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
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INVESTIGATION OF THE CHANGES IN THE AMINO ACIDS CONTENT OF SHRIMPS
AND THE SUITABILITY OF SUCH A CHANGE AS A METHOD FOR THE
ROUTINE ASSESSMENT OF THEIR QUALITY

by

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A Master's Thesis submitted in partial fulfilment of the requirements for the award of a degree of Master of Philosophy of the Loughborough University of Technology.

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Summary

The present work was undertaken to find a suitable objective quality control method for use in the crustacean markets and in similar situations.

Two main types of objective methods used for fish are available - these are the chemical methods measuring either the trimethylamine-nitrogen (TMA-N) or the hypoxanthine (Hy) concentrations. However, the "K value" is a more simple method for evaluating the nucleotide degradation than the measurement of (Hy) alone, hence the "K value" was preferred in this work.

The "Ornithine Equivalent", the separated ornithine and proline, pH, TMA-N and "K value" of pink (Pandalus montagu) and brown (Crangon crangon) shrimps were investigated. These different measurements were assessed as possible methods for the routine quality control of shrimps.

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Contents cont.	<u>Page</u>
<u>PART I</u>	
2.0.0 THE APPLICATION OF CHINARD'S METHOD FOR THE ESTIMATION OF COMBINED ORNITHINE AND PROLINE (I.E. "ORNITHINE EQUIVALENT")	34
2.1.0 Standard procedure for determination of amino acids using Chinard's method	35
2.1.1 Reagents	35
2.1.2 Experimental method	35
2.2.0 The procedures for the application of Chinard's method to pure solutions of amino acids	38
2.2.1 The standard amino acids solutions	38
2.2.2 The procedure for determining the stability of the ornithine-ninhydrin colour	39
2.2.3 The procedure for determining the effect of addition of metal ions to the ornithine standard solutions	39
2.2.4 The procedure for determining the pH effect on the developed colour	40
2.3.0 The results and discussion of the application of Chinard's method to standard amino acids solutions ..	41
2.3.1 The results and discussion of the solvent effect on the ornithine-ninhydrin reaction	41
2.3.2 The results and discussion of the stability of the developed colour	46
2.3.3 The results and discussion of the interference of amino acids on the ornithine-ninhydrin reaction	48
2.3.4 The results and discussion of the pH effect on the amino acids-ninhydrin reaction	48
2.3.5 The results and discussion of the effect of metal ions on the ornithine-ninhydrin complex	52
2.4.0 The application of Chinard's method to shrimp extracts	55
2.4.1 The procedure for the determination of "Ornithine Equivalent" of pink shrimps	55
2.4.2 First batch	
(a) "Ornithine Equivalent" of pink shrimps, "bag stored" as whole unpeeled shrimp during storage in ice	55

Contents cont.	<u>Page</u>
3.7.0 The procedure for trimethylamine-nitrogen measurement	86
3.7.1 Extraction procedure of brown shrimp for TMA-N determination	88
3.7.2 The procedure for the determination of TMA-N of the brown shrimps	88
3.8.0 The procedure for the measurement of "K value"	92
3.8.1 The materials for "K value" measurement	92
3.8.2 Preparation of the anion exchange resin	92
3.8.3 Extraction procedure of the brown shrimp for "K value" determination ..	92
3.8.4 The procedure for "K value" determination of the brown shrimps	93
3.8.5 Calculation of "K value"	94
3.9.0 The procedures for the treatment and measurements of proline, ornithine and pH value of pink shrimp during storage in ice	95
3.9.1 pH measurements of shrimps homogenates	95
3.10.0 The results and discussion of proline changes of fresh brown shrimp and cooked pink shrimps during storage in ice.. .. .	96
3.10.1 "bag stored" samples	96
3.10.2 "open stored" samples	103
3.11.0 The results and discussion of ornithine changes of fresh brown shrimps and cooked pink shrimps during storage in ice	105
3.11.1 "bag stored" samples	107
3.11.2 "open stored" samples	113
3.12.0 The results and discussion of the TMA-N changes of brown shrimps, "bag stored" during storage in ice	117
3.13.0 The results and discussion of pH values changes of pink "open stored" and brown "bag stored" shrimps during storage in ice	119

Contents cont.	<u>Page</u>
3.14.0 The results and discussion of the "K value" changes of brown shrimps, "bag stored" during storage in ice	122
3.15.0 General discussion for the "bag stored" brown shrimps	123
4.0.0 CONCLUSION	127
5.0.0 SUGGESTIONS FOR FUTURE WORK	128
6.0.0 LIST OF REFERENCES	129

List of Figures

<u>Figure</u>	<u>Page</u>
1. Possible adenosine triphosphate (ATP) degradation pathways in fish and shellfish	11
2. Arginine phosphate degradation pathway	23
3. Reaction of α -amino acids with cupric ion	24
4. α -amino acid-ninhydrin reaction	25
5. Reaction of ninhydrin with proline	26
6. Structure of proline-ninhydrin complex	27
7. Chemical structure of metal-amino acid-ninhydrin	30
8. Separation and estimation of proline and ornithine	82

List of Graphs

<u>Graph</u>		<u>Page</u>
1	The calibration curves of ornithine and proline	44
2	"Ornithine Equivalent" changes of pink unpeeled shrimps:- (a) "bag stored" in ice (b) "open stored" in ice	61
3	"Ornithine Equivalent" changes of pink shrimps during storage in ice:- (a) "bag stored" as peeled shrimps (b) "open stored" as whole unpeeled (peeled just before analysis) shrimps	63
4	"Ornithine Equivalent" changes of first and second batches of brown shrimps "bag stored" during storage in ice	68
5	Trimethylamine-nitrogen (TMA-N) calibration curve, measured at 410 nm.	91
6	(a) Proline and ornithine changes of pink shrimps "bag stored" during storage in ice (b) Proline and ornithine changes in pink shrimps "open stored" during storage in ice	99 104
7	(a) Proline and ornithine changes of fresh brown shrimps "bag stored" during storage in ice (b) Proline and ornithine changes of fresh brown shrimps "open stored" during storage in ice	111 114
8	pH values of pink shrimps "open stored" and brown shrimps "bag stored" during storage in ice	120
9	Proline, ornithine, TMA-N and "K value" changes of the fresh brown shrimps "bag stored" during storage in ice	125

List of Tables

<u>Table</u>		<u>Page</u>
1	Chinard's method applied to DL-ornithine with 80% ethanol as solvent	42
2	Chinard's method applied to DL-ornithine with 96% ethanol as solvent	43
3	Chinard's method applied to L-proline with 80% ethanol as solvent	45
4	Stability of the ornithine-ninhydrin colour, measured by Chinard's method	47
5	Absorbances of glycine, L-arginine and L-lysine standard solutions measured by Chinard's method	49
6	Absorbances of mixtures of amino acids measured by Chinard's method	50
7	The effect of pH on colour developed from the amino acid-ninhydrin reaction, using Chinard's method	51
8	The effect of metal ions on the DL-ornithine-ninhydrin reaction measured by Chinard's method	53
9	"Ornithine Equivalent" changes of unpeeled pink shrimps (a) "bag stored" in ice (b) "open stored" in ice	62
10	"Ornithine Equivalent" changes of pink shrimps (a) "bag stored" in ice as peeled shrimps (b) "open stored" in ice as unpeeled shrimps (peeled just before analysis)	64
11	"Ornithine Equivalent" changes of brown shrimps kept in bags during storage in ice (first batch)	69
12	"Ornithine Equivalent" changes of brown shrimps kept in bags during storage in ice (second batch)	70
13	Proline and ornithine recovery from standard solutions of each using Zerolit 225 resin	78
14	Proline and ornithine recovery from standard solutions of each using Zerolit S/F resin	80

List of Tables (cont.)	<u>Page</u>
15 The results of the titration of 5.0 ml of TMA-HCl stock solution (after the distillation by micro-kjeldhal) with 0.1N H ₂ SO ₄	87
16 TMA-N of standard solution related to the absorbances, measured at 410 nm.	90
17 (A) Proline changes in "bag stored" shrimps	97
(B) Proline changes in "open stored" shrimps	98
18 (a) Proline and ornithine changes of pink shrimp, "bag stored", during storage in ice	100
(b) Proline, ornithine and pH changes of pink shrimp, "open stored", during storage in ice	105
19 (A) Ornithine changes in "bag stored" shrimps	108
(B) Ornithine changes in "open stored" shrimps	109
20 (a) Proline and ornithine changes of brown shrimp, "bag stored", during storage in ice	112
(b) Proline and ornithine changes of brown shrimp, "open stored", during storage in ice	115
21 Proline, ornithine, TMA-N, "K value" and pH changes of brown shrimp, "bag stored", during storage in ice	126

1.0.0

INTRODUCTION

1.1.0 The basis of quality control assessment of fish
and shellfish

The potential hazards to the consumers from the mishandling and improper storage of fish and shellfish are important, and the need for methods for monitoring the state of deterioration of the fish and shellfish are becoming essential. This is especially so in the less developed countries where urbanisation is occurring.

Connell⁽¹⁾ defined quality as "all those attributes which the consumer considers should be present." These quality attributes include flavour, odour, appearance and texture. Sundsvold⁽²⁾ defined the term quality as "what is acceptable to the consumer locally or regionally." Kramer and Twigg cited by Sundsvold⁽²⁾ defined the quality of food as "the composite of those characteristics that differentiate individual units of product, and have significance in determining the degree of acceptability of that unit by the buyer." Rock⁽³⁾ defined the "'quality' in the negative sense, as the absence of all defects, and, in the positive sense, as the presence of characteristic excellence." And he concluded that a combination of the two would give an acceptable definition. The term "freshness", which has strong link with fish quality, needs to be defined. Gould and Peters⁽⁴⁾ defined "freshness" as the

"interval from the point of fish death to the first detectable signs of spoilage, after which the term "freshness" no longer applies." It can then be concluded that the degree of freshness of fish may be taken as the degree of acceptability by the consumers.

To measure the degree of quality of fish a scientific index is required. Connell⁽¹⁾ and Connell and Shewan⁽⁵⁾ reviewed most of existing methods of assessing of fish quality, and divided them into two groups:-

- a) sensory methods;
- b) non-sensory methods.

A) Sensory methods

These are dependant on human judgments. However, using people is expensive and their responses can vary (a fish may well be judged as fresh and acceptable by one person yet by another be judged as unacceptable). Two types of sensory methods have been distinguished recently:-

i) Subjective sensory methods

These methods are very useful for rapid examination of fish and shellfish and their products. Since they are

based on untrained personal judgments, the consequences may lead to biased opinions and may result in numerous subjective differences and disagreements about the quality of the samples. Therefore, they are not reproducible tests.

ii) Objective sensory methods

In these methods, biased judgments are minimised by the use of specially trained taste panels who concentrate their judgments on a particular well-defined attribute of the sample. They are reproducible and relatively accurate methods. These methods are difficult to realize, are very expensive in terms of personnel, and they cannot be standardized in different laboratories. Due to the disadvantages of the sensory assessments, there needs to be non-sensory methods to replace or reinforce taste panel methods.

B) Non-sensory methods

An acceptable non-sensory method is one which correlates with an objective sensory method. Gould and Peter⁽⁴⁾ reported that the taste panel itself was the standard, against which the accuracy of a non-sensory method should be judged. These objective tests are capable of being reproduced accurately in

laboratories, and they can stand as international standards of measurement unlike the taste panel judgments. They are normally of lower costs and more convenient than the sensory methods (Connell and Shewan).⁽⁵⁾

The non-sensory methods are grouped in different categories:

i) Mechanical, instrumental and physical methods

In general, these methods are used in determining whether samples lie outside the range of normal acceptability. They do not require highly trained personnel and they can test a large number of samples quickly. They are in principle the most convenient routine tests in the fishery industry. However, reproducible and reliable devices to perform these routine tests have not yet been developed. Such devices are usually based on the physical, chemical and electrical properties of fish muscles. For example, the pH meter (a physicochemical test); the Torry freshness meter (an electrical capacitance test); the penetrometer (a firmness test); the viscosity of flesh homogenate; and others. A large number of readings are usually required on each sample and it is by no means guaranteed that any single reading is a sure index to quality as judged by a sensory method.

ii) Bacteriological methods

These are usually very laborious and time-consuming requiring special care, facilities and skilled personnel. Therefore they may prove too expensive to be useful as a routine test.

There are two bacteriological methods:

- 1) Total number of organisms present in a sample is measured by the Standard Plate Count (SPC); generally used as a quality index for fish and shellfish;

- 2) The other method involves counting special groups of organisms such as pathogens; this is more difficult and complex than the SPC method.

iii) Chemical and biochemical methods

An ideal objective chemical or biochemical test would be one which would give an accurate and reproducible numerical value of quality, based upon the analysis of the changes of certain constituents in fish flesh during the shelf life stages (Bailey et al.)⁽⁶⁾ In addition, the test should be easy to perform, simple and reasonably rapid, to meet the requirement in any laboratory. They include those tests that measure

substances whose accumulation or decline are due to autolytic enzymes or bacterial action or both, during the shelf life of fish or shellfish. Some of the substances measured and used as quality index, may bear some relationship to the quality attributes, e.g. TMA has a "fishy" odour; Hy is said to be bitter; and inosine monophosphate (IMP) and free amino acids enhance sweet meaty flavour in fish flesh.

Three tests have been recognised for determining the spoilage compound changes in fish flesh, these are:

- 1) Hypoxanthine:- based upon the adenosinetriphosphate degradation (see section 1.4.0).
- 2) Total volatile bases (TVB):- based upon measuring the compounds of TMA, dimethylamine (DMA) and ammonia together (see section 1.5.0).
- 3) TMA-N:- based upon trimethylamine oxide (TMAO) reduction to TMA (see section 1.6.0).

Crustaceans have a short shelf life and the flesh deteriorates rapidly, and so there is a real need for a scientific quality index; this need is greater than that for fish. Since the changes in the compounds present resulting from the spoilage are similar to those occurring in fish, most of the existing chemical

methods for measuring fish quality have been used for crustaceans.

In addition to the TMA-N⁽⁷⁾, TVB⁽⁸⁾ and Hy⁽⁹⁾ tests, several other tests have been used for assessing shrimp quality and other crustacean type species. Bailey et al.,⁽⁶⁾ suggested glycogen, lactic acid and acid soluble orthophosphate measurements as prime quality tests; and volatile acid, bacterial plate count, sulfhydryl groups and TMA-N measurements for the onset of spoilage, for shrimps stored in ice. Changes in pH values^(6,10); free α -amino acids-nitrogen (AA-N)⁽¹¹⁾; volatile acids (VA)⁽¹²⁾; picric acid turbidity^(10,13); indole⁽¹⁴⁾; carotenoid levels⁽¹⁵⁾; tyrosine⁽¹⁶⁾; ammonia⁽¹⁷⁾, and TVN/AA-N ratio⁽⁹⁴⁾, have been suggested or used as quality indices for crustaceans during ice storage. Individual amino acid changes during post-mortem storage, such as ornithine has been suggested as a quality index for lobster.

1.2.0 The potential application of chemical tests to crustaceans

The most important changes occurring in shrimps during post-mortem storage, are ATP, TMAO (marine only) and non-protein-nitrogen (NPN) compounds (free amino acids mainly). The following sections review the background of the degradation pathways and the significant changes in each of the components - ATP, TMAO, free amino acids or their derivatives during storage, and their usefulness as quality index for shrimps.

1.3.0 Autolytic enzymes in shellfish

The enzymatic deterioration arise in the first place from the large number of different enzymes naturally present in the flesh. One of the enzymatic reactions is the gradual hydrolysis, during the first hours of death, of glycogen to lactic acid, resulting in a fall in pH. This depends on the species and the conditions of the crustacean flesh. It is an advantage to have the pH as low as possible for as long as possible to reduce microbial activities, but this decline in pH affects the quality, especially the texture which becomes firmer and is accompanied by an increase in the drip loss. In practice, the generation of basic nitrogenous compounds such as trimethylamine and ammonia by bacterial action gradually raises the pH during the period of

rigor mortis. In the shellfish and especially the crustacean, the pH may reach 8.5 in some species, in the spoilage stages.

The gut contains enzymes, which are responsible for the breakdown of food. Post-mortem, these proteolytic enzymes attack the organs themselves and penetrate to the surrounding tissues, thereby causing additional quality deterioration.

The digestive enzymes of some shellfish (for example, the lobsters and rock lobsters) are especially active and are able to attack the flesh even at the point of death. For this reason, these shellfish should be kept alive and in as full vigour as possible until just before processing, if the best quality is to be obtained. Cobb⁽¹⁸⁾ reported that the level of proteolytic enzymatic activity in shrimps varies with the different stages of the moulting cycle and seasons, and that the proteolytic digestive enzymes levels would be higher in feedy (i.e. gut filled with food) than in non-feedy shrimps. Obtaining shrimps at a period of low levels of digestive enzymes activity may allow head-on shrimps stored in ice to be kept for longer periods than normal. Cobb et al.,⁽¹⁹⁾ reported that there were considerable anabolic (i.e. amino acids producing) and catabolic (i.e. amino acids reducing) activities in post-mortem shrimp tails. When shrimps are harvested near their moulting period or taken from organic rich waters, they may blacken excessively, particularly if the washing action of melted ice is absent (Cobb and Vanderzant⁽⁸⁾, Cobb et al.,⁽²⁰⁾, Vanderzant et al.⁽²¹⁾).

