Systematic Analysis of Antibiotics via Agar Gel Electrophoresis and Antimicrobial Spectrum-Candidacy for Detecting Residual Antibiotics in Foods

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ABSTRACT

Systematic analysis of antibiotics via agar gel electrophoresis and antimicrobial spectrum, a method previously developed in our laboratory, was modified to increase its resolution power and sensitivity. Six test organisms were employed in the analysis system so that antibiotics of different chemical groups can be distinguished from each other. Optimal conditions of the height of agar gel and test organism strip, and the width of sample slit were determined and modifications were made which enable the analysis of antibiotics at the concentration levels of food residues. Other features of the analysis system such as feasibility for handling large number of samples in a single procedure and reproducibility were together discussed so as to evaluate its candidacy for detecting residual antibiotics in foods.

Introduction

Antibiotic residues may occur in foods in any of the following ways: foods fermented with antibiotic producing microbes; antibiotics artificially added for the purpose of food preservation; antibiotic supplements in feeds; and antibiotics employed as veterinary medications. These residual antibiotics may cause problems including individual antibiotic toxicity, hypersensitivity, and, probably most serious of all, the hazard of increasing antibiotic resistant strains which may lead to impairment of antibiotic chemotherapy[13]. Therefore, the level of antibiotic residues in foods has been regulated in most countries. To cite as examples, the Joint FAO/WHO Committee on Food Additives recommended the limitation of residual antibiotics to within detectable levels[13]. In our country, antibiotics are not allowed to reside in raw milk[3].

An analytical method qualitatively and quantitatively efficient for detecting the unknown and trace amount of antibiotics in foods is a prerequisite for the control measures. Today such an analytical method is still not available. Thus neither the triphenyltetraazonium chloride test method[1, 11] nor the official plate methods employing Bacillus subtilis or Sarcina lutea as test organism[5, 12] is qualitatively able to distinguish even the antibiotics from other antimicrobial agents. Systematic analysis using paper chromatography[8], thin layer chromatography[7], or electrophoresis[14] is difficult, if not impossible, to be employed. To illustrate the extent of difficulties, Bossuyt *et al.* recently reported a modified method of thin layer chromatography for the detection of 14 antibiotics in milk. For each sample they had to use 6 chromatograms, 3 absorbents, 4 extraction solvents and procedures, 5 developing solvent systems, and 4 test organisms[9]. To adopt such a method for the purpose of general survey of commercial foods is evidently beyond any laboratory capacity. Thus the extent of the residual antibiotics problem has not been truely evaluated yet.

Efforts have been made in our laboratory to meet such a methodological demanding. A method of systematic analysis of antibiotics via agar gel electrophoresis and antimicrobial spectrum (AGE/AS method) was developed[10]. Essentially, the method takes into consideration both the physical-chemical properties and the biological characteristics of the chemically heterogeneous antibiotics to be analyzed. After electrophoretic separation of each sample as a band on agar gel, strips of different test organisms are placed on the agar gel so that inhibi-

tion zones of different size and position characteristic of each antibiotic appear after incubation. From the electrophoresis mobility and antimicrobial spectrum an unknown sample can be compared with those of standard antibiotics and thus possibly identified. This report presents some technical improvements of the method with respect to its resolution power, reproducibility, and sensitivity, so as to assess its candidacy for detecting residual antibiotics in foods.

Materials and Methods

Detailed procedure of the AGE/AS method has been previously reported[10]. Essential features and modifications are described as follows:

Antibiotics

In addition to those antibiotics previous reported, 4 more antibiotics were included in this study. Bacitracin stock solution of 100 U/ml was prepared with 1% phosphate buffer, pH 6.0 (1% PB 6.0), and used within 7 days. Chlortetracycline stock solution of 1 mg/ml was prepared with 0.01 N HCl and used within 4 days. Dihydrostreptomycin stock solution of 1 mg/ml was prepared with 0.1 M phosphate buffer, pH 8.0 (0.1 M PB 8.0), and used within 1 month. Tylosin stock solution was prepared by dissolving each 10 mg of the antibiotic with 1 ml of methanol and further diluted with 0.1 M PB 8.0 to 1 mg/ml and used within 7 days. All of the antibiotics employed were working standards routinely used in our laboratory for official assays. The stock solutions were kept at 4°C. For daily preparation of final dilutions, 0.05 M phosphate buffer, pH 7.0 (0.05 M PB 7.0), was employed.

