THE ISOLATION AND SURVEY OF CUCURBITIN IN THE SEEDS OF CERTAIN SPECIES OF THE CUCURBITACEAE

by

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CHAPTER I

INTRODUCTION

Seeds of Cucurbita species especially C. pepo L., C. maxima Duch. and C. moschata Duch. have been used as anthelmintics in the treatment of tapeworm infections. Their special advantage as a taenifuge over other anthelmintics is their lack of toxicity.

The active principle of pumpkin seeds has for many years eluded a large number of investigators. In 1931, Neely and Davy (32) showed that the active principle of C. pepo fruit was soluble in 75% ethyl alcohol but was insoluble in petroleum ether. It was dialyzable and its activity was reduced by boiling with dilute sulfuric acid. Krayer (23), in 1937 asserted that the active principle of Cucurbita seeds was soluble in water and was heat resistant. Subsequently, Veen and Collier (49) in 1949 used as anthelmintic for human use a purified, deproteinized, stable aqueous extract of the seeds of C. moschata. Colorado et al. in 1950 (6), and Mazzotti et al. in 1951 (26) also used aqueous extracts of pumpkin seeds. In 1956, Valentin and Brockelt (48) prepared from a number of varieties of Cucurbita pepo seeds concentrated extracts which were found to be strongly toxic when tested on earthworms. The active principle was soluble in water but

was insoluble in ether. In 1961, Karamanukian (21) showed that whole decorticated pumpkin (cucurbit) seeds were more active than an aqueous extract from a similar weight of seeds administered to human beings.

In 1961, Fang and associates (11) isolated an active substance from Cucurbita moschata seeds. The active compound was water-soluble and was found to be a free amino acid which had physical and chemical properties different from those of the known naturally occurring amino acids. They named the substance cucurbitine. The structure of this new amino acid was confirmed by degradative reactions, ultra-violet and infrared spectroscopy, as well as by synthesis, to be 3-amino-3-carboxypyrrolidine. These authors claimed that cucurbitin (as now spelled) possessed anthelmintic properties. In vivo, biological testing of cucurbitin showed it to be active in inhibiting the growth of immature Schistosoma japonicum.

Taenifugal properties are not limited to the seeds of Cucurbita species. According to Pefer and coworkers (12), aqueous and hydro-alcoholic extracts, and the oil from watermelon seeds, were found to be vermicidal; while the ether extracts of the seeds had no anthelmintic action. It is the practice of certain communities in Lebanon to use the seeds of Luffa cylindrica (Cucurbitaceae) as a taenifuge.

The purpose of the present investigation is manifold.

With the isolation of cucurbitin by Fang et al. (11) as mentioned above, it was thought appropriate to study the quantitative distribution of this substance in the different cucurbits grown in Lebanon or available on the Lebanese market from foreign sources. It was also interesting to find out whether the activity of some of the commercial varieties of Cucurbita seeds as found by Karamanukian (21) agrees with the relative concentration of cucurbitin in these seeds. To do this, it was necessary to develop a simple method or methods for separating cucurbitin from the numerous free amino acids naturally occurring with it in the seed or in other parts of the fruit, and a simple method for estimating it quantitatively in these parts.

CHAPTER II

PLANT MATERIAL

A large number of cultivars of the seeds of pumpkin, water-melon, and muskmelon are available on the Beirut market. They are designated by the merchants according to the country from where they were imported, as for example, Turkey, Bulgaria, China, etc. The commercial origin, in certain instances, was not necessarily indentical with the habitat. For example, the 'Chinese' commercial samples could have had their origin from any one of the far eastern countries. The different commercial samples used in this work will be designated as cultivars (abbreviated as cv.).

Seeds* of the different Cucurbit cultivars, were grown and their species identified, according to Bailey (1,2), Karamanukian (21), and Whitaker et al. (52,53), taking into consideration the taxonomic characteristics of the leaves, stems and seeds.

These species and their cultivars are as follows:

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* Obtained through the courtesy of Messrs. Hassan and Naja, Foch Street, Beirut.

1. Cucurbita pepo L.

   Pumpkin, Winter Squash.

   a. cv. 'Yugoslavia'.

   b. cv. 'Chinese Shen-Skin'.

   c. cv. 'Bulgaria'.

2. Cucurbita maxima Duch. ex Lam.

   Pumpkin, Winter Squash.

   a. cv. 'Chinese Snow-White'.

Fruits of Cucurbita species available on the market were also used. Seeds were grown and the species identified by examining all the plant parts taxonomically. These seeds belonged to the following species:

3. Cucurbita pepo L.

   Squash, Pumpkin.

   a. cv. 'Turkey'.*

4. Cucurbita maxima Duch. ex Lam.

   Winter Squash, Pumpkin.

   a. cv. 'Ainjar, globular'. *

   b. cv. 'Ainjar, oblong'. *

   c. cv. 'Syria, globular, large fruited' (giant pumpkin). **

5. Cucurbita moschata Duch.

   Winter Squash, Pumpkin.

   a. cv. 'Syria, crook-necked'. **

   b. cv. 'Syria, globular'. **

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* Purchased from Ainjar market, Lebanon.

** Purchased from Beirut market (Nouriye).

Other cucurbitaceous seeds, besides Cucurbita spp., were also used in this investigation.* These included (52,53):

6. Lagenaria leucantha Rusby (Cucurbita lagenaria,L., C. leucantha. Duch., L. vulgaris Ser.)
   Calabash gourd, gourd.
   a. cv. 'Lebanon'.
7. Luffa cylindrica (L.) M.Roem. (L.aegyptica,Mill.)
   Dish-cloth gourd, rag gourd.
   a. cv. 'Lebanon'.
8. Citrullus vulgaris Schrad.
   Water-melon.
   a. cv. 'China'.
   b. cv. 'Turkey'.
   c. cv. 'Lebanon.'
9. Cucumis melo L.
   Musk-melon, Cantaloupe.
   a. cv. 'America, round'.
   b. cv. 'America, oblong.'
10. Cucumis sativus L.
    Cucumber.
    a. cv. 'America'.
    b. cv. 'Lebanon'.

The fruit pulp of the following was also examined:

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* Purchased from Hasbini and Sons, Argentine Str., near Cinema Byblos, Beirut.

11. *Cucurbita maxima* Duch.

    a. cv. 'Ainjar, globular'.

    b. cv. 'Ainjar, oblong'.

Analytical data obtained for the seeds and pulp in the foregoing list will be found in Chapter VII.

CHAPTER III

ANALYTICAL METHODS

In this chapter the different procedures and analytical methods employed in this investigation will be outlined and discussed and the composition of the reagents indicated. The method of extraction of natural cucurbitin, and the preparation of cucurbitaceous extracts are the subjects of Chapters V and VI which constitute a part of the investigation with which this thesis is concerned.

I. Chromatographic Methods

Chromatography stands supreme as the technique for separating and showing the components of a mixture. Compounds containing a particular reactive group will react with the visualization reagent specific for that group. It often happens, in one-dimensional chromatography, that two or more compounds will run together or overlap on the chromatogram. It is essential, therefore, to use a variety of solvents; or resort to two-dimensional chromatography. The latter is especially indicated in amino-acid analysis, where a pair of solvents is used in an attempt to separate such compounds as will normally run together in one-dimensional chromatograms.

As cellulose phosphate is the strongest of the

cation exchange celluloses, it has been studied more extensively than other exchangers, particularly with regard to the separation of amino acid mixtures. Its use in the present investigation finally solved the difficulty of separating cucurbitin from accompanying amino acids by chromatography (See II. p. 17).

A. Paper Chromatography of the Amino Acids of Cucurbita species

Solvent Systems Used in One-Dimensional Paper Chromatography

In view of the extremely limited solubility of amino acids in organic solvents, systems suitable for chromatography must generally contain water (3). Solvents such as methanol, ethanol or acetone are used as markedly polar organic components. Often fairly good separations are achieved with such systems. They give relatively diffuse zones, however, and tend to cause tailing. This tendency is checked by the addition of a few ml. per cent of glacial acetic acid, and by reducing the amounts spotted to about 2 mcg. per amino acid. According to Brenner *et al.* (3) smaller zones result also when methanol or ethanol are replaced by *n*-propanol, *n*-butanol and the like, but this advantage is somewhat offset by the reduced rate of flow which is largely due to increased viscosity. Zones generally spread less, i.e.,

separation is better the higher the solvent viscosity. Phenol, as a solvent, provides a good example of this effect. If viscous alcohols are replaced by non-polar fluids of lower viscosity, a solubilizer such as methanol, pyridine or glacial acetic acid will help restore miscibility with water. With systems of this latter type, separations may be very satisfactory and rapid (3).

Basic amino acids travel much more slowly under acidic conditions that have little if any affect on the mono-aminocarboxylic acids (Consden et al. 1944) (7). This is, indeed, a very good test for basicity. Ammonia, on the other hand, selectively slows acidic amino acids and hastens the passage of basic ones.

The following solvent systems were used ascendingly on Whatman No. 1 chromatographic paper:

1. n-Butanol 40 ml.
   Glacial Acetic Acid 10 ml.
   Water 50 ml.

   The upper phase of this classical mixture, referred to as "Partridge" solvent (33) is a most widely used solvent. It should be aged for at least three days before use.

2. n-Butanol 60 ml.
   Glacial Acetic Acid 15 ml.
   Water 25 ml.

   A monophasic solvent, the composition of which corresponds to that of the upper organic phase of solvent No. 1 (43).

3. n-Butanol 40 ml.
   Glacial Acetic Acid 10 ml.
   Ethanol 95% 10 ml.
   Water 20 ml.

   A monophasic solvent used by Fang et al.(11) for the detection of cucu bitin.

4. n-Butanol 90 ml.
   Glacial Acetic Acid 10 ml.
   Water 29 ml.

   A monophasic solvent used by Dunnill and Fowden (10) for cucurbitin.

5. n-Butanol 40 ml.
   Glacial Acetic Acid 10 ml.

   A solvent system used by Fang et al. (11) to detect cucurbitin.

6. Diethylamine 1 ml.
   Ethanol 77% 100 ml.

   A solvent system used by Fang et al. (11) to detect the postition of cucurbitin on the chromatograms.

7. Phenol Cryst. 75 gm.
   Water 25 ml.

   The molten phenol was mixed with hot water. The mixture was cooled to room temperature before use.

8. Phenol Cryst. 75 gm.
   Water 25 ml.

   The solvent was used here in an atmosphere of ammonia from a strong ammonium hydroxide

solution (10). The solvent was prepared as in No. 7 (above). Concentrated ammonium hydroxide solution was placed in the chamber during the running of the chromatogram. Ammonia causes the phenol to darken from pink to red, to blue, and finally to black in the course of the operation of the chromatogram. Therefore, the solvent was renewed every time a new chromatogram was to be run.

9. n-Propanol 70 ml.
   Concentrated Ammonium Hydroxide Solution 30 ml.

Solvent Systems Used in Two-Dimensional Paper Chromatography

Two-dimensional chromatography improves the separation of substances which would otherwise overlap when chromatographed in one-dimension only in as much as it allows the combination of the resolving powers of two different solvents. Two-dimensional chromatography is recommended exclusively when a mixture of unknown composition is to be analyzed as is the case with pumpkin seed extracts.

In the following solvent systems, system "a" was first used in one direction, then system "b" was used in a direction at a right angle to "a". Both systems were used ascendingly on Whatman No. 3MM chromatographic paper.

10.	"a".	Diethylamine	1 ml.
		Ethanol 77%	100 ml.
	"b".	n-Propanol	70 ml.
		Concentrated Ammonium Hydroxide Solution	30 ml.
11.	"a".	n-Butanol	60 ml.
		Glacial Acetic Acid	15 ml.
		Water	25 ml.
	"b".	n-Butanol	10 ml.
		Pyridine	10 ml.
		Water	10 ml.
12.	"a".	Phenol Cryst.	75 gm.
		Water	25 ml.

