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Simultaneous Detection of Lactone Form and Acid Form Monacolin K by Sensitive TLC Method

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Abstract: A novel and sensitive thin-layer chromatography (TLC) method for specific and simultaneous detection of both lactone form Monacolin K and acid form Monacolin K is developed in this study. The TLC works in the following conditions: The TLC plate was Silica Gel 60, RP-18 F254, 0.5 mm, chemically modified layer, Merck, Germany; The solvent system was chloroform∶methanol∶ammonium hydroxide = 25∶3∶1 (volume ratio); Monitoring the TLC under 365 nm after heating it with a slight coating of the charring reagent 30% (volume fraction) H_2SO_4 ethanol on the TLC plate, allowed the specific detection of Monacolin K and the low detection limit was first time brought down to 30 ng. The present TLC method is suitable for the routine qualitative and semi-quantitative analysis of the bulk materials containing Monacolin K.

Key words: thin-layer chromatograhpy; lactone form Monacolin K; acid form Monacolin K

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Monacolin K (also called lovastatin) possesses various medical functions such as lowering high level blood cholesterol[1~3], anticancer[4,5] and promoting bone formation[6,7]. Before 1994 only pigment red rice was produced but after that the functional red rice which contains monacolin K appeared in the market[8]. Therefore in the market there are both pigmental and functional red rice or their products. The functional red rice or its product was further divided into different grades according to its Monacolin K content, which ranged from 0.1%~1.0% (mass fraction, the following is the same)[9]. So it is very important to develop a rapid and easy method to analyze total Monacolin K in red rice or its products.

Study[3] has proved that there are two forms of Monacolin K: lactone form and acid form (refer to Fig. 1).

In our previous study we have developed a simultaneous detection method for both LFMK and AFMK by RP-HPLC in our lab[10], but HPLC not only takes much time, but also is rather expensive. The method of double wave-length UV spectrophotometry was also reported, but it requires sample pre-treatment through a neutral aluminum oxide column and is therefore quite complicated[11]. So far the best reported TLC system[12] uses silica gel plate, the developing reagent of hexane∶acetone = 1∶1 (volume ratio, the following is the same) and the iodine as the colorant for the separated spots. The observation condition is under daylight. The reported TLC method can detect only LFMK and its lower detection limit was 10 000 ng, while the usual loading amount of standard Monacolin K was 40 000 ng. As to AFMK, since it is not commercially available, moreover, its lower detection limit is as low as that of LFMK with common TLC method (indicated in this

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Biography: Wang Qi-jun (born in 1972), male, Ph.D, lecturer, mainly researches on bioengineering and food ana-lysis.

study), therefore it can not be detected by common TLC methods. In the present study we have developed a sensitive, rapid and economical TLC method which allowed the qualitative, quantitative (approximately) and simultaneous detection of LFMK and AFMK when observed under 365 nm UV light after charred with 30 % H_2SO_4 ethanol. And this method can be used to monitor Monacolin K content during fermentation, and to estimate the quality of red rice or Monacolin K containing medicine.

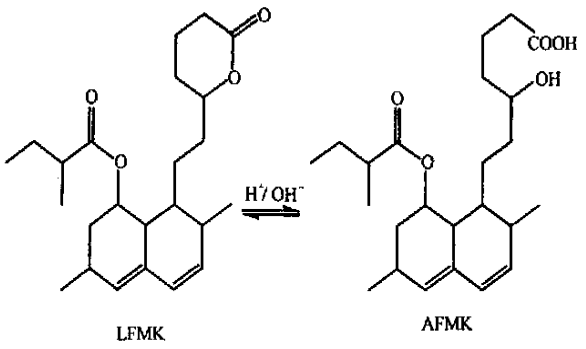

Fig. 1 Chemical structures of LFMK and AFMK

1 Materials and Methods

1.1 Standards and Red Rice Samples

LFMK was purchased from Sigma Chemical Co., New York, USA. Functional red rice of FHQF and the medicinal capsule of Xuezhikang were kindly donated by Professor Zhou Li-ping of Zhejiang Univ. of Tech., Hangzhou, China. The pigmental red rice of HQF was purchased from Taiwan.

