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Dissipation of Oxytetracycline Residue in Royal Jelly[1]

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Abstract : Oxytetracycline (OTC) in royal jelly can be extracted by organic solvents and Amberlite XAD-2 resine column chromotography, and the average of OTC recovery was 64.86±6.14%. The cylinder-plate method is generally used for the assay of OTC residue with *Bacillus cereus* var. *mycoides* (ATCC 11778). Different dosages of OTC were applied to honeybee colonies to control foulbrood. The dissipation curves of OTC residue were achieved by periodically detecting samples of royal jelly. The curve showed that the OTC residue reduced to about 10% of initial residual level within 13 days (4 samplings). After then, the OTC residue dissipated slowly to undetectable level. For hives received 200 ppm (0.125 g) OTC twice, 100 ppm (0.063 g) twice and 50 ppm (0.031 g) once, the periods needed for residual dissiption to lower than 0.05 ppm were 45, 35 and 24 days, respectively.

Key Words : Oxytetracycline, Royal jelly, Residual dissipation.

Introduction

The royal jelly contains 66.05% water, 12.34% proteins, 5.46% lipids, 12.49% sugars, 0.82% dust and 2.84% unknown compounds (Melampy and Jones, 1939). The major lipid component, 10-hydroxy-2-decenoic acid (10-HDA), which easily dissolves in ethyl ether, has been proved as a natural antibiotic in royal jelly (Blum et al., 1959; Dixon and Shuel, 1978; McCleskey and Melampy, 1939). 10-HDA is also the major interference factor in the bioassay of oxytetracycline (OTC) residue.

OTC inhibits protein synthesis in bacteria (Suarez and Nathans, 1965). The bee keepers usually apply this compound on bee hive to prevent the infection of American and European foulbroods, however, this control method always results in antibiotic residue in bee products.

Since OTC is highly soluble in water but not in ethyl ether (Anonymous, 1968), it is easy to separate OTC and 10-HDA into different phases. The cylinder-plate method (Grove and Randall, 1969) is commonly used for the assay of antibiotic residue in food (Anonymous, 1980). To monitor OTC residue periodically and to find out its dissipation patterns in royal jelly, it is necessary to set up a standard bioassay method to analyze the amount of OTC in royal jelly.

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Materials and Methods

Sample preparation

The honey bees, *Apis mellifera* L. were reared at the apiary in Taipei to produce royal jelly.

Three dosages of OTC at 0.5, 1.0 and 2.0g were dissolved in 10 liter of saturated sugar solution (1 kg sugar/1 liter water) and the concentration were 50, 100, 200 ppm, respectively. Each cocktail was then divided into sixteen equal portions for hive treatment, therefore, the dosages of OTC per hive were 0.031g, 0.063g, 0.125 g, respectively. For each treatment, royal jelly randomly collected from 5 hives with the same treatment were mixed as a sample. Three replications were conducted for each dose treatment.

Those hives treated with 50 ppm of OTC once served as the protective application. Two other doses were applied twice, and the second application was carried out on the third day, right after the first sampling. The royal jelly from treated hives were collected twice a week. The samples collected at the 3rd, 6th, 13th, 24th, 31st, 38th and 45th day after the first application were subjected for OTC residue assay.

Extraction and clean-up of OTC residue from royal jelly

The analytical procedures for tetracyclines were listed in scheme 1 (Chung, 1985, personal communication). Ten grams of royal jelly was suspended and centrifuged (1500 × g) with two subsequent volumes (50 ml and 40 ml) of 2% trichloroacetic acid solution. Half of the combined supernatant was transferred into separatory funnel and extracted with 100 ml of ethyl ether first, then 100 ml of n-hexane was added. The pH of water layer was adjusted to 5.0 before loading on XAD-2 column. The sample was eluted at the speed of 36 ml/hr, and 100 ml of distilled water was then used to elute the contaminates at the speed of 60 ml/hr. Finally, 100 ml of methanol was applied at the speed of 60 ml/hr to wash out OTC.