The solvent was used here in the presence of an atmosphere of ammonia from a concentrated solution of ammonium hydroxide.

	"b".	n-Butanol	90 ml.
		Glacial Acetic Acid	10 ml.
		Water	29 ml.

This solvent pair gave the best resolution in the chromatography of the amino acid extracts of cucurbit seeds (10).

Intermediate drying of two-dimensional chromatograms

Intermediate drying presented no problem when highly volatile solvents were used. The chromatogram was left in a well ventilated hood for about 15 minutes, after which it was immediately submitted to the second dimension run. Less volatile solvents such as phenol could be

removed only by leaving the chromatograms overnight, although there was a risk of oxidation of certain amino acids.

B. Thin-Layer Chromatography of the Amino Acids of Cucurbita species.

The main advantage of the use of thin-layer chromatography in amino acid analysis is the time factor. The total time required for a two-dimensional chromatoplate to be developed being between four to five hours, as compared to two or three days required for a paper chromatogram.

Solvent Systems Used in One-Dimensional Thin-Layer Chromatography

i- Adsorbant: Silica Gel G (Merck).

Solvents : the following were used:

13.	Methyl ethyl ketone	10 ml.
	Pyridine	15 ml.
	Water	15 ml.
	Glacial Acetic Acid	2 ml.
14.	Propanol	70 ml.
	Water	30 ml.

Remarks. Separation of the amino acids on Silica gel was poor, and the results with solvents '13' and '14' were not satisfactory.

ii- Adsorbent: Cellulose (S. & S. 123).

Solvents : the following were used:

15.	Methanol	80 ml.
	Water	20 ml.
	Pyridine	5 ml.
16.	n-Butanol	40 ml.
	Glacial Acetic Acid	10 ml.
	Water	50 ml.

The upper organic phase of this solvent was used. (See solvent 1.)

17.	n-Propanol	70 ml.
	Water	30 ml.

This is the same as solvent 14.

Remarks. Although separation on cellulose was far better than on silica gel, one-dimensional chromatograms were not satisfactory because of the large number of amino acids present in the extracts.

Solvent Systems Used in Two-Dimensional Thin-Layer Chromatography

As in the case of two-dimensional paper chromatography, system "a" was used first in one direction, then system "b" in a direction at a right angle to the first.

i- Adsorbent: Silica Gel G (Merck).

Solvents : the following were used:

18.	"a".	n-Butanol	40 ml.
		Glacial Acetic Acid	10 ml.
		Water	10 ml.

"b". n-Propanol 70 ml.
Water 30 ml.

ii- Adsorbent: Cellulose (S. & S. 123).

Solvents : Solvent pair No. 12.

Preparation of Thin-Layer Plates

i- Silica Gel Plates. Glass plates measuring 20 X 20 cm., were coated with a slurry consisting of 1 part by weight of Silica Gel G (Merck) and 2 parts by volume of distilled water, using the Camag coating apparatus, to a thickness of 0.3 mm., were left to dry overnight and later used without activation.

ii- Cellulose Plates. Cellulose layers were prepared by shaking 15 gm. of Cellulose (S. & S. 123) powder very vigorously with 90 ml. of distilled water (39) for 1-2 min. and then coating the plates with the slurry to a thickness of 0.3 mm. The plates were left to dry overnight at room temperature, and used without further treatment.

Intermediate Drying of Two-Dimensional Thin-Layer Plates

The same precautions were observed here as under paper chromatography. See p. 13.

C. Visualization of the Amino Acid Spots on Paper and Thin-Layer Chromatograms

Ninhydrin has retained its postion as the most useful reagent for the visualization of amino acids on

chromatograms (25,27).

The following ninhydrin spray solution was used,

Ninhydrin Spray Solution

Ninhydrin	0.2 gm.
Ethanol 95%	100 ml.

The chromatogram was carefully sprayed with the ninhydrin solution and dried in the air. It was then heated for 2 min. in an oven at 110° C. Cucurbitin gave a light brown color with ninhydrin. If the chromatogram was heated for a longer period, the background assumed a pink coloration.

It is important to note the pH at which the color reaction for developing the spots is carried out.

This color is in no way characteristic of amino acids: peptides and other amino compounds also give the same color.

II. The Use of Ion-Exchange Cellulose Phosphate Papers for the Separation of Mixtures of Amino Acids

Cellulose phosphate is a strong ion exchanger (22), in that it retains a fully dissociated form over the pH range of most chromatographic applications.

The ion-exchange celluloses have made it possible to combine the advantages of ion-exchange principles and paper chromatographic techniques. In the application of the solution under test to the paper, there is no need to restrict the size of the spot: a single application

may be used, where four or five separate applications, with drying in between, might have been required on a "conventional" paper chromatogram. Nor is there any necessity to dry the spot before running the chromatogram.

Techniques for handling ion-exchange papers.

The methods of spot application, development, and final location were similar to those used in conventional paper chromatography (dry-start method). No equilibration period in the solvent atmosphere was necessary. In the application of the solutions under test to the paper there was no necessity to restrict the size of the spot, provided the total weight of solutes added was compatible with the capacity of the paper.

The "dry-start method" was found to impose certain restrictions on the technique associated with the initial preparation of the materials in the dry state. An alternative method of development conveniently distinguished as the "wet-start method", consisted essentially of treating the paper as if it were a column of ion-exchange material. The paper, with the positions for subsequent spot application clearly marked, was washed with a suitable solvent and the excess liquid allowed to drain off. The required volume of solution under test was then applied as a spot in a single application (22).

As exchanger, Whatman Cation Exchanger Cellulose Phosphate Papers P-20, measuring 57 × 23 cm., were

developed descendingly for 7 hours in the following buffers for the separation of the basic amino acids found in the cucurbit extracts.

1. Buffer pH 2.1 (22). Molarity 0.2.

Sodium Acetate	1N	50	ml.
Hydrochloric Acid	1N	52.5	ml.
Water up to		250	ml.

Method of handling: wet-start method.

2. Buffer pH 9.2*. Molarity 0.2.

Sodium bicarbonate	1M	127	ml.
Sodium carbonate	1M	24.4	ml.
Water up to		1000	ml.

Method of handling: dry-start method.

After each chromatogram was developed with the appropriate method, it was allowed to dry overnight. The paper was then sprayed with the ninhydrin spray solution,** and allowed to dry first in the air, then in an oven at 90° C. for 2-3 min. which was the optimum time for color development.

III. Electrophoretic Methods Used in the Investigation of the Basic Amino Acids of Cucurbita Species.

Electrophoresis may be defined as the migration of dissolved or suspended particles under the influence

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* Cyril Long (Ed.), 1961. Biochemists' handbook, E. & F.N. Spon Ltd., London. p. 3[illegible].

** See Chapter II, p. 17.

of an electric field. Electrophoresis may serve as a means for the separation of particles which are different in their electrophoretic behavior. An advantage of paper electrophoresis over paper chromatography in amino acid analysis is that the results can be obtained in a shorter time (44).

Much attention has been paid to the problem of separation of basic amino acids especially at low potential gradients. Jirgl in 1964 (18) tried to solve this problem by means of low-voltage paper electrophoresis at 7 volts/cm., using a tetraborate electrolyte of pH 12.0. Good separation of histidine, lysine, arginine (18) or histidine, ornithine and arginine (19) was obtained, but lysine and ornithine had similar mobilities. However, by using a sodium bicarbonate elctrolyte at pH 7.4-7.5, Jirgl (20) succeeded in 1965 in achieving a separation and a quantitative determination of all these basic amino acids and of citrulline.

Since cucurbitin is a basic amino acid, both the tetraborate electrolyte at pH 12.0 (18) and the sodium bicarbonate buffer at pH 7.4-7.5 (20) were used in this investigation to study cucurbitin and compare its electrophoretic behavior with that of the known basic amino acids.

Reagents:

1. a. Buffer pH 12.0 (18).

Sodium tetraborate 0.05M (9.75 gm. in 500.0 ml. water)	250.0 ml.
Sodium hydroxide 0.2M (4 gm. in 500.0 ml. water)	215.0 ml.
Water	35.0 ml.

b. Neutralizing solution.

Acetone	40 ml.
Glacial Acetic Acid	10 ml.
Formic Acid 85%	10 ml.

2. a. Buffer pH 7.4 (20).

Sodium bicarbonate	6.72 gm.
Water up to	1000.0 ml.

b. Neutralizing solution.

Same as 1b.

3. Ninhydrin Solution (Detecting Solution)

Ninhydrin	0.5 gm.
Acetone	100 ml.

Procedure:

Sheets of Whatman No. 3MM chromatographic paper, measuring 30 X 17.5 cm. were used. The paper was first wetted with the buffer, the excess buffer removed, and the paper equilibrated for 1 hour at 75 volts in a Thomas Electrophoresis Cabinet, Model 20.*

The sample was then spotted and the electropherogram

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* made by Arthur Thomas & Co., Philadelphia, Pa., U.S.A.

developed with the appropriate buffer at 210 volts i.e., 7 volts/cm.

After 120 min. of separation, the electropherogram was withdrawn from the cabinet and dried at about 90° C. for 20 min. It was then passed through the appropriate neutralizing solution and again dried at 90° C. for about 5 minutes. For the detection of the amino acids, the electropherogram was sprayed with the ninhydrin solution. It was first dried in the air, then in the oven at 90° C. Maximum color development was achieved after about 2 minutes of drying in the oven.

IV. Gel Filtration of the Proteins, Peptides and Amino Acids of Pumpkin Seed Extracts

The name 'Sephadex'* describes a group of modified dextrans of microbial origin. Because of the abundant hydroxyl groups present in the reticulated polysaccharide structure, 'Sephadex' strongly swells in water and aqueous solutions, forming swollen gels that are able to sieve or discriminate against the penetration into the gel particles of molecules above a certain size. Generally this size is correlated with molecular weight. Large molecules such as proteins do not enter the gel phase and rapidly emerge in the effluent, while smaller molecules such as amino acids diffuse into the gel particles

* made by Pharmacia, Upsala, Sweden.

and thus migrate at a slower rate than the large ones which become eluted first (37,41).

Technique of Gel Filtration

1. Material used

The dextran gel used was Sephadex G-25, coarse grade, with an exclusion limit of about 5000. It has a particle size of 100-300 microns and water regain of 2.4 ml. of water per gm. of dry material.*

2. Preparation of the Gel Column

Meticulous preparation of the chromatographic bed is necessary if a satisfactory flow rate is to be obtained. (14,41).

a. Preparation of the Gel

The dry 'Sephadex' powder was suspended in 0.1% sodium chloride solution and the suspension stirred for a few minutes to allow it to swell and to prevent clump formation. After a sedimentation time of half to one hour, the fines remaining in the supernatant were removed by decantation. The procedure was repeated five times.** The volume of salt solution used was such that the ratio of supernatant to sediment was at least 10 to 1 (14).

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* Obtained through the courtesy of Dr. Elias Awad, Chemistry Department, School of Arts and Sciences, American University of Beirut.

** Sephadex G-25 swells fairly rapidly, final equilibration being reached within a few hours.

b. Packing of the Column

The column used consisted of a cylindrical glass tube 1.7 cm. in diameter and 70 cm. in length.

The column was set up on a level surface and perfectly aligned vertically. A small piece of glass wool was laid over the outlet and above it a 2 cm. layer of glass beads. The top of the column was connected to a 500-ml. funnel.

The complete assembly was filled with water and the gel slurry was introduced into the funnel. The slurry was mechanically stirred as it was fed slowly into the water column. As the sephadex settled slowly, the outlet was opened to allow a flow rate of 5 to 10 ml. per minute. During the packing, a rising horizontal surface of packed material was considered evidence of good packing. To let the bed stabilize it was percolated overnight with the eluant to be used (14,41).

