# EXTRACTION OF PROTEIN FROM EXPELLER- AND SOLVENT-EXTRACTED COCONUT MEAL BY DILUTE ACID, ALKALI, AND SALT SOLUTIONS

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The extractability of protein (nitrogen) from poonac (the press-cake left after extraction of oil from dried coconut kernel) with dilute aqueous hydrochloric acid, sodium hydroxide, and salt solutions has been investigated. Experiments were carried out on both expeller poonac and solvent-extracted poonac, the protein of the former being more soluble. Approximately 40% and 55% of the poonac protein nitrogen was extractable with 0·15% aqueous acid and alkali, respectively, under optimum conditions. Dilute salt solutions were found to have a comparatively poor solubilising effect under the conditions employed. Increasing the fat content of expeller poonac to 10% increased the nitrogen extracted by acid but not by alkali solutions.

## Introduction

Few experimental studies have been published on the extraction of coconut protein from the residues left after the extraction of oil from dried coconut endosperm ('copra'). Recent reports are those of Sreenivasan[1] and Smith[2]; earlier work has been described by Curtin.[3]

Coconut oil is one of the major exports of Ceylon. 'Poonac', the presscake obtained as a by-product after expulsion of the oil is used in Ceylon chiefly as a constituent of animal feeds and agricultural fertilisers and contains 19–22% protein and 5–10% oil. The chief disadvantage of the crude poonac is its high fibre content (10%). Early work on the material was restricted to determining its biological value in experimental animals,[4,5] but no attempt to prepare from it a product which would be fit for human consumption has come to the notice of the present authors.

A study of the extractability of the protein of poonac has accordingly been carried out, bearing in mind that the procedure applied should ultimately be capable of economic and commercial exploitation and of producing a product of unimpaired nutritional value. The experiments and conclusions reported in this paper constitute the first part of this investigation.

## Experimental

### Materials and Methods

Two 5 lb samples of poonac, one of solvent-extracted and the other of expeller poonac, were obtained from the Oils and Fats Corporation, Seeduwa. A petroleum distillate ('hexane', S.B.P. 62°–82°, Shell Company of Ceylon) is used as solvent in obtaining the solvent-extracted poonac from expeller poonac. The latter is prepared from kiln-dried copra which is subjected to a preliminary process of disintegration, cooking and drying prior to expulsion of the oil in a screw-press. Each sample, was reduced to a fine powder in a laboratory disintegrator ('Atomill', British Jeffrey Diamond Ltd., Wakefield) and finally passed through a 60-mesh sieve. The samples were dried and then stored in an air-tight bottle. Weighed aliquots in duplicate were taken for each experiment, moisture being determined simultaneously.

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*Moisture*

This was determined by drying at 105° to constant weight.

*Fat*

The fat content was determined by exhaustive extraction with petroleum ether (b.p. 60–80°) in a Soxhlet apparatus.

*Solvents*

Aqueous solutions of HCl (0·10–1·00%, wt./vol.); NaOH (0·01–2·00%, wt./vol.); NaCl (0·5–3·9%, wt./vol.); $CaCl_2$ (0·03–0·10%, wt./vol.). All reagents were of analytical reagent quality.

*Extraction of nitrogen by shaking with solvent*

1 g of the material was shaken with the solvent (25 ml) for an interval of time (1 h or longer), the suspension left to stand for a further period, and shaken again at a given temperature (29°, 45°, 60°) for 2 h in an automatic shaking apparatus prior to filtration or centrifugation. Aliquots of the clear filtrate (No. 1 Whatman filter paper) or supernatant were taken for nitrogen determination. Occasionally it proved necessary to centrifuge the suspension and filter the supernatant to obtain a clear extract.

*Extraction of nitrogen by homogenisation with the solvent*

0·2 g (approx.) weighed aliquots of the material were homogenised with solvent (25 ml/g poonac) in a glass homogeniser (thick-walled glass tube, 14·5 × 2 cm with a ground surface on the inside, and provided with a motorised ground glass pestle). Homogenisation was carried out until no large aggregates were visible under the microscope (about 30 min). The suspension was then centrifuged, the supernatant filtered if necessary, and aliquots of the clear extract taken for nitrogen determination.

