

A new method for screening of β_2 -agonists by micellar electrokinetic chromatography^{*}

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Abstract A method for the determination of five β_2 -agonists in pig feed, liver, meat, and urine samples by micellar electrokinetic chromatography was developed. The separation and detection conditions were optimized in terms of SDS concentration, running buffer pH and concentration, capillary temperature. Under the optimized conditions (15 mmol/L borate, pH = 10, 15 mmol/L SDS, and 25 °C), all five analytes in the spiking samples were separated and identified within 18 min. Good linearity ($R^2 > 0.999$) was obtained for the analytes in the range of 0.1 – 40 $\mu\text{g/mL}$ with the limit of detection ($S/N \geq 3$) in the range of 0.05 – 0.08 $\mu\text{g/mL}$. The satisfactory recoveries of cimaterol, ractopamine and clenbuterol were obtained in the range of 93.1% – 95.8%, whereas the recoveries of salbutamol and terbutaline were at 52.3% and 69.4%, respectively. The relative standard deviations of peak area and migration time of the analytes were less than 4.86% and 1.25%, respectively. The method was successfully applied to monitor the presence of the selected β_2 -agonists in pig feed, liver, meat and urine samples.

Key words β_2 -agonist; micellar electrokinetic chromatography; screening

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一种用于 β_2 受体兴奋剂筛查的胶束电动色谱新方法

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摘 要 建立一种快速、简单、有效的胶束电动色谱技术分别检测猪饲料、猪肝、猪肉和猪尿中的 5 种 β_2 受体兴奋剂. 获得优化后的分离缓冲液为 15 mmol/L SDS、15 mmol/L 硼酸钠、pH = 10、以及毛细管温度为 25 °C. 在优化条件下, 5 种 β_2 受体兴奋剂在 18 min 内完成分离, 在

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0.1 ~ 40 $\mu\text{g/mL}$ 浓度范围内具有良好的线性关系 ($R^2 > 0.999$), 其检出限 ($S/N \geq 3$) 在 0.05 ~ 0.08 $\mu\text{g/mL}$ 范围之内. 西马特罗, 莱克多巴胺, 克伦特罗具有较好的回收率 ($n = 3$), 分别为 95.8%, 93.1%, 95.0%; 而沙丁胺醇和特布他林的回收率较低, 分别为 52.3% 和 69.4%. 被测物的峰面积和迁移时间的相对标准偏差分别小于 4.86% 和 1.25%. 利用该方法对 5 种不同猪饲料、猪肝、猪肉和猪尿样品中的沙丁胺醇, 特布他林, 西马特罗, 莱克多巴胺和克伦特罗进行了筛查.

关键词 β_2 受体兴奋剂; 胶束电动色谱; 筛查

β_2 -Agonists were originally developed for the treatment of asthma and bronchial diseases. However, these substances were also reported as efficient partitioning agents capable of promoting the reduction of body fat and enhancing meat growth in livestock [1-2]. There are numerous publications to report the poisoning effects and potential hazards of β_2 -agonists including cardiac palpitation, tachycardia, nervousness, muscle tremors, and confusion [3-5]. If high dosage of β_2 -agonists is used to provoke anabolic effects, the accumulation of β_2 -agonist residues in edible animal tissues will be associated with a potential risk for consumer health. Therefore, most countries and regions have put strict bans on β_2 -agonists as repartitioning agents in livestock to guarantee safety of consumers.

Presently, a wide variety of analytical methodologies have been developed for the determination of β_2 -agonist residues in feed and meat products [6]. There are some approaches aimed for on-site use, such as ELISA for clenbuterol (CLB) [7], amperometric sensor with molecularly imprinted membranes for ractopamine (RAC) [8], and colorimetric assay based on the capability of directly reducing HAuCl_4 into atomic gold (gold nanoparticles) by β_2 -agonists [9]. However, the above mentioned methods inherit their own drawbacks, such as lack of stable antibodies, time-consuming incubation, and inability to conduct multi-target analysis. Additionally, some conventional analytical techniques have also been extensively developed for routine analysis and accreditation, such as high performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS) [10-18].

Although a great amount of information is provided, MS methodologies have to require skilled workers and expensive instruments.