1.2 TLC Plates

K5 Silica Gel 150, 0.25 mm and K6 Silica Gel 60, 0.25 mm were from Whatman, USA. Silica Gel 60, F254, 0.5 mm, Silica Gel 60, F254, 0.5 mm, aluminum sheet and Silica Gel 60, RP - 18, F254, 0.5 mm, chemically modified layer were from Merck, Germany.

1.3 Chemicals

Acetonitrile, methanol, chloroform, hexane and acetone were purchased from Burdick & Johnson USA, ammonium hydroxide was from Sigma Chemical Co. USA. All other chemicals were of analytical reagent grade, and obtained from Yakuri Pure Chemical Co. Ltd., Japan.

1.4 Hydrolysis of LFMK by NaOH

AFMK so far is not commercially available and it is prepared from LFMK in our laboratory. The stan-dard of LFMK was purchased from Sigma, and 1 mg/mL of LFMK in 80 % acetonitrile water solution was used to obtain AFMK in solution by alkaline treatment with 0.1 kg/mol NaOH. The pH value was estimated with pH test paper. AFMK was confirmed by LC/MS.

1.5 Monacolin K Extraction

The procedures of Monacolin K extraction from red rice or its solid products mainly followed the method described by Moon[10]. 0.200 g red rice powder or its product was extracted with 1 mL 60 % acetonitrile water solution in 1.5 mL Enpendorf tube by sonicating for 20 minutes at 25 ℃. The extract was then centrifuged at 12 000 r/min for 5 minutes. The supernatant was stored at 4 ℃ if not directly used for analysis.

1.6 TLC Analysis

TLC plate of 5～8 cm in width, 6 cm in length, was activated at 105 ℃ for 60 minutes, and stored in a silica desiccator. 3～5 μL standards or Monacolin K extract were loaded on a TLC plate. After air dried, the TLC plate was developed in a presaturated solvent chamber. Before the solvent front reaching the end of the TLC plate, the TLC plate was removed from solvent chamber and sprayed with an even coating of 30 % H_2SO_4 ethanol charring reagent with a TLC sprayer (Merck, Germany). Then the TLC plate was observed and photographed under 365 nm after being heated on a hot plate.

2 Results and Discussion

2.1 Preparation of AFMK from LFMK

AFMK was prepared in liquid solution by alkaline hydrolysis of lactone form Moancolin K. The hydrolysis results were shown in Table 1. Treatment No. 3 was used to prepare the standard solution of AFMK. The converted AFMK was confirmed by LC/MS (refer to Fig. 2). The conversion ratio was estimated by HPLC.

Table 1 Alkaline treatments of lactone form Monacolin K

Treatment No.	LFMK[1)] /μL	NaOH[2)] /μL	pH	Conversion ratio / %
1	500	1	7.0	20
2	500	3	7.5	80
3	500	5	8.0	100
4	500	10	9.5	100
5	500	20	10	100
6	400	100	—	100

1) 0.5 mg/mL LFMK in 60 % acteonitrile water;

2) 0.10 kg/mol NaOH.

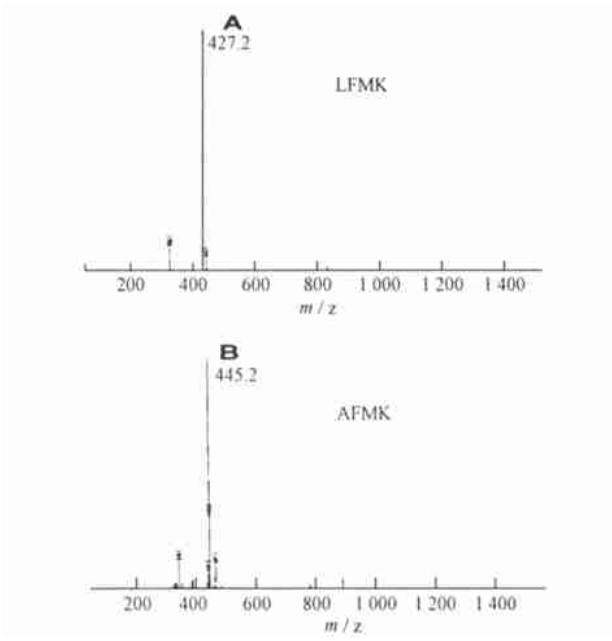

Fig. 2 Mass spectra of LFMK and AFMK

A ($M + Na^+ = 404 + 23$) : from the standard LFMK solution;

B ($M + Na^+ = 422 + 23$) : from the solution of treatment No. 4 in the acid form Monacolin K preparation test

In the experiment it was found that if the amount of NaOH in acetonitrile water solution was more than a certain amount compared to the amount of LFMK, the LFMK could be converted completely into AFMK (refer to treatment No. 3, 4 and 5), but too much alkaline in the mixture could be harmful to both HPLC column and TLC plate. However if the amount of NaOH was not enough, LFMK was only partially converted into AFMK (refer to treatment No. 1, 2), and the conversion ratio increased with the storing temperature and time (data not shown).

