www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-1267-1272 ISSN: 2249-6645

Optimization of media composition for the production of gentamycin by Micromonospora echinospora MTCC 708 using Response Surface Methodology

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ABSTRACT

Response surface methodology(RSM) was applied to determine optimal medium composition for gentamycin production by an indigenous *Micromonospora echinospora* MTCC 708 with a view to reduce the number of experiments and obtaining the mutual interactions between the variables. A 2^4 full factorial central composite design was employed for experimental design and analysis of the results. Four independent variables, viz: concentrations of starch, soya bean meal, zinc sulfate and ammonium phosphate in the production media were tested.The optimum values of the four variables were starch 12.7252g/l, soya bean meal 10.593g/l, zinc sulfate 0.2714g/l and ammonium phosphate 5.4032g/l.The corresponding maximum concentration of gentamycin produced was 408.8844µg/ml. The goodness of the fitness of the model was checked by the determination coefficient R^2 which is 0.94421 and which indicates that 94.421% of the variability in the response could be explained by the model.

Keywords: **Optimization, gentamycin, Response Surface methodology, Micromonospora echinospora, Central Composite Design**

I. INTRODUCTION

Gentamycin is a new broad spectrum, basic, water soluble antibiotic produced by *M.echinospora, M.purpurea and M.rosarea* (K.S.Kim et.al 1990, A.AAbou-Zeid et.al 2002, Juchu etal 2004).It has a wide variety of uses: in the treatment of infections of blood, kidney and lungs (Sarre S.G and Mohn FE.N.Y 1967, Milanesi G. and Ciferri.O1996). The importance of gentamycin is mainly due to its action on pathogenic microorganisms like *Pseudomonas aerusinosa* which is resistant to other antibiotics. It is frequently used in burns, pneumonias, urinary, respiratory and gastrointestinal tract infections. It can also be used in bone, skin, tissues and joint infections caused by susceptible gram positive as well as to prevent fouling of soft contact lenses. The present work deals with the media optimization for the production of gentamycin by *M.echinospora* MTCC 708 using RSM.

RSM is a sequential procedure with an initial objective to lead the experiments rapidly and efficiently along a path of improvement towards the general vicinity of the optimum.It is appropriate when the optimal region for running the process has been identified. This method has been successfully applied to optimize alcoholic fermentation (Bandaru etal 2006), optimize vegetable oil bioconversion (Cheynier.V et.al 1983), biomass production (Moresi M. et.al 1980),α-amylase production (M .Saban et. al 2005) and neomycin production (K.Adinarayana et.al 2003). A detailed account of this technique has been outlined (Cochran.N.G and Cox.G.M 1968). Basically, this optimization process involves three major steps: performing the stastistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model.

Hence the authors report the application of the RSM using the Box-Wilson design (Box.,G.E.P and Wilson, K.B 1951) of experiments to develop a mathematical correlation among the starch, soya bean meal, zinc sulfate, ammonium phosphate and yield of gentamycin.

II. MATERIALS AND METHODS

Microorganism and cultivation:

M.echinospora MTCC 708 supplied by IMTECH Chandigarh, India was used through out the study for the production of gentamycin.

Growth media:

The above organism is maintained on the following ingredients (g/l): glucose 10; soluble starch 20; yeast extract 5; tryptone 5; $CaCO_3$ 1; agar agar 15 and distilled water 1000 ml, maintained at a pH of 7.6 and temperature 28^{o}C.

Inoculum and production media:

The production of gentamycin was carried out using inoculum media and production media. The ingredients of the inoculum media were (g/l): beef extract 3; tryptone 5; glucose 1; soluble starch 24; yeast extract 5; $CaCO_3$ 4 and distilled water 1000ml, maintained at a pH of 7.6 and temperature 30^{o}C. The production medium consists of dextrose 5g/l, soya bean meal 10g/l, corn steep liquor 5g/l, $CaCO_3$ 7g/l and distilled water 1000ml, maintained at a pH of 7.0 and temperature 30$_o$C.