CE is an alternative method for separation of β_2 -agonists due to its high separation efficiency and low waste generation [6, 19-24]. Although various separation modes can be used to manipulate separation [25], most of reported CE methods apply capillary zone electrophoresis (CZE) mode to the separation of multiple β_2 -agonists with relatively low resolution. Micellar electrokinetic chromatography (MEKC) is another commonly used mode in capillary electrophoresis for separation of neutral molecules and charged compounds with similar electrophoretic mobilities and different hydrophobicity. In MEKC, surfactant is added into background electrolyte (BGE) at a level above the critical micelle concentration and surfactant molecules aggregate to form micelles as a pseudo-stationary phase. In general, more hydrophobic analytes have more affinity with micelles, whereas analytes containing more hydrophilic properties have less affinity with micelles. The analyte-to-micelle interaction can be altered in various ways, such as adjustment of buffer pH, change of surfactant, and addition of organic solvent [26]. Once we reported a method for the determination of β_2 -agonists using a microchip capillary electrophoresis with electrochemical detection [27], however, CLB, one of the most strictly banned β_2 -agonists, was unable to be detected due to its relatively high detection potential beyond the scope of the working potential at the gold electrode.

In this paper, the conditions for the separation of β_2 -agonists were investigated and the developed

MEKC method was applied to the determination of salbutamol (SAL), terbutaline (TER), cimaterol (CIM), RAC and CLB in several pig feed, liver, meat, and urine samples. Compared with other methods, this present method is characterized by high-resolution in separation, simple in operation, and low in cost. It provides a straightforward approach for quickly screening multiple β_2 -agonists in real samples.

1 Experiment

1.1 Chemicals and solutions

RAC hydrochloride and TER sulfate were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). SAL sulfate and CLB hydrochloride were obtained from National Institute for Food and Drug Control (Beijing, China). CIM was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) and sodium dodecyl sulfate (SDS) were purchased from Sigma (Shanghai, China). Aqueous solutions were prepared using analytical grade reagents and $18\text{ M}\Omega \cdot \text{cm}$ resistance water (PURELAB Classic, ELGA Lab Water, High Wycombe, UK). Ten mg/mL stock solutions of the β_2 -agonists were prepared by dissolving the individual drug in ultra-pure water, except that CIM was dissolved in methanol. All of them were stored at $4\text{ }^\circ\text{C}$ before use. Unless otherwise stated, the pH of each solution was adjusted with 0.1 mol/L NaOH by a digital pH meter (Denver Instrument, Denver, UT, USA). All chemicals were used as received without further purification. ELISA assay of RAC was conducted using a commercial ELISA kit (Huaan Magnech Bio-TECH Co., Ltd, Beijing, China) according to the manufacturer's instructions.

1.2 Apparatus and procedures

A Beckman-Coulter P/ACE MDQ (Fullerton, CA, USA) capillary electrophoresis instrument equipped with a UV absorbance DAD was used for all the experiments. Electrophoresis was performed in natural fused-silica capillaries (Beckman-Coulter, Fullerton, CA, USA), 60 cm (effective

length 50 cm) $\times 75\text{ }\mu\text{m}$ i. d. ($375\text{ }\mu\text{m}$ o. d.), with the anode and cathode positioned at the inlet and outlet ends of the capillary, respectively. Data collection, processing, and analysis were performed using system 32-karat software (Beckman) and recorded on a personal computer. Unless otherwise noted, samples were introduced into the capillary by 0.5 psi pressure injection for 5 s and subsequently separated by MEKC with an applied potential of 20 kV and a controlled temperature of $20\text{ }^\circ\text{C}$. Direct UV detection was performed with a wavelength of 204 nm , through the capillary at a window located 50 cm from the inlet. The selected wavelength allows direct detection of all five β_2 -agonists, as well as other possible contaminants.

