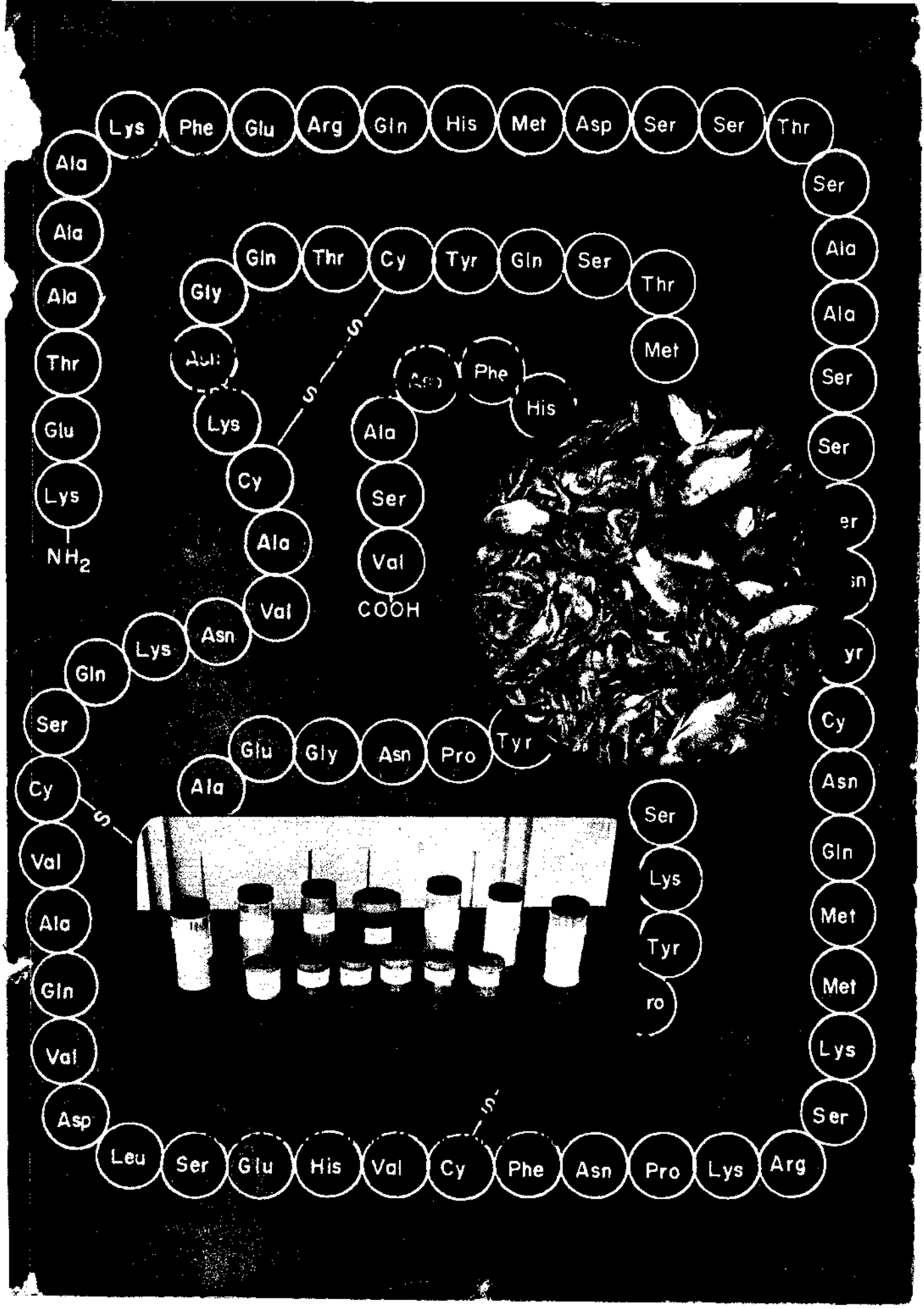
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Gln Thr Cy Tyr Gln Ser Thr Met
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Ala Ser Val COOH
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MANUAL OF RESEARCH METHODS FOR FISH AND SHELLFISH NUTRITION