Preparation of Agar Gel and Electrophoresis

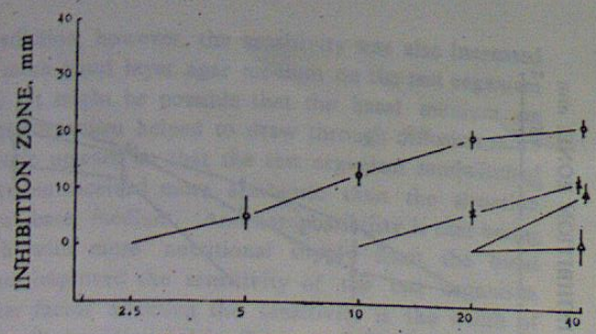
Glass plate regularly used in thin layer chromatography, with dimensions $20 \times 20 \times 0.5$ cm, was employed on which agar gel was prepared. A polyacrylamide frame with the above mentioned outside dimensions was sealed onto the glass plate with a small amount of buffered agar solution. A slits maker for producing 16 slits in two rows in agar gel layer was placed on the glass plate. Buffered agar solution, prepared with various concentrations of agar in 0.05 M PB 7.0, was poured on the glass plate at a temperature of about 60°C. After solidification of the agar layer, the slit maker and frame were removed. Samples of antibiotic solution and a 0.5% trypan blue solution, employed as indicator of electrophoresis mobility, were applied into the slits. Two agar gel plates can be placed in adjacent positions on a cooling plate of electrophoresis chamber. Filter papers or sponges served as electrolyte bridge between the agar gel plates and the 0.05M PB 7.0 in the electrolyte chambers. The agar gel plates were covered with another cooling plate. Electrophoresis was carried out at 20°C, with voltage maintained at 400 volts. Trypan blue served as a visible indicator so that the electrophoresis can be terminated in 30 minutes to 50 minutes when the indicator moved about 3 cm toward the anode.

Bioautography

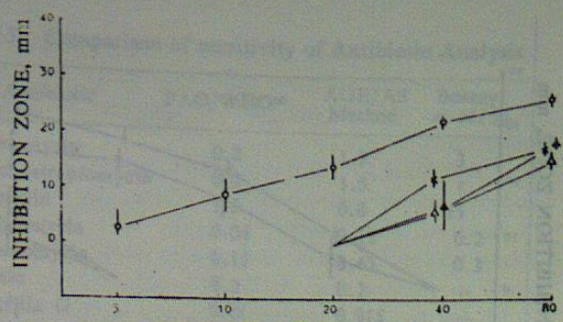
Double-layer agar medium was prepared in petri dishes with test organism inoculated in seed layer. The congealed agar medium was cut into agar strips and inversely placed on each side of and perpendicular to the sample slits. Six rows of test organism strips can be placed side by side for each sample slit. The agar plates were then put into a moisture chamber and incubated at 34°C overnight. A photograph of the agar plate with exhibited inhibition zones of various antibiotics was presented in the previous report[10]. From the position and size of inhibition zones in the test organism strips the electrophoresis mobility can be calculated and the antimicrobial spectrum of different test organisms toward each sample recorded. In addition to 4 test organisms previously employed, Sarcina lutea ATCC 9341 and Micrococcus flavus ATCC 10240 were also used in this study. Triphenyltetraazonium chloride, previously incorporated in seed layer to favor the detection of inhibition zone, was eliminated because of incompatibility with the additional test organisms.