*Nitrogen*

All determinations were carried out by the micro-Kjeldahl procedure according to Chibnall, Rees & Williams.[6]

*Precipitation of protein from the extract with trichloroacetic acid (TCA)*

40% TCA solution in water was added to a measured aliquot of the clear extract till a final concentration of 5% was

attained (5 ml extract + 0·75 ml 40% TCA). The precipitate was washed by centrifugation with a small volume of 5% TCA and transferred to a micro-Kjeldahl flask for protein-N estimation.

*Precipitation of protein from the extract by heat coagulation*

A measured volume of the clear extract was carefully neutralised to the point of maximum turbidity (pH ~ 4·5) and heated to 80–90° when coagulation took place. The solids were separated by centrifugation, washed with water and protein nitrogen determined as before.

*Centrifugation*

This was usually carried out for 10 min in the M.S.E. Minor centrifuge using the maximum speed position. Room temperature which varied between 28° and 30° is recorded as 29°. Where higher temperatures were used for extraction, a constant temperature water bath was employed. All pH determinations were done with a Cambridge pH meter.

*Non-protein N (NPN)*

This was determined by shaking or homogenising weighed aliquots of the sample with 5% TCA (25 ml/g poonac) for 3 h at 29°, and taking suitable aliquots of the clear filtrate for estimation of N.

## Results and Discussion

The figures obtained for moisture, fat and nitrogen contents of the samples of poonac used are shown in Table I.

The effect of concentration of HCl on the extraction of nitrogen from expeller poonac at room temperature is shown in Table II. The extracted N was found to reach a maximum with acid concentrations in the range 0·4–0·5% regardless of the method of extraction employed; however the increase in extracted N with acid concentrations over 0·2% was relatively less.

In the case of solvent-extracted poonac (Table III), the extracted N was very small, a value of the same order as the water-soluble N of the expeller poonac being obtained. Also, variation in the acid concentration had only a relatively small effect on the extractable N. It is shown later (see Table XIII) that the extractable N from the solvent-extracted poonac by salt solutions is also very small. This suggests that the proteins of the poonac have suffered further denaturation in the solvent-extraction process. This could have nutritional implications. Butterworth & Fox[7] have shown that the nutritive value of coconut deteriorates as processing temperature increases. The superior lysine availability of a solvent-extracted coconut meal prepared for human consumption (UNICEF) over two other solvent-extracted commercial samples (France) which they reported indicates that the protein could suffer damage in the extraction process itself.

In view of the insolubility of solvent-extracted poonac protein, it was decided to use only expeller poonac in studying the effect of other variables like the volume of solvent per unit weight of poonac, the temperature of the medium, the fat content of the poonac and the time of contact of the poonac with the solvent. On the basis of the results shown in Table II and some earlier observations obtained before it was realised that temperature could have a marked effect, 0·15% HCl was chosen as the most suitable and economic concentration of acid, as it gave near-maximum N extractability.

**Effect of solvent volume on N extractability**

This is shown in Table IV. The extracted N increased to a maximum at a volume of 25–30 ml/g poonac, and thereafter decreased somewhat. A volume less than 15 ml/g poonac produced a semi-solid mass from which a filtrate or supernatant on centrifugation could not be obtained. With increase in volume, a decrease in pH of the mixture occurred as would be expected from the fact that the buffering effect of the poonac protein would be limited. The maximum N extraction was obtained at pH 1·8–2·0.