Additionally, we found that even under a very strong basic condition like treatment No. 6, LFMK still could not be converted into Monacolin J. The conversion of acid form Monacolin J form lactone form Monacolin K need to be further studied.

2.2 Novel TLC Detection Method for Monacolin K

Fig. 3 shows the result obtained with the TLC plate of Silica Gel 60, F254, 0.5 mm, Merck, Germany and the mobile phase of chloroform : methanol = 9 : 1, 2.0 μL (i.e. around 1 μg total Monacolin K) of was applied to the TLC plate.

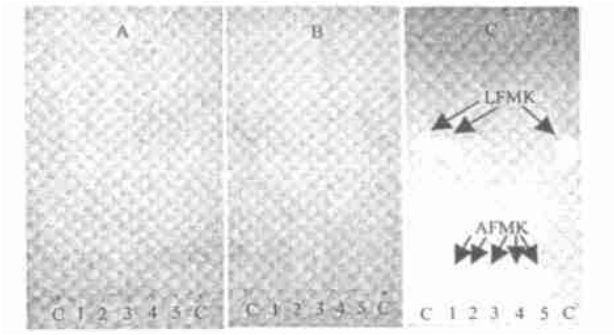

Fig. 3 Novel TLC detection method for Monacolin K

(1) Samples 1～5: corresponding to the alkaline treatment from No. 2 to No. 6 in Table 1; Sample C: the standard lactone form Monacolin K of 1 000 ng (left) and 500 ng (right).

(2) A: observed directly under 365 nm before spraying charring reagent, indicating Monacolin K itself had no fluorescence; B: observed under day light as normal TLC method required after charring treatment, the signal were very faint; C: observed under 365 nm after charring treatment, both LFMK and AFMK now showed bright yellow fluorescence.

(3) The Rf of LFMK was 0.64, the Rf of AFMK was 0.025 (meaningless).

The TLC plate was observed step by step. First, the plate was observed directly under daylight, under 254 nm UV light and under 365 nm UV light respectively, and no spots could be recognized, indicating that Monacolin K had no fluorescence reaction. Second, the TLC plate was observed under daylight, after being sprayed with the charring reagent of 30 % H_2SO_4 ethanol and being heated properly the plate on a hot plate (if over heated, the whole TLC plate will be spoiled), and only faint gray spots (in fact hardly recognizable) shown on the plate. Following the second step, the TLC plate was observed under 254 nm and the signals could not be enhanced by any degree, compared with observing at daylight. However when the TLC was observed under 365 nm, and very bright yellow spots were found, indicating that both LFMK and AFMK after being charred by H_2SO_4 could produce yellow fluorescence under 365 nm.

The result were photographed under 365 nm.

But the problem was that the spots of AFMK even did not move forward in this TLC system. So the further screening work for the proper mobile phases or other TLC plates were required.

2.3 Optimal TLC System

Five kinds of solvent systems, i.e. chloroform : methanol = 9 : 1; hexane : chloroform : methanol = 9 : 3 : 1; hexane : acetone = 1 : 1; chloroform : methanol : ammonium hydroxide = 25 : 3 : 1; he-xane : chloroform : isopropanol = 6 : 3 : 1 and six kinds of TLC plates listed in the section of material and methods were tried for screening the optimal stationary phase and mobile phase for detecting Monacolin K. The most important part of the results were shown in Fig. 4. The optimal TLC plate as Silica Gel 60, RP - 18 F254, 0.5 mm, chemically modified layer, Merck, Germany, and the optimal mobile phase as chloroform : methanol : ammonium hydroxide = 25 : 3 : 1 was screened out. The Rf values for LFMK and AFMK were 0.62 and 0.54, respectively. The low detection limit for LFMK was 30 ng (refer to Fig. 4). It should be pointed out that the mobile phase temperature could slightly affect the Rfs of both LFMK and AFMK, but it would not interfere the re-cognition of Monacolin K.