Results and Discussion

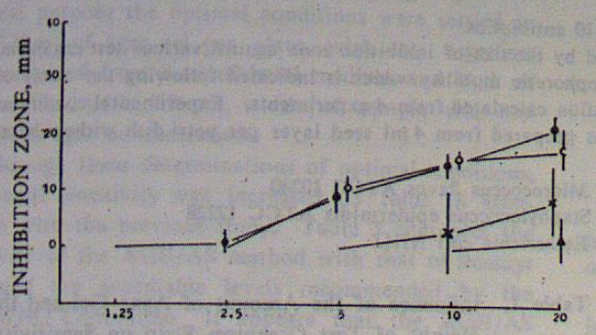
In the previous study, 15 antibiotics were tested to see if they could be distinguished from each other via electrophoresis mobility and antimicrobial spectrum in a single procedure. The antibiotics studied include: ampicillin, chloramphenicol, cloxacillin, erythromycin, gentamycin, neomycin, novobiocin, oleandomycin, oxytetracycline, penicillin G, polymyxin B, streptomycin, and tetracycline. The result indicated that while most of the antibiotics can be distinguished either with electrophoresis mobility or antimicrobial spectrum, chlortetracycline and tetracycline as a group and kanamycin and streptomycin as a group exhibit similar electrophoresis mobility and antimicrobial spectrum. In order to increase the resolution power, two more test organisms were employed so that 6 test organisms can be tested for each antibiotic's antimicrobial spectrum. Additional antibiotics, oxytetracycline and dihydrostreptomycin, were included in this study to see if antibiotics of similar chemical properties and biological origins can be resolved. Bacitracin and tylosin were also included because of their important status as residual antibiotics. In order to increase sensitivity, the analytical system was modified to have 90 ml of agar gel per glass plate instead of the previous 60 ml; the height of the test organism strip was reduced to 4 ml seed layer per petri dish without base layer; and the width of sample slits doubled to 2.8 mm. Agar gel concentration was stayed to 1.5% of the previous study. Fig. 1 summarized the results of 4 trials for each of 10 antibiotics studied. It is evident that resolution power