TABLE I

**Composition of expeller poonac and solvent-extracted poonac**

| | Expeller poonac | Solvent-extracted poonac |
|---|---|---|
| Moisture, % | 8·7 | 9·6 |
| Moisture, maximum absorbed on equilibration, % | 15·5 | 15·7 |
| Fat (on anhydrous basis), % | 9·4 | 3·1 |
| Nitrogen (on anhydrous basis), % | 3·78 | 4·20 |

TABLE II

**Effect of varying the concentration of aqueous HCl on the extraction of nitrogen from expeller poonac (Moisture, 11%)**

The pH values of the solutions are the values obtained after shaking the sample for 3 h with the acid at 29°C

| HCl, % (wt./vol.) | pH of acid | pH of mixture | Extracted N (shaking) (g N/100g) | Extracted N (homogenising) (g N/100g) |
|---|---|---|---|---|
| 0·00 | — | 5·80 | — | 0·34 |
| 0·10 | 1·57 | 2·35 | 0·55 | 0·72 |
| 0·15 | 1·42 | 2·02 | 0·86 | 1·26 |
| 0·20 | 1·26 | 1·75 | 0·99 | 1·36 |
| 0·30 | 1·08 | 1·38 | 1·03 | 1·43 |
| 0·40 | 0·97 | 1·14 | 1·08 | 1·70 |
| 0·50 | 0·88 | 0·99 | 1·05 | 1·72 |
| 0·60 | 0·79 | 0·95 | 1·05 | 1·65 |
| 0·80 | 0·67 | 0·76 | 0·78 | 1·35 |
| 1·00 | 0·56 | 0·66 | 0·56 | — |

TABLE III

**Effect of varying concentration of aqueous HCl on the extraction of nitrogen from solvent-extracted poonac (Moisture, 11%) at 29°C**

| HCl, % (wt./vol.) | Extracted N (shaking) (g N/100 g) | Extracted N (homogenising) (g N/100 g) |
|---|---|---|
| 0·0 | — | 0·28 |
| 0·4 | 0·36 | 0·34 |
| 0·5 | 0·41 | 0·36 |
| 0·6 | 0·37 | 0·37 |
| 0·7 | 0·37 | 0·35 |
| 0·8 | 0·34 | 0·34 |

TABLE IV

**Effect of varying the volumes of 0·15% HCl/g poonac on the extraction of nitrogen from expeller poonac (Moisture, 11%) at 29°C**

| Volume of acid, ml/g | 15 | 17 | 20 | 25 | 30 | 35 | 45 | 50 |
|---|---|---|---|---|---|---|---|---|
| pH of extract | 2·82 | — | 2·50 | 2·02 | 1·84 | 1·78 | 1·75 | 1·62 |
| N (shaking), g N/100 g | 0·33 | 0·38 | 0·81 | 0·88 | 0·89 | 0·84 | 0·75 | 0·77 |

**Effect of temperature and time of contact on N extraction**

This is shown in Table V. The extracted N increased with temperature irrespective of the time of contact. At room temperature (29°), maximum values were obtained after 24 h, while at the higher temperatures, no further increase was obtained after a 3 h period of contact. The N extracted after a 3 h period of shaking at 60° was 177% of the amount extracted at room temperature for a like period. Extraction at higher temperature could therefore obviate the inconvenience of long extraction periods in the cold. The use of temperatures higher than 60° was not investigated due to the likelihood of decreasing the solubility of the poonac protein by increasing denaturation.

**Effect of added oil on N extractability**

The low solubility of solvent-extracted poonac protein suggested that fat played a critical part in determining the solubility of the protein. Preliminary experiments indicated that addition of oil produced no increase in extracted N in the case of solvent-extracted poonac, but that with expeller poonac, a definite increase was obtained. The extractability of nitrogen from expeller poonac was therefore determined in the presence of added aliquots of coconut oil and varying acid concentrations (Table VI). It was found that with 0·10% HCl, further addition of oil did not increase the extractable N. With 0·15% HCl, maximum extraction was obtained when the fat content of the sample was increased from its original figure of 8·4% to 9·2%; with 0·20% HCl, a figure of 11·1% fat gave maximum extraction of nitrogen. This increase in extracted N in the presence of fat is important in that it adds weight to the argument that the lower solubility of the solvent-extracted poonac protein is due to irreversible denaturation of the coconut lipoprotein complex which starts with the drying of the coconut-endosperm and is completed by the solvents used in the extraction process.