3. Gel Filtration

Most of the liquid over the bed was removed and the sample to be filtered was carefully added to the column, dropwise. The flow was started and the sample allowed to enter the bed. At the moment the sample disappeared into the column a few ml. of eluant was added to wash the surface. Finally the space above the bed was filled with the following eluate and elution of the column began. The eluant had the following composition:

1. Buffer pH 6.86*. Molarity 0.025

Potassium dihydrogen phosphate	3.402 gm.
Disodium hydrogen phosphate	3.549 gm.
Water up to	1000.0 ml.

To this buffer solution a sufficient amount of sodium chloride was added to make it 0.2M with sodium chloride.

The eluates were collected in 5-ml. fractions. The rate of elution with the buffer was adjusted so that 20 ml. fractions were collected every 10 minutes.

All experiments were performed at room temperature.

V. Ion Exchange Chromatography of the Basic Amino Acids of Cucurbita Species and the Use of an Amino Acid Analyzer

In ion exchange chromatography, separations are effected by virtue of the formation of reversible heteropolar chemical bonds between the solid stationary phase and the ionic constituents of the sample. Ion exchange chromatography can be used to separate ionic compounds from non-ionic substances and also to fractionate ionic compounds into classes having the same type and number

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* Jenkins, G.C., J.E.Christian and G.P. Hager, 1953. Quantitative Pharmaceutical Chemistry, 4th. edition, McGraw-Hill, N.Y. p. 441

of ionizable groups. The rate of migration of a compound is determined by its net charge, i.e., the sum of the products of the numbers of ionized groups and their respective dissociation constants. Thus it is possible to fractionate a mixture of ionic compounds by proper adjustment of the pH and the ionic strength of the developing solvent.

Methods of ion exchange chromatography for the resolution of mixtures of amino acids and related compounds were developed by Moore and Stein (28,29,34). These methods have been further refined and adapted to an apparatus which automatically records, with quantitative precision, the different components of the mixture as they are eluted from the column (30,45).

This method was used in the present investigation for a dual purpose:

a. for detecting cucurbitin - by determining the sequence in which it appears in the eluate with respect to other amino acids and, under a standard set of conditions, the time interval after which it appears in the eluate.
b. for determining cucurbitin quantitatively in certain species of Cucurbita.

Technique of Ion-Exchange Chromatography Using the Automatic Amino Acid Analyzer

Column chromatography using sulfonated polystyrene

cation-exchange resin (30) with the automatic recording method of Spackman, Stein and Moore (45) was used. The separations were carried out in a Phoenix Amino Acid Analyzer, Model K-5000*. The specifications for the resin, length of column, buffer, ninhydrin reagent, flow rates, and foreruns were the same as those prescribed by Spackman et al. (45), except that 0.1 ml. of octanoic acid per liter of buffer (34) was substituted for the phenol originally used by Spackman as a preservative.

Separations were carried out on the 50 X 0.9 cm. resin column of Amberlite IR-120. The resin particles were separated according to the method of Hamilton (16) and packed as specified (34).

The following buffer, pH 4.26, was used to elute the amino acids from the column:

Sodium Citrate Buffer 0.38N pH 4.26

Citric Acid ($C_6H_8O_7 \cdot H_2O$)	266.0 gm.
Sodium Hydroxide (97%)	156.0 gm.
Conc. Hydrochloric Acid	153.5 ml.
Octanoic Acid	1.0 ml.
Brij-35 Solution **	20.0 ml.
Distilled water up to	10.0 liters.

* Made by Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A., used through the courtesy of Dr. Peter L. Pellett, Department of Food Technology and Nutrition, Faculty of Agriculture, American University of Beirut.

** A brand of polyoxyethylene fatty alcohol ether.

The ninhydrin reagent had the following composition:

Ninhydrin Reagent:

Methyl Cellosolve (Peroxide free)	3 liters
Sodium Acetate Buffer 4N (see below)	1 liter
Ninhydrin	80 gm.
Stannous chloride	1.5gm.

These were dissolved under nitrogen. The details of the procedure are described by Moore and Stein (30).

Sodium Acetate buffer 4N, pH 5.51 ± 0.03

Sodium Acetate. $3H_2O$	1.088 gm.
Glacial Acetic acid	200 ml.
Water up to	2 liters.

The buffer was supplied to the column by a non-corrosive precision metering pump at a constant rate of 30 ml. per hour. The ninhydrin reagent was supplied by another pump at the rate of 15 ml. per hour.

The column effluent and ninhydrin reagent were combined in a continuously flowing stream through a fine Teflon capillary tube. The capillary tube was immersed at 100° C. in the bath. It was long enough to allow the amino acids to remain there during the 15 to 20 minutes necessary for the reaction between the amino acids and the ninhydrin to develop its maximal color intensity. The diameter of the capillary was small enough to prevent remixing of the amino acids as they emerged in sequence from the column. The effluent from the reaction capillary entered the vertical tubular flow cell of a

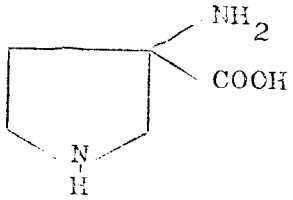
three-channel photometer, where the color was monitored successively at 570, 440, and 570 mμ. The effective fluid depth of the flow cell in the last 570 mμ channel was reduced by an internal glass spacer to approximately one third that of the first 570 mμ channel to enable a greater range of color to be monitored. The change in color detected in each channel was displayed on the moving chart of a three-point strip-chart recorder (27,34). A complete run on the 50 cm. column lasted 22 hours.

CHAPTER IV

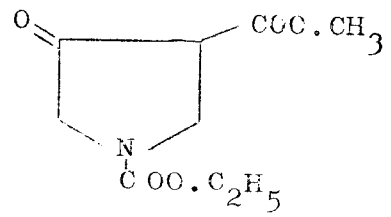
SYNTHESIS OF CUCURBITIN

It was realized, very early in the investigation, that it was necessary to have a reference sample of pure authentic cucurbitin with which to compare natural cucurbitin when the latter is extracted or is to be located on the chromatograms. Since none was available, it was necessary to synthesize it.

Sun *et al.* (46) had shown that cucurbitin was 3-amino-3-carboxyl pyrrolidine (I). They synthesized it (46) using 1-carbethoxy-3-carbomethoxy-4-pyrrolidone (II)

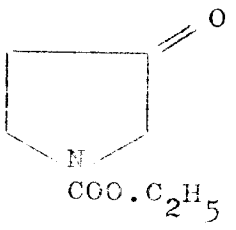


(I)



(II)

as the starting material for the synthesis. Degradation of 1-carbethoxy-3-carbomethoxy-4-pyrrolidone (II) with dilute hydrochloric acid gave 1-carbethoxy-3-pyrrolidone (III). The latter was treated with ammonium chloride and potassium cyanide in aqueous methanol.



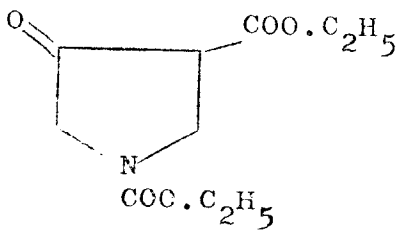
(III)

The organic layer was extracted from the reaction mixture, and was then hydrolyzed with hydrobromic acid to give (±) -3-amino-3-carboxylpyrrolidine (I).

In the present synthesis, N-carbethoxy glycine ethyl ester (IV) was synthesized according to the method of Fischer and Otto (13) from glycine ethyl ester hydrochloride and ethyl chloroformate. This compound was then condensed with ethyl acrylate according to the method of Kuhn and Osswald (24) to yield 1,3-dicarbethoxy-4-pyrrolidone (V). The latter was then used for the synthesis of cucurbitin according to the method of Sun et al. (46).

$CH_3.CH_2.COO.NH.CH_2.COO.C_2H_5$

(IV)



(V)

EXPERIMENTAL

Synthesis of Carbethoxyglycine Ethyl Ester (IV).

A solution of 25 gm. of glycine ethyl ester hydrochloride in 25 ml. of distilled water was strongly chilled and 18.0 ml. of 10N aqueous sodium hydroxide solution and 20 gm. ethyl chloroformate were slowly added into it in an ice-cooled flask. The mixture was stirred continuously while a solution of 10 gm. of anhydrous sodium carbonate in 50 ml. of water was gradually poured in. When carbon dioxide liberation had stopped, the mixture was transferred to a separatory funnel and the upper layer separated and dried with anhydrous sodium sulfate. The oily layer was filtered over anhydrous sodium sulfate and purified by distillation at 90° C. and 1.75 mm.Hg. The distillation lasted about two hours. The ester, weighing 11 gm., crystallized in the cooled receiver in monoclinic prisms, which melted at 26° - 27° C. (value reported in the literature 27° - 28° C.) (13).

Synthesis of 1,3-Dicarbethoxy-pyrrolidone-4 (V).

In a 300 ml. round bottom flask containing 150 ml. sodium-dried benzene, 10.75 gm. of N-carbethoxy glycine ethyl ester and 1.4 gm. of granulated sodium (see below) were added, followed by 6.5 gm. of acrylic acid ethyl ester. The exothermic heterogeneous reaction mixture,

became self-heated and gelatinous, and acquired a blue tinge. It was left to stand at room temperature with continuous shaking for half an hour. The flask was then fitted with an efficient reflux condenser protected from the air by means of a calcium chloride guard tube, and was refluxed for one hour. To remove any remaining free sodium, 1 ml. of absolute ethanol was added. The reaction mixture was transferred to a separatory funnel and extracted with 100 ml. of ether with vigorous shaking. Three hundred ml. of ice-water was added and the mixture shaken again. The aqueous layer was separated and the organic colorless phase was shaken twice with 100 ml. of ice-water and the washings combined with the aqueous layer. The combined aqueous liquids were washed with 100 ml. ether, and then poured over a mixture of 6 ml. concentrated sulfuric acid and 100 gm. ice. The resulting reddish-brown oil was made to separate from the aqueous solution with saturated sodium chloride solution, and was then extracted with five 100 ml. portions of chloroform until no red-violet color was obtained with ferric chloride test solution. The combined chloroform extracts were washed with 50 ml. cold saturated sodium chloride solution containing 0.25 gm. sodium bicarbonate. Finally, the oil was dried with anhydrous sodium sulfate and filtered through anhydrous sodium sulfate twice. The chloroform was distilled under vacuum. The residue, a

reddish-brown thick oil, was fractionated under high vacuum at 225° - 240° C. and 1.2 mm. Hg. The compound (V) distilled over and solidified in the cooled receiver as a white crystalline mass. It weighed 2.6 gm. Its semicarbazone was prepared as described below and was crystallized from ethanol yielding small needles which melted at 204° - 205° C. (reported value 208° - 209° C.) (24).

a. Preparation of Granulated Sodium (51).

Five grams of clean sodium (most conveniently weighed under sodium-dried ether after the surface coating had been cut out with a knife) was introduced into a 125-ml. round bottom pyrex flask containing about 100 ml. of sodium-dried xylene sufficient to cover the sodium completely. The flask was fitted with a reflux condenser, to reduce the danger of fire, and was placed on a sand bath supported on a ring. The sand bath was heated cautiously and the "ring" of condensed vapor of the xylene was carefully watched. When the sodium began to melt, the flame was extinguished. The flask was quickly stoppered, completely wrapped in a thick towel, and shaken vigorously for 30-60 seconds. The content of the flask was then cooled to room temperature. The sodium was thus obtained in the form of small spheres whose size was controlled by the duration and speed of the shaking.

b. Preparation of the Semicarbazone (42).