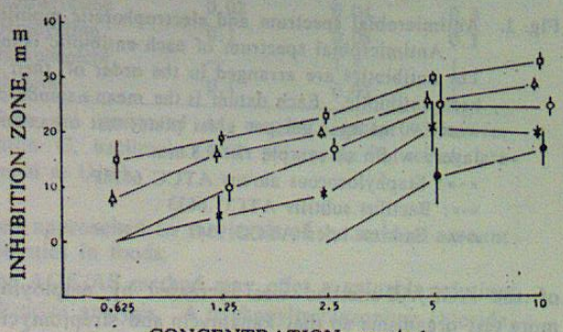
(a) Streptomycin −25.1±1.3 mm



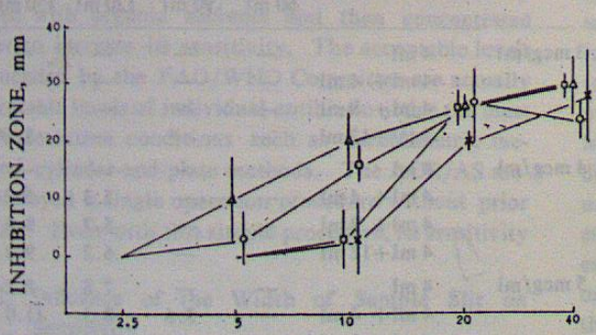
(b) Dihydrostreptomycin −24.2±0.94 mm



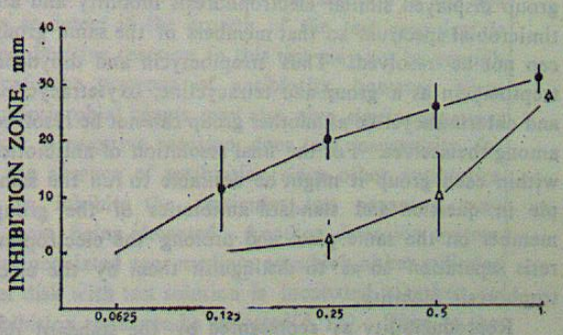
(c) Kanamycin −20.5±0.29 mm



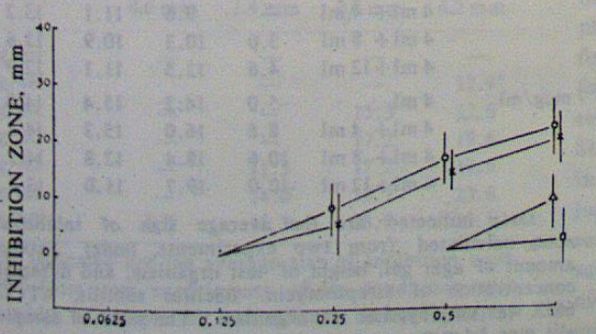
(d) Tylosin −18.2±0.51 mm



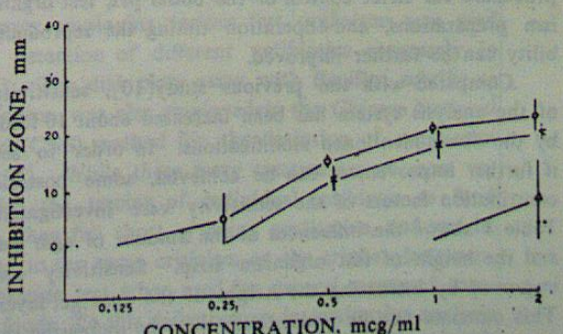
(e) Chloramphenicol −10.2±0.26 mm



(f) Bacitracin −9.5±1.17 mm



(g) Tetracycline −8.4±0.75 mm



(h) Oxytetracycline −7.5±1.67

Fig. 1

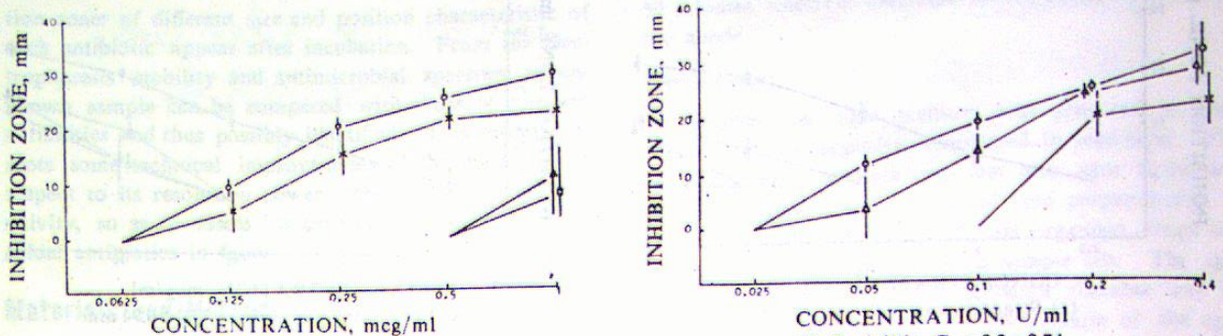


Fig. 1. Antimicrobial spectrum and electrophoretic mobility of 10 antibiotics.

Antimicrobial spectrum of each entibiotic is indicated by the size of inhibition zone against various test organism. The antibiotics are arranged in the order of their electrophoretic mobility which is indicated following the name of each antibiotic. Each datum is the mean±standard deviation calculated from 4 experiments. Experimental conditions were: 90 ml agar gel per glass plate; test organism strips prepared from 4 ml seed layer per petri dish without basal layer; width of sample slit 2.8 mm.

×-×: Staphylococcus aureus ATCC 6538P
□-□: Micrococcus flavus ATCC 10240
○-○: Bacillus subtilis ATCC 6633
●-●: Staphylococcus epidermidis ATCC 12228
△-△: Sarcina lutea ATCC 9341
▲-▲: Escherichia coli NIHJ

of the AGE/AS method was increased by employing more test organisms so that kanamycin and streptomycin can now be distinguished by the antimicrobial spectrum. However, antibiotics belonging to a particular chemical group displayed similar electrophoresis mobility and antimicrobial spectrum so that members of the same group can not be resolved. Thus streptomycin and dihydrostreptomycin as a group and tetracycline, oxytetracycline and chlortetracycline as another group can not be resolved among themselves. For the final resolution of antibiotics within each group it might be desirable to run the sample in question and standard antibiotics of the group member on the same plate and prolong the electrophoresis separation so as to distinguish them by the electrophoresis mobility.

Reproducibility as represented by the standard deviation indicated in Fig. 1 seems to be generally satisfactory. It is expected that by adopting a standard procedure for strict control of the buffer pH, test organism preparations, and operation timing the reproducibility can be further improved.