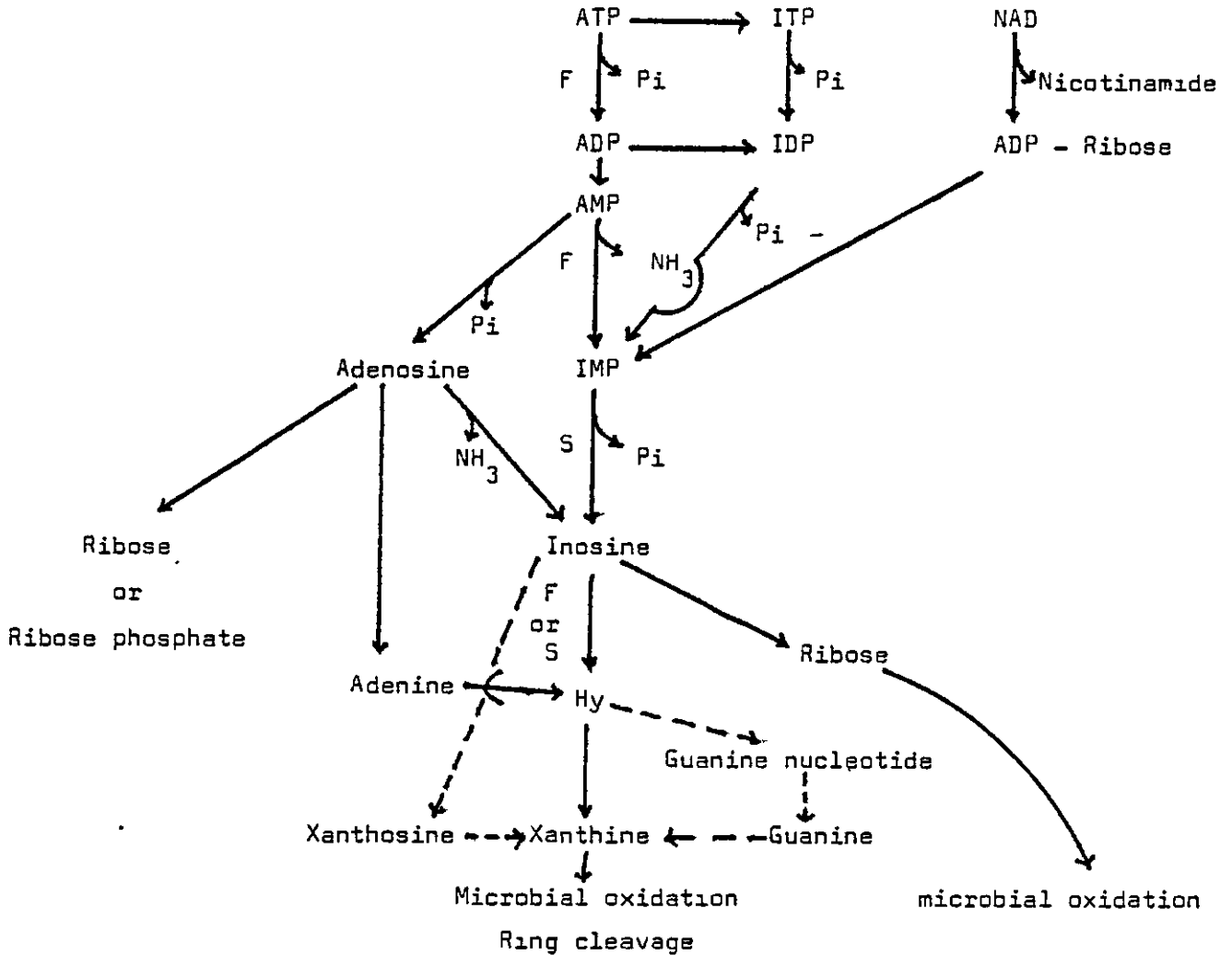
1.4.0 Nucleotide and nucleoside degradations of fish and shellfish

In live, respiring muscles a high level of adenosine triphosphate (ATP) is maintained by the oxidation of organic compounds. When the animal dies and oxygen is no longer available to the cells, adenosine triphosphate (ATP), along with creatine phosphate (CP), and arginine phosphate (AP) can no longer be maintained by anaerobic glycolysis. As the ATP decreases rapidly, the muscle passes into rigor-mortis, a state characterized by a sharp increase in the modulus of elasticity of the muscles followed by the development of a rigid texture.

Several pathways of degradation of ATP in fish and shellfish muscles have been observed and the following full-scale (figure 1) diagram has been suggested. (22,23,24,25,26,27) The scheme illustrated is a composite one (based on the previous references) and not all the compounds named are detectable or present in each species of fish or shellfish. The relative activities of the different enzymes involved in the sequence determine not only which route is followed in a particular species but also which substances may accumulate at the end or on the way, as well as the rates at which they will increase and decrease in concentration. Bacterial enzymes can also be involved, particularly

Figure 1 Possible ATP degradation pathways in fish and shellfish

(Broken lines indicate routes of minor importance)



- ITP = Inosine Triphosphate
- IDP = Inosine Diphosphate
- IMP = Inosine mono phosphate
- ADP = Adenosine Diphosphate
- AMP = Adenosine monophosphate
- NAD = Nicotinamide adenine dinucleotide
- Pi = inorganic phosphate
- F = fast reaction rate
- S = slow reaction rate

in the later stages of spoilage, with the ultimate breakdown of hypoxanthine presumably to uric acid.

It has been reported by several investigators^(28,29,30) that except in unusual circumstances, ATP is rapidly degraded in fish and shellfish muscle post-mortem as in the pathway:



Fraiser et al.,⁽³¹⁾ found that ATP was dephosphorylated and deaminated to IMP followed by gradual dephosphorylation to IMP in turn to Inosine, which is then degraded by Hy. The low levels of ADP and AMP indicated that these nucleotides could not have been present in relaxed cod muscle in amounts great enough to be detected during the post-mortem period.

Kassemsarn et al.,⁽²²⁾ found a little adenine in lemon sole muscle, indicating that ATP dephosphorylation route to adenine in this species was possible.

Fraiser et al.,⁽³²⁾ found that autolytic enzymic dephosphorylation of IMP and the production of Hy were rapidly completed in red fish muscle (without the accumulation of inosine). This completion of Hy was reached several days before the point of inedibility was attained due to the onset of bacterial spoilage.

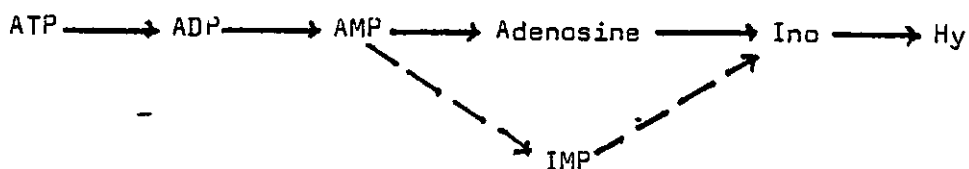
Raja and Moorjari⁽³³⁾; and Dingle and Hines⁽³⁴⁾ found that the rates of breakdown of ATP, AMP, IMP and inosine and the accompanying accumulation of hypoxanthine varied considerably in the muscle of the species studied, when stored in ice. It has been observed that ATP, ADP, AMP and IMP vary in concentration among fish muscles depending on the condition of the fish before slaughter, and that the rate of degradation changes according to the storage temperature, species of fish, type of muscle and the condition of the fish before death.

Flick and Lovell⁽⁹⁾ found that ATP degradation in the tail of unexercised brown shrimp (Panaeus aztecus) from the Gulf of Mexico followed the route:



This pathway is in agreement with Tarr and Comer⁽³⁵⁾ and Dingle et al.⁽³⁶⁾ They reported post-mortem degradation of ATP in the frozen pink shrimp (Pandalus borealis) and in the tail muscle of lobster (Homarus americanus) respectively. This route is similar to the one found in fish and different from the marine invertebrates as proposed by Arai.⁽²⁷⁾ Arai⁽²⁷⁾ reported

that ATP might be degraded in prawn as follows:



He suggested that the route passing through IMP was unlikely. Stone⁽³⁷⁾ found that the major pathway for ATP degradation in pink shrimp (Pandalus borealis) resulted in the formation of IMP and Hy. Groninger and Brandt⁽³⁸⁾ found as a result of heat processing and storage that ATP and ADP were degraded to AMP, inosine and hypoxanthine. Only small amounts of IMP were found in cooked crabmeat. Porter⁽³⁹⁾ studied the nucleotide in King crab and found:

ATP, ADP, AMP, IMP, NAD, GMP (Guanine monophosphate),
GTP (guanine triphosphate) and other nucleotide compounds.

1.4.1 Nucleotides degradation and their use for quality index

The sequence of degradative changes in the muscle of fish, crustaceans and molluscs which start with the loss of the ATP at capture continues during post-mortem storage. In most species studied, hypoxanthine concentrations have been found to rise at a reasonably steady rate throughout the period of storage at chilled temperatures, although a few exceptions have been found. In these, the Hy levels either increases so rapidly that they have reached maximum values long before the fish is inedible (Fraiser et al.,⁽³²⁾) or they increase so slowly that little change is detectable despite the apparent extensive spoilage. The dephosphorylation and deamination of ATP during post-mortem, liberates IMP, which contributes to flavour (Kuninaka et al.⁽⁴⁰⁾) A subsequent enzymic degradation of IMP is responsible partly for the progressive loss of sweet, meaty flavour during storage (Kassemsarn et al.⁽²²⁾). Hy, Ino + Hy and IMP have been suggested by several workers as an index of freshness or quality for fish and shellfish. The rates of the nucleotide change in particular species will be a major factor in determining its suitability as an index of quality. Most species have been found to be suitable subjects, since a large proportion of the nucleotide changes take place during the period of time in which the species is acceptable for edible purposes. For others, the degradative rates are too rapid or too slow and render them unsuitable.

The dephosphorylation of IMP is usually complete well within the period of edibility; hence its usefulness is limited to the early storage life of the products. Unlike IMP, Hy accumulates in most species not only throughout the edible storage life but well beyond the limit of acceptability.

⁽⁴¹⁾ Flick found that in brown Gulf shrimp (Penaeus aztecus) the Ino appeared within 24 hours and increased from 0.95 $\mu\text{mol/g}$ to 5.22 $\mu\text{mol/g}$ after 10 days of storage at 0°C. Hy was detected after 48 hours of storage and after 10 days the Hy content of the brown Gulf shrimp reached 4.52 $\mu\text{mol/g}$; he suggested that the Hy may be useful quality index for ice stored shrimp. Flick and Lovell⁽⁹⁾ found similar results in the tail muscle of unexercised brown shrimp.

The Japanese workers Ehira and Uchiyama⁽⁴²⁾ found the Hy index to be of little value in most of their commercial fish as the Hy production is less than the Ino, as an end-product in the nucleotides degradation. Ehira and Uchiyama⁽⁴²⁾ studied 98 fish species and determined the molar ratio of Ino:Hy or Hy:Ino when either ratio was 5:1 or larger, the former was arbitrarily defined as Ino forming type species whilst the latter was defined as the Hy forming species. For those Ino forming type species, the Japanese and U.K. workers developed a new freshness index, the so-called "K value", which is the ratio (expressed as a percentage) of inosine plus hypoxanthine to the total amount of ATP related compounds measured by the absorbance of the components

at 250 nm in a perchloric acid extract (Saito et al.)⁽⁴³⁾.

These authors, however, used column chromatography for the separation of the Ino + Hy fraction, a procedure too complicated for routine use. Jones and Murray⁽²⁹⁾ further developed and simplified the procedure by separation of the mononucleotides from Ino and Hy by shaking with the anion-exchange resin Dowex 1x8 at pH 6.50. The percentage of 248 nm absorbing material in the extract after resin treatment gave a measure of nucleotide dephosphorylation.

The enzyme assay using xanthine oxidase is widely used for Hy estimation.^(37,44) It is based on the conversion of Hy to uric acid by the enzyme and measuring the acid at 290 nm. Later, it has been modified by involving the indicator dye dichlorophenol indophenol into the reaction mixture, which contains a neutralised acid extract of fish muscle, phosphate buffer and the xanthine oxidase. The changes in the dye colour can be measured colorimetrically.^(45,46) Another modification to the enzyme assay test for Hy has been developed by Jahns et al.⁽⁴⁷⁾ it was an enzyme strip test. The principle was to soak the strips in a buffer and xanthine oxidase solution (under specific conditions) which were then dried and stored until required. On soaking a strip with the sample extract, a colour is produced which can be compared with a standard coloured disc or chart. It has not

been thoroughly investigated, but it could provide the basis of the routine quality test in the fishery industry, because of the simplicity and rapidity of its application.

1.5.0 Total volatile bases in fish and shellfish
and its use as a quality index

Total volatile bases (TVB) is a much used chemical method of determining the quality of many fish and fish products. TVB includes TMA, DMA and ammonia. Ammonia may arise from:

- 1) the bacterial degradation of proteins and amino acids; (57)
- 2) from the deamination of urea; (21) and
- 3) from AMP to IMP or adenosine to Ino. (22)

Ammonia was found to be produced in shrimp muscle during the post-mortem period at the rate of approximately 1 mg/day.

Ammonia was also leached simultaneously from shrimp at a logarithmic rate resulting in little apparent increase (Cobb et al. (19)). Production of ammonia during post-mortem storage resulted in pH change in rock lobster muscle. (57)

Ammonia production from bacterial urease may become important during the latter stages of storage in ice, particularly if sufficient urea has not been leached out from fish and shellfish muscle.

For the gadoids species (temperate) of fish, limits of 30-40 mg TVN/100g have been suggested as the upper limit of their quality index. Several investigations using tropical species of fish have shown that even for samples from the same batch, there are considerable variation in TVB content during storage in ice. Mowlah⁽⁴⁸⁾ reported that prawn muscle change in TVB during storage in ice for 20 days showed wide and irregular variations. Cobb, B.F. et al⁽⁴⁹⁾ reported similar results for shrimp.

Total volatile nitrogen is determined by two main basic methods, these are the micro-diffusion and distillation techniques. Careful standardisation of the method for TVB determination is required in order to enable inter-laboratory comparisons to be made.

1.6.0 Trimethylamine and its usefulness as a quality index

Measuring spoilage in the chilled state of fish depends upon the complex series of changes in the flesh constituents brought about by autolytic enzymes and putrefactive micro-organisms. The best known of these compounds is the base trimethylamine, which is derived partly by intrinsic enzyme activity but certainly by bacterial action from TMAO by triamine oxidase (Connell⁽¹⁾). TMAO is found in the tissue of many fish and shellfish. Only marine species contain TMAO; fresh water fish species rarely have any in the tissue, and if any is present it is derived from the feed (Simidu⁽⁵⁰⁾). The intrinsic enzymes (probably TMAO reducing enzyme) convert TMAO to DMA and formaldehyde.

TMA is produced in increasing amounts as spoilage advances, and accordingly there have been many attempts to use the TMA content as an index of quality. It is clear that TMA is not suitable for frozen fish or heavily salted fish (because of negligible bacterial effect under such conditions), or fresh water fish.

Burt et al.,⁽⁵¹⁾ found that the TMA index ($\log_{10} (\text{TMA} + 1)$) of cod muscle showed a linear relationship with time in ice for storage period from 7 to 20 days. Differences in the TMA content between fish from different fishing grounds at different

seasons were found to be considerable. Bethea and Ambrose⁽¹⁰⁾ found that the TMA-N (mg-N/100g) values of iced headless brown shrimp were of use in assessing the quality, but only after the prime quality had disappeared and off-flavours had become apparent to the taste panel. TMA-N remained at about 1 mg-N/100g of shrimp until the 8th day. It then started to increase, and rose rapidly from the 12th to the 16th day, after which it levelled off. Fieger and Friloux⁽⁷⁾ found that TMA-N values of ice stored headless shrimp were of value in indicating whether spoilage had occurred, but did not give information of pre-spoilage changes. Farooqui et al.,⁽⁵²⁾ found that the TMA-N values of shrimp started increasing after 4 days of storage in ice and suggested that if the TMA-N content was more than 2.5 mg/100g of shrimp, the sample was considered to be of poor quality. A level between 5 to 10 mg TMA-N/100g has been proposed as the upper limit allowable for cod measured by Dyer's method (quoted from Tozawa et al.⁽⁵³⁾). Other acceptable levels of TMA-N have been suggested for different species in different countries. It is clear that to obtain reproducible and comparable results, very careful standardisation of raw material and procedure is necessary.

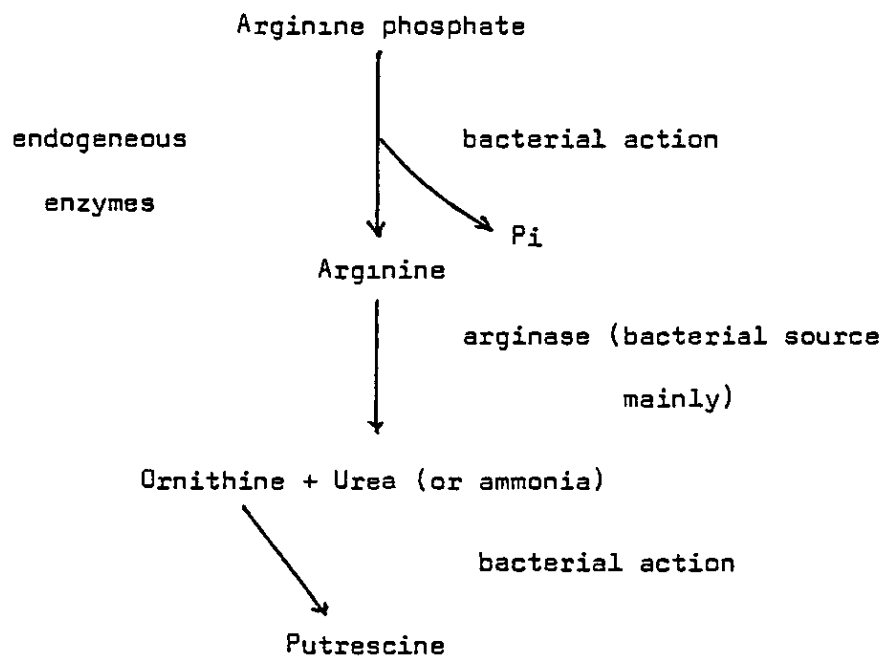
1.7.0 The post-mortem arginine phosphate degradation

A wide range of biochemical changes occur from the time shrimps are removed from sea water to the time of their consumption. These changes are brought about mostly by the combined action of endogenous enzymes present in the muscle and exogenous enzymes produced by bacteria. In general, crustacean flesh spoiled at a faster rate than that from fish under similar conditions.⁽⁵⁴⁾ The high rate of spoilage has been ascribed to a higher content of α -amino acids in the non-protein nitrogen of crustacean muscle. Loss of α -amino acids from shrimps has been correlated with deterioration of shrimps flavour.⁽⁵⁵⁾ Cobb et al.,⁽¹⁹⁾ found that the amino acids; arginine, taurine, proline and glycine contributed 93% of the total free amino acids of white shrimp (Penaeus setiferus). Glycine alone comprised 67% of the total free amino acids.