1.3 Sample preparation

Five pieces of pig liver and lean meat were purchased from several local supermarkets. Five branded feed samples (made of corn and soybean) were purchased from a local market. Five urine samples from Beijing Black Pigs (weight from 90 to 120 kg) were collected from a local slaughter farm. One gram of each solid sample (grinded feed, pig liver and meat) was distributed into 5.0 mL of 0.1 mol/L HCl and the mixture was blended thoroughly with vortexing, whereas 5.0 mL of urine sample was acidified with the concentrated HCl. After a while, the acidic extract solution was centrifuged at $3\text{ }000\text{ r/min}$ for 10 min . Next, 3.0 mL of the resultant supernatant was collected and the pH was adjusted to neutral with NaOH (5.0 mol/L), and then centrifuged at $3\text{ }000\text{ r/min}$ for 10 min . After the pH of the collected supernatant was adjusted to 10 with NaOH, sodium chloride was added to make the solution saturated. Then the solution was centrifuged for 10 min ($12\text{ }000\text{ r/min}$), and 2.0 mL of the supernatant was extracted with ethyl acetate-hexane ($9:1, v/v$) in triplicate. The combined organic liquid was dried under a nitrogen flow. The residue was reconstituted with 1.0 mL BGE, and then centrifuged at $12\text{ }000\text{ r/min}$ for 10 min . Finally, the supernatant was transferred to a sample vial for injection.

2 Results and discussion

Because the selected β_2 -agonists are banned by most of authorizations for usage in animal feeding, the separation step is particularly important to samples containing more than one of these substances. To optimize the conditions of the separation of the five β_2 -agonists, the effects of the concentration of SDS and BGE, buffer pH, and capillary temperature on migration time were studied.

2.1 Effect of SDS concentration

β_2 -Agonists possess a common β_2 -hydroxylamine group on the side chain. However, they are differentiated from each other by varied substituents on the aryl moiety, such as substituted anilines (CLB and CIM) and substituted phenols (SAL, TER, and RAC). The chemical structures of these selected drugs are shown in Fig. 1. The

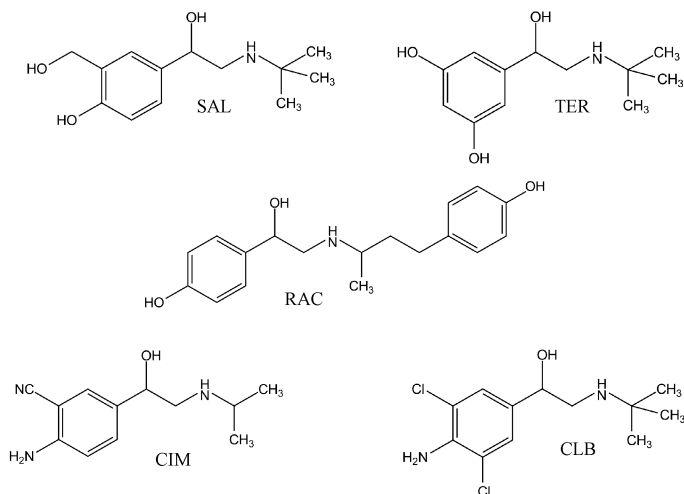


Fig. 1 Molecular structures of the selected β_2 -agonists

2.2 Effect of borate concentration and pH

The buffer concentration or ionic strength greatly affects EOF, current generated in capillary, and micelle aggregation number of surfactant^[28]. In order to investigate the effect of borate concentration on the separation of the analytes, we studied the range of 5-20 mmol/L borate (pH 10) with 15 mmol/L SDS. As shown in Fig. 2B, the higher borate concentration resulted in the larger migration

separation of the five β_2 -agonists was based on their differential partitioning between micellar phase and aqueous phase. The effect of SDS concentration was studied in the range of 0-25 mmol/L with 15 mmol/L borate (pH 10). As shown in Fig. 2A, SAL and CIM co-eluted with TER and RAC at CZE mode (without SDS), respectively. When the SDS concentration was progressively increased, the baseline separation of the five drugs was achieved. With increasing SDS concentration, the longer migration times were obtained while the overall resolution and peak symmetry were also improved. For instance, the asymmetries of CIM and CLB were changed from 2.57 and 5.83 at 5 mmol/L SDS to 1.35 and 0.88 at 15 mmol/L SDS, respectively. Considering the peak symmetry and the analysis time, the optimum concentration of SDS was selected as 15 mmol/L in the following experiments.

times. For instance, the migration time of CLB changed noticeably from 9.10 min at 5 mmol/L borate to 19.50 min at 20 mmol/L borate. Meanwhile, the overall peak resolution became large with the increase of borate concentration. To balance the resolution and analysis time, 15 mmol/L was selected as the optimum borate concentration.