The N precipitable as protein from these extracts is shown in Table VII. It will be seen that 71% of the extractable N (90% of the protein N) can be precipitated by TCA. It was found in further experiments that corresponding amounts of protein could also be recovered by heat coagulation under carefully controlled conditions.

The NPN of poonac amounts to about one-tenth of the total nitrogen. The bulk of this consists of free amino acids.[8] It was found somewhat unexpectedly that elimination of the water-soluble constituents of the poonac improved the extraction of the poonac protein (Table VIII). The nitrogen extracted by 0·15% HCl from washed poonac was found to be almost completely recoverable as protein; and the total extractable protein N was thereby increased from 1·06 to 1·24 g N/100 g poonac, an increase of 17%. The figures also show that the water-soluble protein of the poonac is only a minute fraction of the total protein, amounting to ~ 0·4 g protein/100 g poonac.

By first extracting the water-soluble N and then extracting the residual N with acid, the total extractable nitrogen was also increased, a figure of 1·62 g N (Table VIII) being obtained as against 1·49 g N (Table VII)/100 g poonac. These results suggest that there are certain water-soluble constituents in the poonac which decrease the solubility of the poonac proteins in this acid medium. Tables IX, X, XI and XII give the figures of nitrogen extracted from expeller poonac using dilute aqueous NaOH solutions as solvent under conditions similar to those employed when dilute HCl was used. Though

TABLE V

**Effect of temperature and time of contact on the extraction of nitrogen from expeller poonac (Moisture, 11%) by 0·15% HCl**

Values given represent g N extracted/100 g poonac

| Extraction time, h | 29°C | 45°C | 60°C |
|---|---|---|---|
| 3 | 0·85 | 1·28 | 1·51 |
| 24 | 1·06 | 1·29 | 1·52 |
| 48 | 1·08 | 1·25 | 1·48 |

TABLE VI

**Effect of fat content of the poonac on the extraction of nitrogen from expeller poonac (Moisture, 11%; fat, 8·4%) by varying concentrations of aqueous HCl at 29°C**

| HCl, % (wt./vol.) | Weight of oil added /g poonac, mg | Fat in sample (calculated), % | Extracted N, g/100 g poonac |
|---|---|---|---|
| 0·10 | — | 8·4 | 0·55 |
| | 15·8 | 9·8 | 0·48 |
| | 27·2 | 10·8 | 0·38 |
| | 40·9 | 12·0 | 0·40 |
| 0·15 | — | 8·4 | 0·86 |
| | 9·3 | 9·2 | 1·14 |
| | 19·8 | 10·2 | 1·10 |
| | 29·0 | 11·1 | 1·09 |
| 0·20 | — | 8·4 | 0·99 |
| | 17·6 | 9·8 | 1·23 |
| | 29·1 | 11·1 | 1·30 |
| | 37·3 | 11·7 | 1·19 |
| 0·30 | — | 8·4 | 1·03 |
| | 17·1 | 9·9 | 1·21 |
| | 27·7 | 10·8 | 1·22 |
| | 37·0 | 11·7 | 1·25 |

TABLE VII

**Recovery of protein from 0·15% HCl extract of expeller poonac (Moisture, 11%) under conditions for maximum extraction**

1 g poonac and 25 ml 0·15% HCl were shaken for 3 h, left to stand for a further 18 h and then heated in a thermostatically controlled water bath at 60°C with shaking for 3 h before filtration and precipitation of protein with TCA

| Total extracted N | NPN | Protein N by difference | N in protein ppt | % Total N recovered | % Protein N recovered |
|---|---|---|---|---|---|
| | (g N/100 g poonac) | | | | |
| 1·49 | 0·32 | 1·17 | 1·06 | 71 | 90 |