Hiltz and Bishop⁽²⁸⁾ reported that the arginine phosphate (AP) initial concentration in leg muscle of the queen crab (Chionoecetes opilio) was 28.4 μ mol/g. The AP level dropped rapidly within one day of ice storage and AP was completely hydrolysed by cooking the crab meat in boiling water for 7 minutes. Sidhu et al.,⁽⁵⁶⁾ found that AP and arginine concentrations of rock lobster (Jasus novae-hollandiae) were 26.5 (\pm 1.3) and 36.8 (\pm 1.7) μ mol/g respectively measured at 20 minutes post-mortem. AP degraded to arginine very rapidly at high temperature

(20°C). It took 6 hours at 20°C, but 72 hours at 0°C for the AP to drop to a level of approximately 1 μ mol/g. From both treated (with antibiotic) and untreated samples Sidhu et al.,⁽⁵⁶⁾ deduced that the breakdown of AP was by endogeneous enzymes and by bacterial action. The arginine level increased at the initial stages of post-mortem in rock lobster due to the degradation of AP, but arginine started to fall sharply as a result of bacterial action at 24 hour post-mortem (20°C) and produced ornithine and ammonia. Sidhu et al.,⁽⁵⁷⁾ reported that arginine did not breakdown and there was no accumulation of ornithine in the rock lobster sample treated with antibiotic (oxytetracycline).

The following diagram shows arginine phosphate degradation:-



Pi = inorganic phosphate

Figure 2 Arginine phosphate degradation pathway

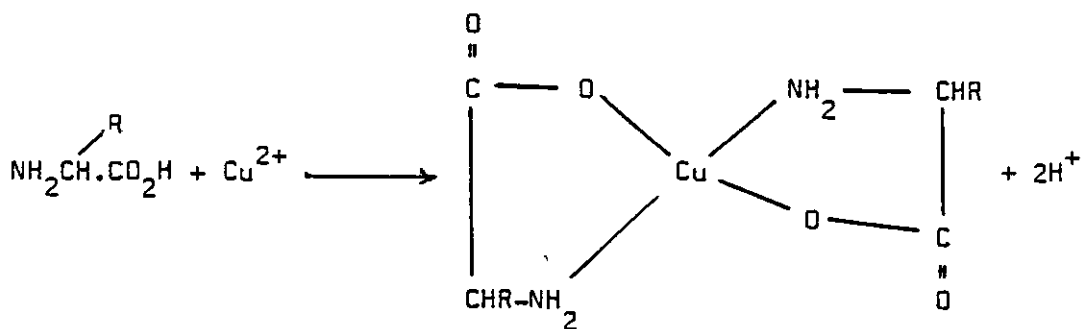
1.8.0 The quantitative colorimetric estimation of α -amino acids

There are two main quantitative colorimetric methods for estimating the α -amino acids, these are:-

- 1) the copper complex method;
- 2) the α -amino acid-ninhydrin reaction.

1.8.1 The copper complex method

The principle of this method is that two molecules of an α -amino acid and one cupric ion chelate and result in deepening of the blue colour of the solution and the formation of the blue-coloured amino acid-copper complex. This colour can be estimated quantitatively with reference to a standard curve.



α -amino acid cupric-ion amino acid-copper complex (blue)

Figure 3 Reaction of α -amino acids with cupric ion

(Greenstein and Winitz⁽⁵⁸⁾).

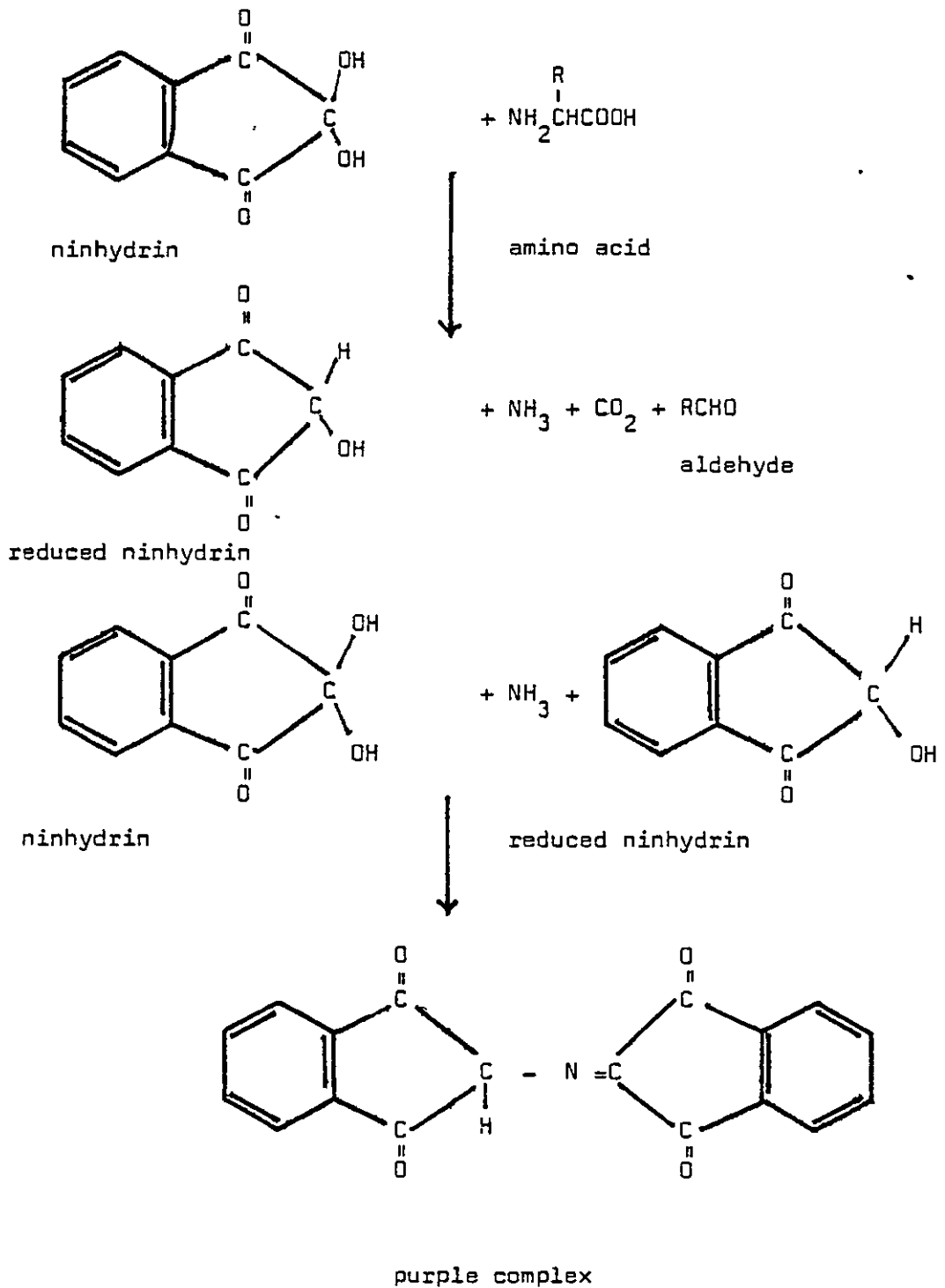


Figure 4 α -amino acid-ninhydrin reaction

1.8.2 The α -amino acids-ninhydrin reaction

The reaction between α -amino acids and ninhydrin (figure 4) (triketohydrindene hydrate) is one of the most commonly used methods for the detection and estimation of amino acids. The amino acids (in aqueous solutions close to neutrality) are involved in oxidative decarboxylation upon heating with ninhydrin. This is followed by a condensation of the diketohydrindamine and ammonia formed with a second molecule of ninhydrin to yield a purple complex (diketohydrindylidene diketohydrindamine) with a characteristic absorption band at 570 nm (Greenstein and Winitz⁽⁵⁸⁾; Tayler⁽⁵⁹⁾).

With excess ninhydrin, proline and hydroxyproline form a yellowish-red colour. Carbon dioxide is evolved, not ammonia, and two molecules of ninhydrin combine with the decarboxylated residue of the proline or hydroxyproline as follows:-

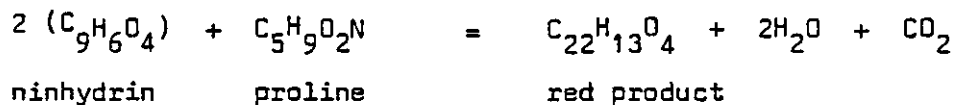
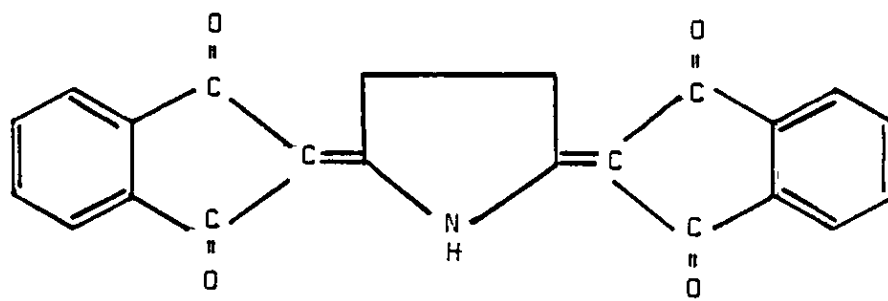
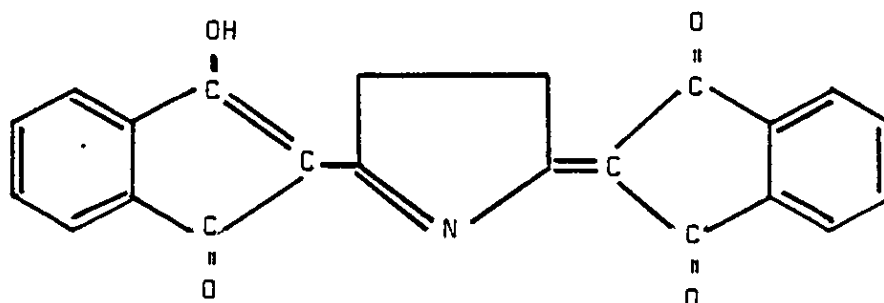


Figure 5 Reaction of ninhydrin with proline

proline first condenses with one molecule of ninhydrin, forming a yellow product, then with a second molecule forming a red complex. Grassmann and Von Arnim⁽⁶²⁾; and Troll and Lindsley⁽⁶³⁾ suggested the following structures for the proline and hydroxyproline reaction with ninhydrin in neutral (I) and acid conditions (II):-



I



II

Figure 6 Structures of proline-ninhydrin complex

Van Slyke et al.,⁽⁶⁴⁾ reported that the purple complex of the α -amino acid-ninhydrin reaction, did not form at pH below 2.5, but the red product of proline and hydroxyproline, and ornithine occurred at pH 1. Troll and Cannan⁽⁶⁵⁾ reported that the yellow solutions of proline and hydroxyproline-ninhydrin reaction possess a broad spectrum-band with the maximum at 440 nm, in contrast with the purple-red solutions which had an absorption maxima in the region of 550 to 570 nm.

The reaction between α -amino acids and ninhydrin proceeds with the formation of products (i.e. an aldehyde, ammonia, carbon dioxide, and coloured compound) each of which may provide a quantitative measure of the initial amount of α -amino acids.

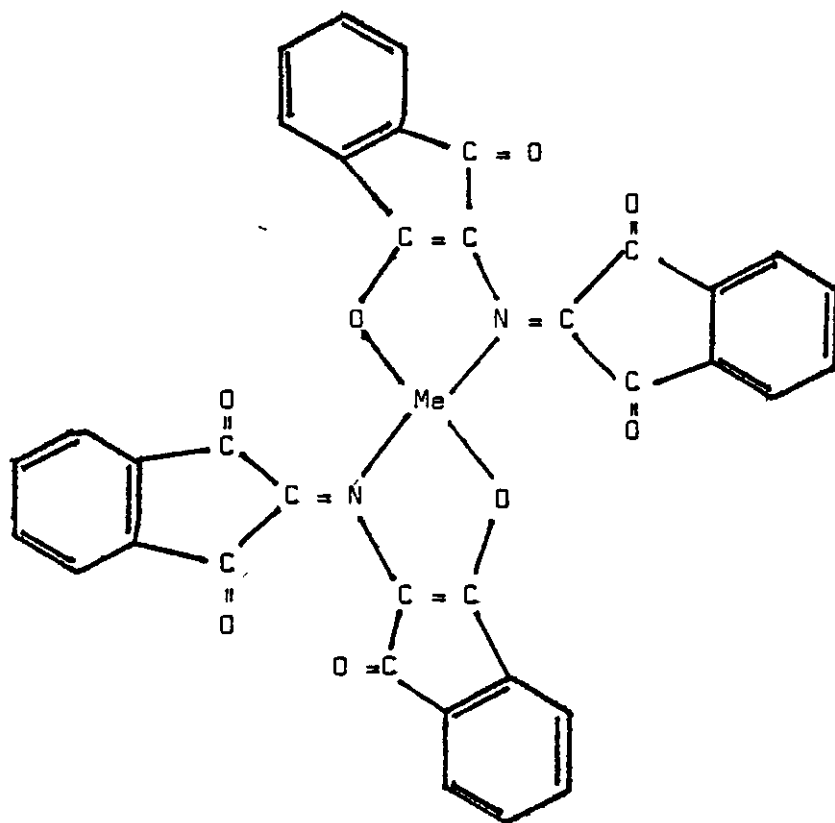
A reproducible colorimetric method has been developed by Moore and Stein⁽⁶⁶⁾ for quantitative estimation of amino acids, based on the purple colours induced by the ninhydrin, which exhibits a maximum absorption at 570 nm, for the α -amino acid and at 440 nm for proline and hydroxyproline. Chinard⁽⁶⁷⁾ developed a photometric estimation for proline, ornithine, lysine and hydroxylysine in pure solution, based on the colour formed by the reaction of these amino acids with ninhydrin at approximately pH 1.0. Schweet⁽⁶⁸⁾ described a modified Chinard's method, based on the formation of a red colour when proline and pipercolic acid react with ninhydrin in glacial acetic acid under nearly anhydrous conditions. Troll and Lindsley⁽⁶³⁾ modified

Chinard's method⁽⁶⁷⁾ for proline (cysteine interferes slightly) by removing the basic amino acids (ornithine, lysine and hydroxylysine) by shaking the solutions of these amino acids with permutit resin.

1.8.2.1 The effect of metals on the amino acids-ninhydrin reaction

In paper chromatography, ninhydrin has been used almost exclusively for the identification and qualitative assay of amino acids. The purple pigment (diketohydrindylidene diketohydrindamine), which is formed in this reaction faded rapidly on filter-paper chromatograms. It was found by Kawerau and Wieland⁽⁶⁹⁾, Giri *et al.*,⁽⁷⁰⁾ that the colour initially purple could be made more stable by coupling it with a metal ion, to form a stable red pigment. This occurred with all the α -amino acids except proline and hydroxyproline, which formed yellow metal complexes.⁽⁶⁹⁾

The red complex salt was formed from two molecules of the purple pigment with one atom of the metal being linked between the two nitrogen atoms. Hence, it was described as triketo-hydrindyliden-2-amino-1-oxy-indenon (3)-metal. The following structural formula has been suggested by Wieland:-⁽⁷¹⁾



Me = Metal ion

Figure 7 Chemical structure of metal-amino acid-ninhydrin

Kawerau and Wieland⁽⁶⁹⁾ found that the red copper pigment, was relatively stable on dry filter-paper when it was out of contact with other ions, but it was very unstable in aqueous solutions. The free hydrogen ions caused rapid bleaching of the red copper complex, Meyer and Ricklis⁽⁷²⁾ suggested that several cations impair the ninhydrin reaction with amino acids. Kalant⁽⁷³⁾ overcame the inhibition problem of the metals by using a citrate buffer. Copper sulphate appeared to improve the

colour development slightly (Giri et al.,⁽⁷⁰⁾ Kalant⁽⁷³⁾). Kalant⁽⁷³⁾ showed that cobalt citrate and ferrous sulphate did not improve the colour intensity, when the Yemm and Cooking⁽⁷⁴⁾ method was used. Manganese ions (from $MnCl_2 \cdot 4H_2O$) were found by Singh et al.,⁽⁷⁵⁾ to enhance the colour formed when amino acids reacted with ninhydrin, based on the method of Lee and Takahashi⁽⁷⁶⁾. All the amino acids showed colour enhancement in the presence of Mn^{2+} except L-proline and hydroxyproline, presumably because proline and hydroxyproline do not form a blue pigment with ninhydrin, but give a yellow colour. L-ornithine showed a two-fold enhancement. The sensitivity of the method was significantly increased by the presence of manganese ions in the reaction mixture at levels of $5 \mu g$ per 2 ml of the reaction mixture.

1.9.0 The separation of amino acids by ion exchangers

The separation of amino acids by ion exchange techniques has been widely used to permit quantitative estimation of the acids. The determination of the amount of each amino acid, or a group of the acids in a mixture involved quantitative separation using ion exchange under either "batch" or "column" conditions. A "batch" procedure involved shaking or stirring the resin with the solution of acids according to published procedures. A "column" procedure involved passing the solution through a packed column of the resin.

Folin and Bell⁽⁷⁷⁾ and Folin⁽⁷⁸⁾ used the batch procedure for separating ammonia from urine using synthetic zeolite with the trade name "Permutit". The permutit resin is a sodium salt of aluminate silicate, and has the approximate composition (Whitehorn⁽⁷⁹⁾) $(\text{SiO}_2)_2, \text{Al}_2\text{O}_3, \text{Na}_2\text{O} \cdot 0.6\text{H}_2\text{O}$.