Compared with the previous study[10], sensitivity of the analysis system has been increased about 10 folds by the above-mentioned modifications. In order to see if further improvement can be achieved, some possible contribution factors of the sensitivity were investigated. Table 1 shows the influences of the amount of agar gel and the height of test organism strip. Sensitivity was improved by increasing the height of the agar gel layer. This outcome can be understood, since by increasing the height of the agar gel layer the amount of sample applied in the sample slit was also proportionally increased, and thus larger quantity of antibiotic became available for the upward diffusion to the test organism strips. Contrary

Table 1. Influence of the Amount of Agar Gel and the Height of Test Organism Strip on Sensitivity

Ant. Conc.[a]	T. O. Strip[b]	Agar Gel[c] 60 ml	90 ml	120 ml	150 ml
3 mcg/ml	4 ml	—	—	—	—
	4 ml+ 4 ml	—	—	—	—
	4 ml+ 8 ml	—	—	—	—
	4 ml+12 ml	—	—	—	1.6[d]
4 mcg/ml	4 ml	—	—	—	—
	4 ml+ 4 ml	—	—	5.3	5.7
	4 ml+ 8 ml	—	—	5.7	9.0
	4 ml+12 ml	—	—	6.2	9.0
5 mcg/ml	4 ml	—	—	7.8	8.2
	4 ml+ 4 ml	—	3.6	8.3	11.0
	4 ml+ 8 ml	—	4.1	9.3	12.7
	4 ml+12 ml	4.2	3.8	8.5	13.0
6 mcg/ml	4 ml	—	5.0	9.8	11.1
	4 ml+ 4 ml	—	9.6	11.1	13.2
	4 ml+ 8 ml	3.0	10.3	10.9	13.6
	4 ml+12 ml	4.6	12.3	11.1	12.9
7 mcg/ml	4 ml	5.0	14.2	15.4	14.0
	4 ml+ 4 ml	8.8	16.0	15.3	14.5
	4 ml+ 8 ml	10.6	18.4	12.8	14.2
	4 ml+12 ml	10.0	19.7	11.0	13.2

Data indicated are the average size of inhibition zone, calculated from two experiments, under various amount of agar gel, height of test organism, and different concentration of streptomycin. Bacillus subtilis, ATCC 6633, was employed as test organism. The width of sample slit was 1.4 mm.

[a] Concentration of streptomycin.
[b] Test organism strips prepared from the indicated amount of seed layer+basal per petri dish.
[c] Amount of agar gel per glass plate.
[d] Size of inhibition zone given in mm.

to prediction, however, the sensitivity was also increased with more basal layer agar medium on the test organism strips. It might be possible that the basal medium on the test organism helped to draw through diffusion more antibiotic upward so that the test organism sandwitched in between received more antibiotic than the situation without basal medium. Another possibility is that better growth with more nutritional supply from the basal medium improved the sensitivity of the test organism. Another factor affecting the sensitivity is the width of sample slit, as presented in Table 2. Concentration of agar in the agar gel layer, in the range from 1% to 3%, did not seem to affect sensitivity significantly. For practical purpose the optimal conditions were settled to have 120 ml of agar gel per plate, with test organism strips prepared from 8 ml of basal medium and 4 ml of seed medium, with 2.8 mm width of sample slit, and with 1.5% agar concentration.

Through these determinations of optimal conditions, the overall sensitivity was increased 33 folds in comparison with the previous study. Table 3 compares the sensitivity of the AGE/AS method with that of Bossuyt *et al.* and the acceptable levels recommended by the FAO/WHO Committee. It appears that the AGE/AS method has reached a sensitivity in between them. It should be understood, however, that in the system employed by Bossuyt *et al.*, each sample has to be separately extracted with organic solvents and then concentrated in order to increase its sensitivity. The acceptable levels recommended by the FAO/WHO Committee are actually the detectable levels of individual antibiotic under the most sensitive detection conditions such as turbidimetric methods and cylinder-and-plate methods. The AGE/AS method employed a single operation procedure without prior extraction. Even with this simple procedure, its sensitivity has been approached to the levels of the residual amount of antibiotics in foods.

Table 2. Influence of the Width of Sample Slit on Sensitivity

Antibiotic Conc.	Sample Slit Width			
	0.6 mm	1.4 mm	2.8 mm	4.2 mm
0.5 mcg/ml	—	—	—	—
1.0 mcg/ml	—	—	—	12.9[a]
1.5 mcg/ml	—	—	15.3	12.0
2.0 mcg/ml	—	—	17.2	19.6
3.0 mcg/ml	—	11.3	17.2	19.6
4.0 mcg/ml	—	14.0	18.7	23.6
6.0 mcg/ml	6.0	16.3	23.0	25.1

Data indicated are the average size of inhibition zone, calculated from two experiments, under conditions with various width of sample slit and different concentration of streptomycin. Bacillus subtilis ATCC 6633 was employed as test organism. Agar gel plates were prepared with 120 ml per plate. Test organism strips were prepared from 8 ml basal layer and 4 ml seed layer per petri dish.
[a] Size of inhibition zone given in mm.