The semicarbazone reagent was prepared by dissolving 1 gm. semicarbazide hydrochloride and 1.5 gm. sodium acetate (anhydrous) in 10 ml. of ethanol. About one mg. of the compound whose semicarbazone derivative was to be prepared, was dissolved in 0.5 ml. alcohol in a test tube and 5 ml. of the freshly prepared semicarbazide reagent was added. The mixture was vigorously shaken, and the test tube was placed in a beaker of boiling water for 10 minutes. The tube was then placed in the refrigerator. Crystals of the semicarbazone of the compound settled down, were collected, dried and their melting point determined.

Synthesis of 1-carbethoxy-pyrrolidone-4 (III).

A mixture of 2.5 gm. of 1,3-dicarbethoxy-pyrrolidone-4 (V) and 15 ml. concentrated hydrochloric acid was heated for about 15 minutes over a steam bath. Then the HCl was removed *in vacuo*. The oily residue was taken up with three 30 ml. portions of chloroform and the combined chloroform solutions washed successively with three 20 ml. portions of water and three 10 ml. portions of concentrated solution of sodium bicarbonate. After drying over sodium sulfate, the chloroform was distilled *in vacuo*. The remaining orange-brown colored oil (1.5 gm) was fractionated at 122° - 132° C. and 12 mm. Hg.

The compound distilled over as a colorless oil. A yield of 0.8 gm. was obtained.

The semicarbazone of the product was prepared in dioxane and recrystallized from the same solvent. It melted at 175° C. (value reported by Kuhn and Osswald (24) was 178° - 179° C.).

Synthesis of (±)-3-Amino-3-carboxylpyrrolidine or Cucurbitin (I).

1-carbethoxy-3-pyrrolidone (III) (0.72 gm.), in 1.2 ml. methanol, free of acetone, was mixed with 0.31 gm. potassium cyanide and 0.30 gm. ammonium chloride dissolved in lukewarm water, in a 50-ml. Erlenmeyer flask fitted with a ground glass stopper. The flask was carefully stoppered by greasing the stopper and keeping it in place by the use of an adhesive tape. The content of the flask was stirred with a magnet stirrer and maintained at 45° - 50° C. for 4 hours. It was then extracted with three 10-ml. portions of benzene. The benzene was removed under vacuum leaving an oil (0.35 gm.), which was refluxed with 3 ml. of 48% hydrobromic acid for 3 hours. The hydrobromic acid was distilled off under reduced pressure. A solid separated which was dissolved in 0.5 ml. warm water. The solution was mixed with 5 ml. of 95% ethanol. After allowing the mixture to stand in a refrigerator overnight, (±)-3-amino-3-carboxypyrrolidine

hydrobromide (0.28 gm.) crystallized out.

An aqueous solution of the hydrobromide of cucurbitin was passed through an Amberlite CG-45 (Type I, OH^- form, 100-200 mesh) column measuring 20 X 1.5 cm. The column was eluted with water until the effluent gave a negative test with ninhydrin. It was concentrated nearly to dryness on a flash evaporator,* at 50° C., under vacuum . The residue was dissolved in 10 drops of water, treated with 10 times its volume (5 ml.) of ethanol. The alcoholic solution was subsequently treated with perchloric acid (60%) until the pH of the solution became 5. Cucurbitin perchlorate (0.24 gm.) crystallized out upon cooling in the refrigerator. A further crop of cucurbitin perchlorate was obtained on evaporation of the mother liquor.

Cucurbitin perchlorate decomposed above 270° C. without giving a sharp melting point. (reported value: above 275° C. (11) or 280° C. (46)).

The structure of cucurbitin perchlorate was confirmed by infrared spectroscopy. The IR spectrum of the synthesized cucurbitin perchlorate was taken in Nujol (liquid paraffin), using a Unicam Ultraviolet Spectrophotometer SP. 800** (See Fig.1). Absorption bands at

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* made by Buchler Instruments, N.J., U.S.A.

** Used through the courtesy of Dr. D. McLaren, Department of Clinical Nutrition, Faculties of Medical Sciences, American University of Beirut.

1640 cm^{-1} , 3100 - 3115 cm^{-1}, and 2500 -2550 cm^{-1} , corresponding to the carboxyl, the priamry amino and the secondary amino groups, respectively, were obtained, which is in agreement with the data given by Sun *et al.* (46) and Fang *et al.* (11).

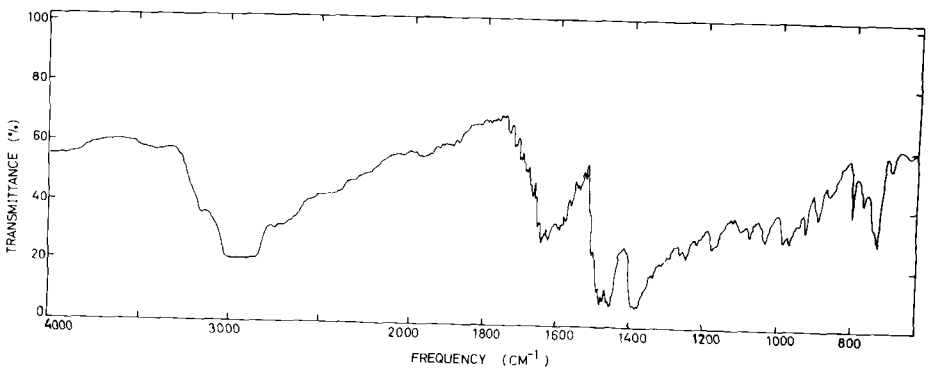

Figure 1.- Infrared spectrum of cucurbitin.

Cucurbitin, the free base, was easily obtained by passing a solution of cucurbitin perchlorate in water, through an Amberlite CG-45 (Type I, OH^- form, 100-200 mesh) column measuring 20 X 1.5 cm. The effluent was evaporated to dryness, using a flash evaporator. The cucurbitin was further dried in a vacuum desiccator

oven* at 40° C. for 5 hours, and stored in a desiccator.

The following chemicals were used in the synthesis of cucurbitin:

Acrylic acid ethyl ester (Fluka), ammonium chloride (Merck), ethyl chloroformate (Merck), glycine ethyl ester hydrochloride (Fluka), hydrobromic acid (Riedel-Dehaen), perchloric acid (Merck), potassium cyanide (May & Baker), semicarbazide hydrochloride (K. & K. Laboratories), sodium metallic (Fischer), sodium acetate (BDH-Analar grade).

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* Heated Vacuum Desiccator, Precision Scientific Co., Chicago, U.S.A.

CHAPTER V

NATURAL CUCURBITIN

EXTRACTION AND CHARACTERIZATION

The isolation of natural cucurbitin from defatted *Cucurbita moschata* Duch. seeds was reported by Fang *et al.* in 1961 (11). A total free amino acid extract was obtained by passing an aqueous extract through a cation-exchange resin and eluting the amino acids adsorbed on the resin with ammonia. According to these authors, cucurbitin precipitated as a perchlorate salt when a concentrated total extract was treated with perchloric acid. Essentially the same method of isolation was used in the present work. It was found, however, that cucurbitin perchlorate did not precipitate in a pure form; a few amino acids in relatively small amounts, came down with it as an impurity. Therefore, a mixed melting point of the natural cucurbitin perchlorate with synthetic cucurbitin perchlorate could not be taken. To characterize natural cucurbitin it was therefore necessary to resort to other methods of identification. This resulted in the establishment of a here-to-fore unpublished data on cucurbitin. Among the methods used in the present investigation for the characterization of natural cucurbitin, the following may be mentioned:

paper chromatography, thin-layer chromatography, cellulose phosphate ion-exchange chromatography, electrophoresis, and examination in an automatic amino acid analyzer. See Chapters III & VII.

Experimental

The plant material used in the preparation of natural cucurbitin consisted of the seeds of *Cucurbita maxima* cv. 'Chinese Snow-White' which is available on the Beirut market and is claimed by the merchants to be active as a taenifuge.

Defatting of Seeds:

Three hundred forty seeds weighing 113.5 gm. were decorticated by a laboricus separation of the testa from the seeds. The kernels weighed 90 gm. These were finely ground and divided into two equal parts. Each part was defatted with petroleum ether, b.p. 30-40° C. for 20 hours. The petroleum ether extract was evaporated to constant weight at 105° C. The fat content of the seeds averaged 46.61% calculated on the basis of the air-dried decorticated kernels. This defatting method was also used for the determination of the fat content of other seeds. See Chapter VII.

Preparation of natural cucurbitin

Twenty five grams of the decorticated and defatted seeds were first extracted with 200 ml. of water at

50° C. with constant stirring for about 4 hours, the mixture was centrifuged, the supernatant reserved, and the residue extracted in a similar manner, with three 100 ml. portions of water at 50° C. for about 40 minutes each time. The combined supernatants and washings measured 500 ml. The turbid liquid was clarified by adding to it 500 ml. of 95% alcohol to precipitate the suspended protein material and the whole kept in the refrigerator overnight.

The following day, the mixture was centrifuged and the clear supernatant was collected. The alcohol was distilled off under reduced pressure, at 40° C., using a flash evaporator. The aqueous solution was passed through a column of Dowex 50W-X8 (H^+ form, 200-400 mesh) measuring 75 X 2.2 cm. The column was washed with 200 ml. of water, and then eluted with 1% ammonium hydroxide solution, until the effluent gave a negative test with ninhydrin. The eluate was evaporated to dryness under reduced pressure, at 50° C., using a flash evaporator. The syrupy residue, measuring 0.2 ml., was dissolved in the same flask in twice its volume of warm water and to this 6 ml. (10 times its volume) of 95% alcohol was added. The solution was then treated with 60% perchloric acid, drop by drop, until the pH of the solution became 5. Cucurbitin perchlorate precipitated on allowing the flask to stand in the refrigerator for 2 days.

The perchlorate was dissolved in water, and the solution passed through a column of Amberlite CG-45 (Type I, OH^- form, 200-400 mesh), measuring 20 X 1.5 cm. Evaporation of the eluate, under reduced pressure, gave cucurbitin.

CHAPTER VI

PREPARATION OF SEED AND PULP EXTRACTS FOR ANALYSIS

A. Preamble and Summary

Free amino acids are markedly hydrophilic compounds which dissolve only slightly in non-aqueous solvents. Furthermore they are also amphoteric.

Aqueous extracts of plant tissues generally contain, in addition to free amino acids, peptides, proteins, carbohydrates, salts and emulsified lipids.

The presence of salts in amino acid solutions render difficult the preparation of paper chromatograms that can be easily interpreted. The effect of salts has been described by Consden and Gordon (8). Desalting of amino acid solutions by a suitable ion exchange resin (4,35,40) is applied routinely prior to quantitative estimation of amino acids. Occasionally, desalting by means of an ion-exchange resin results in losses (9): for example, arginine, and to some extent lysine, are not retained by strongly basic resins; on the other hand, they are incompletely eluted from acid resins.

Proteins and polysaccharides may be precipitated by addition of organic solvents: alcohol (50) and acetone

are mostly used, as they are easily removed after the process. Proteins (albumins) are also coagulated by warming to 60° C., or higher. Furthermore, proteins, unlike amino acids, are only slightly retained on highly-cross-linked ion exchange resins of the strong base or the strong acid type.

Gel filtration with Sephadex (36,38,47) is a new method for the separation of high molecular from low molecular weight compounds. Sephadex is an insoluble cross-linked dextran which greatly swells with water. Large molecules are excluded from entering the gel particles while smaller molecules or inorganic salts diffuse without hindrance into the gel particles. If an aqueous solution of a mixture of such substances is passed through a Sephadex column, the larger molecules will be eluted first. Smaller molecules are retained for some time and can be eluted quantitatively with more water or a dilute salt solution.