The separation of amino acids by column procedures is widely used, especially with a wide range of ion exchangers, at wide range of pH and temperatures. Whitehorn⁽⁷⁹⁾ recommended the use of column procedure rather than the batch procedure when he used the permutit resin for separating several amines. Moore and Stein⁽⁸⁰⁾; and Hamdy et al.,⁽⁸¹⁾ used the column procedure for separating a wide range of amino acids quantitatively. They

gave full details including the methods of elution, and the estimation of the amino acids by ninhydrin reaction.

Piez et al.,⁽⁸²⁾ described a method of separating the proline and other cyclic imino acids with Dowex 50X12 resin and their subsequent estimation. Archibald⁽⁶⁰⁾ used the Decalso resin for separating arginine from glutamine. Troll and Lindsley⁽⁶³⁾ used permutit resin for separating the basic amino acids from the others, especially proline, using the batch procedure in the pH range 1 to 7, at room temperature. The batch procedure is a simple and rapid technique and may be used at room temperature. However, not all ion exchange resins can be used in this procedure. The column procedure is generally more complex and expensive, and requires trained personnel.

PART I

2.0.0 THE APPLICATION OF CHINARD'S METHOD FOR THE ESTIMATION
OF COMBINED ORNITHINE AND PROLINE (I.E.. "ORNITHINE EQUIVALENT")

2.0 (A) Instrumental data

Throughout this thesis, the absorbances were measured using a Perkin-Elmer Model 402 spectrophotometer. In all measurements 1 cm silica cells were employed.

2.0 (B) pH meter

An EIL pH meter Model 388, with a glass electrode was used throughout this work.

2.0 (C) Chemicals

All the chemicals used in this thesis were standard grade, and supplied by British Drug Houses (BDH), England.

2.1.0 Standard procedure for determination of amino acids
using Chinard's method (67)

2.1.1 Reagents:-

i) Acid mixture

6M phosphoric acid H_3PO_4 was mixed with glacial acetic acid in the proportion of 40/60 (v/v).

ii) Ninhydrin solution

25 mg of ninhydrin (triketohydrindene) were dissolved per one ml of acid mixture (as above) and heated up to $70^{\circ}C$ to ensure that the ninhydrin was dissolved, and allowed to cool to room temperature prior to use. This reagent solution was made fresh daily.

2.1.2 Experimental method:-

Pyrex test tubes (20 ml) were used.

First test tube:- to this was added a mixture made up of

- i) 2.0 ml of the solution to be analysed;
- ii) 2.0 ml of glacial acetic acid;
- iii) 2.0 ml of ninhydrin solution.

Second test tube:- to this was added a mixture as given below and was used as the sample blank

- i) 2.0 ml of the solution to be analysed;
- ii) 2.0 ml of glacial acetic acid;
- iii) 2.0 ml of acid mixture without ninhydrin.

Third test tube:-

This contained the reagent blank, which consisted of:-

- i) 2.0 ml of the solvent of the amino acid (80% ethanol);
- ii) 2.0 ml of ninhydrin solution;
- iii) 2.0 ml of glacial acetic acid.

After mixing the contents of each test tube, the tubes were capped and then heated in a boiling water-bath at 100°C for 60 minutes. 2.0 ml of glacial acetic acid was then added to each tube and the tubes were cooled to room temperature. The volume of each was adjusted to 10.0 ml in a volumetric flask with glacial acetic acid. The absorbances were measured against the reagent blank at 515 nm. Measurements were taken within half an hour after developing the colour.

Note:

In the reported Chinard⁽⁶⁷⁾ procedure, the volume of each solution to be analysed; the glacial acetic acid, and the ninhydrin solution were each 1.0 ml and the total volume was made up to 5.0 ml in a volumetric flask. In the present work, although the volumes were doubled, the ratio of the reagent and sample was the same as in the reported procedure.

The sample blank was found to have no effect on the measurement of the standard amino acids or the amino acids in the shrimps extracts.

2.2.0 The procedures for the application of Chinard's method
to pure solutions of amino acids

(A) Amino acids used:-

DL-ornithine monohydrobromide;

L-proline (Hydroxyl-L-proline free);

L-Histidine monohydrochloride;

L-Arginine;

Glycine, and

L-lysine;

were used.

All the amino acids were supplied as chromatographically
pure (B.D.H.)

2.2.1 The standard amino acids solutions

i) Stock solutions

They were made by dissolving 50.0 mg of each amino acid
separately in 80% ethanol and 96% ethanol, and each made up to
100.0 ml in a volumetric flask with 80% and 96% ethanol, as
appropriate.

The resulting concentration of each solution was 500 μ g/ml
of 80% ethanol or 96% ethanol.

ii) Working solutions

A range of concentrations were made by diluting the stock solutions separately to the required concentrations with 80% ethanol and 96% ethanol as appropriate (see Tables 1, 2, 3 and 5). 2.0 ml of each working solution of the amino acids were subjected to the Chinard procedure (as in 2.1.2).

iii) Mixture of amino acids

Mixtures of stock solutions of the amino acids were made at the ratios required and diluted with 80% ethanol to the required concentrations (see Table 6). 2.0 ml of each mixture were subjected to the Chinard procedure (2.1.2).

2.2.2 The procedure for determining the stability of the ornithine-ninhydrin colour

A range of ornithine standard solutions were measured by the Chinard procedure and the developed colours were left for 1, 2, 4, 6 and 24 hours (see Table 4).

2.2.3 The procedure for determining the effect of addition of metal ions to the ornithine standard solutions

Manganous chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) and cupric sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were used as sources of Mn^{2+} and Cu^{2+} ions. They

were dissolved separately in distilled water. 20.0 mg of each of the salts were separately dissolved and diluted up to 100.0 ml in volumetric flask to obtain concentrations of 200 $\mu\text{g/ml}$ as stock solutions. A mixture of ornithine and the salts stock solutions were made at the required ratio and diluted with 80% ethanol to the required concentrations (see Table 8).

2.2.4 The procedure for determining the pH effect on the developed colour

Mixtures of the standard amino acids solutions, the ninhydrin solution and glacial acetic acid were adjusted to the pH required by using a buffer solution ($\frac{1}{5}\text{M KH}_2\text{PO}_4 + 1\text{M KOH}$). These solutions were then separately subjected to further Chinard procedure analysis (see Table 7).

2.3.0 The results and discussion of the application of
Chinard's method on standard amino acids solution

The objective of the experiments in this section was to determine the parameters of the amino acid-ninhydrin reaction for its application on the shrimp extracts.

2.3.1 The results and discussion of the solvent effect on
the ornithine-ninhydrin reaction

Results are shown in Tables 1 and 2.

Ethanol was suggested as a solvent for precipitating the protein and extracting the free amino acid (Welcher⁽⁸³⁾). Therefore, it was necessary to investigate the effect of this solvent on the reaction of the free amino acids with the ninhydrin, under the Chinard's procedure conditions.

Table 1 Chinard's method applied to DL-ornithine

Solvent used: 80% ethanol

DL-ornithine conc. in $\mu\text{g/ml}$	* Absorbance readings			
	X_1	X_2	X_3	Mean \bar{X}
2	0.038	0.038	0.038	0.038
5	0.100	0.098	0.100	0.099
10	0.200	0.200	0.208	0.203
20	0.438	0.455	0.438	0.444
30	0.705	0.685	0.675	0.688

absorbance error ± 0.003

Regression equation obtained was

$$Y = 0.01830534 + 0.02333622X$$

Correlation coefficient $R = 0.9994875$ ($P < 0.01$)

The regression equation was used to calculate the amount of ornithine from the absorbance readings,

where $X =$ amount of ornithine in $\mu\text{g/ml}$

$Y =$ absorbance reading at 515 nm

Values of the correlation coefficient (r) were calculated by a computer. The calculated r values were compared at appropriate significance level with the tabulated r values in the standard statistical tables (Statistical Tables, second edition, Ed. by Murdoch J. and Barnes J.A., Macmillan Press Ltd., 1970).

Table 2 Chinard's method applied to DL-ornithine

Solvent used: 96% ethanol

DL-ornithine conc. in $\mu\text{g/ml}$	* Absorbance readings					mean \bar{X}
	X_1	X_2	X_3	X_4	X_5	
0.5	0.005	0.020	0.010	0.010	-	0.011
1.0	0.010	0.030	0.010	0.010	0.015	0.015
2.0	0.030	0.040	0.030	0.020	0.030	0.030
5.0	0.078	0.090	0.070	0.070	0.078	0.077
10.0	0.170	0.180	0.170	0.165	0.185	0.174
15.0	0.251	0.262	-	0.280	0.275	0.267
20.0	0.380	0.420	0.365	-	0.385	0.388
25.0	0.525	0.545	0.505	0.525	0.503	0.521
30.0	0.605	0.670	0.630	0.655	0.616	0.635

* (See Table 1)

Regression equation for ornithine $Y = -0.01901339 + 0.02109789 X$

Correlation coefficient $R = 0.996829$

$Y =$ absorbance reading at 515 nm

$X =$ amount of ornithine in $\mu\text{g/ml}$

Graph 1 The calibration curves of ornithine and proline

Solvent used:- 80% ethanol

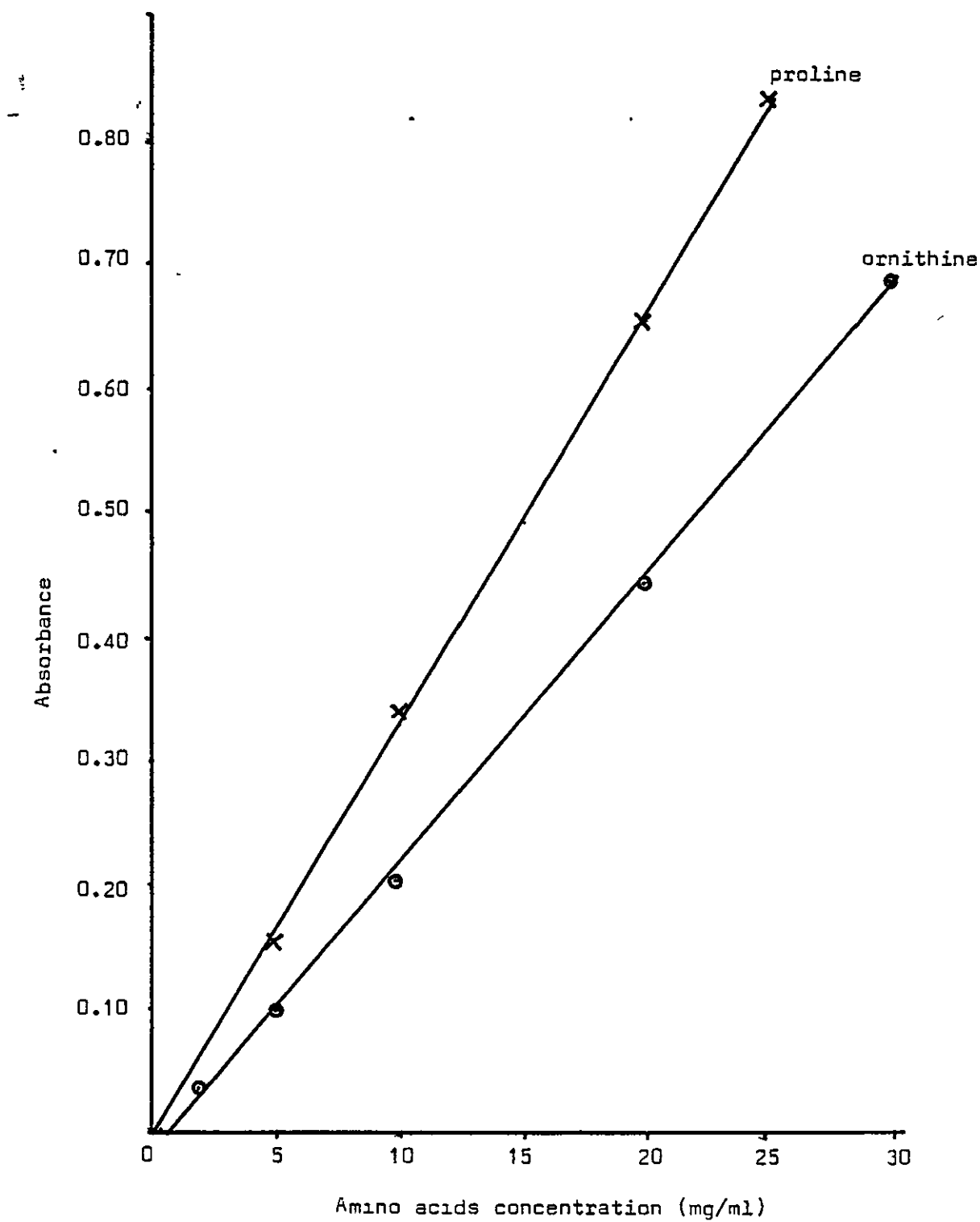


Table 3 Chinard's method applied to L-proline

Solvent used: 80% ethanol

Proline conc. in $\mu\text{g/ml}$	* Absorbance readings			
	X_1	X_2	X_3	mean \bar{X}
5	0.155	0.150	0.160	0.155
10	0.340	0.335	0.345	0.340
20	0.650	0.645	0.670	0.655
25	0.840	0.835	0.840	0.838

* (See Table 1)

Regression equation obtained was

$$Y = -7.300018 \times 10^{-3} + 0.03362 X$$

Correlation coefficient $R = 0.9995016$ ($p < 0.01$)

where

Y = absorbance reading at 515 nm

X = amount of proline in $\mu\text{g/ml}$.

Hence 80% ethanol appeared to be the best solvent for this reaction ($r = 0.9995$ $P < 0.01$). The higher solvent concentration of 96% ethanol used with the ornithine showed an inhibiting effect on the colour intensity along the concentration range used ($r = 0.9968$ $P < 0.01$) (Table 1 and 2). A linear relationship was obtained by Chinard⁽⁶⁷⁾ for proline and ornithine at concentrations between 0.02-0.1 $\mu\text{mol/ml}$ of the amino acid. The concentration of amino acid used here in this thesis was between 2-30 $\mu\text{g/ml}$.

2.3.2 The results and discussion of the stability of the developed colour

Results are shown in Table 4.

The colour intensity of ornithine-ninhydrin reaction showed a fading of its initial reading after one hour, this initial reading refers to that obtained within $\frac{1}{2}$ hour of its development. The rate of colour fading was greater at low amino acid concentrations (rate loss was about 20% of the initial reading, after one hour at 2 $\mu\text{g/ml}$). The average rate of colour loss (read 1 hour after the initial reading) for high amino acid concentration was about 4% of the initial reading. Hence, it was concluded that the colour intensity must be read

Table 4 Stability of the ornithine-ninhydrin colour measured by
Chinard's method

Solvent used: 80% ethanol

Ornithine concentrations ($\mu\text{g/ml}$)	* Absorbance					
	initial reading	1 hr	2 hr	4 hr	6 hr	24 hr
2	0.035	0.028	0.025	0.025	0.025	0.025
10	0.200	0.180	0.170	0.170	0.170	0.170
20	0.430	0.410	0.390	0.390	0.385	0.380
30	0.680	0.650	0.645	0.635	0.630	0.625

* (See Table 1).

as soon as possible, within half an hour of developing the colour and at a high concentration of the amino acid or sample to be analysed to avoid any fading in the developed colour. However, the results in this thesis were similar to those of Chinard's⁽⁶⁷⁾ observations, where he found that the colour was stable for at least an hour.

2.3.3 The results and discussion of the interference of amino acids on the ornithine-ninhydrin reaction

Results are shown in Tables 5 and 6.

Glycine and arginine showed no 'interference' with the method. The lysine 'interference' was shown to be negligible. Chinard's⁽⁶⁷⁾ experiments showed similar results. Lysine and arginine are present as free amino acid in the shrimp extractive, (Cobb, et al.,⁽¹⁹⁾). Other than these slight 'interferences', the method was shown to be reproducible for ornithine and proline for the 80% ethanol.

2.3.4 The results and discussion of the pH effect on the amino acids-ninhydrin reaction

Results of this experiment are shown in Table 7.

Table 5 Absorbances of Glycine, L-Arginine and L-lysine standard solutions estimated by Chinard's method

Solvent used: 80% ethanol

amino acids	amino acid conc. in $\mu\text{g/ml}$	* Absorbance				mean \bar{X}
		X_1	X_2	X_3	X_4	
L-lysine						
	25	0.080	0.060	0.070	0.070	0.070
	40	0.130	0.130	0.120	0.125	0.126
	80	0.245	0.235	0.250	0.250	0.245
L-Arginine						
	10	0.000	0.000	0.000	0.000	0.000
	500	0.015	0.025	0.025	0.028	0.023
Glycine						
	20	0.000	0.000	0.000	0.000	0.000
	250	0.000	0.000	0.000	0.000	0.000
	500	0.0	0.010	0.010	0.010	0.008

* (See Table 1).

Table 6 Absorbances of mixtures of amino acids measured by Chinard's method

Solvent used: 80% ethanol

test tube no.	ornithine	proline	lysine	glycine	arginine	Absorbance			Calculated absorbance
	$\mu\text{g/ml}$ mixture solution					X_1	X_2	mean \bar{X}	*
1	10	10	-	-	-	0.55	0.56	0.555	0.543
2	10	-	40	-	-	0.38	0.39	0.385	0.329
3	10	-	-	250	-	0.30	0.315	0.308	0.203
4	10	-	-	-	250	0.31	0.32	0.315	0.214
5	-	10	40	-	-	0.505	0.51	0.508	0.466
6	-	10	-	250	-	0.45	0.455	0.452	0.340
7	-	10	-	-	250	0.44	0.445	0.442	0.351
8	-	-	40	250	-	0.12	0.13	0.125	0.126
9	-	-	40	-	250	0.15	0.145	0.148	0.137
10	-	-	-	250	250	0.015	0.015	0.015	0.011

* The absorbance of each mixture was calculated by combining the individual absorbance of each amino acid from Tables: 1, 3 and 5.