Issued on the occasion of the Workshop on
METHODOLOGY FOR FISH AND SHELLFISH NUTRITION
organised by
The Centre of Advanced Studies in Mariculture,
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COCHIN

PREFACE

The Centre of Advanced Studies in Mariculture established at the Central Marine Fisheries Research Institute has been conducting Workshops in Research Methodologies on specialised disciplines with a view to enhance the competence of the scientific workers specialising in researches connected with mariculture. The main emphasis in mariculture research has been directed towards the development of economically viable culture techniques for culturable species of fish and shellfish, with a view to augmenting the fish and shellfish production of the country. In order to develop low-cost technologies the essential operational inputs have to be rationally utilized.

It has been well established that feeding constitutes the major cost of production, often exceeding 50 per cent of the operating costs in intensive aquaculture operations. Two main factors affecting the cost of feeding are composition of the diet and efficiency of feed conversion. In order to develop least-cost formula diets of high conversion efficiency, knowledge of the nutritional requirements of the different species during the different phases of the life cycle and the nutritive value of the complex feed ingredients available in the country to the candidate species is a prerequisite.

The existing information on the nutritional requirements of cultivated species of fish and shellfish in India, is meagre and recently research has been intensified in this area. If researches on this field could be carried out using standardised experimental procedures, the data obtained on the nutritional requirements of the different species could be stored in a fish and shellfish nutrition data bank, from where data could be disseminated to the users such as feed manufacturers, farmers, extension workers and research workers as and when required. It is also necessary that the data collected on the chemical composition of the feed ingredients and their nutritive value for the species should be based on standard chemical methods and experimental procedures so that the data could be stored in

the data bank which eventually could become a National Fish Feed Information Centre.

To undertake studies on the above lines, especially by the technicians and research workers entering afresh into the field, the need of practical guides describing the research techniques and methods, planning of investigations, collection of data and their interpretation need not be emphasized. Keeping this in view, the present manual on Research Methods in Fish and Shellfish Nutrition is issued by the Centre of Advanced Studies in Mariculture on the occasion of the Workshop on Methodology of Fish and Shellfish Nutrition.

Dr. Akio Kanazawa, Professor of Nutritional Chemistry, University of Kagoshima, Japan and Consultant in Fish and Shellfish Nutrition at the CAS in Mariculture, has been kind enough to cooperate with the Scientists of CAS in Mariculture of the Central Marine Fisheries Research Institute in the preparation of this manual. There are chapters in this manual covering various methods on composition analysis of feeds, including growth inhibitors and toxins; determination of digestibility coefficient; protein evaluation; bioenergetics; determination of essential amino acid requirements using radioisotope method; research test diets for fishes and prawns; feed formulation methods; experimental design, etc. Methods of preparation of microparticulate diets, phytoplankton and zooplankton culture methods, etc. are also included to facilitate larval nutrition studies. Many of the methods given in the manual have been standardized for fish and shellfish nutrition studies in India and abroad. The users can also gain maximum benefit by suitable modifications of other methods which are given as guidelines.

I would like to thank all the scientific and technical staff especially Shri S. Ahamed Ali, Dr. K. Alagarswami, Shri D.C.V. Easterson, Shri C.P. Gopinathan, Shri T. Jacob, Shri M.S. Muthu, Dr. R. Paul Raj, Dr. A.G. Ponniah and

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CHAPTER 5

DETERMINATION OF GROWTH INHIBITORS AND TOXINS*

1 INTRODUCTION

Certain feedstuffs contain natural toxins that, at high enough levels, are growth inhibitors and sometimes fatal to the animal consuming them. Principal among these are: (a) Urease-an enzyme found in raw soybean which produces toxicity through the hydrolysis of urea to ammonia; (b) gossypol-an endogenous toxin present in the gland of cottonseed which persists during production of the meal unless removed by a special process, or, unless, the cotton seed is a glandless variety; (c) Isothiocyanates-cyanogenic glycosides are found in linseed and cassava; (d) aflatoxin is a class of extremely potent toxins produced by the mould Aspergillus flavus. Aflatoxin may be present in any materials produced and stored under hot and humid conditions and is usually found in groundnut cake, palm cake, copra cake and maize.