In the preparation of aqueous extracts for paper and thin-layer chromatography, the protein was excluded by extracting with 75% ethyl alcohol. After evaporation of the alcohol, the aqueous liquid was passed through a column of sulfonated polystyrene resin (Dowex 50W-X8, H^+ form), which was subsequently thoroughly washed with water. All the ninhydrin-positive material was held by the column. The amino acids were then displaced from the

column by 2N ammonium hydroxide solution without fractionation and the eluate was concentrated by evaporation *in vacuo* at 50° C., to remove the ammonia.

In studying the free amino acids, notably the basic ones on the *Automatic Amino Acid Analyzer*, a protein-free extract of the seed was prepared. Finely ground decorticated and non-defatted seeds were extracted with 10-fold their amount of 1% picric acid, according to the method of Hamilton (15). The picric acid precipitate was removed by centrifugation. The excess picric acid in the protein-free extract was removed by passing the extract through a Dowex 2-X8 column. The colorless filtrate was concentrated and adjusted to pH 2 with 1N hydrochloric acid.

For the *electrophoretic* studies of the basic amino acids, the protein-free extracts obtained by precipitating the protein with picric acid as described above were used.

For chromatography on *ion-exchange cellulose phosphate papers*, which combines the advantages of the use of ion-exchange resins and paper chromatography, prior desalting of the extracts was not necessary. Aqueous protein free extracts were prepared by precipitating the soluble proteins with picric acid, and using the aqueous extract of the total free amino acids for analysis.

To find out whether or not cucurbitin was found in

the water-soluble peptides and proteins, the aqueous extracts were passed through a Sephadex G-25 column. The eluate, containing the soluble peptides and proteins, was concentrated to a small volume and the soluble peptides and proteins, was concentrated to a small volume and the soluble peptides and proteins hydrolyzed with 6N hydrochloric acid for 24 hours at 110 ± 1° C. Cucurbitin was not affected by this treatment. The hydrolyzate, after suitable treatment, was then ready for chromatographic analysis.

To find out the best extraction method which will extract all the free cucurbitin found in the seed, extracts were prepared from the defatted Cucurbita maxima cv. 'Chinese Snow-White', using water at room temperature, water at 50° C., and alcohol 60%, and stirring the suspension continuously for 24 hours. After centrifuging, washing the residue, and again centrifuging, the operation being repeated for two more times, the combined washings and supernatant were concentrated in vacuo and the extracts examined. Extraction of the residues with the same solvents was continued for another 24 hours and the combined washings and supernatant were again examined. To find out whether or not cucurbitin was found in the bound form in the residual protein, the residue was hydrolyzed as indicated above, the acid subsequently removed in vacuo at 50° C. The aqueous

extract of the hydrolyzed protein was then examined for the presence of cucurbitin.

It was found that cucurbitin was best extracted from the decorticated and defatted seed with water at 50° C. for 24 hours. Subsequently, all the cucurbitaceous seeds examined were first defatted and then extracted with water at 50° C. for 24 hours.

B. Experimental

Preparation of the Amino Acid Fraction for Paper and Thin-Layer Chromatography.

1. Extraction of the Amino Acids from Seeds:

One gram of finely ground decorticated and defatted seeds in a 50-ml. Erlenmeyer flask was shaken continuously with 25 ml. of alcohol 75%, for 24 hours, using a Wrist Action Shaker. The clear supernatant extract obtained after centrifuging was distilled *in vacuo* at 50° C. using a flash evaporator. The aqueous solution was applied to a small column of Dowex 50W-X8 (H^+ form, 200-400 mesh) measuring 10 X 1.7 cm., to retain the amino acids; after thorough washing with 50 ml. of water, the amino acids were eluted from the column with 2N ammonium hydroxide solution until the eluate was ninhydrin-negative. About 25 ml. of 2N ammonium hydroxide solution was used. The amino acid fraction was then

concentrated *in vacuo* to 5 ml. using a flash evaporator at 50° C. For spotting on paper and on thin plates 50 µl and 30 µl of this solution were used respectively.

2. Extraction of Amino Acids from "Leoseed"* Extract.

One ml. of "Leoseed" was diluted with 19 ml. of alcohol 50%, mixed thoroughly, and centrifuged. The alcohol from the supernatant was distilled off, and the aqueous solution was applied to a Dowex 50W-X8 column (H^+ form, 200-400 mesh) measuring 25 X 1.7 cm. to retain the amino acids. After thorough washing with 100 ml. of water, the amino acids were eluted with 2N ammonium hydroxide solution as outlined above. The amino acid fraction was concentrated to 10 ml. For spotting on paper, 25 µl, and on thin plates 10 µl were used.

Preparation of the Amino Acid Extracts for Extracts for Examination on Ion-Exchange Paper Chromatography.

A. Extraction of Cucurbitin and the Free Amino Acids from Seeds.

Separate 0.5 gm. quantities of powdered decorticated and defatted seed of *Cucurbita maxima* cv. 'Chinese Snow-White' were extracted separately with 25 ml. of water at room temperature, 25 ml. of water at

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* A proprietory preparation sold in Lebanon known since 1954 as "Leo's Pumpkin Seed Extract".

50° C., and with 25 ml. of ethyl alcohol 60%, for 24 hours with continuous stirring with a magnetic stirrer in a 50-ml. Erlenmeyer flask. The supernatants were removed by centrifugation. Each residue was washed with three 15-ml. portions of water or alcohol 60%, centrifuging after each addition. The supernatant and washings from each set were combined together and reduced to a volume of 5.0 ml., by evaporating the solution under reduced pressure at 50° C.

The residue in each case was extracted by the same method for another day. The supernatant and washings from each set were combined and reduced to a volume of 5.0 ml.

When the extracts were chromatographed on ion-exchange Cellulose Phosphate Paper P-20, measuring 57 X 23 cm., in quantities of 10-20 microliters and the extraction with the three solvents compared it was found that water at 50° C. was the best solvent for extracting cucurbitin from the defatted seed. It was also found that extraction for 24 hours at 50° C. was sufficient to extract all the cucurbitin.

B. Preparation of the Seed Protein Hydrolyzate

The washed residues were dried and transferred separately to 20 ml. pyrex glass ampuls each containing 12 ml. of 6N hydrochloric acid. The ampuls were sealed and placed in an oven maintained at 110 ± 1° C.,

for 24 hours to hydrolyze the proteins.

At the end of 24 hours, the ampuls were removed from the oven, left to cool and their contents evaporated separately in the flash evaporator to dryness. One ml. of water was added to each residue and the mixture evaporated to dryness. This was repeated twice to get rid of all traces of hydrochloric acid. The brown residue was dissolved in 3 ml. of water, the solution was clarified with activated charcoal (BDH), filtered and made up to 5.0 ml. with water.

1. Ion-exchange Cellulose Phosphate Paper Chromatography:

Ten to 50-µl quantities of the aqueous extracts were chromatographed on ion-exchange Cellulose Phosphate Paper P-20 sheets as described in Chapter III, p. 17.

2. Results:

No cucurbitin could be detected on the chromatograms of the residual protein hydrolyzates. False results were obtained when the residue was not washed properly.

C. Extraction of Cucurbitin and the Free Amino Acids from the Pulp of Cucurbita maxima.

Two grams of the finely ground dried pulp, representing 40 gm. of the pulp, was extracted with 40 ml.

water at 50° C. for 24 hours, with continuous stirring with a magnetic stirrer, in a 125-ml. Erlenmeyer flask. The supernatant was removed by centrifugation. The residue was washed with three 20-ml. portions of water, centrifuging after each addition. The supernatant and washings were combined together and reduced to a volume of 1 ml. For examination on ion-exchange cellulose phosphate paper (as described in Chapter III, p. 17.), 50-100 µl. portions were chromatographed.

Preparation of the Amino Acid Fractions for Examination on Paper Electrophoresis, and in the Amino Acid Analyzer.

Two grams of finely ground decorticated seeds in a 50-ml. Erlenmeyer flask was extracted at room temperature with 25 ml. of 1% aqueous picric acid solution for 24 hours on a shaker. The picric acid precipitate was removed by centrifugation. The excess picric acid in the supernatant was removed from the protein-free extract by passing the solution through a Dowex 2-X8 (Ionic form Cl^-, 20-50 mesh) column* measuring 4 x 1.7 cm. The colorless eluate was reduced to 2 ml. under reduced pressure and enough water and 1N hydrochloric acid were

* In preparing the column, the finer particles of the resin were removed by elutriation. The resin column was washed with 15 ml. of 1N hydrochloric acid then with water until the effluent was neutral.

added to bring its volume to 10.0 ml. and its pH to 2 ± 0.1.

For electrophoretic examination of seed extracts, 20-40 µl of the extracts were spotted.

For analysis using the automatic Amino Acid Analyzer a quantity equivalent to 0.15-0.25 mg. N was used.

Preparation of the Soluble Protein Fraction.

Three 0.5 gm. quantities of decorticated, defatted, seeds of Cucurbita maxima cv. 'Chinese Snow-White' were extracted with 25 ml. water at room temperature, 25 ml. of water at 50° C. and with 25 ml. of ethanol 60%, respectively, for 40 hours. The suspension was centrifuged and the supernatant was filtered twice through a sintered glass funnel of medium porosity. The alcohol, where used, was distilled off. The aqueous solutions, were made up to 25 ml. (30% of the column bed volume), and were passed through a column of Sephadex G-25. (See Chapter III, pp. 22-25). The column was subsequently eluted with 0.025M phosphate buffer, pH 6.86, containing 0.2M NaCl. (See Chapter III, p.25).

The eluates were collected in 5-ml. fractions, until no color with a solution of 0.1% ninhydrin in acetone was observed. The rate of elution with the buffer was adjusted so that 20-ml. fractions were collected every 10 minutes.

All experiments were performed at room temperature.

The fractions were tested for the presence of proteins and peptides by the following tests:

a. Modified Biuret Test

Coghill (5) reports the following modification of the biuret reaction by T.B. Osborne as affording a very sensitive biuret test for proteins and peptides.

In this method, one drop of 1% copper sulfate solution was added to 2 ml. of the solution to be tested. One ml. of ethanol and an excess of potassium hydroxide pellets were added. The latter dissolved and the saturated potassium hydroxide solution salted ethanol out of solution, which brought with it all the biuret color present. According to this method, less than 50 μg. of protein gives a distinct pink tinge to the ethanol layer.

b. Nigrosin Test

According to Morris and Morris (31), the dye solution is best made up as follows:

0.01% solution of nigrosin in:

Glacial Acetic Acid	10 ml.
Methanol	50 ml.
Water	50 ml.

For testing, samples from each tube were spotted on paper, which was dried and passed through the nigrosin solution. The excess of the dye was removed by washing with tap water, and the paper dried. The dark greyish-blue color of the spots, after drying the paper, was a

positive test for protein.

The fractions containing soluble proteins or peptides were examined separately, for presence of cucurbitin, by ion exchange cellulose phosphate paper chromatography.

The results of these tests are given in the following table:

Table I.- Fractionation of the Aqueous and Hydroalcoholic Extracts of the Seed of Cucurbita maxima cv. 'Chinese Snow-White' on Sephadex G-25 Column.

Extraction	Positive - Biuret Reaction Fractions	Positive - Nigrosin Test, Fractions	Positive Cucurbitin Fractions
Water at room Temp.	8 - 12	8 - 12	12
Water at 50° C.	7 - 11	7 - 11	10,11
Alcohol 60%	8 - 11	8 - 11	12

The contents of the tubes which showed a positive reaction for protein and peptide and a negative test for cucurbitin were combined, evaporated to dryness under reduced pressure and hydrolyzed with 6N hydrochloric acid as outlined for the hydrolysis of reserve protein (See p. 50), the hydrolyzate after proper treatment (See p. 51) was ready for examination.

Chromatography of the hydrolyzates of the soluble

peptides and proteins on cellulose phosphate ion-exchange papers in quantities of 20-40 µl. showed that cucurbitin was absent.