Table 3. Comparison of sensitivity of Antibiotic Analysis

Antibiotic	FAO/WHO[10]	AGE/AS Method	Bossuyt *et al.*[5]
Streptomycin	0.2	1.5	3
Dihydrostreptomycin	0.2	1.5	1
Neomycin	1.5	0.6	15
Erythromycin	0.04	0.12	0.2
Oleandomycin	0.15	0.45	0.3
Tylosin	0.2	0.2	—
Penicillin G	0.01	0.015	—
Bacitracin	0.7	0.04	0.128
Polymyxin B	2	1.2	—
Tetracycline	0.1	0.08	0.3
Chlortetracycline	0.02	0.04	0.3
Oxytetracycline	0.1	0.08	0.3
Chloramphenicol	—	1.5	1
Novobiocin	0.1	0.03	1

Data are presented as mcg/ml, except those for penicillin G, bacitracin, and polymyxin B which are presented as U/ml.

The AGE/AS method may offer a valuable solution in qualitative analysis. A triphenyltetraazonium chloride test has been employed for the detection of antibiotics in milk[11]. In this test system test organism is inoculated into milk and incubated prior to the addition of the dye solution to detect if the growth of the test organism is inhibited. Positive reaction in this test may not be enough to indicate the presence of antibiotics, however, since other antimicrobial agents may also give similar reaction. Moreover, a negative reaction does not necessarily indicate the absence of antibiotics, since other antibiotics unable to inhibite the particular test organism may escape from being detected. Another detection system employs inoculated agar medium on which either cylinder or paper disk with test solution is incubated together so that inhibition zone can be detected[5, 12]. Thus in the A. O. A. C. Methods of Analysis a disk plate assay employing Bacillus subtilis as test organism was used for the detection of penicillin in milk[1], and a cylinder plate assay employing various test organisms was used for the detection of different antibiotics separately in feeds[6]. The disk plate assay with Bacillus subtilis as test organism was also employed in the Chinese National Standard (CNS) method for the detection of antibiotics in milk[4]. While these assay systems are of most valuable for the tracing of individual antibiotic as food residues, they fall short of being qualitative, and can be subjected to the same criticism as the triphenyltetraazonium chloride test when used for general survey of commercial foods. To this defectiveness in qualitative analysis of antibiotics the AGE/AS method could offer to amend.

In addition to its feasibility for qualitative analysis, the AGE/AS method is also semiquantitative in the sense of its providing the data of the size of inhibition zones

from which the amount of antibiotics present may be roughly estimated. Further determination of the exact quantity can then be easily carried out via the official potency assays.

From these overall features including the qualitative resolving power, semiquantitative estimation, feasibility for handling large number of samples in a single procedure, and sensitivity at the residual antibiotic levels in foods, it appears that AGE/AS method stands for a choice for detecting residual antibiotics in foods.

Seriousness of the problem concerning residual antibiotics in Taiwan area have been suggested by some research and survey activities. Thus Tsen *et al.* reported in 1974 that 17.12% of fresh milk in Taipei area showed positive reaction in triphenyltetraazonium chloride test[2]. Recent survey carried out in our laboratory, employing the CNS method, also indicated incidences as high as 30% in Taiwan area (unpublished data). While taking into consideration the defect inherited in these methods that these data may not be truly representative of the rate of residual antibiotics, a qualitative and quantitative survey system employing methods such as the AGE/AS technique is urgently needed for confirmation and for further tracing study.

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寒天凝膠電氣泳動及抗菌譜之抗生素系統分析法—用以檢驗食品中殘留抗生素之可能性

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摘　要

原由本實驗室發展之寒天凝膠電氣泳動及抗菌譜之抗生素系統分析法，經改良以增進其定性效果及敏感度，供試菌種增加爲6種後已可區分不同類之抗生素，寒天凝膠之厚度，供試菌種帶之厚度，及樣品縫之寬度等之最佳條件經定出而使本分析法之敏感度達到食品中抗生素殘留量之範圍，加上一次操作可處理多數量檢體之可行性及再顯性等條件一起評價，顯示利用本分析法以檢驗食品中殘留抗生素之可能性。