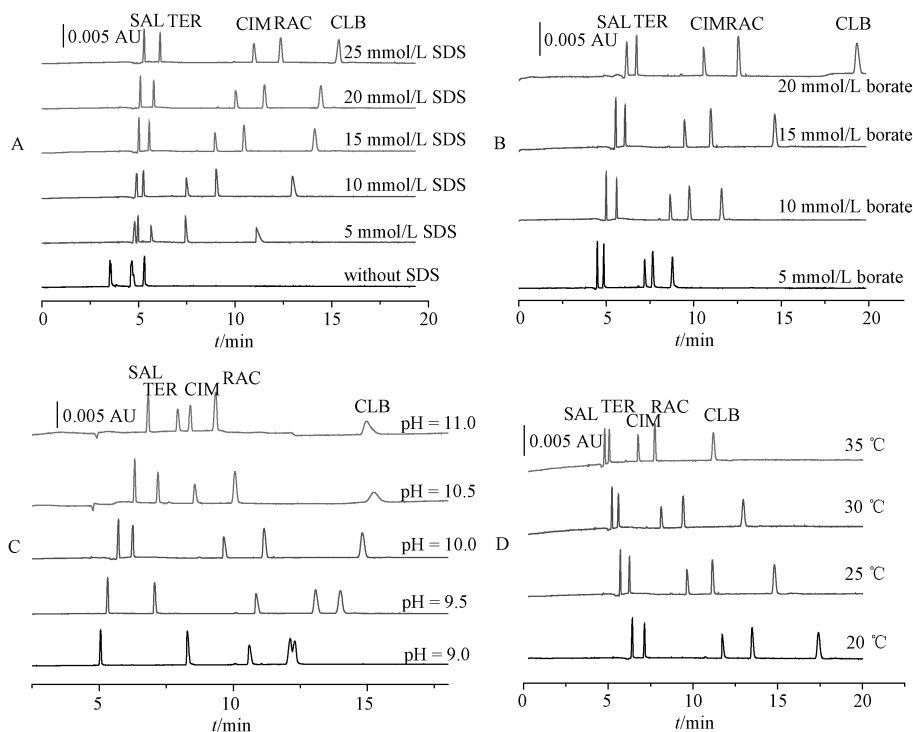
Change in buffer pH determines the zeta potential (EOF) of capillary wall and the charge degree of analyte. The effect of pH on the separation

of the analytes was investigated at the pH value ranging from 9.0 to 11.0. As shown in Fig. 2C, the resolution between RAC and CLB was improved largely when the pH value changed from 9.0 to 10.0. When the pH value was larger than 10, the peaks of the analytes, except for CLB, tended to cluster, while the peak of CLB became broader with a less change in migration time. Accordingly, the optimum pH value was selected as 10, at which the five β_2 -agonists were baseline separated with high resolution in general.

2.3 Effect of temperature

The capillary temperature is an important parameter that considerably affects the resolution, efficiency, and analysis time. Therefore, the effect

of capillary temperature on the separation was investigated between 20 °C and 35 °C. As shown in Fig. 2D, the migration time and resolutions generally decreased with increasing the temperature. This behavior can be mainly attributed to the decrease in BGE viscosity which results in the increase in electrophoretic and electro-osmotic mobility. Although a significant decrease in the separation time was achieved at 35 °C, the optimum temperature was chosen at 25 °C to prioritize separation resolution over separation time in consideration of the interfering matters from sample. Meanwhile, the lower temperature is helpful to avoid the detrimental formation of bubbles.



Other conditions: separation voltage = + 20 kV, injection = 5 s, 0.5 psi, detection wavelength = 204 nm. Note: the separation order of the analyses was the same in all electropherograms.