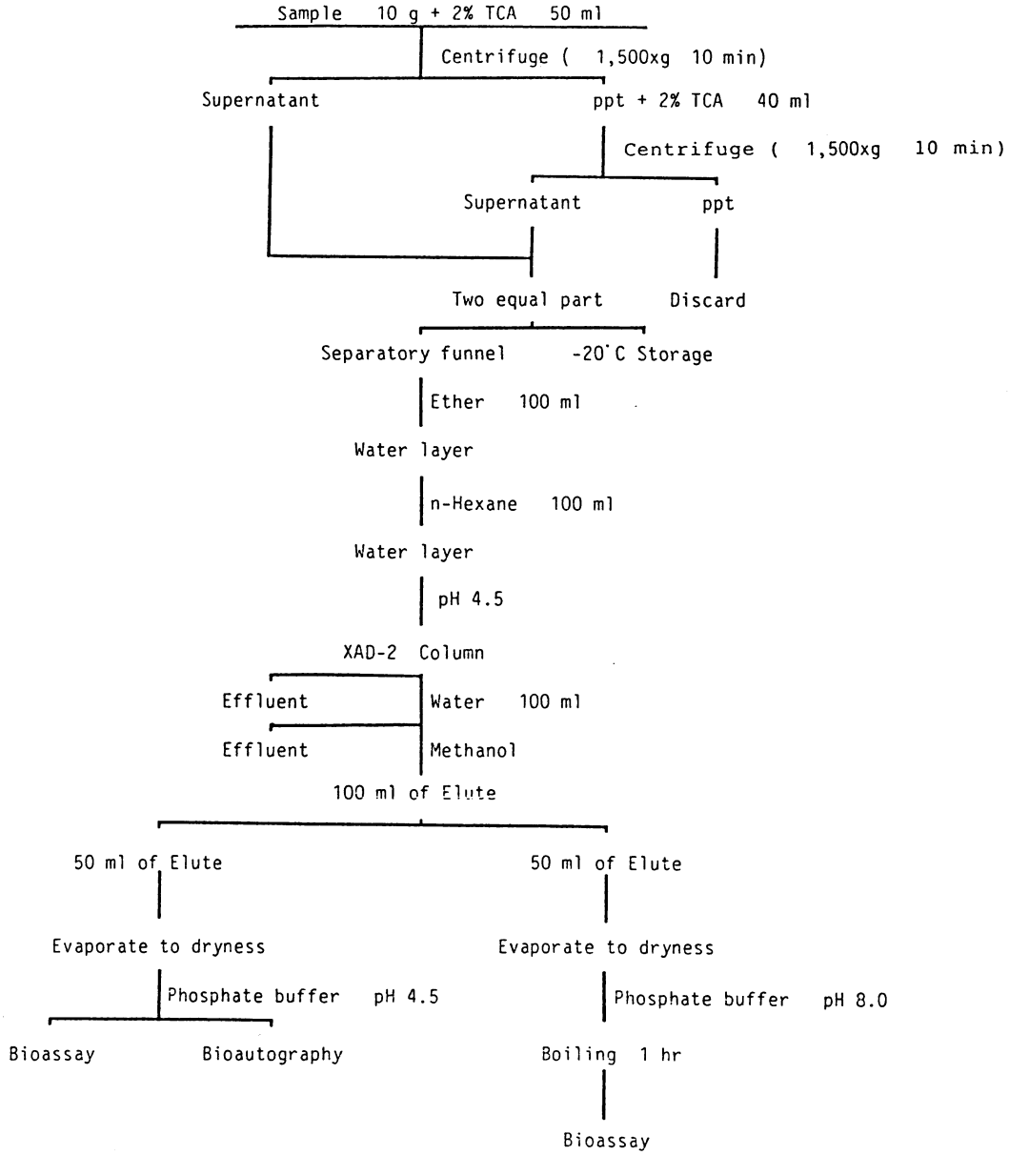
The methanol elution was divided into two equal portions and were concentrated into dryness with rotary evaporator. One portion of the elute was resuspended with 0.1 M phosphate buffer (pH 8.0) to the initial volume (1.8 ml) of the sample. The solution was heated on 100°C water bath for one hour to breakdown OTC and served as the check to identify other interference. The other portion was resuspended by 0.1 M phosphate buffer (pH 4.5) and used for microbioautography and cylinder-plate assay.