2 DETERMINATION OF UREASE ACTIVITY IN SOYBEAN MEAL

2.1 Apparatus

(a) Water bath at 40°C, capable of maintaining temperature within ± 1°C, with shaking device,

(b) Conical flasks, 125 ml

(c) Volumetric flasks, 25 ml and

(d) Spectrophotometer.

2.2 Reagents

(a) Dimethylaminobenzaldehyde solution (DMAB):

Dissolve 16g DMAB in 1 litre 95% ethyl alcohol, and add 100 ml concentrated hydrochloric acid (stable for one month)

(b) Pyrophosphate buffer:

Dissolve 23.3g $Na_4P_2O_7 10H_2O$ in approximately 980 ml distilled water. Add 3 ml of concentrated

* Prepared by R. Paul Raj and Syed Ahamed Ali, Central Marine Fisheries Research Institute, Cochin-18.

HCl and then additional HCl until the pH of the buffer is 7.7 - 7.8. Dilute to 1 litre.

(c) Buffered urea solution:

Dissolve 04g urea in 1 litre pyrophosphate buffer (stable for one week).

(d) Zinc acetate solution:

Dissolve 22.0g zinc acetate $2H_2O$ in distilled water, add 3 ml of glacial acetic acid, and dilute to 100 ml.

(e) Potassium ferrocyanide solution:

Dissolve 10.6g $K_4Fe(CN)_6 3H_2O$ in distilled water, and dilute to 100 ml.

(f) Charcoal

2.3 Procedure

Accurately weigh 1 g of soybean meal into a conical flask and 50 ml of the buffered urea solution. Incubate in water bath for exactly 30 min at 40°C with shaking. Remove from water bath and quickly add 0.5 ml each of concentrated HCl, ferrocyanide solution, zinc acetate solution and 0.1g of charcoal. Shake for 15 min. and filter. If the filtrate is coloured, repeat the procedure using more charcoal. Pipette 10 ml aliquots of the filtrate and the DMAB solution into a 25 ml volumetric flask and make up to volume with distilled water. Make up also a reagent blank (10 ml DMAB made up to 25 ml with water) and a urea blank (10 ml buffered urea solution and 10 ml DMAB made upto 25ml with water). Prepare a standard curve by pipetting aliquots of buffered urea solution from 2 to 12 ml into 25 ml volumetric flasks, adding 10 ml of DMAB and make up to volume.

Mix flasks well, stand in water bath at 25°C for 10 min. and then read at 430 mμ. Calculate urease activity as mg/litre urea in urea blank less mg/litre urea in sample.

3 DETERMINATION OF FREE GOSSYPOL IN COTTONSEED MEAL

3.1 Apparatus

(a) Mechanical shaker
(b) Spectrophotometer
(c) Conical flasks, 250 ml capacity
(d) Volumetric flasks, 25 and 250 ml and
(e) Water bath (boiling)

3.2 Reagents

(a) Aqueous acetone, 7 parts acetone, 3 parts distilled water (v/v)

(b) Aqueous acetone - aniline solution:

To 700 ml acetone and 300 ml distilled water add 0.5 ml redistilled aniline. Prepare solution daily.

(c) Aqueous isopropyl alcohol solution: 8 parts isopropyl alcohol, 2 parts distilled water (v/v)

(d) Aniline:

Distill reagent grade aniline over a small quantity of zinc dust, discarding the first and last 10 percent of the distillate. Store refrigerated in a brown glass stoppered bottle. Stable for several months.

(e) Standard gossypol solution:

(i) Dissolve 25 mg of pure gossypol in aniline-free acetone and transfer to a 250 ml volumetric flask using 100 ml of acetone. Add 75 ml of distilled water, dilute to volume with acetone, and mix.

