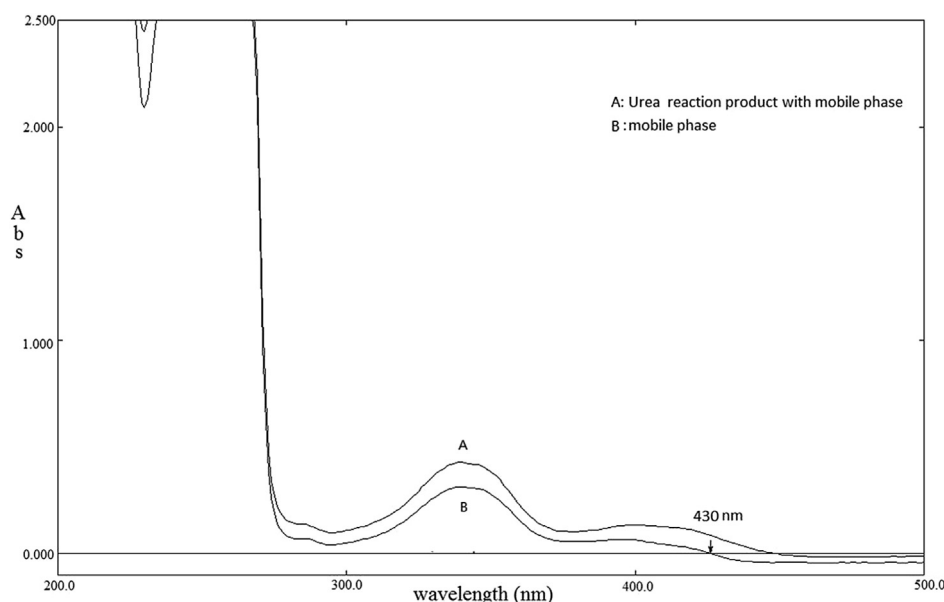


Fig. 2 – The UV-visible spectra of the urea standard solution [(A) urea-PDAB-HCl in the mobile phase and the (B) PDAB solution in the mobile phase. HCl = hydrochloric acid; PDAB = p-dimethylaminobenzaldehyde; UV = ultraviolet.

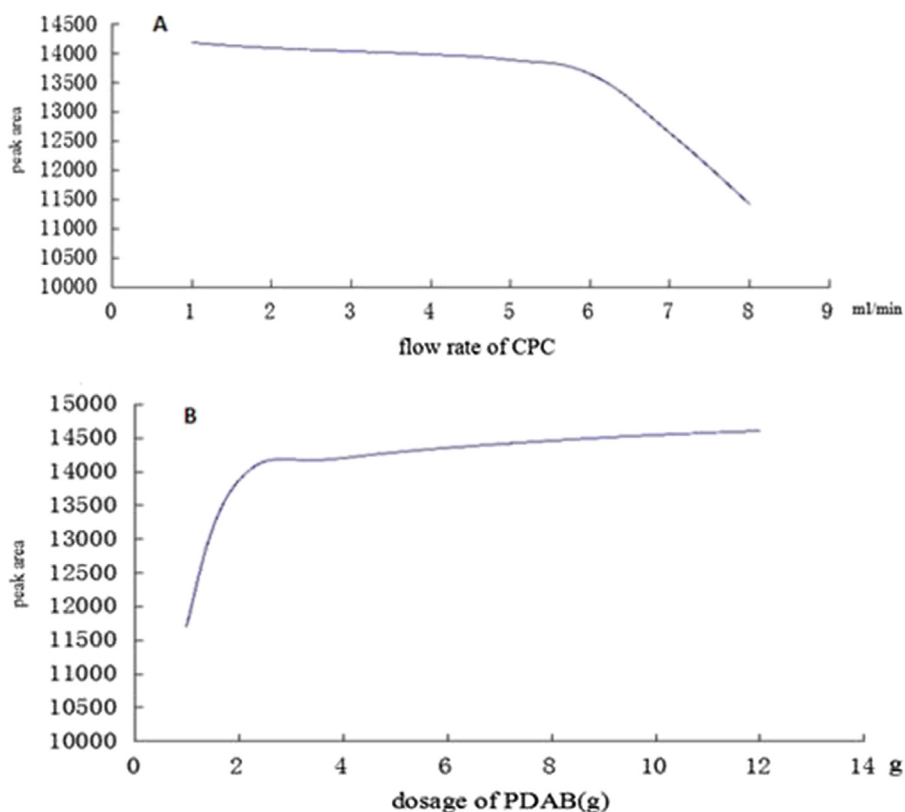


Fig. 3 – (A) The influence of the CPC flow rate and (B) the PDAB dosage on the peak areas in CPC of the urea derivatization product. CPC = centrifugal partition chromatography; PDAB = p-dimethylaminobenzaldehyde.

Generally, the slower the mobile phase flows, the more volume of stationary phase is washed out. In view of these two factors, the flow rate was finally set at $5 \text{ mL} \cdot \text{min}^{-1}$.

Fig. 3B illustrates the relationship between the peak area of the urea derivatization product in CPC at 430 nm and PDAB dosage, indicating that the peak area remained steady within the PDAB dosage of ~2–12 g, and rose slightly with increased PDAB dosage. Therefore, considering the baseline noise and derivatization reaction, the PDAB dosage PDAB was finally chosen to be 2 g.