TABLE VIII

**Effect of washing expeller poonac (Moisture, 11%) with water on the extraction of its protein with 0·15% aqueous HCl at 60°C**

1 g poonac was shaken with 100 ml water for 3 h. After filtering and washing the residue, it was shaken with 25 ml 0·15% HCl for 1 h and then heated at 60°C for 2 h with shaking before filtration and N estimation in the extract. Protein was precipitated with TCA. N in a suitable aliquot of the water-soluble fraction was also determined. Figures represent g N/100 g poonac.

| Water-soluble N | Extracted N | Protein N | |
|---|---|---|---|
| 0·34 | 1·28 | 1·24 | (97% of the extracted N) |

aqueous NaOH (0·15%, wt./vol.) extracted more nitrogen than the same concentration of HCl, there was the disadvantage that the extract developed a brown colour, the colour increasing in intensity with increase in strength of alkali and temperature. Unlike acid extraction, alkali extraction was not improved by increasing the fat content of the sample. Shaking the sample for periods longer than 3 h produced only a small increase in the extracted N (Table X). The values in Table XII suggest that this increase does not reflect increased solubilisation of protein but rather a breakdown of protein to soluble nitrogenous products. The amount of total N recovered as protein was not increased by prolonging the time of contact of the sample with the alkali solution.

Table XIII shows that dilute salt solutions are comparatively poor extractants of poonac N. Aqueous $CaCl_2$ solutions were not as effective as aqueous NaCl solutions. Here again it is seen that solvent-extracted poonac proteins are more insoluble in the salt solution. When allowance is made for the NPN present, a maximum solubility of protein (corresponding to ~ 0·3 g protein N/100 g poonac) was obtained with 3% aqueous NaCl solution. The nitrogen extractability with aqueous NaCl of expeller poonac, however, showed a marked difference in that maximum solubility (corresponding to ~ 0·6 g protein N/100 g poonac) was achieved with only 0·06% aqueous NaCl solution. The advantage of salt extraction is that no chemical reactions take place during the extraction process, and dialysis of the extract gives a relatively white protein product.

With all solvent media, it was found that the degree of comminution of the poonac was important in relation to the extractable N. If the sample was merely shaken, and not homogenised, the extracted N was less. The effect of homogenisation tended to disappear at higher temperatures of extraction and with longer periods of contact. Improving contact of the poonac proteins with the extracting medium is therefore very important. It is likely that a higher state of subdivision than the 60-mesh product used would have helped considerably in the solubilisation of the poonac protein. These results indicate that it should be possible to devise a fairly simple and economic process for extraction of coconut protein from poonac and similar oilseed residues.

Values for the essential amino acids in three coconut protein isolates prepared by different methods have been reported by Sreenivasan.[1] Table XIV shows the highest values obtained for each amino acid in these preparations, together with values determined by one of the authors (Baptist, unpublished data) in crystalline coconut globulin (N = 17·5%, anhydrous basis) prepared from poonac. The composition of the FAO reference protein[9] is also given for comparison.

The lysine and tryptophan contents/g food N are higher than those of cereals, leafy vegetables, and other oilseed proteins.[9,10] From Table XIV, 'chemical score' values of 82 and 71 are obtainable for the coconut protein isolate and for coconut globulin, the limiting amino acids being methionine and tryptophan, respectively. In comparison, the biological value of poonac protein for the rat has been reported to be 71[5] (solvent-extracted meal) and 77[4] (expeller meal).