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-1267-1272 ISSN: 2249-6645

Assay of gentamycin

At the end of the incubation period, the amount of gentamycins produced by *M.echinospora* 708 were biologically determined by E.coli as the assaying organism (Indian Pharmacopia 1996).

III. EXPERIMENTAL DESIGN AND OPTIMIZATION

Central composite experimental design (CCD) (Box.,G.E.P and Wilson, K.B 1951) was used in the optimization of gentamycin production. Starch(g/l), soya bean meal(g/l), zinc sulfate (g/l), ammomium phosphate(g/l) were chosen as the independent variables. Yield of gentamycin (Y, µg/ml) was used as the dependent variable. For statistical calculations the variables Xi were coded as xi according to Eq. (1)

$$x_i = (X_i - \bar{x}_i)/(\Delta x_j)\ (i = 1, 2, 3,\ldots, k) \quad \ldots\ldots\ldots\ldots(1)$$

where x_i is dimensionless value of an independent variable, X_i is real value of an independent variable, $\bar{x}_i$ is real value of the independent variable at the center point and Δx_j is step change.

RSM includes full factorial CCD and regression analysis. Also this method evaluates the effective factors and building models to study interaction and selects optimum conditions of variables for a desirable response. The full CCD, based on three basic principles of an ideal experimental design, primarily consists of (1) a complete 2n factorial design, where n is the number of test variables, (2) n0 center points ($n_0 \geq 1$) and (3) two axial points on the axis of each design variable at a distance of 2 ($2^{n/4}=2$ for n = 4) from the design center. Hence, the total number of design points (30) is $N = 2^n + 2n + n0$. Using CCD, a total number of 30 experiments with different combinations of starch, soyabean meal, zinc sulfate and ammonium phosphate were performed (Table 2 and 3). The response was taken as the maximum citric acid production which was observed at fourth day.

A 24 – factorial central composite experimental design, with eight axial points ($\alpha = \sqrt{3}$) and six replications at the center points ($n_0 = 6$) leading to a total number of thirty experiments was employed (Table 2) for the optimization of the parameters. The second degree polynomials (Eq. (2)) were calculated with the statistical package (Stat-Ease Inc, Minneapolis, MN, USA) to estimate the response of the dependent variable.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_{12} + b_{22}X_{22} + b_{33}X_{32} + b_{44}X_{42} + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where Y is predicted response, X_1, X_2, X_3, X_4 are independent variables, b_0 is offset term, b_1, b_2, b_3, b_4 are linear effects, b_{11}, b_{22}, b_{33}, b_{44} are squared effects and b_{12},b_{13}, b_{14}, b_{23}, b_{24}, b_{34} are interaction terms.

IV. RESULTS AND DISCUSSION

The four factors which highly influence the fermentative production of gentamycin are starch, soyabeanmeal, zincsulfate, ammonium phosphate. Hence these four factors are considered as major constituents of the medium.

The experimental design matrix was given in Tables 1 and 2. Thirty experiments were performed using different combinations of the variables as per the CCD. Using the results of the experiments the following second order polynomial equation giving the amount of gentamycin as a function of starch (X_1, g/l), soyabeanmeal (X_2, g/l), zinc sulfate (X_3, g/l) and ammonium phosphate (X_4, g/l) was obtained:

$$Y_i = -631.47 + 9.68X_1 - 3.53X_2 + 156.35X_3 - 11.03X_4 - 753.21X_{12} - 1487.18X_{22} + 93.59X_{32} - 8.53X_{42} + 5X_1X_2 + 50X_1X_3 + 2.5X_1X_4 + 100X_2X_3 - 2.5X_2X_4 - 25X_3X_4 \quad \ldots\ldots (3)$$