Preparation of OTC standard solution

The OTC standard solution (1000 ppm) was prepared by dissolving 52.8 mg OTC (94.7 % W/W) purchased from Sigma Co. in 50 ml of 0.01 N HCl solution and was divided into fifty equal portions and then stored at −10°C. The solution for testing calibration curve was diluted from this standard solution.

Mass rearing of tested organism

Bacillus cereus var. *mycoides* (BCM, ATCC 11778) was adopted as the testing organism. Colonies were periodically transferred to new slant medium (DIFCO antibiotic medium # 1) to maintain the strain.



Scheme 1. Analytical procedures for detecting oxytetracycline residue in royal jelly

For mass production, colonies of BCM were washed from slant medium by sterilized saline solution. The solution was transferred onto a layer of DIFCO antibiotoc medium # 1 in a 1 liter flask, and was cultured on 29°± 1 °C for 14 days for sporulation. The sporulated BCM was washed out with sterilized saline solution. The mixture was repeatedly treated by heat shock, 65°C for 30 min, then 1500 x g centrifugation, till the supernatant was clear. The precipitate was resuspended with saline solution for assaying.

Assay of OTC residue

A method modified from cylinder-plate method (Grove and Randall, 1969) was adopted for assaying OTC residue, and DIFCO antibiotic medium # 8 was used to culture BCM. The test plates were prepared by pouring 5 ml basal layer medium and 5 ml seed layer medium containing BCM on a 90 mm petri dish sequentially. The steel cylinders, 6 mm inner diameter × 10 mm height, were vertically placed on the test plate, and were used as the sample holder. For the assay of OTC standard curve, five steel cylinders were placed onto each of the five test plates, then, the cylinders were applied with 0.28 ml of 0.05, 0.1, 0.2, 0.4, 0.8 ppm OTC test solutions, respectively, and cultured at 29°±1°C for 16—18 hr. The diameter of inhibition rings were measured. The standard curve was obtained by calculating these data with linear regression. Recovery of OTC can be determined with known amount of OTC in royal jelly by passing through the same extraction and clean-up procedures.

For the assay of samples, five plates each contained eight cylinders were prepared. Among these 8 cyliners, 3 of them were treated with the 3 sample solutions, another 3 of cylinders were pipetted with the same sample solutions pre-heated in 100°C water-bath. Blank buffer was added into one of the remaining cylinders, while 0.1 ppm OTC standard solution was pipetted into the other one. The interpolation method was used to propose the amount of OTC residue in samples. If the inhibition ring of heat-treated sample was smaller than that of 0.05 ppm OTC standard, it was considered to be free from contamination, and the result was acceptable. Whenever the sample inhibition ring is larger than that of 0.8 ppm OTC standard solution, it will be resampled and tested with diluted sample solution, which should fall into 0.05—0.8 ppm OTC standard range.

Bioautography

The samples were spotted on chromatographic paper (Whatman # 1 , 160 x 200 mm), and developed with mixtures of n-butanol, acetic acid and H_2O (4 : 1 : 5). The developing height was 150 mm. After air-dried, the paper was then pressed against a aluminum plate (250 × 170 × 30 mm) of the DIFCO antibiotic medium # 8 containing BCM for 30 minutes. The plate medium was then cultured at 29°± 1 °C for 16—18 hr. The Rf value of inhibitory zones for the OTC identification and the presence of interferences were also checked.

Results and Discussions

The Rf value of OTC residue extracted from royal jelly by paper chromatography

was 0.42, while that of the natural antibiotic (10−HDA) in royal jelly was 0.88. These compounds were easily separated and detected by bioautography. The results of bioautography and heat-treated samples demonstrated that the methods used for OTC extraction and clean-up were efficient in separating OTC from the natural antibiotic in royal jelly.

The OTC standard curve of known dose (Line I) and the extracted amount of OTC spotted into royal jelly (Line II) were presented in Figure 1. The percentage of recovery was calculated and served as a correction constant. The average recovery of OTC is 64.86 ±6.14%.