### Application in human feeding

In converting the tasteless coconut protein to an acceptable human food, it should prove possible to increase the biological

TABLE IX

**Effect of varying concentrations of aqueous NaOH at 29°C on the nitrogen extracted from expeller poonac (Moisture, 11%)**

| NaOH, % (wt./vol.) | Extracted N (shaking) (g N/100 g poonac) | Extracted N (homogenising) (g N/100 g poonac) |
|---|---|---|
| 0·01 | 0·45 | 0·64 |
| 0·02 | 0·51 | — |
| 0·03 | 0·64 | 0·93 |
| 0·04 | 0·76 | — |
| 0·05 | 0·88 | 1·47 |
| 0·06 | 1·04 | — |
| 0·07* | 1·18 | — |
| 0·08 | 1·31 | — |
| 0·09 | 1·52 | — |
| 0·10 | 1·56 | 1·78 |
| 0·15 | 1·76 | — |
| 0·30 | 2·11 | 2·05 |
| 0·50 | 2·22 | 2·08 |
| 1·00 | 2·28 | 2·27 |
| 2·00 | — | 2·58 |

* Above this concentration, the extract took on an increasingly brown colour

TABLE X

**Effect of temperature and time of contact on the nitrogen extracted from expeller poonac (Moisture, 11%) by 0·15% aqueous NaOH**

The experimental procedure was identical with that for Table V. Values given represent g N extracted/100 g poonac

| Extraction time, h | 29°C | 45°C | 60°C |
|---|---|---|---|
| 3 | 1·76 | 1·92 | 2·13 |
| 24 | 1·83 | 1·98 | 2·25 |
| 48 | 1·88 | 2·03 | 2·26 |

TABLE XI

**Effect of the fat content of poonac on the nitrogen extracted from expeller poonac (Moisture, 11%) by 0·15% aqueous NaOH**

| NaOH, % (wt./vol.) | Weight of oil added/g poonac, mg | Fat in sample (calculated), % | Extracted N /100 g poonac, g |
|---|---|---|---|
| 0·15 | — | 8·4 | 1·78 |
| 0·15 | 24 | 10·7 | 1·67 |

TABLE XII

**Protein (5% TCA precipitable) nitrogen in 0·15% aqueous NaOH extract at 60°C of expeller poonac (Moisture, 11%)**

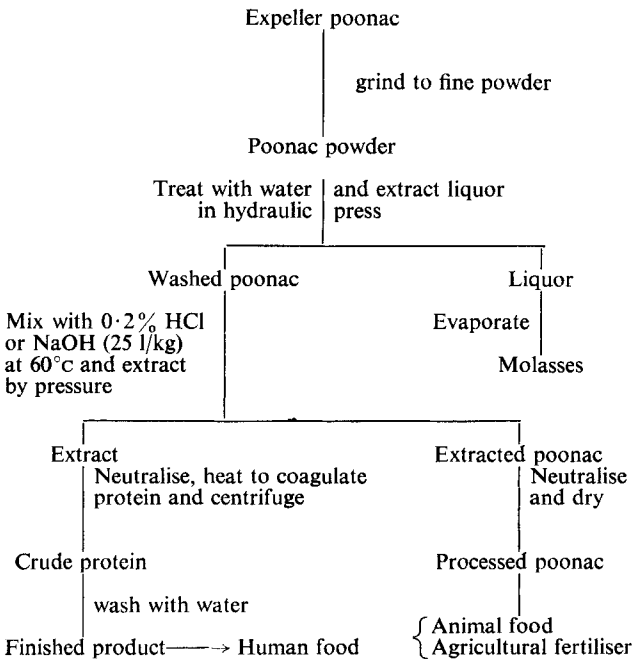
1 g poonac was kept in contact with 25 ml 0·15% NaOH for intervals of 1 h and 22 h, and heated with shaking in a thermostatically controlled water bath for a further 2 h before centrifugation. Total N and protein N was determined in the supernatant.

| Time of contact, h | Extracted N | NPN | Protein N by difference | Protein N in TCA ppt | Total N recovered % | Protein N recovered % |
|---|---|---|---|---|---|---|
| | (g N/100 g poonac) | | | | | |
| 3 | 2·17 | 0·32 | 1·85 | 1·71 | 78·6 | 92·4 |
| 24 | 2·10 | 0·32 | 1·78 | 1·65 | 78·2 | 92·7 |

value of the product still further by admixture with small amounts of animal protein such as milk or fish protein, or of vegetable protein such as leaf protein, manufactured by alternative processes already worked out for such materials.