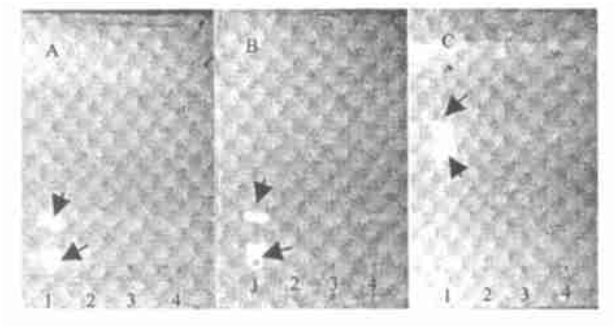

Fig. 4 Screening of the optimal TLC system for Monacolin K detection

(1) Samples on all of 3 plates were the same: 1# 500 ng LFMK plus 500 ng AFMK, 2# 20 ng LFMK, 3# 30 ng LFMK, 4# 50 ng LFMK. In each plate, the upper arrow pointing to LFMK, and the lower arrow pointing to AFMK.

(2) Plate A: Silica Gel 60, F254, 0.5 mm, Merck, Germany, Rf = 0.18 and 0.00; Plate B: Silica Gel 60, F254, 0.25 mm, aluminum sheet, Merck, Germany, Rf = 0.18 and 0.02; Plate C: Silica Gel 60, RP - 18 F254, 0.5 mm, chemically modified layer, Merck, Germany, Rf = 0.62 and 0.54.

The low detection limit for AFMK was also estimated, and the result was shown in Fig. 5. The low detection limit was also around 30 ng. It was quite close to the low detection limit of HPLC/UV detection method, around 10～20 ng. We supposed that such lower detection limit of this TLC system perhaps were also suitable for the other Moncolin K analogs like Monacolin J, L, X, M and so on, since they share the basic common chemical structure [13].

Fig. 5 Sensitivity estimation of the screened TLC system for AFMK

AFMK contents of spots 1, 2, 3, 4, 5, 6, 7, 8 were 300, 150, 100, 50, 30, 20, 15, 10 ng, respectively.

In this experiment we found that after developing TLC plates of the kind F254 even in different mobile phases, sprayed with 30 % H_2SO_4 ethanol then heated on a hot plate or in an oven of 80～100 ℃, when observed under 365 nm, the bright yellow spots of both LFMK and AFMK would appeared.

2.4 Validity of the Screened TLC Method

Considering a lot of other compounds in red rice, in order to confirm whether this yellow fluorescence was specific for Monacolin K or not, two functional red rice samples of FHQF and Xuezhikang and one pigmental red rice of HQF as well as standard Monacolin K were applied to the screened TLC system. After developed, the TLC was also observed step by step. The results were shown in Fig. 6.

It was found that this novel TLC method could greatly improve the resolution and sensitivity for Monacolin K detection. Lots of other compounds in red rice samples especially in pigmental red rice had fluorescence when observed under 365 nm before spraying charring reagent (refer to Fig. 6 C). After spraying