CHAPTER VII

ANALYTICAL DATA AND RESULTS

Table II.- Average R_f Values of Cucurbitin in One-Dimensional Whatman Paper No.1 Chromatograms as Determined Ascendingly.

No.	Solvent Systems*	R_f
1.	n-Butanol-Acetic Acid-Water (4:1:5)	0.18
2.	n-Butanol-Acetic Acid-Water (60:15:25)	0.12
3.	n-Butanol-Acetic Acid-95% Ethanol-Water (4:1:1:2)	0.20
4.	n-Butanol-Acetic Acid-Water (90:10:29)	0.13
5.	n-Butanol-Acetic Acid (4:1)	0.00
6.	Diethylamine (1%) in Ethanol (77%)	0.70
7.	Phenol-Water (3:1) in the presesence of Ammonia Vapors.	0.72
9.	n-Propanol-Concentrated Ammonium Hydroxide (7:3)	0.27

* See Chapter III, pp. 10-12.

Table III.- Average R_f Values of Cucurbitin on Two-Dimensional Whatman No. 3MM Paper Chromatograms.

No.	Solvent Systems*	R_f	Ratio of R_f (a) to R_f (b)
10.	a. Diethylamine (1%) in 77% Ethanol	0.70	$\frac{0.70}{0.26}$
	b. n-Propanol-Concentrated Ammonium Hydroxide (7:3)	0.26	
11.	a. n-Butanol-Acetic Acid-Water (60:15:25)	0.13	$\frac{0.13}{0.19}$
	b. n-Butanol-Pyridine-Water (1:1:1)	0.19	
12.	a. Phenol-Water (3:1) in the presence of Ammonia Vapors.	0.71	$\frac{0.71}{0.12}$
	b. n-Butanol-Acetic Acid-Water (90:10:29)	0.12	

* See Chapter III, p. 13.

Table IV.- Average R_f Values of Cucurbitin on One-Dimensional Cellulose (S. & S. 123) Thin-Layer Chromatograms.

No.	Solvent Systems*	R_f
15.	Methanol-Water-Pyridine (80:20:5)	0.20
16.	n-Butanol-Acetic Acid-Water (4:1:5)	0.17
17.	n-Propanol-Water (7:3)	0.23

* See Chapter III, p. 15.

Table V.- Average R_f Values of Cucurbitin on Two-Dimensional Silica Gel G Thin-Layer Chromatograms.

No.	Solvent System*	R_f	Ratio of R_f (a) to R_f (b)
18.	a. n-Butanol-Acetic Acid-Water (4:1:1)	0.12	$\frac{0.12}{0.20}$
	b. n-Propanol-Water (7:3)	0.20	

* See Chapter III, pp.15,16.

Table VI.- Average R_f Values of Cucurbitin on Two-Dimensional Cellulose (S. & S. 123) Thin-Layer Chromatograms.

No.	Solvent System*	R_f	Ratio of R_f (a) to R_f (b)
12.	a. Phenol-Water (3 : 1) in the presence of Ammonia Vapors.	0.80	$\frac{0.80}{0.14}$
	b. n-Butanol-Acetic Acid-Water (90:10:29)	0.14	

* See Chapter III, pp. 13.

Table VII.- Relative Ninhydrin Color Intensity of Cucurbitin Spots on Two-Dimensional Whatman No. 3MM Paper Chromatograms, as obtained with Solvent System No. 12* and 0.05 ml. of Seed Extract.**

No.	Plant	Relative Strength
	Cucurbita pepo L.	
1a.	cv. 'Yugoslavia'	+
1b.	cv. 'Chinese Shen-Skin'	+
1c.	cv. 'Bulgaria'	+
3a.	cv. 'Turkey'	++
	Cucurbita maxima Duch.	
2a.	cv. 'Chinese Snow-White'	++
4a.	cv. 'Ainjar globular'	+++
4b.	cv. 'Ainjar oblong'	+++
4c.	cv. 'Syria globular, large fruited'	+++

* See Chapter III, pp. 13-

** See Chapter VI, pp. 48-49.

Table VIII.- Average $R_{Leucine}$ Values of Cucurbitin and Other Basic Amino Acids on Ion-Exchange Cellulose Phosphate Paper P-20. Developing Buffer*: 0.2M, pH 2.1

Amino Acid**	$R_{Leucine}$
Leucine	1.00
Lysine	0.23
Cucurbitin	0.20
Ornithine	0.19
Arginine	0.18
Histidine	0.15

* See Chapter III, p. 19.

** Loading: 5 mcg. of basic amino acids, 7 mcg. of cucurbitin.

Table IX.- Average $R_{Histidine}$ Values of Cucurbitin and Other Basic Amino Acids on Ion-Exchange Cellulose Phosphate Paper P-20. Developing Buffer*: 0.2M, pH 9.2

Amino Acid**	$R_{Histidine}$
Histidine	1.00
Cucurbitin	0.92
Lysine	0.72
Ornithine	0.65
Arginine	0.63

* See Chapter III, p. 19.

** Loading: 5mcg. of basic amino acids, 10 mcg. of cucurbitin.

Table X.- Estimated Cucurbitin Content of *Cucurbita* Seeds, as Determined on Ion-Exchange Cellulose Phosphate Paper P-20 and 0.2M, pH 9.2 buffer*, in mg. of Cucurbitin per Gram of the Decorticated and Non-Defatted Kernel.

No.	Plant	Average Cucurbitin Content in mg.
	Cucurbita pepo L.	
1a.	cv. 'Yugoslavia'	2.5
1b.	cv. 'Chinese Shen Skin'	4
1c.	cv. 'Bulgaria'	3
3a.	cv. 'Turkey'	10
	Cucurbita maxima Duch.	
2a.	cv. 'Chinese Snow-White'	8
4a.	cv. 'Ainjar,globular'	19
4b.	cv. 'Ainjar,oblong'	25
4c.	cv. 'Syria, large fruited'	24
	Cucurbita moschata Duch.	
5a.	cv. 'Syria, crook-necked'	12
5b.	cv. 'Syria, globular'	6
	"Leoseed" **	
	Estimated cucurbitin content per bottle	1.5 gm.

* See Chapter III, p. 19.

** A proprietory preparation sold in Lebanon. It is marketed in one dose bottles each containing the equivalent of 500 gm. of decorticated pumpkin seeds in 30 ml. of extract (equivalent to some 700 gm. of whole seed).

Determination of Cucurbitin Content of Seed Extracts using Ion-Exchange Cellulose Phosphate Paper.

Five to ten microliters of the seed extract, prepared as outlined in Chapter VI, pp. 49-50, was spotted on ion exchange Cellulose Phosphate Paper P-20, and eluted with 0.2M, pH 9.2 buffer, (See p.19). The developed and visualized cucurbitin spots were compared on the same chromatogram with 30 and 40 mcg. quantities of synthetic cucurbitin which were developed simultaneously with those of the seed extract. The amount of cucurbitin in each seed extract was estimated visually and the result reported as mg. of cucurbitin per gram of the undefatted kernel of the seed. The average results of two or more determinations per each seed extract are given in Table X, as mg. of cucurbitin per gram of decorticated, non-defatted seed.

Distribution of Cucurbitin in the Fruit of Cucurbita species:

1. Seed extracts were prepared as described in Chapter VI, and examined for presence of cucurbitin. It is evident from Table XI that water-extracts of the seeds contained cucurbitin.

2. Hydrolysates of water-soluble peptides and proteins of the seeds, prepared as outlined in

Chapter VI, pp. 53-56, showed absence of cucurbitin.

3. Hydrolyzates of the residual proteins, prepared as described in Chapter VI, pp. 50-51, showed that cucurbitin was absent from the reserve seed protein.

4. Water extracts of the dried pulp, prepared as outlined in Chapter VI, pp. 51-52, were found to be free of cucurbitin, even when examined in the maximum possible concentration.

Table XI.- Data on the Distribution of Cucurbitin in the fruit of Cucurbita maxima.

Seed of C. maxima cv. 'Chinese Snow-White'.	
Cucurbitin in total water-soluble extract	Present
Cucurbitin in water-soluble peptides and proteins	Absent
Cucurbitin in reserve seed protein	Absent
Pulp of C. maxima cv. 'Ainjar globular' and cv. 'Ainjar oblong'.	
Cucurbitin in total water-soluble extract	Absent

Table XII.- Average Shell and Fat Content* of, and Cucurbitin Distribution** in, the Various Seeds of the Cucurbitaceae.

No.	Plant	Per Cent[a] Shell Content	Per Cent[b] Fat Cont.	Cucurbitin Distribution[c]
	Cucurbita pepo L.			
1a.	cv. 'Yugoslavia'	33.6	45.2	+
1b.	cv. 'Chinese Shen-Skin'	34.2	39.4	+
1c.	cv. 'Bulgaria'	34.0	37.0	+
3a.	cv. 'Turkey'	33.7	36.0	+
	Cucurbita maxima Duch.			
2a.	cv. 'Chinese Snow-White'	33.8	48.6	+
4a.	cv. 'Ainjar, globular'	20.7	47.2	+
4b.	cv. 'Ainjar, oblong'	22.5	45.2	+
4c.	cv. 'Syria, globular large-fruited'	31.5	31.5	+
	Cucurbita moschata Duch.			
5a.	cv. 'Syria, crook-necked'	29.7	40.0	+
5b.	cv. 'Syria, globular'	33.7	37.0	+
	Citrullus vulgaris Schrad.			
8a.	cv. 'China'	59.3	40.6	-
8b.	cv. 'Turkey'	59.4	40.1	-
8c.	cv. 'Lebanon'	62.6	43.3	-

No.	Plant	Per Cent[a] Shell Content	Per Cent[b] Fat Cont.	Cucurbitin Distribution[c]
	Cucumis melo L.			
9a.	cv. 'America, round'	38.0	47.1	-
9b.	cv. 'America, oblong'	34.9	48.6	-
	Cucumis sativus L.			
10a.	cv. 'America'	39.1	52.1	-
10b.	cv. 'Lebanon'	26.9	38.2	-
	Lagenaria leucantha Rusby			
6a.	cv. 'Lebanon'	49.1	45.1	-
	Luffa cylindrica (L.) M.Roem			
7a.	cv. 'Lebanon'	44.8	39.1	-

* Fat content as determined by the method outlined in Chapter V, p. 41.

** As screened by chromatography on Whatman ion-exchange cellulose phosphate papers P-20, as outlined in Chapter III, pp. 17-19.

[a] Calculated on the basis of the whole air-dry seeds.

[b] Calculated on the basis of the air-dry kernels.

[c] + = present; - = absent.

Quantitative Determination of Cucurbitin and Other Basic Amino Acids on the Phoenix Amino Acid Analyzer.

Determination of the Total Nitrogen Content of the Free Amino Acid Extract.*

Principle:

All the nitrogen in the extract was converted to ammonium sulfate by digestion with sulfuric acid at elevated temperature. The ammonium nitrogen was then quantitated photometrically by the Bertholet color reaction (17) namely by treating it with phenolnitro-prusside and alkaline hypochlorite reagents.** The blue color produced was compared with that obtained with a standard.

Procedure:

One half ml. of the extract was placed into each of two 50 ml. Kjeldahl digestion flasks. To each flask 1.0 ml. of 10N sulfuric acid solution and 2 glass beads were added. The flasks were heated carefully on a digestion rack, in the hood, until all the water had been removed and dense fumes of sulfur trioxide appeared. Each flask was then covered with a marble and digested

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* For the preparation of extracts, see Chapter VI, pp. 52-53.

** Supplied through the courtesy of Mr. A. Shahinian, Biochemistry Department, American University of Beirut.

for ten more minutes. The flasks were allowed to cool. To each, 5 ml. of water and 4 ml. of 2.5N sodium hydroxide solution were added to neutralize the sulfuric acid. The content of each flask was diluted with water to the 50-ml. mark and mixed by inversion.