(ii) Take 50 ml of solution (a) add 100 ml pure acetone, 60 ml of distilled water, mix, and dilute to 250 ml with pure acetone. Solution (b) contains 0.02 mg gossypol/ml and is stable for 24 h in darkness.

3.3 Procedure

Grind sample to pass 1 mm screen taking care not to overheat. Take approximately 1g of the sample and add 25 ml of pure acetone. Stir for a few minutes, filter, and devide filtrate into two. To one portion add a pellet of sodium hydroxide and heat in a water bath for a few minutes. A light yellow extract which does hot change colour with sodium hydroxide indicates that the cottonseed meal is untreated and procedure (1) should be used. A deep orange red colour in the tube containing sodium hydroxide indicates the presence of dianilinogossypol and this requires that procedure (2) be used.

3.3.1 Procedure (1):

Weigh 0.5 to 1g of sample, depending on expected gossypol content, into a conical flask and add glass beads. Pipette in 100 ml of aqueous acetone solution, stopper the flask, and shake for one hour. Filter, discarding the first few ml of filtrate, and then pipette out duplicate aliquots into 25 ml volumetric flasks. (Take aliquots from 2 to 10 ml again depending on expected gossypol content). Dilute one of the aliquots to volume with aqueous isopropyl alcohol (solution a), while to the other aliquot (solution b) add 2 ml redistilled aniline, heat in a boiling water bath for 30 min together with a reagent blank containing 2 ml of aniline and a volume of aqueous acetone solution equal to the sample aliquot. Remove solution b and the blank, add sufficient aqueous isopropyl alcohol to effect homogeneous solution, and cool to room temperature in a water bath. Dilute to volume with aqueous isopropyl alcohol.

Read samples at 400 mμ. Set instrument to 0 absorbance with aqueous isopropyl alcohol, and determine absorbance of solution 'a' and reagent blank. If the reagent blank is below 0.022 absorbance proceed

as below, otherwise repeat the analysis using freshly distilled aniline.

Determine the absorbance of solution b, with the reagent blank set at 0 absorbance. Calculate the corrected absorbance of the sample aliquot: the corrected absorbance is the absorbance of solution b minus the absorbance of solution a. Determine the mg of free gossypol present in the sample solution using the calibration curve (see below).

3.3.2 Procedure (2):

Weigh 1g of sample into a conical flask, and add 50 ml aqueous acetone, shake, and filter as above. Pipette duplicate aliquots of the filtrate (from 2 to 5 ml, depending on expected free gossypol level) into 25 ml volumetric flask. Dilute one of the aliquots to volume (solution a) with aqueous isopropyl alcohol and leave for at least 30 min before reading on the spectrophotometer. Treat the other aliquot (solution b) as in procedure (1), determine the absorbances of solutions a and b as before, and calculate the apparent content of gossypol in both solutions a and b by using the calibration curve (see below).

3.3.3 Preparation of calibration curve:

Pipette duplicate 1, 2, 3, 4, 5, 7, 8 and 10 aliquots of the 0.02 mg/ml gossypol standard into 25 ml volumetric flasks. Dilute one set (solution a) to volume with aqueous isopropyl alcohol and determine absorbances as previously. To the other set (solution b) add 2 ml of redistilled aniline and proceed as previously. Prepare one reagent blank, using 2 ml aniline and 10 ml of aqueous acetone, heated together with the standards. Determine absorbances as in procedure (1) and calculate the corrected optical density for each standard solution:

Corrected absorbance = (absorbance solution b - absorbance solution a). Plot the standard curve, plotting corrected absorbance against gossypol concentrate in the 25 ml volume.

3.4 Calculation

Calculate free gossypol percent in normal meals as:

$$\text{Free gossypol \%} = \frac{5G}{WV}$$

where G - is the graph reading
W - sample weight
V - aliquot volume used

For chemically treated meals:

$$\text{Free gossypol \%} = \frac{5\ (B - A)}{WV}$$

where A - mg apparent free gossypol in sample aliquot (a)
B - mg apparent free gossypol in sample aliquot (b)
W - sample weight
V - aliquot volume used

4 THIOGLUCOSIDE DETERMINATION

The method described will give approximate thioglucoside content but does not allow the individual thioglucosides and isothiocyanates to be determined.