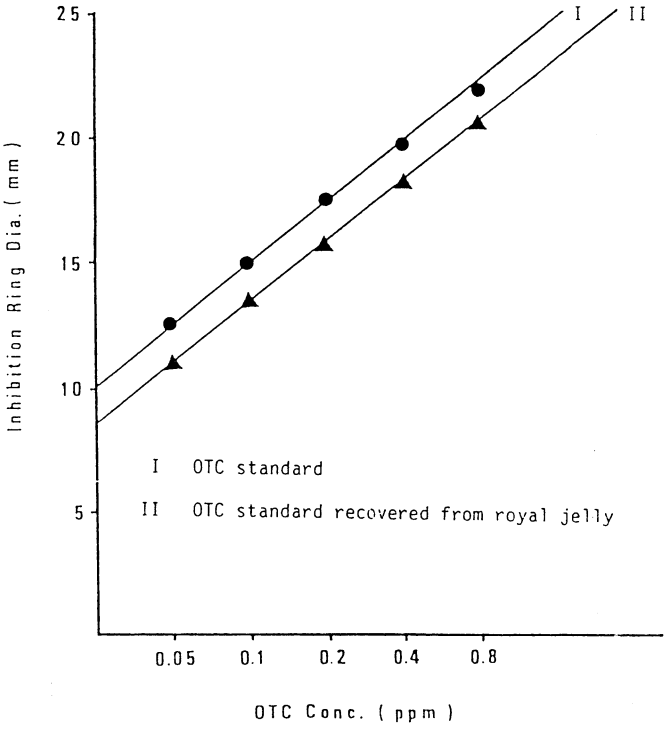


Fig 1. Comparison of OTC standard and its recovery from royal jelly.

Fig. 2 showed the dissipation patterns of OTC residue in royal jelly. For hives received 200 ppm OTC twice, the residue in royal jelly became undetectable or below 0.05 ppm after 45 days. It took 35 days for those hives applied with 100 ppm OTC twice to degrade to undetectable concentration. Twenty-four days were needed for those hives treated by 50 ppm OTC once to dissipate to undetectable level.

The persistence of OTC residue was closely related to the dosage applied to the hive. Besides, the OTC residue was much higher at the first sampling after the OTC was applied. After then, the residue reduced sharply to a comparatively low level and dissipated slowly to undetectable concentration. The high level of OTC residue in royal jelly might directly derive from sugar solution, while the slow dissipation of OTC residue might due to the contamination of OTC which was persisted in the hive.