The predicted levels of gentamycin using the above equation were given along with experimental values in Table 3. The coefficients of the regression model (Eq.(3)) calculated are listed in Table 4, in which they contain four linear, four quadratic and six interaction terms and one block term. The effects of all four parameters i.e. starch, soya bean meal, zinc sulfate, ammonium phosphate and their interactions with each other on gentamycin concentration were found to be significant ($p \leq 0.05$). The parity plot showed a satisfactory correlation between the values of experimental values and predicted values (Fig. 1), wherein, the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the experimental and predicted values was less. And also the goodness of the model could be checked by different criteria. The coefficient of determination, R^2 is 0.94421 which implies that 94.42% of the variability in the response could be explained by the model.The corresponding analysis of variance (ANOVA) was presented in Table 5. The predicted optimum levels of starch, soyabeanmeal, zinc sulfate, ammonium phosphate were obtained by applying the regression analysis to the Eq. (3). The predicted and experimental gentamycin concentration at the optimum levels were also determined by using Eq. (3). Fig 2-7 represent the response surface and contour plots for the optimization of medium constituents of gentamycin production. The optimum medium constituents for higher metabolic production can be attained at the concentration of 12.72516(g/l) of starch, 10.59384(g/l) of soyabeanmeal, 0.27144(g/l) of zinc sulfate and 5.40323 (g/l) of ammonium phosphate. At these optimum medium concentrations maximum gentamycin production of 408.8844µg/ml was obtained.

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-1267-1272 ISSN: 2249-6645

V. Figures and Tables

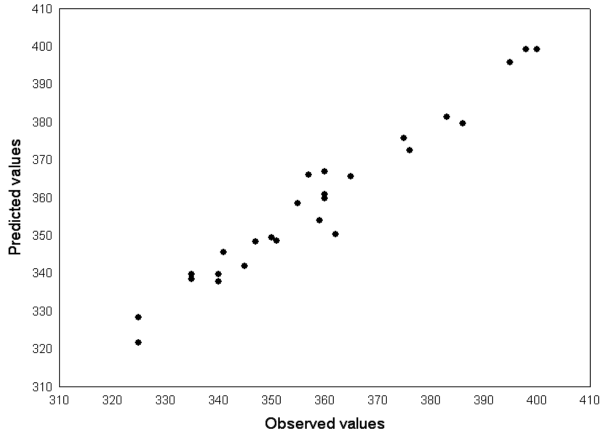

Figure 1. parity plot

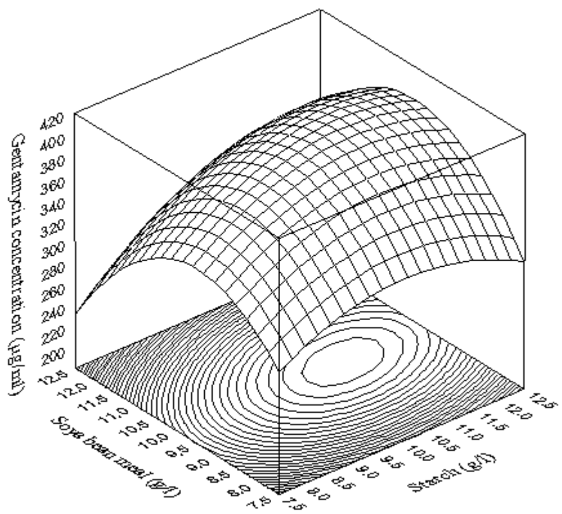

Figure 2. Response Surface and Contour plots for the production of Gentamycin using Starch and Soya bean meal

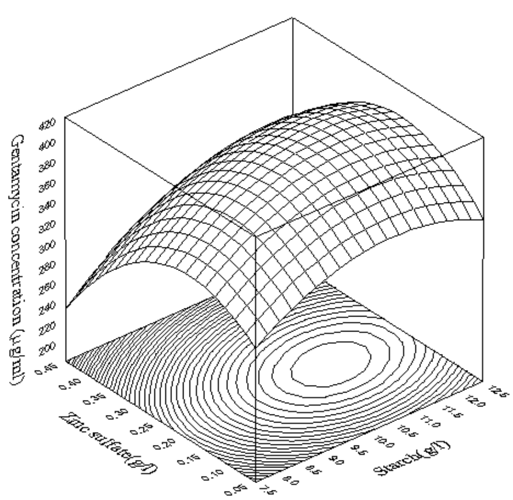

Figure 3. Response Surface and Contour plots for the production of Gentamycin using Starch and zinc sulfate