3.1.3. Selection of acid reagent and concentration

With other conditions unchanged, urea reacted with hydrochloric acid and sulfuric acid in a 40°C water bath for 10 min, and the product maintained the same UV absorbance. Considering that sulfuric acid might cause corrosion of the CPC rotor and increase baseline noise, we chose hydrochloric acid as the acid reagent. Fig. 4 shows that the peak area of the urea derivatization product remained steady within the concentration of hydrochloric acid at $\sim 0.2\text{--}1.2 \text{ mol} \cdot \text{L}^{-1}$. To decrease equipment corrosion, the concentration of hydrochloric acid in the mobile phase was set at $0.2 \text{ mol} \cdot \text{L}^{-1}$.

3.2. Validation of the analytic method

3.2.1. Linearity and recovery rate

Plotting the peak areas of the derivatization product in CPC versus the concentrations of the urea standard solution resulted in linear regression analysis curves (Table 2). The

results show that the linearity is fine, and the CPC method is qualified for quantitative analysis in the concentration range required.

The data from the recovery experiment for urea are presented in Table 3, and the results show that urea recovery is $>98\%$, which is adequate to meet the requirement of content determination.

3.2.2. Precision, repeatability, and stability

The RSD of urea content determination was calculated at 1.2%, and the average repeatability of urea content determination was 98.5%, with an RSD of 0.7%. The results indicated that the precision of the apparatus and the repeatability of the method were both adequate, and that the test solutions could remain stable for up to 24 h under the test conditions.

3.3. Comparison between CPC and colorimetric methods for urea determination

3.3.1. Comparison of the two derivatization modes

This study conducted urea derivatization in two modes: inside and outside of the rotor. Urea cream was dissolved in an aqueous solution and determined by the CPC method with two derivatization modes (Table 4), with the results indicating almost no difference between the two derivatization modes.

3.3.2. Comparison of CPC and colorimetric methods

The content of urea in urea cream using the colorimetric method was analyzed according to the literature [14] (Table 4).

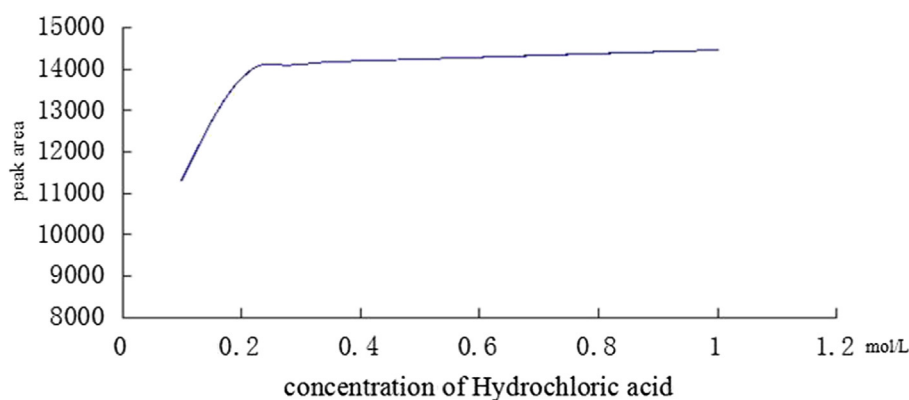


Fig. 4 – The influence of the concentration of HCl on the peak areas in the CPC of the urea derivatization product. CPC = centrifugal partition chromatography; HCl = hydrochloric acid.

Table 2 – Linearity of urea determination in urea standard solution by the CPC method.

Compound	Linear equation	Linear range (mg·mL ⁻¹)	R ^{2a}
urea	Y=29.0X+0.310	~0.1006–0.5032	0.9982
CPC = centrifugal partition chromatography. ^a Average of duplicate determinations.			

The results indicated that the content values of urea obtained using the colorimetric method were lower than those using the CPC method, which might be attributed to the loss of urea during the complicated pretreatment process. In the CPC method, however, the stationary phase containing *n*-hexane dissolved the cream matrix, while urea and its derivatization product could not dissolve in the stationary phase and was extracted completely by the mobile phase.

4. Discussion

In this study, urea in urea cream was determined by using CPC with a biphasic solvent system of *n*-hexane and methanol containing hydrochloric acid and PDAB. The solvent system, detection wavelength, flow rate, and derivatization methods were studied, with the results indicating that the CPC method was a good option for content determination of the urea cream by exhibiting acceptable validation results.

Compared with spectrophotometry and urease methods, the CPC method has advantages in sample pretreatment,

Table 3 – The data for the urea recovery experiment using the CPC method.

Proportion of added standard	Recovery (%) ^a	RSD (%) ^{fa}
80%	98.1	0.6
100%	98.9	0.5
120%	98.0	0.5
CPC = centrifugal partition chromatography. ^a Average of triplicate determinations.		

Table 4 – The data of urea content determination in urea cream (n=5) using the CPC method with two derivatization modes and colorimetry.

Batch	CPC (g·g ⁻¹)		Colorimetry (g·g ⁻¹)
	Derivatization inside of rotor	Derivatization outside of rotor	
1	0.0987±0.0012	0.0989±0.0011	0.0958±0.0009
2	0.0991±0.0009	0.0993±0.0010	0.0964±0.0011
3	0.0992±0.0013	0.0993±0.0014	0.0957±0.0012
CPC = centrifugal partition chromatography.			

since the stable and uniform emulsion samples can be directly injected into the apparatus without pretreatment. Furthermore, the *in situ* derivatization simplifies the analytical procedures, thus minimizing systematic error. Therefore, the content determination of cream or ointment products using the CPC method has specific methodological advantages over traditional quantitative analysis methods.

In summary, the CPC analysis suggested in this paper is a precise, reliable, and user-friendly method for cream analysis, and is more advantageous than other methods based on its simplicity and low expense, given that it is free of sample pretreatment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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