Table 7 The effect of pH on colour developed from the amino acid-ninhydrin reaction using the Chinard's method

pH	Colour development of the ninhydrin with the amino acids					Reagent blank
	Ornithine	Proline	Lysine	Glycine	Arginine	
1.20	reddish	reddish	orangish	colourless	colourless	
1.50	reddish	reddish	orangish	colourless	colourless	
2.25	reddish	reddish	orangish	colourless	colourless	
4.00	blue	blue		blue	blue	
4.40	blue	blue				
7.00	yellow	yellow	yellow	yellow	yellow	yellow

Proline and ornithine gave a red colour at pH near 1. However, raising the pH above 7.0 caused the colour of the ninhydrin-amino acid complex to deviate from the normal blue complex (the yellow pigment at pH above 7 may be caused by the ninhydrin present). It was found that pH 2.25 was the highest pH level at which Chinard's⁽⁶⁷⁾ method could be applied. Above pH 2.25, other amino acids interfere and the method would no longer be specific for proline, ornithine and lysine. Van Slyke⁽⁶⁴⁾ reported that the blue pigment of the amino acid with ninhydrin reaction (other than proline and hydroxyproline) could not be produced under pH 2.5, and that proline reacted at pH 1 to give the red pigment.

2.3.5 The results and discussion of the effect of metal ions on the ornithine-ninhydrin complex

Results are shown in Table 8.

It has been reported by Kalant⁽⁷³⁾ and Singh *et al.*,⁽⁷⁵⁾ that the amino acid-ninhydrin pigment (blue/purple) could be enhanced by the addition of some cations such as Cu^{2+} , Mn^{2+} , Fe^{2+} . Singh *et al.*,⁽⁷⁵⁾ found that the Mn^{2+} ions enhanced the L-ornithine-ninhydrin complex colour by a factor of two at pH 5.5 in citrate buffer, but proline did not show any colour enhancement. The reason could be that the proline-ninhydrin

Table 8 The effect of the metal ions on the DL-ornithine-ninhydrin reaction measured by Chinard's method

Metal ion	*amount of metal (salt) added μg	Absorbance readings		
		ornithine concentration in $\mu\text{g/ml}$		
		2	10	30
Mn^{++}	0.0	0.035	0.200	0.680
$(\text{MnCl}_2 \cdot 4\text{H}_2\text{O})$	0.8	0.035		
	1.2	0.030		
	2.0	0.030		0.675
	4.0	0.035		
	10.0	0.030	0.195	0.670
	14.0	0.035		
	20.0	0.035	0.190	
	30.0	0.035	0.190	0.670
Cu^{++}				
$(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})$	0	0.035	0.200	0.680
	20	0.030	0.195	0.630
	40	0.030	0.200	0.64

* (See 2.2.3).

reaction formed a yellow pigment and not a purple pigment in the stated condition. The addition of Mn^{2+} or Cu^{2+} to the ornithine-ninhydrin reaction mixture (in the work done here) did not show any colour enhancement at any of the amino acid concentrations used. The reason may be due to the low pH (near 1) of the reaction, which might impair the amino acid-ninhydrin-metal complex.

2.4.0 The application of Chinard's method to shrimp extracts

2.4.1 The procedure for the determination of
"Ornithine Equivalent" of pink shrimps

Pink shrimps (Pandalus montaquii) purchased locally, were produced by J. Van Smirren Ltd., Boston, England, as cooked, salted frozen shrimps, packed in polyethylene bags. The date of catching and subsequent history of treatment were not known. The shrimps were mixed thoroughly before they were prepared for sampling.

2.4.2 First batch

(a) "Ornithine Equivalent" of pink shrimps, "bag stored"
as whole unpeeled shrimp during storage in ice

The frozen cooked shrimps were left at room temperature for half an hour to thaw. Samples of between 5 and 8 shrimps were dried by filter paper and weighed (about 14g), and kept in polyethylene bags as whole unpeeled shrimps. The bags were kept in a box of ice and then stored in a fan cooled Foster Refrigerator at 0° to 5°C. Samples were taken in duplicate for the "Ornithine Equivalent" determination at the stated intervals.

- (b) "Ornithine Equivalent" of pink shrimps, "open stored"
as whole unpeeled shrimp during storage in ice

The frozen cooked shrimps were prepared, sampled and analysed as in 2.4.2 (a), the shrimps were not kept in bags, but stored in the box of ice as open whole unpeeled shrimps. The samples were weighed immediately before carrying out the analysis for "Ornithine Equivalent".

2.4.3 Second batch

- (a) "Ornithine Equivalent" of pink shrimps, "bag stored"
as peeled shrimp during storage in ice

The frozen cooked shrimps were prepared and analysed as in 2.4.2 (a), except that the samples were peeled and weighed, then kept in bags as peeled shrimps (tails only).

- (b) "Ornithine Equivalent" of pink shrimps, "open stored"
as whole unpeeled shrimp (peeled just before analysis)
during storage in ice

The frozen cooked shrimps were treated as in 2.4.2 (a), except that the samples were kept in ice as "open-stored" whole unpeeled shrimp. The samples were peeled and immediately analysed for "Ornithine Equivalent".

2.5.0 The procedure for the determination of "Ornithine Equivalent" of brown shrimps

Brown shrimps (Crangon crangon) were caught in March 1980 off Cleethorpes beach, England, in shallow water (2-3 feet deep) using a push net. The fresh shrimps were brought to the laboratory in sea water within two hours of the catch. The sea water was drained off, and the shrimps were washed with cold tap water. The shrimps were divided into two batches.

2.5.1 First batch treatment

- (a) "Ornithine Equivalent" of brown shrimps, "bag stored" as whole unpeeled shrimps during storage in ice

The fresh brown shrimps were sampled and stored as in 2.4.2 (a).

2.5.2 Second batch treatment

- (a) "Ornithine Equivalent" of brown shrimps, "bag stored" as whole unpeeled shrimps during storage in ice

The fresh brown shrimps were frozen at -35°C and stored at -30°C for two weeks. The frozen shrimps then were thawed and treated as in 2.5.1 (a), and analysed for the "Ornithine Equivalent" as "bag stored" samples.

2.6.0 The procedure for the "Ornithine Equivalent" measurement

2.6.1 The extraction method for the free amino acids of the shrimps

- i) About 14g of shrimp samples, peeled or unpeeled, were homogenised with 50 ml of 96% ethanol for 2 minutes, using Ato-mix blender (Measuring and Scientific Equipment Ltd.)
- ii) For the samples stored in bags, any residue in the bags was washed out with (80%) ethanol. The solution so obtained was added to the homogenate;
- iii) The homogenates were filtered under suction using Whatman No. 42 filter paper, and the protein precipitates were washed with 80% ethanol;
- iv) The filtrates were made up to the volume in 100 ml volumetric flasks with 80% ethanol;
- v) The extracts were diluted up to 50-fold (especially the "bagged samples") with 80% ethanol. 2 ml (in duplicate) of each of these diluted extracts were taken for the analysis of amino acids as "Ornithine Equivalent" by the Chinard procedure, as in section 2.1.0.

2.6.2 "Ornithine Equivalent" calculation

The concentration of the amino acids in the extract; measured by Chinard procedure, and referred to as "Ornithine Equivalent" was calculated from the Ornithine calibration curve. Hence it is referred to as the "Ornithine Equivalent" and expressed as m mol of ornithine/100g wet sample.

$$\text{Conc. } (\mu\text{g/ml} \times \text{dilution factor} \times \frac{100 \text{ g}}{\text{sample weight}} \times \frac{1}{213.09} \times \frac{1}{1000} =$$

"Ornithine Equivalent" m mol/100g

Where:

The relative molecular mass of ornithine monohydrobromide
= 213.09

Conversion factor from μ mol to m mol

$$= \frac{1}{1000}$$

2.7.0 The results and discussion of the "Ornithine Equivalent" changes in pink shrimps (Pandalus montaquii), peeled and unpeeled, "open" and "bag stored" samples during storage in ice

The objective of this set of experiments was to investigate the "Ornithine Equivalent" changes of "open" and "bag stored" shrimps and the leaching effect of ice.

The results for the (a) whole unpeeled, and (b) peeled pink shrimps when "open" or "bag stored" are shown in graphs 2 and 3; and Tables 9 and 10. The "Ornithine Equivalent" of the peeled and unpeeled, open stored shrimps (graph 2 and 3) fell sharply in the early stages of ice storage. This decline in the "Ornithine Equivalent" could be due mainly to the washing effect of the melting ice on the free amino acids ornithine and proline. In contrast, the bag stored peeled and unpeeled samples which were protected from the leaching effect of ice, did not show the sharp decline in the "Ornithine Equivalent" values. Iyenger et al.,⁽⁸⁴⁾ reported that the leaching effect would depend upon

- 1) the amount of shrimps stored;
- 2) the size of the shrimps, the smaller the shrimps, the greater the leaching effect;
- 3) the amount of ice used.

Cobb et al.,⁽¹⁹⁾ showed that the rate of loss of free amino acids from white shrimps (Penaeus setiferus) was increased with

Graph 2 "Ornithine Equivalent" changes of pink unpeeled shrimps

(a) "bag stored" in ice

(b) "open stored" in ice

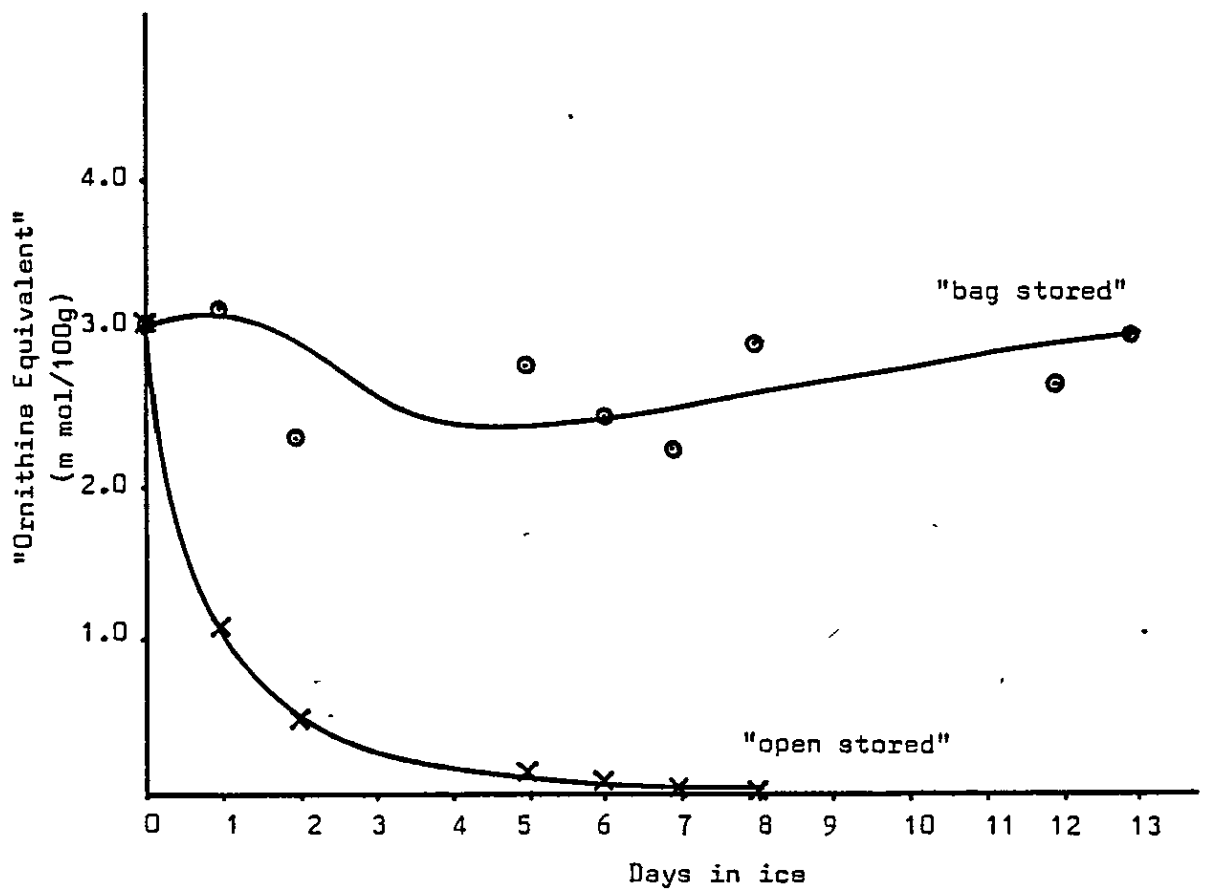


Table 9 "Ornithine Equivalent" changes of pink shrimps

(a) "bag stored" in ice as whole unpeeled shrimps

(b) "open stored" in ice as whole unpeeled shrimps

Storage period (days)	"Ornithine Equivalent" m mol/100g					
	(a) "bag stored"			(b) "open stored"		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	2.941	3.205	3.073	2.941	3.205	3.073
1	3.121	3.314	3.218	1.109	1.076	1.092
2	2.254	2.355	2.305	0.455	0.470	0.462
5	2.820	2.813	2.816	0.102	0.156	0.129
6	2.793	2.125	2.459	0.075	0.090	0.083
7	2.243	-	2.243	0.053	0.046	0.050
8	3.084	2.749	2.916	0.045	0.026	0.036
12	2.628	2.715	2.672	-	-	-
13	2.913	3.026	2.970	-	-	-

Graph 3 "Ornithine Equivalent" changes of pink shrimps

- (a) "bag stored" in ice as peeled shrimps
- (b) "open stored" in ice as whole unpeeled shrimps
(peeled just before analysis)

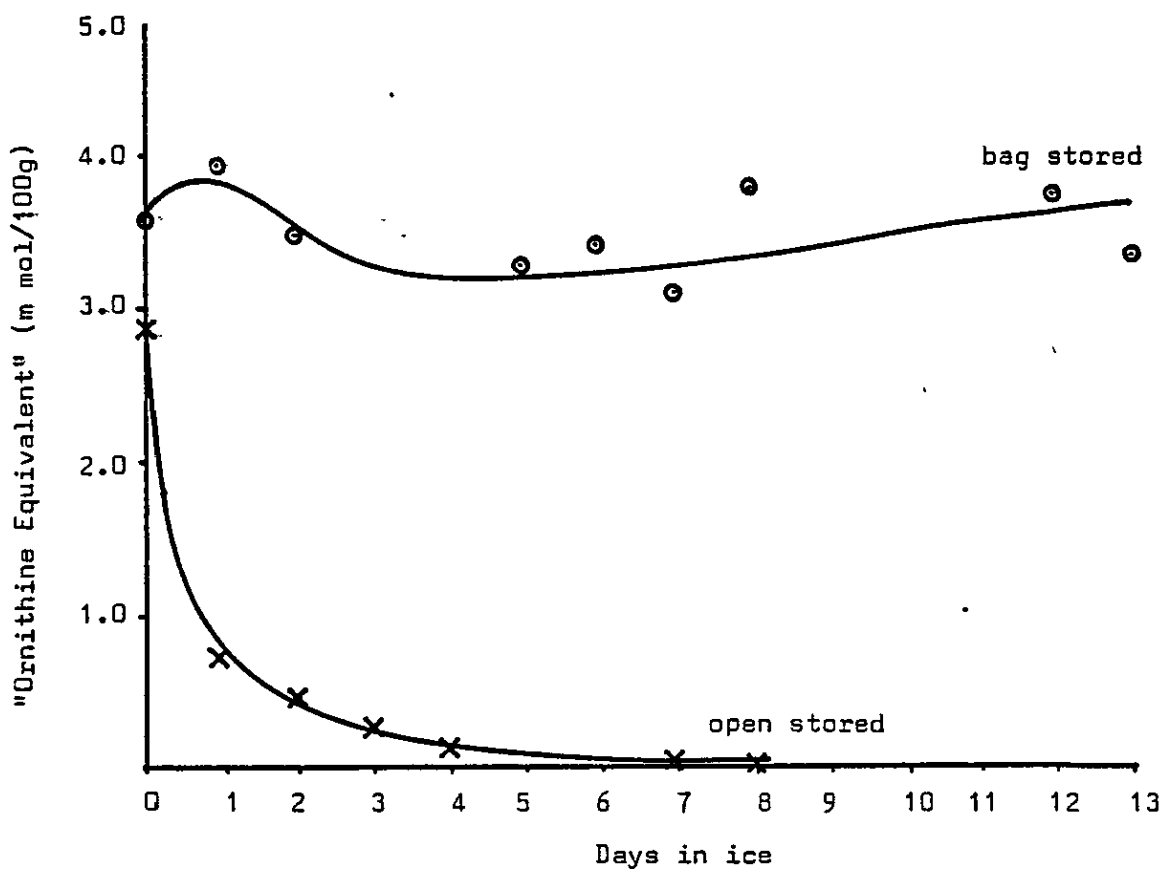


Table 10 "Ornithine Equivalent" changes of pink shrimps

- (a) "bag stored" in ice as peeled shrimps
 (b) "open stored" in ice as whole unpeeled shrimps
 (peeled just before analysis)

Storage period (days)	"Ornithine Equivalent" m mol/100g					
	(a) "bag stored"			(b) "open stored"		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	3.225	3.938	3.581	2.808	2.880	2.845
1	3.778	4.126	3.953	0.712	0.731	0.721
2	3.563	3.398	3.480	0.431	0.469	0.450
3	-	-	-	0.234	0.262	0.248
4	-	-	-	0.108	0.118	0.113
5	3.226	3.335	3.28	-	-	-
6	3.517	3.331	3.424	-	-	-
7	3.255	2.902	3.079	0.031	0.032	0.031
8	3.870	3.646	3.758	0.015	0.017	0.016
12	3.565	3.933	3.749	-	-	-
13	3.358	3.284	3.321	-	-	-

the increasing rate of the ice melt and obtained 81% lower ornithine in ice stored than in "bag stored" shrimps.