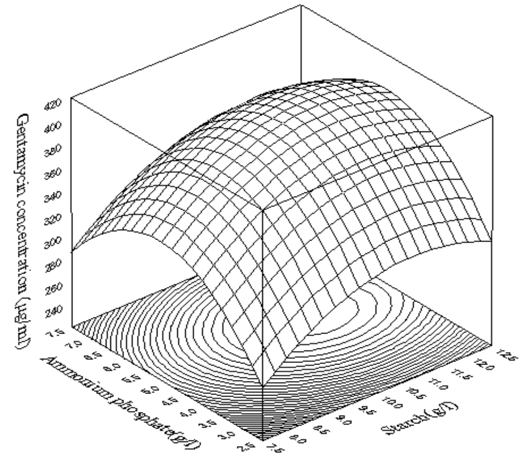

Figure 4. Response Surface and Contour plots for the production of Gentamycin using Starch and Ammonium Phosphate

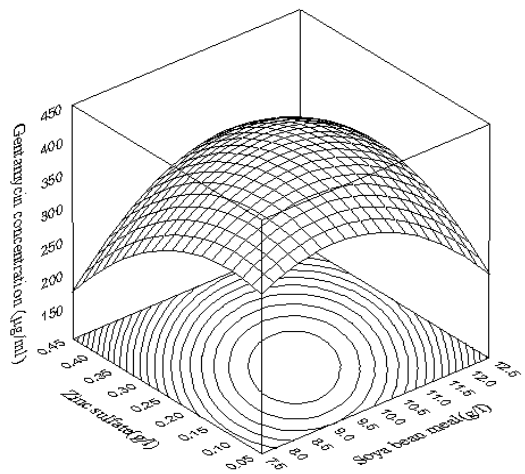

Figure 5. Response Surface and Contour plots for the production of Gentamycin using Soya bean meal and zinc sulfate

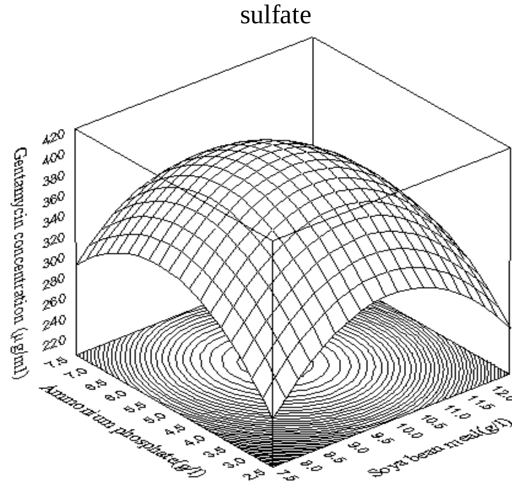

Figure 6. Response Surface and Contour plots for the production of Gentamycin using Soya bean meal and Ammonium Phosphate

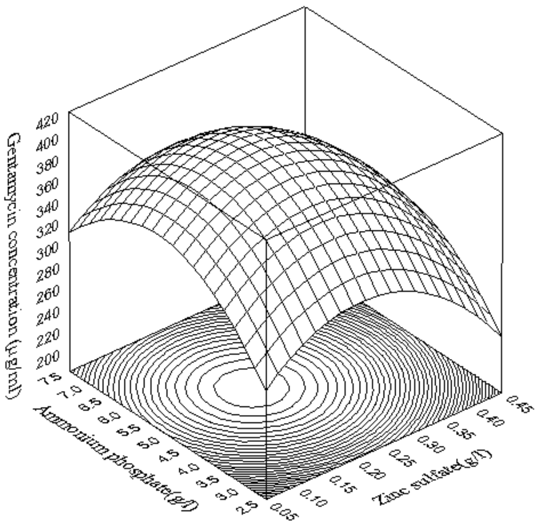

Figure 7. Response Surface and Contour plots for the production of Gentamycin using Zinc sulfate and Ammonium phosphate