Fig. 2 Effects of the different variables on the separation of the five β_2 -agonist: SDS concentration (A), borate concentration (B), pH (C), and capillary temperature (D)

2.4 Method validation

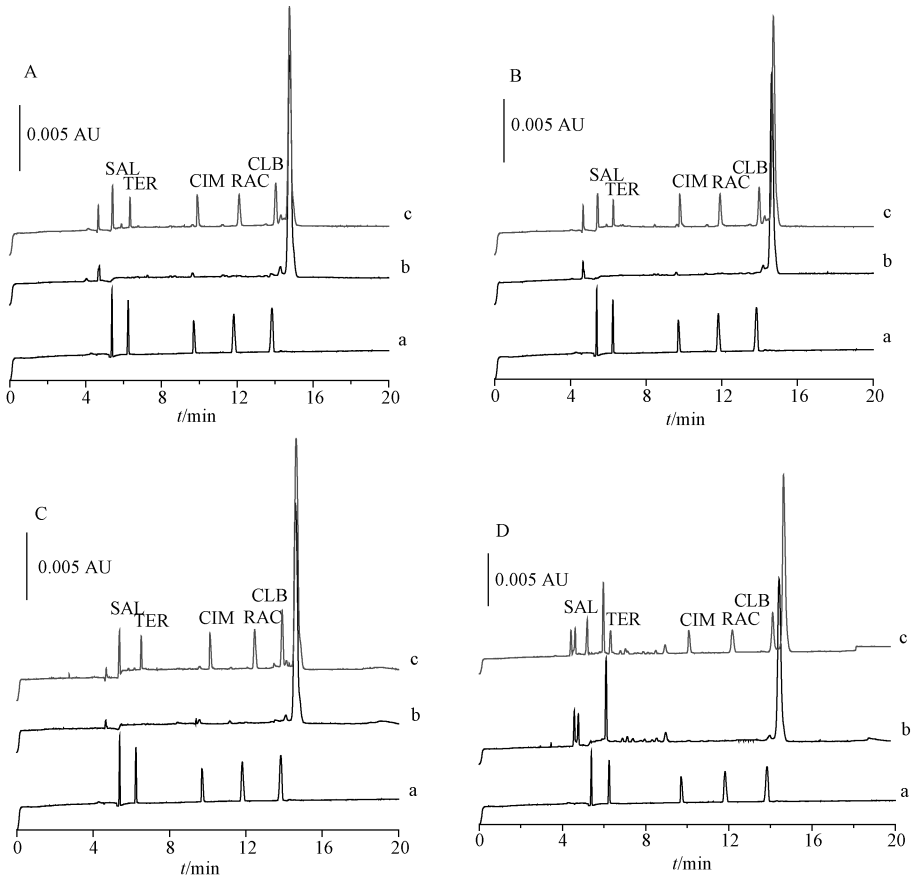
The method validation was performed according to the FDA guideline: Guidance for Industry-Bioanalytical Method Validation. Under the optimum conditions (15 mmol/L borate, pH = 10, 15 mmol/L

SDS and 25 °C), the repeatability of the proposed method was evaluated by measuring intraday and interday precisions in terms of peak area and migration time at three different levels (0.1, 1.0, and 10.0 $\mu\text{g/mL}$). Briefly, the intraday RSDs of

peak area and migration time were less than 3.45 and 0.97, respectively. The interday RSDs of peak area and migration time were lower than 4.86 and 1.25, respectively. The linear relationships between the peak area and the analyte concentration were obtained in the 0.1- 40.0 $\mu\text{g/mL}$ range with the correlation coefficients larger than 0.99 and the LODs lower than 0.08 $\mu\text{g/mL}$. Although the LODs reported here are higher than those reported by others^[11-13], the method shows the capability to detect even lower amount of these compounds in the real sample by increasing the amount of sample for preparation.