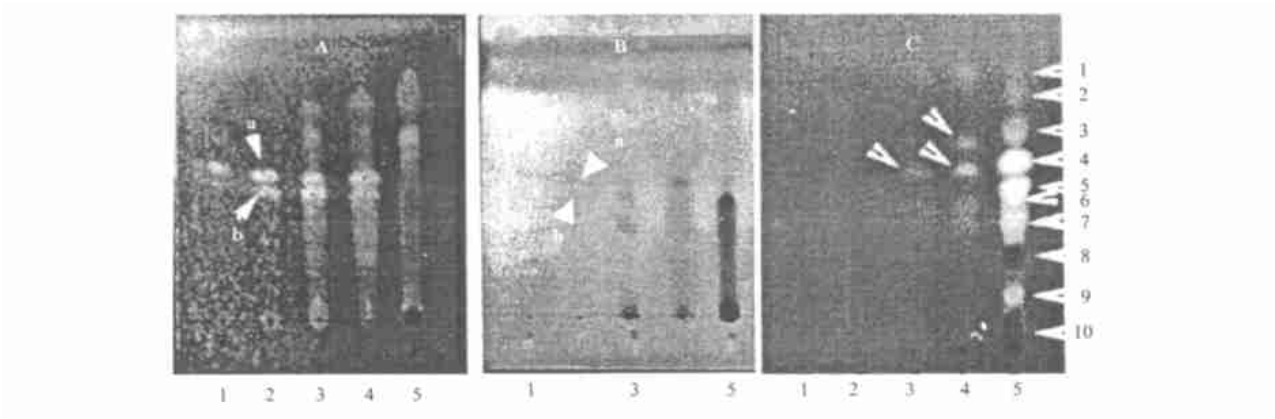

Fig. 6 Validity of this novel TLC method estimated with red rice samples

(1) A, B, C was from same one developed TLC plate but observed under different conditions. Samples of 1,2,3,4,5: were 300 ng LFMK, 500 ng AFMK plus 500 ng LFMK; red rice powder FHQF (4 μL of 5 times dilute); Xuezhi Kang (4 μL of 5 times dilute); red rice powder HQF (4 μL spotting from 5 X dilute), respectively. In pattern A and B, arrow "a" pointed to LFMK; and arrow "b" pointed to AFMK. In photo C, all the arrows pointed to non-Monacolin K compounds.

(2) A: observed at 365 nm after spraying 30 % H_2SO_4 ethanol and heating; B: observed under day light, after spraying 30 % H_2SO_4 ethanol and heating; C: directly observed at 365 nm before spraying 30 % H_2SO_4 ethanol, indicating many other compounds in red rice has fluorescence.

the charring reagent and heating, when observed under daylight as done in most reported TLC methods, there were many gray spots in both functional and pigmental rice. Therefore even with the standard Monacolin K as control, it was difficult to judge that in pigmental rice there was no Monacolin K. Furthermore, the standards spots of 500 ng Monacolin K were very weak too (refer to Fig. 6 B). However, when observed under 365 nm after being heated, the bright yellow Monacolin K spots became very clear and the standard of 300 ng Monacolin K now could be seen clearly, and what is more important, other interfering spots were not the problem any more (refer to Fig. 6 A).

When this method is used, attention has to be paid to the following two critical points. One is even spray of proper amount of 30 % H_2SO_4 ethanol. Too much H_2SO_4 will spoil the whole TLC plate, and too little will also decrease the detection sensitivity. The other is even heating on a hot plate, keeping a close eye to the spots color changing with the help of hand UV light while heating. At first, about 7～10 (depending on different fermented red rice) colorful and obvious spots of other components turned more and more faint while the spots of both LFMK and AFMK became more and more clear with the color changing from none to white to blue and then to yellow, under 365 nm. This color changing just met our needs: interfering component spots faded away and the target components spots turned strong.

In conclusion, the developed TLC system greatly improved the resolution of Monacolin K in red rice and for the first time allowed the simultaneous detection of both LFMK and AFMK. The low detection limit was brought down to 30 ng, which was quite near to that of RP-HPLC method (10～20 ng)[10]. Owing to the simplicity and rapidity of TLC, this method would be very useful monitoring Monacolin K production during the fermentation process or estimation of the Monacolin K content in red rice samples.

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薄层色谱法同步检测酸式和内酯式莫奈呵啉 K

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摘 要: 通过实验筛选出了适于特异性的同步检测酸式和内酯式莫奈呵啉 K的薄层色谱法(TLC). 最佳薄层色谱条件为: 薄层板采用德国 Merck 公司的化学修饰薄层板 Silica Gel 60, RP - 18 F254, 0.5 mm; 展开剂系统采用 V(氯仿) V(甲醇) V(氨水) = 25 3 1; 炭化试剂采用 30 %(体积分数) 的硫酸乙醇溶液; 紫外检测波长为 365 nm. 该法实现了酸式和内脂式莫奈呵啉 K的检测特异性与同步性, 并将检测灵敏度提高到绝对检测量 30 ng. 该法适用于任何含莫奈呵啉 K的样品的检测, 特别适用于大批量经常性的定性或半定量莫奈呵啉 K的检测.

关键词: 薄层色谱; 内酯式莫奈呵啉 K; 酸式莫奈呵啉 K

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