A further consideration is that the poonac residues remaining after protein extraction still contain over half their original protein content, and could be re-utilised after appropriate treatment for the same purposes for which the original product was used, namely, as a constituent of animal feed or agricultural fertiliser.

A flowsheet that could form the basis of a technological process is shown in the following scheme:




**Acknowledgments**

The authors thank the Oils and Fats Corporation, Ceylon, for the gift of samples of the poonac used in this study. One of the authors (J.C.) also thanks the Ceylon Association for the Advancement of Science for the award of a Research Studentship during the tenure of which a part of this work was carried out.

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Received 1 May, 1968;
amended manuscript 6 September, 1968

TABLE XIII

**Effect of varying concentrations of salt solutions at 29°c on the nitrogen extracted from expeller poonac (Moisture, 9·6%) and solvent-extracted poonac (Moisture, 11·0%)**

| Concentration of salt in solution %, (wt./vol.) | N extracted by aqueous NaCl (homogenising) g/100 g expeller poonac | N extracted by aqueous $CaCl_2$ (shaking) g/100 g expeller poonac | N extracted by aqueous NaCl (homogenising) g/100 g solvent-extracted poonac |
|---|---|---|---|
| 0·00 | 0·34 | — | — |
| 0·03 | — | 0·39 | — |
| 0·04 | 0·78 | — | — |
| 0·05 | 0·79 | 0·39 | — |
| 0·06 | 0·89 | — | — |
| 0·08 | 0·72 | — | — |
| 0·10 | 0·57 | 0·38 | — |
| 0·50 | 0·45 | — | 0·27 |
| 1·0 | 0·35 | — | 0·28 |
| 2·0 | — | — | 0·49 |
| 3·0 | — | — | 0·59 |
| 3·5 | — | — | 0·54 |
| 3·9 | — | — | 0·34 |

TABLE XIV

**Essential amino acids in coconut protein**

(Values represent g amino acid/16 g N)

| Amino Acid | Coconut globulin (Baptist) | Coconut protein isolate (Sreenivasan) | Reference protein (FAO) |
|---|---|---|---|
| Lysine | 4·5 | 5·1 | 4·2 |
| Methionine | 2·0 | 1·8 | 2·2 |
| Tryptophan | 1·0 | 1·6 | 1·4 |
| Threonine | 3·4 | 2·9 | 2·8 |
| Isoleucine | 4·5 | 3·5 | 4·2 |
| Valine | 6·6 | 4·8 | 4·2 |
| Phenylalanine | (5·1)* | 5·1 | 2·8 |
| Leucine | (7·2)* | 7·0 | 4·8 |

* From, Horn, M. J., Jones, D. B., & Blum, A. E., *J. biol. Chem.*, 1948, **176,** 682; 1949, **177,** 699

**References**

1. Sreenivasan, A., PAG (WHO/FAO/UNICEF). Nutrition Document, 1963, R.9/ADD.5 August 1963 Meeting, Geneva
2. Smith, R. H., *Adv. Chem. Ser.*, 1966, **57,** 133
3. Curtin, L. V., 'Processed plant protein foodstuffs', 1958, p. 645 (ed. Altschul, A. M.) (New York: Academic Press)
4. Mitchell, H. H., & Villegas, V., *J. Dairy Sci.*, 1923, **6,** 222
5. Mitchell, H. H., Hamilton, T. S., & Beadles, J. R., *J. Nutr.*, 1945, **29,** 13
6. Chibnall, A. C., Rees, M. W., & Williams, E. F., *Biochem. J.*, 1943, **37,** 354
7. Butterworth, M. H., & Fox, H. C., *Brit. J. Nutr.*, 1963, **17,** 445
8. Baptist, N. G., *J. exp. Bot.*, 1963, **14,** 29
9. 'Protein Requirements', 1957 (Rome: F.A.O.)
10. Baptist, N. G., *Brit. J. Nutr.*, 1954, **8,** 205, 218