Cobb et al.,⁽¹⁹⁾ also suggested that the size of the shrimps and the ice particle size (which could affect the flow of the melt ice water) could affect the loss of the free amino acids.

The "Ornithine Equivalent" of the "bag stored" unpeeled and peeled samples showed a slight increase and then a decline in the early storage period, but started to increase gradually on the 6th day until the samples reached the organoleptically unacceptable stage on the 12th day. There are 2 possible reasons for the rise and decline over the short period.

- 1) a possible ornithine increase, as the arginine content increased from the arginine phosphate degradation (this was for a short period depending on the initial arginine phosphate content Sidhu et al.⁽⁵⁷⁾);
- 2) the possible effect or "interference" of proline (see results in latter experiments graph 6(a)) changes during storage in ice, superimposed on the ornithine changes. Proline has been reported to

show 20% decrease during ice storage in bags
(Cobb et al. (19)).

The rate of proline decrease could be greater than the rate of the ornithine increase, hence the small decline in the "Ornithine Equivalent" before the accumulative arginase activity due to the bacterial load built up became apparent, giving the rise in the curve until the 12th day.

The initial "Ornithine Equivalent" values of peeled "bag stored" shrimps was higher than the unpeeled "bag stored" samples. The possible reasons are that:-

- 1) the same sample weight was obtained for the peeled and unpeeled samples, hence 100% muscle tissue was used from the peeled samples while the weight of the head and body shells were included in the unpeeled samples. The shell cutin may have little free amino acid, or it may be difficult to extract;
- 2) it could also be due to natural variations (seasonal different harvesting grounds, etc.,) hence the difference in the initial free amino acid content.

2.8.0 The results and discussion of the "Ornithine Equivalent" changes of brown shrimps (Cranqon cranqon) "bag stored" in ice

Results of the two experiments on fresh brown shrimp are shown in graph 4, and Tables 11 and 12.

From the previous experiments on the commercial pink shrimps, stored open or in bag, the "Ornithine Equivalent" decreased sharply in the open stored samples due to the leaching effect of melting ice water. Hence it was decided to carry out the following experiments on the fresh brown shrimp, unpeeled and stored in bag to minimise the leaching effect.

The "Ornithine Equivalent" of brown shrimp showed a similar pattern during ice storage to those of the "bag stored" pink shrimps, relatively steady increase in both brown shrimp batches, from the 5th day of the storage in ice. The second run had relatively higher "Ornithine Equivalent" values than the first run. This could be due to the freezing effect of these samples prior to ice storage, which might increase the free amino acid content by the denaturation of the protein (especially the proline and arginine).

Vanderzant et al.,⁽²¹⁾ reported that the ornithine and lysine values of shrimp (Penaeus vanami) increased during the 14 days of sterile ice storage while the proline decreased sharply. As mentioned in the previous results section 2.7.0.

Graph 4 "Ornithine Equivalent" changes of first and second batches of brown shrimp (Cranon cranon) "baq stored" during storage in ice

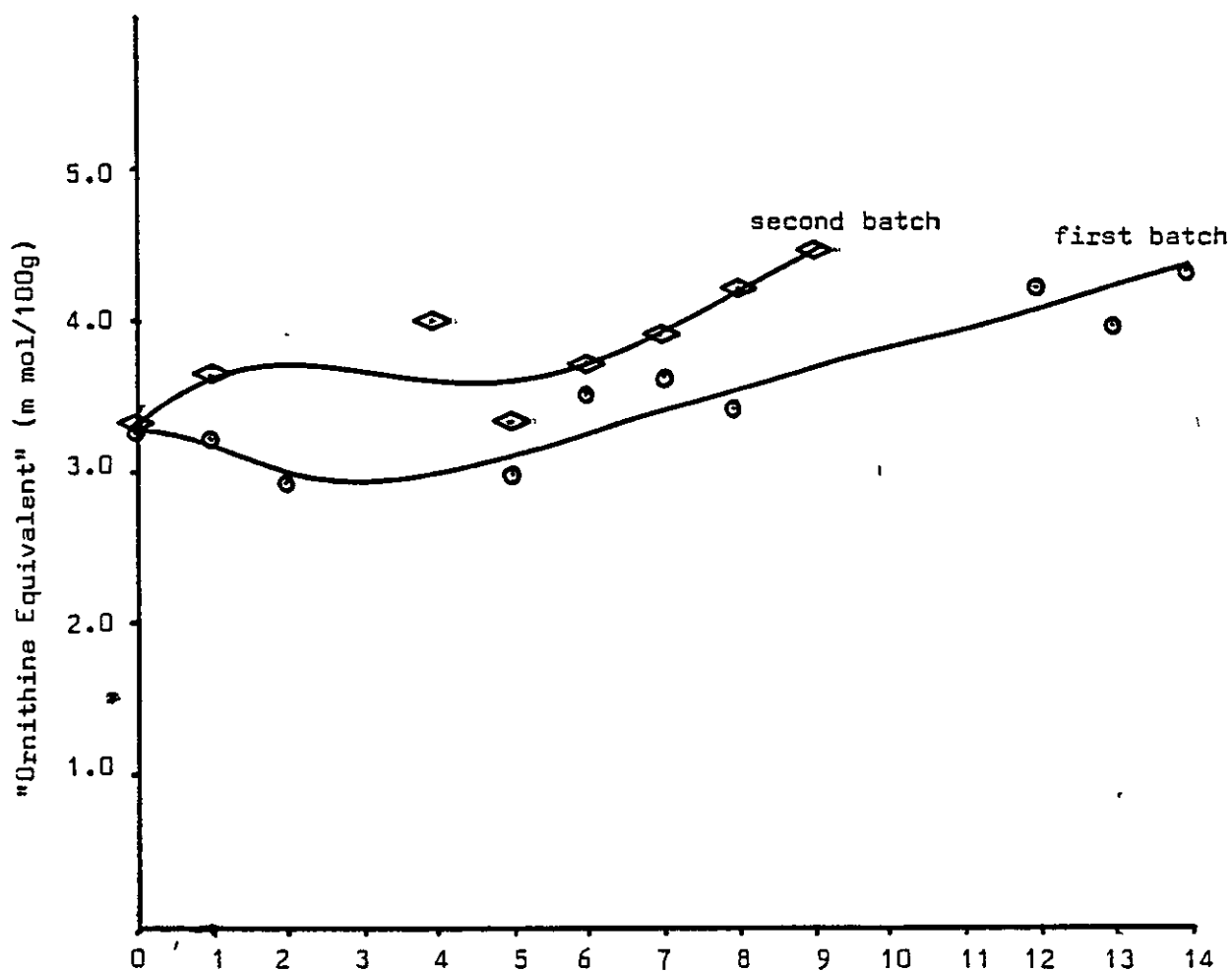


Table 11 "Ornithine Equivalent" changes of brown shrimp
(Crancon crancon) kept in bags during storage in
ice (first batch)

Storage period (days)	"Ornithine Equivalent"		
	m mol/100g		
	x_1	x_2	mean \bar{x}
0	3.257	3.241	3.247
1	3.238	3.187	3.212
2	2.844	2.976	2.910
5	2.909	2.997	2.953
6	3.326	3.585	3.500
7	3.599	3.661	3.629
8	3.513	3.280	3.397
12	4.339	3.977	4.158
13	4.103	3.775	3.939
14	4.453	4.180	4.316

Table 12 "Ornithine Equivalent" changes of brown shrimp
(Cranqon cranqon) kept in bags during storage
in ice (second batch)

Storage period (days)	Ornithine Equivalent [*] m mol/100g			
	X ₁	X ₂	X ₃	mean \bar{X}
0	2.923	3.331	3.631	3.295
1	3.403	3.471	4.002	3.625
4	4.149	3.633	4.195	3.992
5	2.977	3.506	3.460	3.314
6	4.294	3.489	3.277	3.687
7	3.685	3.849	4.075	3.870
8	3.949	4.070	4.488	4.169
9	4.769	4.110	4.440	4.440

The increase in the ornithine concentration and the decrease in the proline concentration, when super-imposed on each other might account for the observed results in both the pink and brown shrimps.

It could be perhaps concluded so far, that the "open stored" pink and brown shrimp "Ornithine Equivalent" values decreased mainly due to the leaching effect of melt ice water. The "bag stored" commercial pink shrimp (with no leaching effect) showed only a small rate of ornithine increase. This could be due to the inactivation of endogenous and bacterial enzymes as the commercial samples were cooked (boiled in brine) and the salt had a preservative effect. However, the "bag stored" brown shrimps showed steady significant increase on the 2 batches after the 5th day in ice. The results of Cobb et al.,⁽¹⁹⁾ showed that ornithine had a 96% increase after 5 days open stored in ice in white shrimp (Penaeus setiferus).

The deteriorative changes in open and bag stored shrimps were not an apparent indication of spoilage and could not be used as a quality index because the changing rate was too small to be useful as quality index. However, a more precise measurement of the separate ornithine and proline concentrations was required and this was investigated as described in the next section.

PART II

3.0.0 THE APPLICATION OF CHINARD'S METHOD FOR SEPARATE ESTIMATION OF ORNITHINE AND PROLINE AND COMPARISON WITH TMA-N, "K VALUE" AND pH VALUES DURING STORAGE IN ICE

3.1.0 The procedures for the proline and ornithine separation by cation exchange resins from standard solutions

Zerolit 225 and Zerolit S/F resins were used in this experiment. They were manufactured by the Permutit Company Ltd., U.K., and obtained from the British Drug Houses (BDH) Chemical Ltd., Poole, U.K.

3.2.0 Application of Zerolit 225 (SRC 13 standard resin)

This resin has the following properties as detailed by the manufacturer:

Type	:	cation exchange resin;
Functional group	:	$R-SO_3^- H^+$ (sulfonated polystyrene);
Form	:	Na^+ (as commercially supplied);
Water regain	:	0.68 to 0.85
Mesh size	:	14 to 52
Capacity	:	4.8 meq./g dry resin 2.1 meq./ml of wet resin
pH range	:	0 - 14

3.2.1 Resin treatment:-

i) Sodium Form (Na^+)

The commercial resin was washed several times with 1 M HCl and 2 M NaOH alternately, and rinsed with distilled water between the washes. Then, it was washed with 2 M HCl and distilled water and finally with 2 M NaCl and distilled water until the washing water was neutral to methyl orange.

ii) Hydrogen Form (H^+)

As in (i) section 3.2.1 except that the final wash with 2 M NaCl was omitted.

3.2.2 Application and treatment of Zerolit S/F ('Decalso' F):-

This is a sodium aluminosilicate cation exchange resin, and is also called Permutit resin. It has a mesh size of 60 to 85.

i) Sodium Form (Na^+)

The commercial resin was washed several times with 1% NaOH (w/v) and distilled water. Finally the resin was washed with 2 M NaCl and distilled water. The resin was allowed to dry at room temperature.

ii) Hydrogen Form (H^+)

The resin was washed with 1% NaOH (W/V), followed by several washes with distilled water. Finally it was washed with 2% acetic acid (V/V) and distilled water until the washings were no longer alkaline to phenolphthaline. The resin was then dried at room temperature.

3.3.0 The procedure for the recovery of proline or ornithine from standard solutions by use of the Zerolit 225 cation exchange resin

Standard solutions of each amino acid (containing 2 to 30 $\mu\text{g/ml}$) were adjusted to the required pH using a phosphate ($\text{M}/5 \text{ KH}_2\text{PO}_4$) buffer (plus 1 M KOH or 1 M HCl). The following procedure was then carried out:-

- i) 10 mL of the pH adjusted standard solution of proline or ornithine was shaken with one gram of dry Zerolit 225 resin (Na^+ or H^+ form) for 5 minutes;
- ii) the solution was filtered under gravity using Whatman No. 1 filter paper;
- iii) the absorbances of the adjusted standard solutions before and after shaking it with the resin, were measured by the Chinard method.

The recovery of the amino acid was expressed as a percentage, calculated as follows:-

$$\frac{A_2}{A_1} \times 100 = \% \text{ recovery of the amino acid.}$$

where:- A_1 is the absorbance of the adjusted solution
before shaking it with the resin .

A_2 is the absorbance of the same adjusted solution
after shaking it with the resin.

3.3.1 The procedure for the recovery of proline or
ornithine from the standard solutions by the use
of the Zerolit S/F cation exchange resin

i) Procedure was as in 3.3.0.

ii) The following buffer solution was used for the
shrimps extracts

Buffer solution:- 100 ml of $M/5$ KH_2PO_4 + 10 ml of
6M H_3PO_4 were made up to 200 ml with distilled water.

3.4.0 The results and discussion of proline and ornithine recovery (%) from standard solutions using the cation exchange resins

There are many types of cation exchange resins commercially available that can be used for the column methods of separation of the amino acids. The "batch" technique was chosen as it was a simple, effective and rapid technique. Standardisation of the experimental conditions was necessary to obtain a high absorption of the basic amino acids in the sample extract and a high recovery of the proline at the same time. Troll and Lindsley⁽⁶³⁾ modified the Chinard's⁽⁶⁷⁾ method by the use of the resin Permutit for this separation.

3.4.1 The results and discussion of separation and recovery with Zerolit 225 resin

The results are shown in Table 13.

The resin was used in both the sodium and hydrogen forms at different pHs of the amino acid solutions (pure standards). It can be observed that with the sodium form the recovery of proline was higher at pHs near to neutrality than at low pHs. The ornithine recovery showed similar results. The hydrogen form gave slightly less ornithine and proline recovery than the sodium form, at the same stated pH.

Table 13 Proline and Ornithine recovery from standard solutions of each using cation exchanger, Zerolit 225

pH	Sodium Form (Na ⁺)		Hydrogen Form (H ⁺)	
	% proline recovery	% ornithine recovery	% proline recovery	% ornithine recovery
1.20	34	10	28	18
1.80	29	19	32	
2.00	20	24	24	-
2.20	15	18	-	-
2.75	24	17	23	12
3.00	31	14	-	-
3.50	28	10	35	15
4.20	38	14	-	-
4.50	32	23	42	20
5.00	37	31	-	-
5.40	53	40	38	17
5.90	48	44	-	-
6.00	42	32	47	10
6.10	54	41	-	-
6.30	62	34	52	21
6.60	64	37	-	-
7.00	59	60	-	-

Results are the mean of triplicates.

It was concluded that Zerolit 225 (Na^+ and H^+ forms) was not suitable for the separation of the ornithine and proline at any pH, using batch technique. Therefore it was decided to use the Permutit resin (ZeroLit S/F).

3.4.2 The results and discussion of separation and recovery of proline and ornithine with Zerolit S/F "Decalso" resin

The results are shown in Table 14.

Folin and Bell⁽⁷⁷⁾ and Whitehorn⁽⁷⁹⁾ reported that this permutit (sodium alumino silicate) could be used for separating ammonia and other basic amines and amino acids. Troll and Lindsley⁽⁶³⁾ used the permutit to separate the ornithine and lysine from the proline at pH range 1 to 7.

It was found that the proline recovery (%) was relatively higher in the Zerolit S/F sodium form at the stated pH range than in the hydrogen form. The ornithine recovery obtained was the lowest in the sodium form at pH around 6.30-6.50. Therefore, it was decided to use the Zerolit S/F sodium form for the separation of proline from the basic amino acids (lysine, ornithine and arginine) at pH 6.30 (proline isoelectric point). At lower pH than 6.30, using the sodium form, this was found to be unsatisfactory because of high ornithine recovery, even though the proline recovery was high. The results were reproducible at pH 6.30.

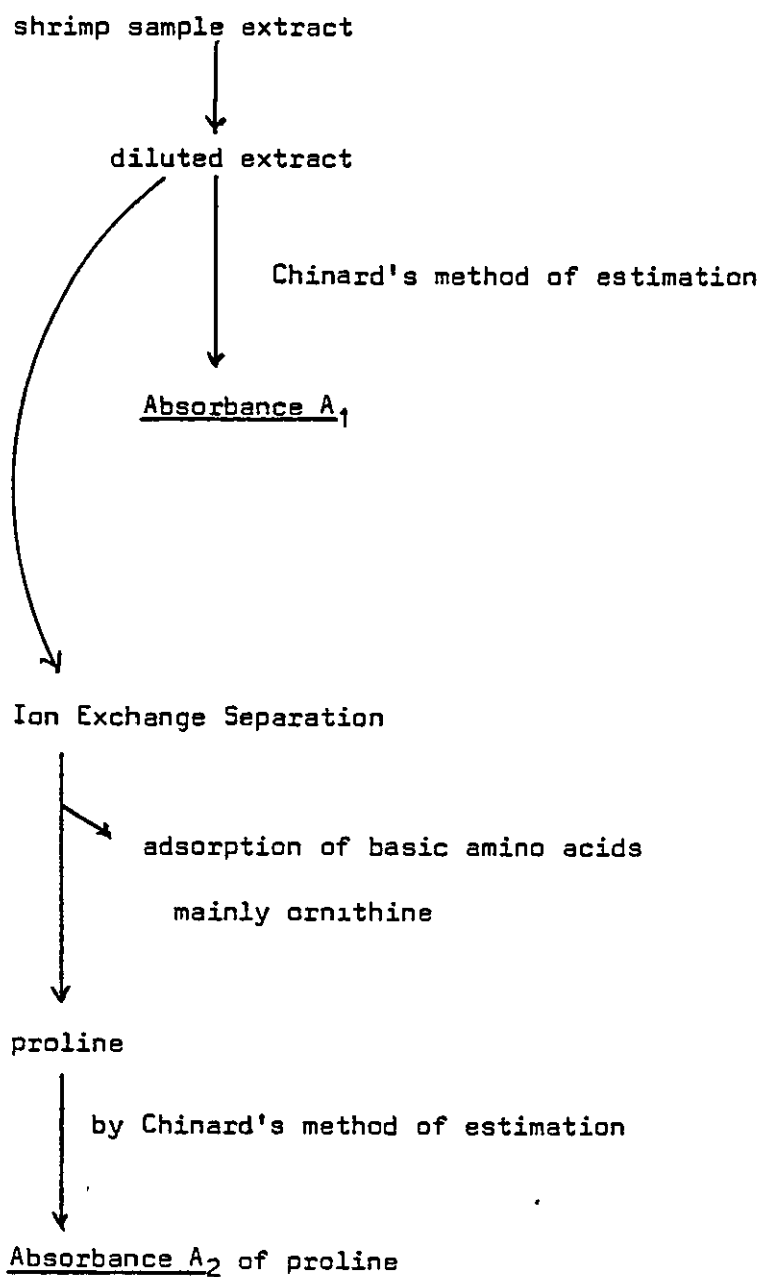
Table 14 Proline and ornithine recovery from standard solutions of each using cation exchanger Zerolit S/F (Decalso F)

pH	Sodium Form (Na ⁺)		Hydrogen Form (H ⁺)	
	% proline	% ornithine	% proline	% ornithine
	recovery	recovery	recovery	recovery
1.20	85	22	-	-
1.80	90	25	-	-
2.00	83	27	78	20
2.20	87	30	-	-
2.75	80	29	80	19
3.00	71	15	83	14
3.50	85	21	82	30
3.70	67	20	60	20
4.00	74	8	-	-
4.20	70	5	74	25
4.50	77	7	-	-
5.00	77	8	80	13
5.20	73	7	75	15
5.50	79	8	-	-
5.80	73	9	67	11
5.90	83	11	-	-
6.00	71	8	70	13
6.10	81	4	71	16
6.30	80	3	70	11
6.40	83	4	66	12
6.50	70	3	77	14
6.60	70	6	70	20
6.90	84	16	-	-
8.00	70	77	78	55

3.5.0 The procedure for separating proline from ornithine
in shrimp extracts

The objective of the following experiments was to eliminate the possible effect of proline on the measurement of ornithine by the Chinard⁽⁶⁷⁾ method. Hence a different approach was adopted, by separating the basic amino acids (ornithine mainly and lysine) on the cation exchange resin Zerolit S/F (Decalso F).