Table 1 Independent variables in the experimental plan

Variables	Coded levels				
	-2	-1	0	1	2
Starch (g/l)	8	9	10	11	12
Soya bean meal (g/l)	8	9	10	11	12
Zinc sulphate (g/l)	0	0.1	0.2	0.3	0.4
Ammonium phosphate (g/l)	3	4	5	6	7

`Table 2 The central composite design matrix employed for four independent variables (actual values given in Table 1)

Run No.	Starch	Soya bean meal	Zinc sulfate	Ammonium phosphate
1	-1	-1	-1	-1
2	-1	-1	-1	1
3	-1	-1	1	-1
4	-1	-1	1	1
5	-1	1	-1	-1
6	-1	1	-1	1
7	-1	1	1	-1
8	-1	1	1	1
9	1	-1	-1	-1
10	1	-1	-1	1
11	1	-1	1	-1
12	1	-1	1	1
13	1	1	-1	-1
14	1	1	-1	1
15	1	1	1	-1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0

Table 3 Experimental and the predicted values for the production of gentamycin

Starch	Soya bean meal	Zinc sulfate	Ammonium phosphate	Production of Gentamycin (µg/ml)	
				Experimental	Predicted
9	9	0.1	4	360	359.8590
9	9	0.1	6	376	372.5256
9	9	0.3	4	340	337.7436
9	9	0.3	6	347	348.4103
9	11	0.1	4	325	321.6923
9	11	0.1	6	325	328.3590
9	11	0.3	4	341	345.5769
9	11	0.3	6	362	350.2436
11	9	0.1	4	357	366.0256
11	9	0.1	6	386	379.6923
11	9	0.3	4	359	353.9103
11	9	0.3	6	365	365.5769
11	11	0.1	4	345	341.8590

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-1267-1272 ISSN: 2249-6645

11	11	0.1	6	350	349.5256
11	11	0.3	4	375	375.7436
11	11	0.3	6	383	381.4103
8	10	0.2	5	355	358.5641
12	10	0.2	5	395	395.8974
10	8	0.2	5	360	360.8974
10	12	0.2	5	335	338.5641
10	10	0.4	5	335	339.7308
10	10	0.4	5	340	339.7308
10	10	0.2	3	351	348.5641
10	10	0.2	7	360	366.8974
10	10	0.2	5	398	399.3333
10	10	0.2	5	398	399.3333
10	10	0.2	5	400	399.3333
10	10	0.2	5	400	399.3333
10	10	0.2	5	400	399.3333
10	10	0.2	5	400	399.3333

Table 4 Coefficients, *t*-statistics and significance probability of the model

Term	Coefficient	Value	Standard error of coefficient	t-value	*P*-Value
Constant	B_0	-1220.6	244.863	-4.9848	0.000163
Starch	B_1	78.60	26.7427	2.9390	0.010159
Soya bean meal	B_2	191.93	26.7427	7.1769	0.000003
Zinc sulfate	B_3	-705.71	217.090	-3.2507	0.005375
Ammonium phosphate	B_4	122.09	22.7107	5.3759	0.000077
Starch x starch	b_{11}	-5.53	1.0741	-5.1443	0.000120
Soya bean meal x soya bean meal	b_{22}	-12.40	1.0741	-11.544	0.000000
Zinc sulfate x zinc sulfate	b_{33}	-1612.1	120.612	-13.366	0.000000
Ammonium phosphate x ammonium phosphate	b_{44}	-10.40	1.0741	-9.6829	0.000000
Starch x soya bean meal	b_{12}	3.50	1.3987	2.5023	0.024394
Starch x zinc sulfate	b_{13}	25.00	13.9870	1.7874	0.094095
Starch x ammonium phosphate	b_{14}	0.25	1.3987	0.1787	0.860535
Soya bean meal x zinc sulfate	b_{23}	115.00	13.9870	8.2219	0.000001
Soya bean meal x ammonium phosphate	b_{24}	-1.50	1.3987	-1.0724	0.300480
Zinc sulfate x ammonium phosphate	b_{34}	-5.00	13.9870	-0.3575	0.725716