The selectivity was evaluated by analyzing the five β_2 -agonists in spiking pig feed, pig liver, pig meat, and pig urine samples, respectively. As shown in Fig. 3, the electropherograms showed clear separation of the analytes in the spiking samples and indicated no interference peak from sample. The

recovery experiments were performed by spiking the analytes at three different levels (1.0, 5.0, and 20.0 $\mu\text{g/g}$) in pig feed, pig liver, pig meat, and pig urine samples, respectively. At each of the levels, a set of three blank samples were spiked with all the compounds before sample preparation, while another set of three blank samples were prepared according to the above described procedure of the sample preparation. The recovery values were calculated by the comparison of the detected amount of analytes with the actually added amount in spiking sample. The mean recoveries of CIM, RAC, and CLB were 95.8%, 93.1%, and 95.0%, respectively, while the mean recoveries of SAL and TER reached around 52% and 69%, respectively. The reason for the lower recovery is that the less hydrophobic propriety of SAL and TER results in the larger distribution into water phase during the liquid-liquid extraction.



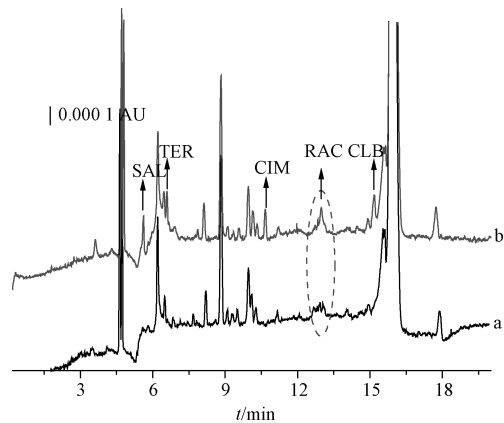
Conditions: 15 mmol/L borate (pH = 10), 15 mmol/L SDS, separation voltage = + 20 kV, injection = 5 s, 0.5 psi, temperature = 25 °C, and detection wavelength = 204 nm.

Fig. 3 Electropherograms corresponding to spiking samples of pig feed (A), pig liver (B), pig meat (C), and pig urine (D); a mixture of standards at 5.0 $\mu\text{g/mL}$ level (a), blank sample (b), and spiking sample (c)

2.5 Real sample analysis

To demonstrate the applicability of this method, pig meat, liver, urine, and feed samples were collected and analyzed to screen the presence of these β_2 -agonist residues. The results of sample analysis were summarized in Table 1. Although the use of β_2 -agonist in animal feeding to enhance lean meat production has been strictly banned in China, it is worth noting that the low amounts of β_2 -agonist residues still exist in the randomly collected real samples. Additionally, Fig. 4 shows an example of the identification and quantification of the selected β_2 -agonists, in which the peak of RAC was confirmed by the addition of the standards in the sample. Meanwhile, ELISA assay of RAC was conducted to further evaluate the accuracy of the

method. These obtained results show that both the methods are comparable.



Other conditions were the same as given in Fig. 3.

Fig. 4 Electropherograms corresponding to samples of meat (a) and the spiking meat sample (b) with a mixture of the standards at 1.0 $\mu\text{g/mL}$ level

Table 1 Assay results of the sample solutions ($\mu\text{g/mL}$, $n = 3$)

Samples *	SAL	TER	CIM	RAC **	CLB
Meat 1	—	—	—	0.55 (0.53)	—
Meat 3	2.63	—	—	—	0.99
Meat 4	2.56	—	—	—	—
Meat 5	0.13	—	—	—	—
Liver 1	—	—	—	0.22 (0.21)	—
Liver 4	—	—	—	—	0.22
Liver 5	—	—	—	—	0.42
Urine 1	—	—	—	0.45 (0.41)	—

Notes: * The samples were listed in Table 1 with the positive results, except for those with negative results. **The contents of RAC in the parentheses were detected by ELISA.

3 Conclusions

The proposed method provides a simple MEKC method for the determination of five β_2 -agonists (SAL, TER, CIM, RAC, and CLB) in pig feed, liver, meat, and urine sample. Under optimum conditions (15 mmol/L borate, pH = 10, 15 mmol/L SDS, and 25 $^{\circ}\text{C}$), the five β_2 -agonists were baseline separated within 18 min. Through a simple liquid-liquid extraction, the complex matrixes in pig feed, liver, meat, and urine samples were effectively removed, avoiding obvious disturbances on the identification and quantification of the β_2 -agonists. Importantly, this method excludes the need of expensive MS instrumentation and has the ability to screen multiple β_2 -agonists simultaneously. It provides an effective and convenient approach for

determination of β_2 -agonists in real samples.

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