The estimation of the combined proline and ornithine ("Ornithine Equivalent") in the shrimp extract before the cation exchange separation and the estimation of the proline content in the same extract after the separation, could perhaps give a more "accurate" measurement of the ornithine value. The principle of this exercise is illustrated in the following diagram:-



$$A_1 - A_2 = A_3 \text{ (Absorbance of ornithine).}$$

Figure 8 Separation and estimation of proline and ornithine

3.6.0 The procedure for the determination of proline and ornithine in brown shrimps

3.6.1 First batch treatment

Brown shrimp, Cranqon cranqon, were caught off the Cleethorpes beach in July 1980 and prepared as in 2.5.0, except that the batch was divided into 2 groups:-

- a) first group was sampled in bags and stored in ice;
- b) second group was kept in ice (open stored).
They were iced as whole unpeeled shrimp.

3.6.2 Free amino acids extraction and separation and determination in first batch

The extraction procedure was as in 2.6.1 for steps i to iv.

- (v) 25 ml of the solution from the volumetric flask was now treated by adjusting its pH to 6.30 with the buffer solution (see 3.3.1 (ii)), and made up to 50 ml in a volumetric flask with 80% ethanol. This adjusted solution was diluted up to 25 times;
- (vi) 10 ml of the adjusted solution was treated as in 3.3.2 (i) using the reference Chinard method in section 2.1.0. The absorbance at 515 nm, of the

adjusted extract solution before the Zerolit S/F cation exchange resin treatment gives the measure of proline plus ornithine. The absorbance of the same solution after the resin treatment, using the same determination method and wavelength, gives the measure of proline alone. The absorbance of ornithine was calculated by subtracting the absorbance of the solution after the resin treatment from the absorbance of the same solution before the resin treatment. (See Figure 8).

Proline and ornithine values were expressed as m mol/100g sample as follows:-

$$C_1 \times \text{dilution factor} \times \frac{100\text{g}}{\text{sample weight}} \times \frac{1}{115.13} \times \frac{1}{1000}$$

= m mol proline/100g sample.

$$C_2 \times \text{dilution factor} \times \frac{100\text{g}}{\text{sample weight}} \times \frac{1}{213.09} \times \frac{1}{1000}$$

= m mol ornithine/100g sample

where C_1 and C_2 are the concentration ($\mu\text{g/ml}$) of proline and ornithine from their calibration curves respectively.

The relative molecular mass of L-proline = 115.13

The relative molecular mass of ornithine monohydrobromide
= 213.09

The results are shown in Tables 20(a) and 20(b).

3.6.3 Second batch treatment of the brown shrimps

The shrimp were caught in September 1980. They were frozen at -35°C and stored at -30°C for 5 days; they were then thawed, weighed and stored in bags as whole, unpeeled shrimps. Proline, ornithine, TMA-N; "K value"; and pH value were determined in duplicates (see 3.6.4; 3.7.0; 3.8.0 and 3.9.1 respectively). Results are shown in Table 21 and graph 9.

3.6.4 Amino acids extraction and determination for the second batch

Proline and ornithine were determined as in 3.6.2.

3.7.0 The procedure for trimethylamine-nitrogen measurement

TMA-N was determined by the picrate salt method as modified by Murray and Gibson⁽⁸⁵⁾.

Materials

Sodium sulphate : granular (anhydrous)

Toluene : free from sulphur, dried over Na_2SO_4

Formalin 50% : commercial formalin (40% formaldehyde) was shaken with magnesium carbonate, filtered and 100 ml of the filtrate made up to 200 ml with distilled water.

Picric acid stock solution : 2.0g of picric acid was dissolved in dry toluene and made up to 100 ml with dry toluene.

Picric acid reagent : 10.0 ml of the stock solution was made up to a litre in a volumetric flask with dry toluene.

Trimethylamine TMA stock solution : 688 mg of TMA-HCl was dissolved in distilled water, 1 ml of 2.5 N HCl added and the solution made up to 100 ml with distilled water. This solution contained 1.01 mg TMA-N/ml. The basic nitrogen content of 5 ml of stock solution was checked by adding 10 ml of 10% NaOH solution and distilling into 10 ml of 4% boric acid in a Micro-Kjeldahl distillation apparatus. Final titration was with 0.1N H_2SO_4 . The results of the titration and the calculation are shown in the following table.

Table 15

The results of the titration of 5 ml of TMA-HCl stock solution (after the distillation by Micro-Kjeldhal) with 0.1N H₂SO₄

<u>ml of 0.1N H₂SO₄</u>	<u>mean</u>
3.59	
3.60	3.59
3.58	

Calculation of TMA-N in the TMA stock solution

$$1.0 \text{ ml of } 0.1\text{N H}_2\text{SO}_4 = 0.0014 \text{ g - N}$$

$$3.59 \times 0.0014 (\text{g-N}) \times 1000 = 5.026 \text{ mg TMA-N/5ml}$$

$$5.026 \div 5 = 1.005 \text{ mg TMA-N/ml}$$

TMA-HCl stock solution

This is approximately 1.01 mg TMA-N/ml.

TMA-HCl working solution : 1.0 ml of the TMA-HCl stock solution

and 1.0 ml of 2.5N HCl were made up to

100 ml with distilled water. This solution

contained 10.1 g TMA-N/ml and was used for

the calibration curve (graph 5).

Potassium hydroxide solution (45%) 800g of KOH pellets were dissolved and made up to 1000 ml with distilled water.

3.7.1 Extraction procedure of the brown shrimp for TMA-N determination

- i) The whole unpeeled shrimps (25g) were taken out of the bag and placed in the blender.
- ii) The bag was washed thoroughly with 10.0 ml aliquot of 5% TCA solution to recover, if any, TMA in the drip.
- iii) 90.0 ml of 5% TCA was added to the sample and homogenised for 2 minutes.
- iv) The homogenate was filtered through the Whatman No. 542 filter paper under vacuum.

3.7.2 The procedure for the determination of TMA-N of the brown shrimps

- i) To 20 ml of the filtrate, 5 ml of formalin (50%) was added in a 50 ml flask.
- ii) 0.5-4.0 ml of the formalin extract was taken in a test tube (20 ml) and made up to 4 ml with distilled water.
- iii) 1 ml of formalin (50%) and 10 ml of toluene were added, followed by 3 ml KOH (45%) solution.
The tube was stoppered.

- iv) The tube was inverted and returned to its original position by hand for a total of forty times over a period of less than one minute. The phases were then allowed to separate for 10 minutes.
- v) 7-9 ml of the toluene layer (upper layer) were transferred by pipette to another tube containing approximately 100-200 mg anhydrous sodium sulphate. The tube contents were shaken to dry the extract, which was removed as required by a suction pipette.
- vi) 5.0 ml of picric acid reagent was placed in a 75 ml conical flask, followed by 5.0 ml of the dried toluene extract. The flask contents were mixed by agitating it gently by hand.
- vii) 4.0 ml of distilled water was used for the blank.
- viii) Standard solutions of TMA-N containing 5.05 to 40.4 g TMA-N/ml were used for the calibration curve.

The blank and the standard were each carried through all the above steps (i) to (vi). The absorbances were measured against the reference blank at 410 nm.

TMA-N was expressed as mg TMA-N/100g sample as follows:

$$C \times \frac{25}{20} \times \frac{120}{1} \times \frac{100g}{\text{sample weight}} \times \frac{1}{1000} = \text{mg TMA-N/100g sample}$$

where

C is the concentration of TMA-N ($\mu\text{g/ml}$) from the calibration curve.

TMA-N values were corrected for 80% moisture content of the shrimp (Cobb, et al.,⁽¹⁹⁾ and Cobb and Vanderzant⁽⁹⁴⁾), therefore the total volume of the homogenate from 25g, was 120 ml.

Table 16 Trimethylamine-nitrogen (TMA-N) of standard solution related to the absorbances measured at 410 nm

TMA-N concentrations ($\mu\text{g/ml}$)	Absorbance			
	X_1	X_2	X_3	mean \bar{X}
5.05	0.105	0.115	0.11	0.110
10.10	0.220	0.230	0.22	0.223
20.20	0.420	0.420	0.430	0.423
30.30	0.620	0.625	0.618	0.621
40.40	0.835	0.830	0.830	0.832

The regression equation for the TMA-N calibration curve

obtained was

$$Y = 0.0125548 + 0.02023787X$$

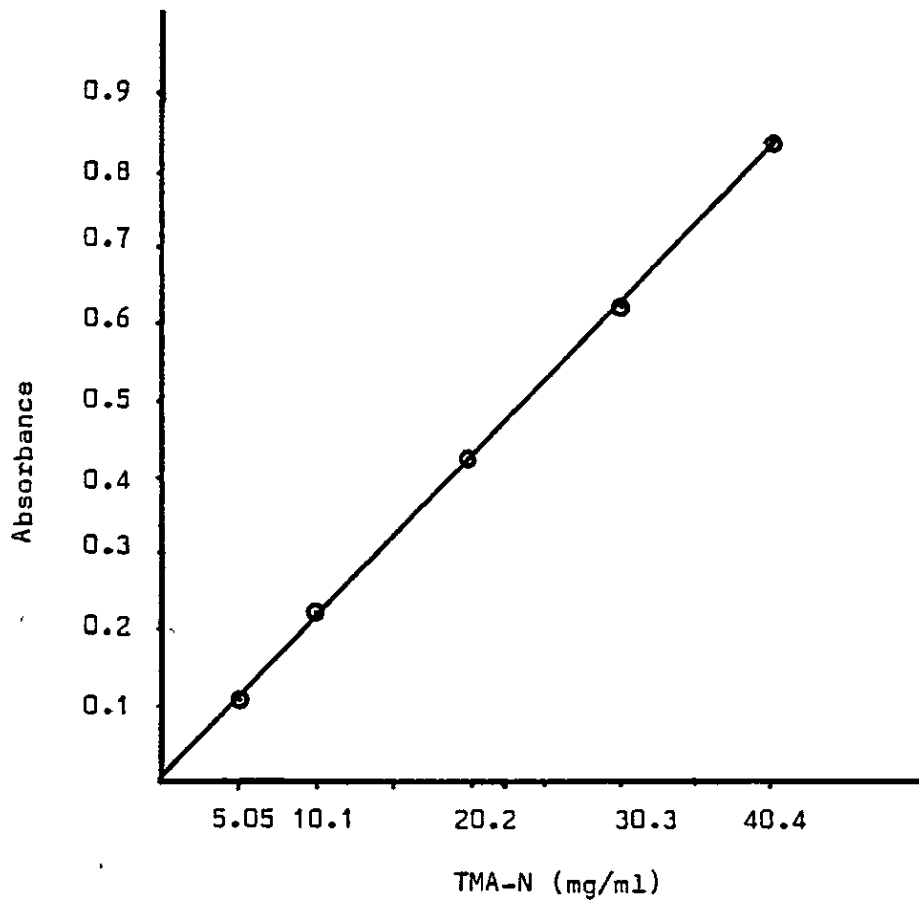
Y = absorbance readings at 410 nm

X = amount of TMA-N in $\mu\text{g/ml}$

Where correlation coefficient $R = 0.9998$ ($P < 0.01$)

The regression equation was used to calculate the TMA-N in the shrimp extracts.

Graph 5 TMA-N calibration curve, measured at 410 nm.



3.8.0 The procedure for the measurement of "K value"

The method, as developed by Saito et al.,⁽⁴³⁾ and modified by Jones and Murray⁽²⁹⁾ was used.

3.8.1 Materials

0.6M perchloric acid

30% KOH (W/v)

Dowex 1 x 8 (formate) anion exchange resin, 200-400 mesh size. This was supplied by British Drug Houses, England.

3.8.2 Preparation of the resin

Dowex 1 x 8 was obtained in the chloride form. The resin was washed several times with 2M NaOH and 2M HCl alternately and with distilled water between the washes. Finally, it was washed with a solution of sodium formate and with water, until the washing water had an absorbance reading at 250 nm of less than 0.02. The resin was prepared as a stock suspension (6.0g \pm 0.1/100 ml).

3.8.3 Extraction procedure of the brown shrimps for "K value" determinations

- i) 25g of the whole unpeeled shrimps were homogenised in 50 ml cold 0.6M perchloric acid for 2 minutes;

- ii) the homogenate was filtered under vacuum, with the Whatman No. 42 filter paper;
- iii) 25 ml of the filtrate was adjusted to pH 6.5 with 30% KOH ($\frac{w}{v}$) and made up to 100 ml with distilled water;
- iv) the adjusted solution was filtered under atmospheric pressure using the Whatman No. 541 filter paper;
- v) the filtrate was diluted 25 times with distilled water.

3.8.4 The procedure for "K value" determination of the brown shrimps

- i) 10.0 ml of the adjusted and diluted extract was added to 1.0 ml of the stock suspension of the resin in the first test tubes;
- ii) to the second test tube, 10.0 ml of the diluted extract and 1.0 ml of distilled water were added;
- iii) to the third test tube, 10.0 ml of distilled water and 1.0 ml of a suspension of the resin were added and used as the reference blank;
- iv) the tube contents were shaken at room temperature for 5 minutes, filtered under atmospheric pressure, using the Whatman No. 1 filter paper;

- v) the absorbances of the filtrates were read at 250 nm against the reference blank.

3.8.5 Calculations of "K values"

The "K value" was calculated using the equation:-

$$K = \frac{A_2}{A_1} \times 100$$

A_1 is the absorbance of the adjusted extract before the resin treatment, at 250 nm

A_2 is the absorbance of the same extract after the resin treatment, at the same wavelength

The absorbance of the filtrate after the resin treatment (A_2) gives a measure of inosine plus hypoxanthine. The filtrate's absorbance before resin treatment (A_1) gives a measure of the total adenosine triphosphate related compounds such as ATP, ADP, AMP and IMP.

3.9.0 The procedures for the treatment and measurement of proline, ornithine and pH value of iced pink shrimps

Pink shrimp, Pandalus montaquui, were obtained in August 1980. They were prepared and treated as in 2.4.2. The samples were stored as whole unpeeled shrimp for the "bag" and "open" storage. Proline and ornithine samples were analysed as in 3.6.2 (for the results see Tables 18(a) and 18(b)) and the pH values of the "open stored" samples measured as in 3.9.1.

3.9.1 pH measurement of shrimp homogenates

The pink and brown shrimps were "open" and "bag" stored respectively in ice as whole unpeeled shrimp.

- i) 10g of shrimps were homogenised with 100 ml of distilled water for 2 minutes using the Ato-mix blender.
- ii) The pH was measured directly in the homogenate at room temperature see 2.0.0 (B).

3.10.0 The results and discussion of proline changes of fresh brown shrimp (Crangon crangon) and cooked pink shrimp (Pandalus montaquii) during storage in ice

3.10.1 (a) "bag stored" samples

The proline results for the "bag stored" pink and brown shrimps (See Tables 18(a) and 20(a)) are shown in graphs 6a and 7a respectively.

The proline contents for the "bag stored" pink and brown samples showed a short period of increase. (about 2 days in the pink shrimps and 4 days in the brown shrimps) and then a general decrease up to the 12th or 13th day for storage in ice. The initial value of the proline concentration in the pink shrimps (4.201 m mol/100g) was higher than that for the brown shrimps (2.620 m mol/100g).

This could be due to:-

- 1) the background history of the commercially processed pink shrimps was not known;
- 2) natural variations (such as different seasons, fishing grounds, species, etc.)

The proline content subsequently declined in the pink shrimps to 26.7% of its initial value on the 13th day while the

Table 17(A) Proline changes in "bag stored" shrimps

Proline in m mol/100g sample

0 day	6th day	% change*	13th day	% change*	species used	reference
4.201	3.392	+ 19.3	3.081	+ 26.7	Pandalus montagui	present work
2.620	3.309	+ 26.3	3.290 (12 days)	- 25.6	Crangon crangon	present work
1.96	1.57	+ 19.9			Penaeus setiferus	Cobb <u>et al.</u> (19)
1.76			2.616 (14 days)	- 48.6	Penaeus setiferus [†] *	Cobb <u>et al.</u> (8)

** Shrimp juice inoculated with several different bacterial species and stored at 5°C for the length of time stated.

Zero day was the control with no inoculation.