Table 5 Analysis of variance (ANOVA) for the entire quadratic model

Source of variation	Sum of squares (SS)	d.f.	Mean squares (MS)	*F*-value	Probe >*F*
Model	16725.17	14	1194.655	38.16	0.000

Error	469.53	15	31.302
Total	17194.70		

$R = 0..98625$; $R^2 = 0.97269$; Adjusted $R^2 = 0.94720759$

$F_{0.01(14,15)} = S^2_r / S^2_e = 38.16582 > F_{0.01(14,15)Tabular} = 3.56$

$P_{model} > F = 0.000000$

VI. Conclusion

The one factor at a time operation used in optimization process which involves changing one parameter at a time, while keeping the others at fixed levels is laborious and time consuming. This method requires a complete series of experiments for every factor of interest. Moreover, such a method does not provide means of observing possible factors interactions. In contrast, CCD offers a number of important advantages. For instance, the researchers could easily determine factor effects with considerable less experimental effort, identify factors, find optima, offer grater precision and facilitate system modeling.

Thus, the present study using the RSM with CCD enables to find the importance of factors at different levels. Thus the optimum values of medium constituents found are starch 12.7252(g/l), soya bean meal 10.5938(g/l), zinc sulfate 0.2714(g/l) and ammonium phosphate 5.4032(g/l) and the gentamycin production obtained with the above optimum values are 408.8844μg/ml. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for gentamycin production. The results of this study have clearly indicated RSM is an effective method for maximum production of gentamycin using *Micromonospora echinospora* MTCC 708.

References

[1] A. A. Abou-Zeid, A.I. Eissa and H. M. Salem. The fermentative production of gentamycin by Micromonospora purpurea. J. Appl. Chem. Biotechnol. 24(655-661),1974.

[2] K.S.Kim. Effect of intercalating dyes on the production of antibiotics by M.purpurea and M.rosarea. Enzyme and Microbial technology.22(564-570),1990.

[3] Juchu, Wenze Niu, Siliang Zhang, Huitta Yingping Zhuang and Yourong Li.Effect of metal ions on the binding of gentamycin to the peptidoglycon of M.echinospora Process Biochemistry.39(1145-1150),2004.

[4] Sarre, S.G.; Mohn,F.E.N.Y. Acad. Sci.1967, 29(5),575.

[5] Milanesi. G.; Ciferri, O. Biochemistry 1996,5(12),3926.

[6] Veera Venkata Ratnam Bandaru, Subba Rao Somalanka, Damodara Rao Mendu, Narasimha Rao Madicherla, Ayyanna Chityala. Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of Zymomonas mobilis using response surface methodology.Enzyme and Microbial Technology.38(209-214)2006.

[7] Cheynier, V., Feinberg, M., Chararas, C. & Ducauze, C. 1983 Application of response surface methodology to evaluation of bioconversion experimental conditions. Applied and Environmental Microbiology 45, 634–639.

[8] Moresi, M., Colicchio, A. & Sansovini, F. 1980 Optimization of whey fermentation in a jar fermenter. European Journal of Applied Microbiology and Biotechnology 9, 173–183.

[9] M.Saban Tanyildizi, Dursun Ozer and Murat Elibol. Optimization of α-amylase production by bacillus species using response surface methodology. Process Biochemistry. 40(2291-2296), 2005.

[10] K.Adinarayana, P.Ellaiah, B.Srinivasulu, J.Lakshminarayana and KVVSN.Bapiraju. Optimization of media and culture conditions for neomycin production using response surface methodology. Ind. J.of Biotechnol.2(564-570), 2003.

[11] Cochran, N.G. & Cox, G.M. 1968 Experimental Designs. John Wiley and Sons Inc., p. 611.

[12] Box, G.E.P. & Wilson, K.B. 1951 On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society (Series B) 13, 1–45.

[13] Indian Pharmacopia.1996.Gentamycin injection. Microbial assay of antibiotics.Method A, appendix 9.1, 230-231. vol 2.