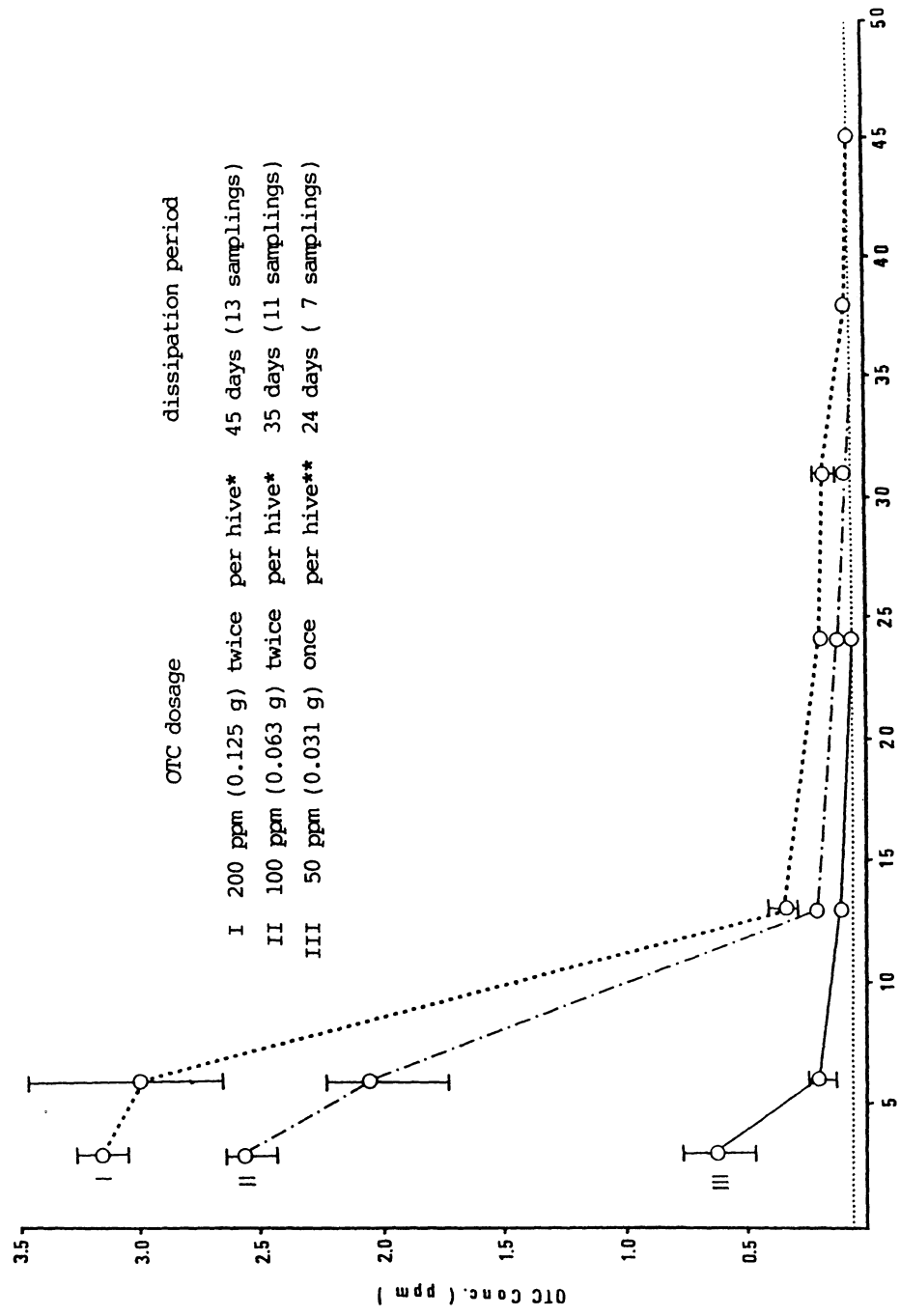


Fig 2. Decay rates of OTC residue in royal jelly
* Two applications at the 1st and 3rd day, respectively
** One application at the 1st day

Conclusions

The OTC and natural antibiotics in royal jelly can be separated due to difference of solubility in ethyl ether and water, which makes the OTC residue assay possible. By using bioautography and heat treatment, the 10-HDA contaminated sample can be easily detected. The average OTC recovery was 64.86 ±6.14%. Since the standard deviation was less than 10%, it was suggested that the procedures for OTC purification in this study were reliable.

Concentration of OTC in royal jelly reached the highest level soon after the treatments, which might directly derive from sugar solution. While the low level and slow dissipation of OTC might due to contamination of OTC that persisted in the hive. The dissipations of OTC residue were correlated to the amount of OTC that applied in hives. For hives received 200 ppm (0.125 g), 100 ppm (0.063 g) and 50 ppm (0.31 g) OTC, the periods needed for residues dissipated to undetectable level were 45, 35 and 24 days, respectively.

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蜂王乳內抗生素殘量消退之測定[1]

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摘 要

蜂王乳內殘留之羥四環素（Oxytetracycline, 簡稱 OTC），其純化可經由乙醚的萃取，Amberlite XAD－2 之層析，再經濃縮而得。本實驗結果顯示，蜂王乳內 OTC 之平均回收率爲64.86±6.14%。其檢定，則用敏感之細菌品系，*Bicllus cereus* var. *mycoides*（ATCC 11778），以圓筒平板法（Cylinder-plate method），依所產生抑制環大小作殘量檢定。

飼育之蜂群（*Apis mellifera* L.）經分組後，分別施用不同劑量的 OTC 後，定期抽樣檢驗其蜂王乳內 OTC 殘量而得其消退曲線。曲線中顯示，在前13天內，OTC 的殘量迅速降到初次測試殘量的10%左右，此後，則以平穩而緩慢的方式消退。此消退資料可作爲訂定蜂王乳安全採收期的參考依據。

關鍵詞：羥四環素、蜂王乳、殘量消退。

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