One ml. from each flask was pipetted into each of two test tubes. One ml. of a standard solution containing 4 mcg. nitrogen per ml. was also placed into each of two other test tubes. Into another tube which was to serve as a blank, 1.0 ml. of distilled water was placed. To each one of the five foregoing tubes one ml. of phenol-nitroprusside reagent and 1ml. of sodium hypochlorite reagent were added and the contents mixed well. The tubes were incubated at 37° C. for 20 minutes. To each was then added 10 ml. of water and the tubes covered with parafilm and their contents mixed by inversion.

The absorbance at 630 mμ was determined, using Spectronic-20*, after adjusting the instrument to zero with water.

The quantity of nitrogen, in micrograms per ml. of extract, was calculated and the average value of four determinations per sample was recorded in Table XIII, p. 71.

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* Manufactured by Bausch and Lomb.

Table XIII.- The Total Nitrogen Content of Seed Extracts in mcg. Nitrogen per ml. of Extract Corresponding to 0.2 gm. of Kernels.

No.	Plant*	mcg. Nitrogen/ml.
	Cucurbita pepo L.	
1a.	cv. 'Yugoslavia'	225
1b.	cv. 'Bulgaria'	199
1c.	cv. 'Chinese Shen-Skin'	157
	Cucurbita maxima Duch.	
2a.	cv. 'Chinese Snow-White'	156

* See Chapter II, p.4.

Sample Fed to the Amino Acid Analyzer.

Depending on the nitrogen content of the extract, as determined above, a quantity of the extract containing the equivalent of 0.15 to 0.25 m g. of nitrogen, was fed to the Analyzer.

Operation of the Analyzer

(See Chapter III, pp.25-29).

Calculation of the Individual Amino Acid Content per Unit Volume of Extract.

The amount of each component amino acid in a sample analyzed by the Amino Acid Analyzer is determined

by measuring the area enclosed by its corresponding peak on the chromatogram. The height times width (HW) method of integration is rapid, satisfactorily accurate, and hence is used for the integration of the peaks (34).

The width is measured at half the height, in terms of time, by counting the number of dots printed above the half-height of the peak. The counting of the dots is facilitated by the fact that every fourth dot of each of the three curves is black.

Procedure:

The base line to 0.001 absorbance units was subtracted from the height of the peak, and then multiplied by half. Starting from the half height mark, and skipping the first black dot, the black dots were counted. To this figure the number of individual dots of the same color between the half height mark and the first black dot on both sides were added, the first black dot on the ascending side being included. To the value obtained the results of the estimations of the distance in tenths of dot separation between the half height marks on both sides and the first individual dot up the curve were added. The sum then obtained was the Width (W) of the peak.

From the formula $\mu M = \frac{H.W}{C_{H.W}}$, the constant ($C_{H.W}$) for cucurbitin was calculated by putting 1 μM concentration on the column and calculating the height times width.

By dividing the height times width value obtained by this constant, it was possible to find out the concentration of cucurbitin present in the extracts studied.

Amino Acid Analyzer Data on Cucurbitin and Certain Basic Amino Acids

Elution volume of cucurbitin has not been previously reported. The position of cucurbitin on the chromatogram obtained by using the Phoenix Amino Acid Analyzer was found to overlap the ethanolamine peak. (See Fig. 2). the higher 440 to 570 mμ color absorption ratio for cucurbitin (calculated to be 0.6) differs so significantly from that of other amino acids that it can serve as a characteristic for identification. Five membered imino acids have a greater absorption at 440 mμ than at 570 mμ. Cucurbitin having both an imino and an amino group shows an intermediate color absorption at 440 and 570 mμ. This generally provides an easy means of recognizing the compound when considered with its elution volume (See Table XIV).

Elution volumes of amino acids, reported by various investigators, presumably using almost identical equipment and conditions may vary. These variations may be the result of differences in column length and diameter, resin cross linkage and particle size, and rather subtle differences in flow rates. Thus markers with previously

reported elution volumes were run.

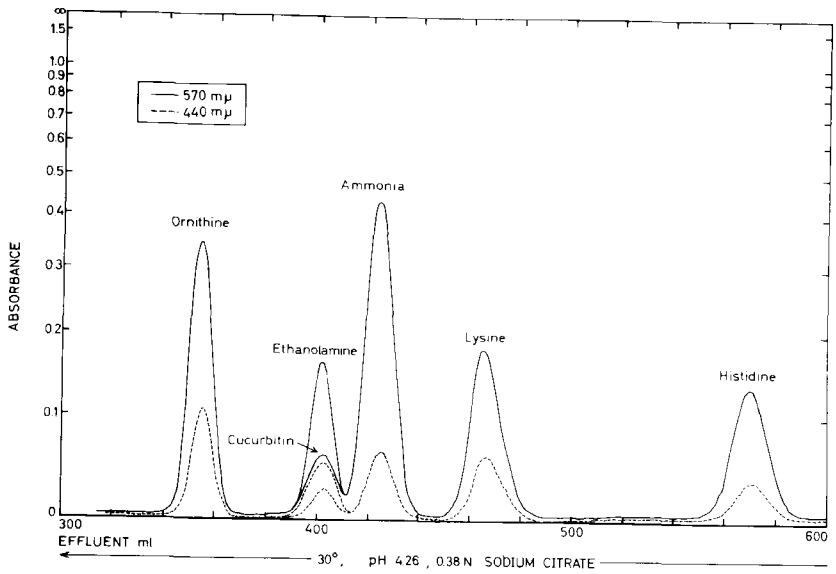

Figure 2.- Position of cucurbitin on the chromatogram of the amino acid analyzer. Elution of the basic amino acids from 50 X 0.9 cm. column of Amberlite IR-120 at 30° C. and at flow rate of 30 ml. per hour. Loading: 0.5 μmole of the basic amino acids and 1.5 μmole of cucurbitin.*

* The ammonia peak is from ammonia which comes partly from the air during the preparation of the buffer, partly from the glassware used in the preparation of the buffer, and partly from the reagents themselves.

Table XIV.- Elution Volume and Time for Cucurbitin, the Basic Amino Acids, and Ethanolamine.

Amino acid	Effluent in ml., and Time in min.					
	At first appearance of amino acid		At peak		last appearance in effluent	
	Effluent in ml.	Time in min.	Effluent in ml.	Time in min.	Effluent in ml.	Time in min
Ornitine	340	170	356	178	368	184
Cucurbitin	384	192	402	201	412	206
Lysine	450	225	472	236	448	244
Histidine	552	276	570	285	588	294
Ethanolamine*	392	196	402	201	412	206

* Ethanolamine is absent in Cucurbita extracts. It is used here only as a marker, since it falls exactly in the same position as cucurbitin.

Table XV.- Quantitative Estimation of Cucurbitin and Other Basic Amino Acids Occurring Free in Commercially Available Seeds of *Cucurbita*, as calculated from Data Obtained on the Phoenix Amino Acid Analyzer.*

Plant	Basic Amino Acids in mg per Gm. of Decorticated Whole Seeds.				Cucurbitin content calc. on the basis of the whole seed
	Ornithine	Cucurbitin	Lysine	Histidine	
1. *Cucurbita pepo*					
a. cv. 'Yugoslavia'	0.08	2.22	-	-	1.47
b. cv. 'Chinese Shen-Skin'	0.02	3.16	0.05	0.05	2.08
c. cv. 'Bulgaria'	-	0.38	0.27	-	0.25
2. *Cucurbita maxima*					
a. cv. 'Chinese Snow-White'	0.08	6.71	0.01	0.02	4.44

* See Chapter III, pp. 25-29.

Table XVI.- Paper Electrophoresis Data on Cucurbitin and Certain Basic Amino Acids.

Amino Acid	Buffer pH 7.4			Buffer pH 12.0		
	Distance* in cm.	M Histidine**	Mobility, u $\frac{cm^2}{volts.sec.}$ ***	Distance* in cm.	M Histidine**	Mobility, u $\frac{cm^2}{volts.sec.}$ ***
Arginine	1.9	0.31	3.7×10^{-5}	2.5	0.35	4.9×10^{-5}
Lysine	2.8	0.46	5.5×10^{-5}	5.5	0.77	10.8×10^{-5}
Ornithine	4.0	0.67	7.9×10^{-5}	5.6	0.79	11.2×10^{-5}
Histidine	6.0	1.00	11.9×10^{-5}	7.1	1.00	14.1×10^{-5}
Cucurbitin	7.8	1.30	15.5×10^{-5}	8.8	1.24	17.5×10^{-5}

* Distance travelled from the starting point in cm.

** Mobility relative to Histidine.

*** The mobility, u, of a molecule is defined by the relation, $u = \frac{V}{E}$, where V is the velocity in centimeters per second when acted upon by an electric force, E, measured in volts per cm.

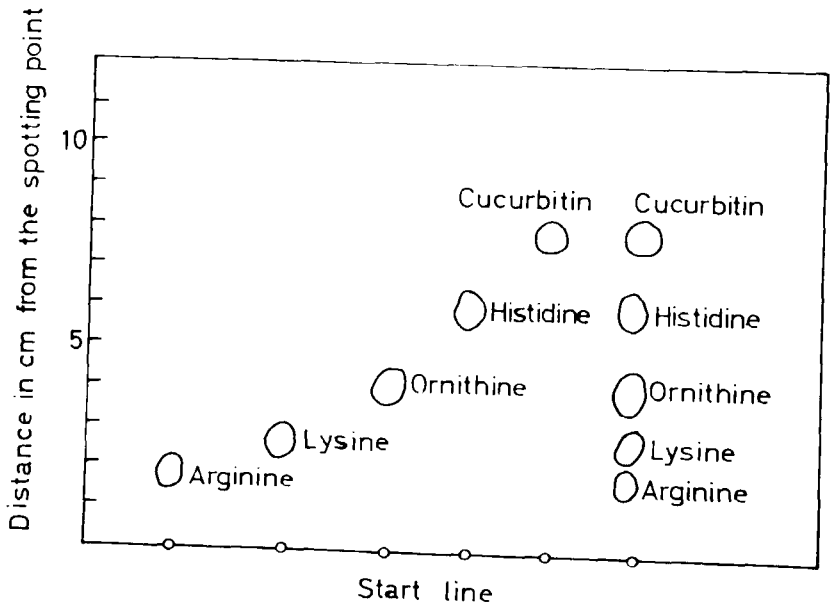

Figure 3.- Electrophoretic separation of Cucurbitin from some basic amino acids in the 8 X 10^{-2} M sodium bicarbonate solution, pH 7.4.
Time of separation was 120 min. at 210 volts and potential gradient 7 volts/cm. Whatman No.3 paper was used. 10 mcg. of each of the basic amino acids and 20 mcg. of cucurbitin were spotted.*

* Samples were applied on the negative (cathode) end.

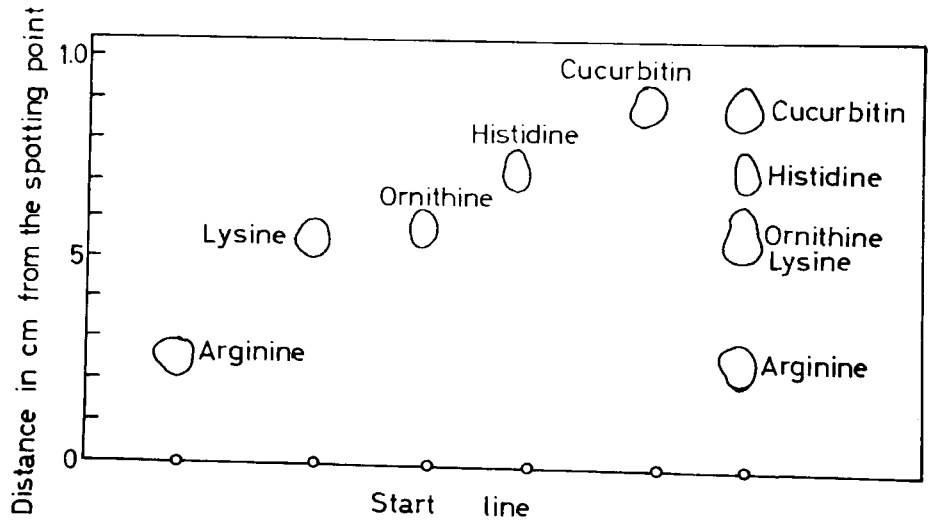

Figure 4.- Electrophoretic separation of Cucurbitin from some basic amino acids in the tetraborate electrolyte, pH 12.0.