4.1 Apparatus and Reagents

(a) Barium chloride (5% solution)
(b) Volumetric flasks, 600 ml and
(c) Steam bath.

4.2 Procedure

To 10g meal (de-fatted by Soxhlet extraction) add 250 ml distilled water, hydrolyse at 54°C for 1 h and then

boil for 2 h, keeping volume constant. Filter, retaining filtrate, and wash residue three times with 50 ml hot water. Add washings to initial filtrate and make up volume to 600 ml. Precipitate barium sulphate by heating and adding excess barium chloride solution. Leave on a steam bath for a few hours and then filter. Ash in a muffle furnace and then weigh precipitate.

4.3 Calculation

Calculate approximate thioglucoside content as:

% thioglucoside

$$= \frac{\text{(M.wt. thioglucoside) (Wt. of Ba } SO_4)}{\text{(M. wt. } BaSO_4\text{) (Sample Wt.)}} \times 100$$

5 AFLATOXIN ANALYSIS

A method of aflatoxin analysis is outlined below which is suitable for materials such as groundnut meal, coconut meal, and palm kernel meal. For full details of the method, and for alternative procedures reference should be made to Methods of Aflatoxin Analysis by B.D. Jones (1972).

5.1 Apparatus

(a) Thin layer chromatography plates, 20 X 20 cm
(b) UV lamp, peak emission at 365 nm
(c) Bottles, wide-mouthed, 250 ml
(d) Micropipettes, and
(e) Shaking device.

5.2 Reagents

(a) Chloroform (reagent grade)
(b) Diethyl ether (reagent grade)
(c) Chloroform/methanol mixture (95/5 v/v)
(d) "Celite", diatomaceous earth
(e) Kieselgel 'G' (Merck)

(f) Qualitative standard:

Helps to distinguish aflatoxin spots from other fluorescent spots which may be present. A groundnut meal containing aflatoxins B, obtained from the Tropical Products Institute, London, can be used for this purpose.

5.3 Procedure

Weigh 10 ml of material into a wide mouthed bottle and thoroughly mix in 10 ml of water. (If high fat material is used, a prior Soxhlet extraction with petroleum ether will be necessary). Add 100 ml of chloroform, stopper with a chloroform resistant bung, and shake for 30 min. Filter the extract through "Celite", take 20 ml of filtrate and make up to 25 ml (solution a). Take another 20 ml of filtrate and concentrate to 5 ml (solution b).

Prepare thin layer plates by shaking Kieselgel 'G' (100 g) with water (220 ml) for 20 min and apply the mixture to the plates with a suitable apparatus to a depth of 500 µ. Leave for 1 h, then dry at 100°C. Spot 10 and 20 µl of solution b, and 5 and 10 µl of solution a onto a plate, together with a qualitative standard spot, in a line 2 cm from the bottom of the plate and at least 2 cm in from each side. Carry out the spot application in subdued light.

Develop the plate in diethyl ether to a height of 12 cm. Allow to dry in subdued light then redevelop the plate in chloroform methanol (95/5, v/v) to a height of 10 cm from the baseline. Examine the plate in a dark room, 30 cm from the UV source. The presence of a blue fluorescent spot at Rf 0.5 to 0.55 indicates aflatoxin B (check that the standard spot also lies in this range). The presence of a second spot at Rf 0.15 to 5 indicates aflatoxin G. The toxicity level of a sample can then be classified in terms of aflatoxins B and G according to Table 1.

Table 1. Toxicity Levels for Aflatoxins B and G

Vol. applied (µl)	Concentration of aflatoxins (µg/kg)		Toxicity level of fluorescence observed
	No fluorescence	With fluorescence	
5 µl (soln. a)	≤ 1000	≥ 1000	Very high
10 µl (soln. a)	≤ 500	500 - 1000	high
10 µl (soln. b)	≤ 100	100 - 500	medium
20 µl (soln. b)	≤ 50	50 - 100	low

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