Time of separation was 120 minutes at 210 volts and potential gradient 7 volts/cm. Whatman No. 3MM paper was used. 10 mcg. of each of the basic amino acids and 20 mcg. of cucurbitin were spotted.*

* Samples were applied on the negative (cathode) end.

CHAPTER VIII

DISCUSSION

The purpose of the present investigation was to study the distribution of cucurbitin both qualitative and quantitative in the different cucurbits grown in Lebanon or available on the Beirut market from foreign sources. To do this, it was necessary at first to develop a simple and definitive method for detecting its presence in plant extracts and for separating it from the numerous free amino acids with which it occurs in the aqueous or hydroalcoholic extracts of cucurbit seeds. Quantitative determination of cucurbitin would then follow easily. However, quantitative determinations would not be significant before a proper method of extraction of cucurbitin from plant tissues has been found. Since no authentic sample of cucurbitin was available, it was necessary to resort to synthesis. Thus, the compound was synthesized and its properties compared with those published in the literature: melting point, behavior on a few chromatographic media, and its infrared spectrum.

Synthesis. Certain raw materials needed for the synthesis were not available and so these were also synthesized namely: N-carbethoxyglycine ethyl ester, and 1,3-dicarbethoxy-4-pyrrolidone. In addition to synthesizing

cucurbitin, new analytical data were established for it such as: new chromatographic data (See Tables II-IX), electrophoretic data (See Table XVI), and data on its behavior on the Phoenix Amino Acid Analyzer (See Tables XIV, XV, and Fig. 2.).

Extraction. Extraction of the cucurbitin from plant tissues was best accomplished by extraction with water at 50° C. Extraction with water at room temperature was the next best (See Chapter VI, pp. 49-50).

Chromatography. In general, chromatographic methods on paper, on silica gel G and on cellulose thin-layers, particularly on one-dimensional chromatograms, were unsatisfactory, as it was not possible to separate from each other all the 17 to 20 free amino acids* found in the seed extracts. Two-dimensional chromatography on Whatman No. 3MM paper gave the best resolution possible with this method when phenol-ammonia (Solvent System No. 12a, p. 12) was used as the first eluant and n-butanol-acetic acid-water (Solvent System No. 12a, p. 13) was used as the second solvent. This method however, was time consuming, requiring three days to obtain a result. It could not be used for quantitative estimation of cucurbitin as the colors with ninhydrin faded slightly before a second chromatogram could be made ready for

* A few of these amino acids have unusual structure, while a few others are of unknown structure (10).

comparison with it.

Electrophoresis. Paper electrophoresis of the free amino acid extracts was successful in separating cucurbitin and the other basic acids from each other, when carried in buffer systems at pH 7.4 (Chapter III, p.21) or pH 12 (Chapter III, p.21). Tailing occurred in buffer pH 7.4 but not in buffer pH 12 when the total amino acid extract was subjected to electrophoresis. When cucurbitin or cucurbitin mixed with other basic amino acids was subjected to electrophoresis in either buffer system, no tailing occurred. Electrophoresis clearly demonstrated the presence of the following basic amino acids, besides cucurbitin, in the free amino acid extracts of Cucurbita seeds: histidine, lysine and ornithine. All three amino acids were present in very small amounts in the Chinese varieties of C. pepo and C. maxima and were well separated on buffer pH 7.4. However, lysine could not be separated from ornithine in buffer pH 12. Lysine and histidine were absent in Yugoslavian seeds of C. pepo; histidine and ornithine were absent in the Bulgarian seeds of C. pepo. These results were confirmed by chromatography on ion-exchange cellulose phosphate paper and by the use of the Amino acid analyzer (see below). Electrophoretic data are given in Table XVI, p. 77 and in Figs. 3 and 4 pp. 78-79.

Paper electrophoresis could not, however, solve the

problem of quantitative estimation of cucurbitin: minimum spotting gave very faint spots of inconstant color density and heavier spotting resulted in tailing. Quantitative results were inconsistant when carried out with known concentrations.

Ion Exchange Cellulose Phosphate Paper P-20. The solution of the problem of characterizing and quantitating cucurbitin found in plant tissues was finally solved by resorting to chromatography on ion exchange cellulose phosphate paper at pH 9.2 (See Chapter III, p. 19). Chromatography on Whatman Cellulose Phosphate Paper P-20, using a carbonate-bicarbonate buffer at pH 9.2 (See Table IX, p. 62) effected a beautiful separation of all the basic amino acids from the other amino acids, and of the basic amino acids, including cucurbitin, from each other.

The basic amino acids separated in good distinct spots which were reproducible in size and color intensity (after visualization with ninhydrin solution). This method therefore provided a good and reasonable means for quantitatively estimating cucurbitin densitometrically either visually or instrumentally. It also provided an easy and quick method for screening plant extracts for the presence of cucurbitin. Chromatographic and quantitative data are given in Tables IX, X pp. 63,64.

Amino Acid Analyzer. The use of an amino acid analyzer

(See Chapter III, pp. 25-26) provides not only an accurate means of determining cucurbitin quantitatively but also provides an accurate means for its qualitative analysis. Using other amino acids as markers, it was found that cucurbitin appeared in the effluent immediately after ornithine and immediately before lysine. It occured on the same spot as ethanolamine. Since ethanolamine was absent in the plant tissues it could be used as a marker also. Data are given in Table XIV p. 75, and Fig. 2, p. 74.

It may be noted here that the quantities of cucurbitin found in Chinese Snow-White seeds as determined by two separate methods: visually on cellulose phosphate paper and instrumentally on the amino acid analyzer, were comparable: namely 8 and 6.71 mg. per gram of decorticated seed, respectively.

Distribution of Cucurbitin in the Cucurbitaceae. Among the seeds of Cucurbita species examined, those of C. maxima were the richest in cucurbitin (Table VII, p.61, X, p. 64). This is in agreement with the potency of the seeds of C. maxima varieties, studied clinically by Karamanukian (21). It was stated (21) that seeds grown in the Middle East, especially in Lebanon, were highly active as taenifugal agents. This is in accord with the high cucurbitin content of the seeds of C. maxima grown in Lebanon and Syria.

A number of cultivars of some genera of the Cucurbitaceae were screened for cucurbitin: Cucurbita pepo, C. moschata, C. maxima, Citrullus vulgaris, Cucumis sativus, Cucumis melo, Luffa cylindrica, and Lagenaria leucantha. Cucurbitin was present only in Cucurbita species and its cultivars, (See Table XII, pp. 67-68) and absent in the other genera studied. The presence of cucurbitin in Cucurbita provides a chemotaxonomic indicator of importance in the classification of the genera of the Cucurbitaceae.

On the Beirut market, seeds of C. maxima cv. 'Chinese Snow-White' are sold as a taenifuge. These seeds have a moderate content of cucurbitin, 6.71 mg. per gram of decorticated whole seeds. The seeds from locally grown fruits are not sold on the Beirut market as a taenifuge. These seeds are kept by the cultivators for replanting in the following season and are not sold to any large extent on the open market.

The seeds of C. pepo cv. Turkey have about the same cucurbitin content as the C. maxima cv. 'Chinese Snow-White', but the former are not available on the market. Relatively a few fruits of C. pepo Turkish variety are grown to maturity. This is only done to obtain the seeds for planting. The majority of cultivated fruits are harvested unripe as a variety of squash, for cooking as a vegetable.

Extracts from watermelon seeds were reported to have vermicidal action (12). However, no cucurbitin could be detected in watermelon seeds. Either the cultivars examined were chemical races deficient in cucurbitin or the anthelmintic property of watermelon seeds (12) was due to another active substance other than cucurbitin. It could be also that the reported vermicidal action rested on insufficient evidence.

The seeds of Luffa cylindrica are used by certain communities in Lebanon as a taenifuge. No cucurbitin was detected in the Luffa seeds examined. However, there is no substantiated clinical evidence as to the efficiency of Luffa seeds as a taenifuge.

The pulp of Cucurbita pepo was reported by Neely and Davy (32) to have anthelmintic action. However, the pulp tested by ion-exchange cellulose phosphate paper chromatography, was found to be free of cucurbitin.

The soluble protein and peptide hydrolyzate of Cucurbita maxima seed extract was free of cucurbitin. The residual seed protein hydrolyzate was also devoid of cucurbitin. Cucurbitin, therefore, occurs only as a free amino acid in the seed. This fact makes it readily available when the seed is eaten or extracted with water.

The cucurbitin content of "Leoseed" ^{R} was found to be 1.5 gm. per the 30 ml. dose of the extract, as estimated by chromatography on cellulose phosphate paper. This provides an adequate dose for an effective treatment in tapeworm infections.

CHAPTER IX

SUMMARY

Several techniques and methods were used in this ivestigation. A few hundred chromatograms of all types were performed, various extraction methods were used, synthetic reactions were carried out, and experience in the use of a number of instruments was obtained.

The findings may be summarized as follows.

1. Cucurbitin was synthesized.
2. Natural cucurbitin was isolated from Cucurbita maxima seeds and compared with synthetic cucurbitin, chromatographically.
3. Cucurbitin was found to occur in the kernel of the seed of Cucurbita species and was absent from the pulp of the fruit. In the seed, cucurbitin occurs only in the free state and is not combined with other amino acids, neither in the water-soluble peptides, nor in the reserve protein.
4. Cucurbitin was found to be stable to strong hydrochloric acid. It was best extracted from the kernels of Cucurbita species with water at 50^{o} C.

5. A simple method was developed for detecting cucurbitin in plant tissues and for estimating it quantitatively. Aqueous extracts were chromatographed on ion exchange Cellulose Phosphate P-20 papers, eluted with a carbonate-bicarbonate buffer at pH 9.2, and visualized with ninhydrin reagent. Quantitatively, the developed spots could be estimated densitometrically or visually.
6. New analytical data were determined for cucurbitin: R_f values in different media, electrophoretic mobilities, order of elution in ion-exchange resin columns using the Phoenix Amino Acid Analyzer, etc.
7. Quantitative determination of cucurbitin was accomplished using the Amino Acid Analyzer and calculating the value from the tracings recorded by the instrument.
8. Cucurbitin was found to occur in the seeds of Cucurbita species and to be absent from the seeds of Citrulius vulgaris, Cucumis sativus, Cucumis melo, Lagenaria leucantha, and Luffa cylindrica.
9. Seeds of Cucurbita maxima contained the highest amount of cucurbitin. Those used in Lebanon as a taenifuge originated from China and contained

a moderate quantity of cucurbitin. Seeds from locally-grown fruit, not generally available commercially, were richer in cucurbitin content. Seeds of Cucurbita pepo grown locally and used mostly for planting purposes, contained about the same amount of cucurbitin as the chinese variety of Cucurbita maxima.

10. A market preparation of pumpkin seed extract was found to contain about 1.5 gm. of cucurbitin per dose of 30 ml.

11. Effectiveness of the different commercial varieties of pumpkin seed as a taenifuge appears to be proportional to its cucurbitin content, when results of clinical trials reported in the literature were compared with the results of this investigation.

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