* The percentage change was calculated as follow :

$$\frac{\text{The difference between the initial and the last value}}{\text{The initial value}} \times 100$$

e.g. Proline % change of, Pandalus montagui, at the 6th day $\frac{4.201-3.392}{4.201} \times 100 = 19.3$

Results of workers were converted to the same measurement units of m mol/100g sample.

Table 17(B) Proline changes in "open stored" shrimps

0 day	6th day	% change*	13th day	% change*	species used	reference
4.201	0.127	+ 97	0.01	+ 99.8	Pandalus montagui	present work
2.620	1.57	+ 40.1	0.264 (12 days)	+ 89.9	Crangon crangon	present work
1.96	0.91	+ 53.6			Penaeus setiferus	Cobb <u>et al.</u> (19)
8.09	7.20 (7 days)	+ 11	3.91 (14 days)	+ 51.2	Penaeus vanami	Vanderzant <u>et al.</u> (21)
			4.00 (21 days)	+ 50.6	Penaeus vanami	Vanderzant <u>et al.</u> (21)
2.00	0.66 (11 days)	+ 67	0.70 (14 days)	+ 65	Penaeus setiferus	Cobb <u>et al.</u> (19)

Results of workers were converted to the same measurement units of m mol/100g sample.

* See table 17(A).

Graph 6(a) Proline and ornithine changes of whole unpeeled pink shrimp (*Pandalus montaquui*) "bag stored" during storage in ice

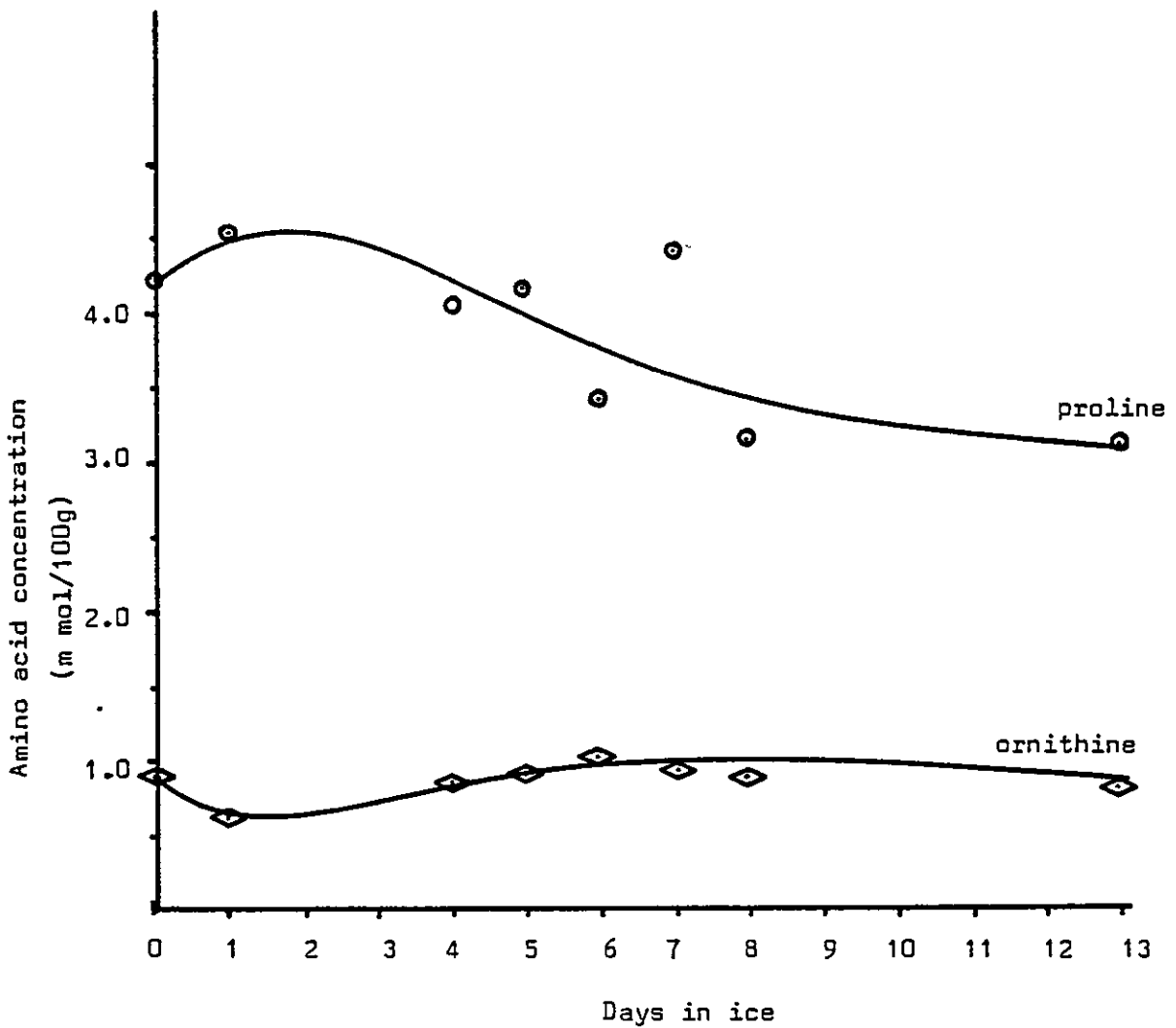


Table 18(a) Proline and ornithine changes of whole unpeeled pink shrimp (*Pandalus montagu*) "bag stored" during storage in ice

Storage period (days)	Whole unpeeled shrimp in bag m mol/100g					
	Proline			Ornithine		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	3.997	4.404	4.201	0.748	0.954	0.851
1	4.617	4.440	4.529	0.516	0.732	0.624
4	3.976	4.077	4.027	0.834	0.831	0.833
5	4.150	4.127	4.139	0.858	0.882	0.870
6	3.241	3.542	3.392	0.982	1.001	0.992
7	4.223	4.532	4.378	0.824	0.995	0.909
8	3.450	2.770	3.110	0.905	0.799	0.852
13	2.751	3.410	3.081	0.372	1.171	0.772

brown shrimps increased to 26.3% of its initial value on the 6th day and then decreased before increasing ^{by} 25.6% of its initial value on the 12th day. As shown in Table 17A, the results of Cobb et al.,⁽¹⁹⁾ showed a decrease of 19.9% on the 6th day. However Cobb et al., inoculated shrimp juice with specific bacterial strains and obtained an increase in proline of 48.6% of its initial value on the 14th day.

Sidhu et al.⁽⁵⁷⁾ found that the proline content of rock lobster at 4 hour post mortem was 13 μ mol/g (1.3 m mol/100g), but after 48 hours (2 days) post mortem at 20 C, the proline content increased to 18.1 μ mol/g (1.81 m mol/100g). Gallagher and Brown⁽⁸⁶⁾ showed that shrimp (Artemia salina) cooked in brine had an initial value of 5.20g/100g protein (9.03 m mol/100g sample) of proline. Chinamma et al.,⁽¹¹⁾ reported that crab (S. serrata) had an initial content of 2.00 mg % (0.0174 m mol/100g) proline (measured by standard microbiological assay methods). Variations in 5 species of cooked crab muscle and significant differences e.g. between whether male or female or whether the sample tested was from the left or right leg, have been reported by Konosu et al.⁽⁸⁷⁾

There seemed to be great variations among the workers on the initial proline content and subsequent changes in the crustacean

post mortem. The possible reasons could be:-

- 1) different species;
- 2) handling history of the shrimps after they were harvested;
- 3) bacterial load and type of bacteria species
(related to the feeding grounds) also affect certain free amino acid content, as shown by Cobb et al. (19)

3.10.2 The results and discussion of proline changes of fresh brown and cooked pink shrimps during storage in ice

(b) "open stored"

The proline results of the brown and pink shrimps (see Tables 18(b) and 20(b)) are shown in graphs 6(b) and 7(b).

The proline concentration of the brown and pink shrimps declined throughout the period of ice storage. The rate of decline was greater in the pink than in the brown shrimps with 99.8% and 89.9% decrease for the pink and brown respectively after 13th and 12th days in ice storage. The initial proline value of the fresh brown shrimp was half the initial value of the cooked commercial pink shrimp. The slower decline in the brown shrimp could be due to:-

- 1) the slow rate of melting ice which could affect the leaching rate of free amino acids;
- 2) the rate of proline production by endogenous and bacterial enzymes⁽⁸⁾ could be equal to the rate of proline lost by the melt ice washing, hence the decline is kept at a slow rate;
- 3) natural variations.

Cobb et al.,⁽¹⁹⁾ reported that the proline values in which *white* shrimp (Penaeus setiferus) decreased during storage in ice (slow

Graph 6(b) Proline and ornithine changes in pink shrimp "open stored"
during storage in ice

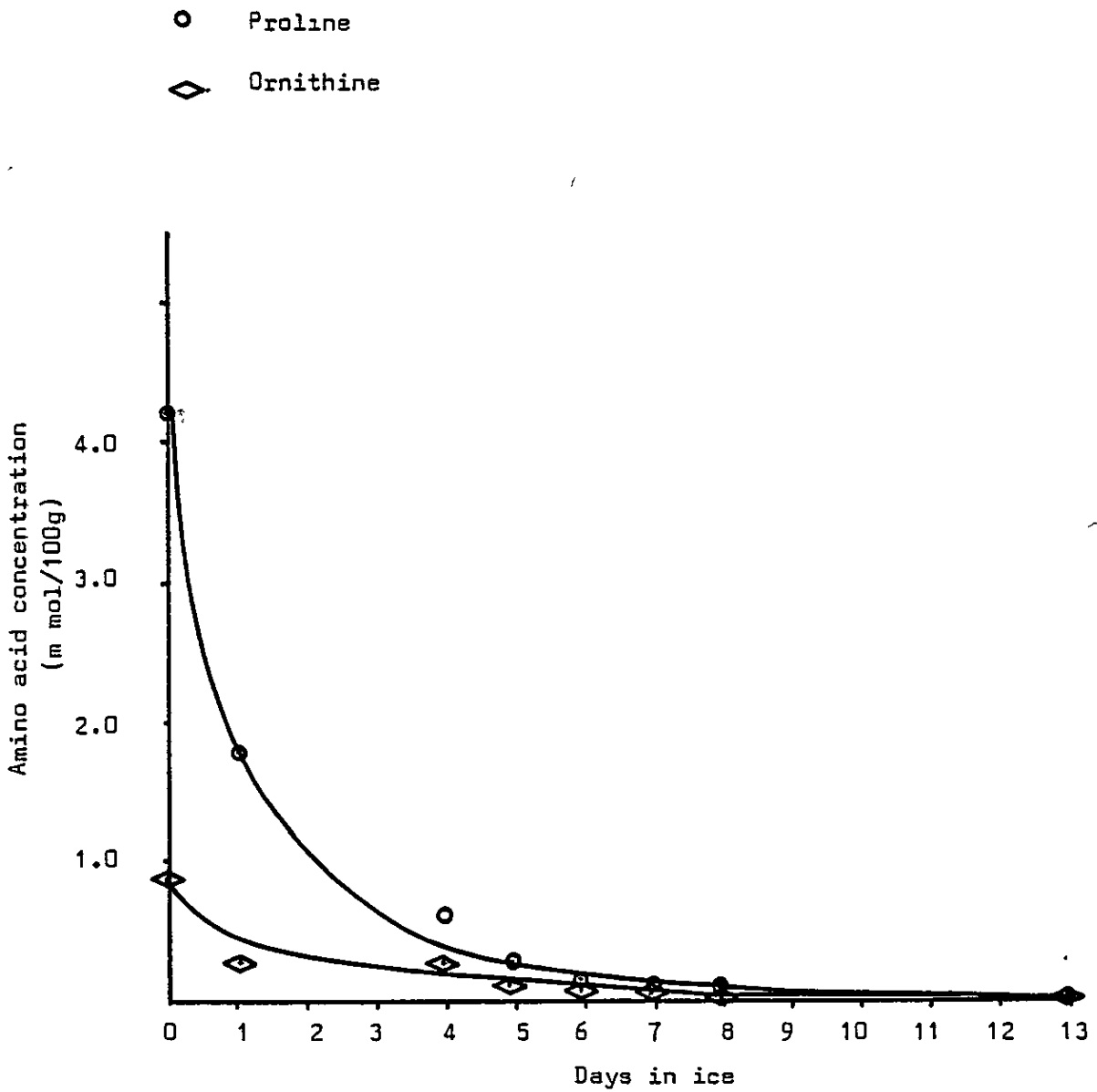


Table 18(b) Proline, ornithine and pH changes in pink shrimp
(Pandalus montagu) "open stored" during storage in ice

Storage period (days)	Whole unpeeled shrimp in open m mol/100g								
	Proline			Ornithine			pH reading		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	3.997	4.404	4.201	0.748	0.954	0.851	7.35	7.40	7.38
1	1.798	1.753	1.776	0.221	0.206	0.213	7.80	7.73	7.77
4	0.396	0.800	0.598	0.214	0.207	0.211	8.20	8.15	8.18
5	0.258	0.222	0.240	0.062	0.048	0.055	8.32	8.30	8.31
6	0.141	0.112	0.127	0.039	0.032	0.036	8.30	8.40	8.35
7	0.092	0.110	0.101	0.025	0.032	0.030	8.25	8.35	8.30
8	0.070	0.090	0.080	0.029	0.038	0.023	8.45	8.42	8.43
13	0.010	0.021	0.016	0.00	0.008	0.004	-	-	-

ice melt rate) and the percentage change for 6 days was 53.6% compared to 40.1% obtained for the brown shrimp (Crangon crangon) in this experiment (see Table 17B). Vanderzant et al.,⁽²¹⁾ also reported 11% decrease on the 7th day in the proline content of white shrimp (Penaeus vanami) during ice storage. Hence it cannot be stressed too often, that melting ice water has a great leaching effect on the free amino acid content during long periods of storage in ice. This contrasts with the rise in proline in the initial period in the "bag stored" brown shrimps found in this work, and the increases reported by other workers⁽⁸⁾ also for "bag stored" shrimps.

3.11.0 The results and discussion of ornithine changes in cooked pink (*Pandalus montaquii*) and brown (*Cranqon cranqon*) shrimp during storage in ice

3.11.1 (a) "bag stored" samples

The results of ornithine in "bag stored" pink and brown samples are shown in graphs 6(a) and 7(a) respectively. The tabulated results are in the Tables 18(a) and 20(a).

The pink shrimp could have undergone deterioration during commercial processing, and this may account for its initially high ornithine content (0.851 m mol/100g) as compared to the fresh brown shrimp (0.524 m mol/100g), which were analysed within a few hours of the catch. The low ornithine in the brown shrimp could be due to:-

- 1) the arginine content was in the initial stage of degradation to ornithine;
- 2) the bacterial load was low at the start of post mortem;
- 3) low arginase activity because of the low pH at the beginning of post mortem storage (lactic acid production from glycolysis would reduce the pH).

The ornithine values in pink shrimp decreased initially and then increased up to the 6th day (16.6% increase), thereafter

Table 19(A) Ornithine changes in "bag stored" shrimps

Ornithine in m mol/100g

0 day	6th day	% change*	13th day	% change*	species used	reference
0.851	0.992	- 16.6	0.772	+ 9.3	Pandalus montagui	present work
0.524	1.324	- 152.7	1.819 (12 days)	- 247.1	Crangon crangon	present work
0.02	0.58	- 2800			Penaeus setiferus	Cobb <u>et al.</u> ⁽¹⁹⁾
0.718			1.832 (14 days)	- 155.2	Penaeus setiferus	Cobb <u>et al.</u> ⁽⁸⁾

* See table 17(A).

Results from other workers were converted to the same measurement units of m mol/100g.

Table 19(B) Ornithine changes in "open stored" shrimps

Ornithine in m mol/100g

0 day	6th day	% change*	13th day	% change*	species used	reference
0.851	0.036	+ 95.8	0.004	+ 99.5	Pandalus montagui	present work
0.524	0.650	- 24.1	0.228 (12 days)	+ 56.5	Crangon crangon	present work
0.02** (antibiotic)	0.54	- 2600			Penaeus setiferus	Cobb <u>et al.</u> (19)
0.36	0.58	- 61.1	1.14 (14 days)	+ 216.7	Penaeus vanami	Vanderzant <u>et al.</u> (21)
0.02** (sterile)			0.25	- 1150	Penaeus setiferus	Cobb <u>et al.</u> (19)

** shrimps were stored in sterile ice or dipped in chloramphenicol to retard bacterial growth.

Results from other workers were converted to the same measurement units of m mol/100g.

* See Table (17(A)).

the ornithine level gradually decreased until the 13th day (9.3% decrease) when they were organoleptically unacceptable. A possible reason for this slow rate of ornithine decrease to the 13th day may probably be due to the rate of ornithine production being less than the rate of the ornithine degradation to putrescine and urea by bacterial action.

The ornithine values of brown shrimps increased significantly throughout the 12 days storage in ice, they were regarded as unacceptable at the 7th day with an ornithine value of 1.63 m mol/100g. The flesh was greenish black in colour and the head was loose, due probably to autolytic enzyme activity. The ornithine value was 1.63 m mol/100g on the 7th day with an increase of 211.1%. Cobb et al.,⁽¹⁹⁾ showed that white shrimp (Penaeus setiferus) had an initial ornithine value of 0.02 m mol/100g (as measured by the amino autoanalyser) and increased to 0.58 m mol/100g (2800%) on the 6th day of ice bag storage (as shown in Table 19A). Cobb et al.⁽⁸⁾ also showed that the ornithine value in white shrimp had shown a 155.2% increase on the 14th day of ice storage, as compared to the ornithine increase of 152.7% on the 6th day and 247.1% on the 12th day of ice storage of brown shrimp in this experiment.

The significant increase in the ornithine content of raw, fresh shrimps when stored in bags, in ice, could possibly be used as an indicator of shrimp quality.

Graph 7(a) Proline and ornithine changes of whole, fresh, unpeeled brown shrimp "baq stored" during storage in ice

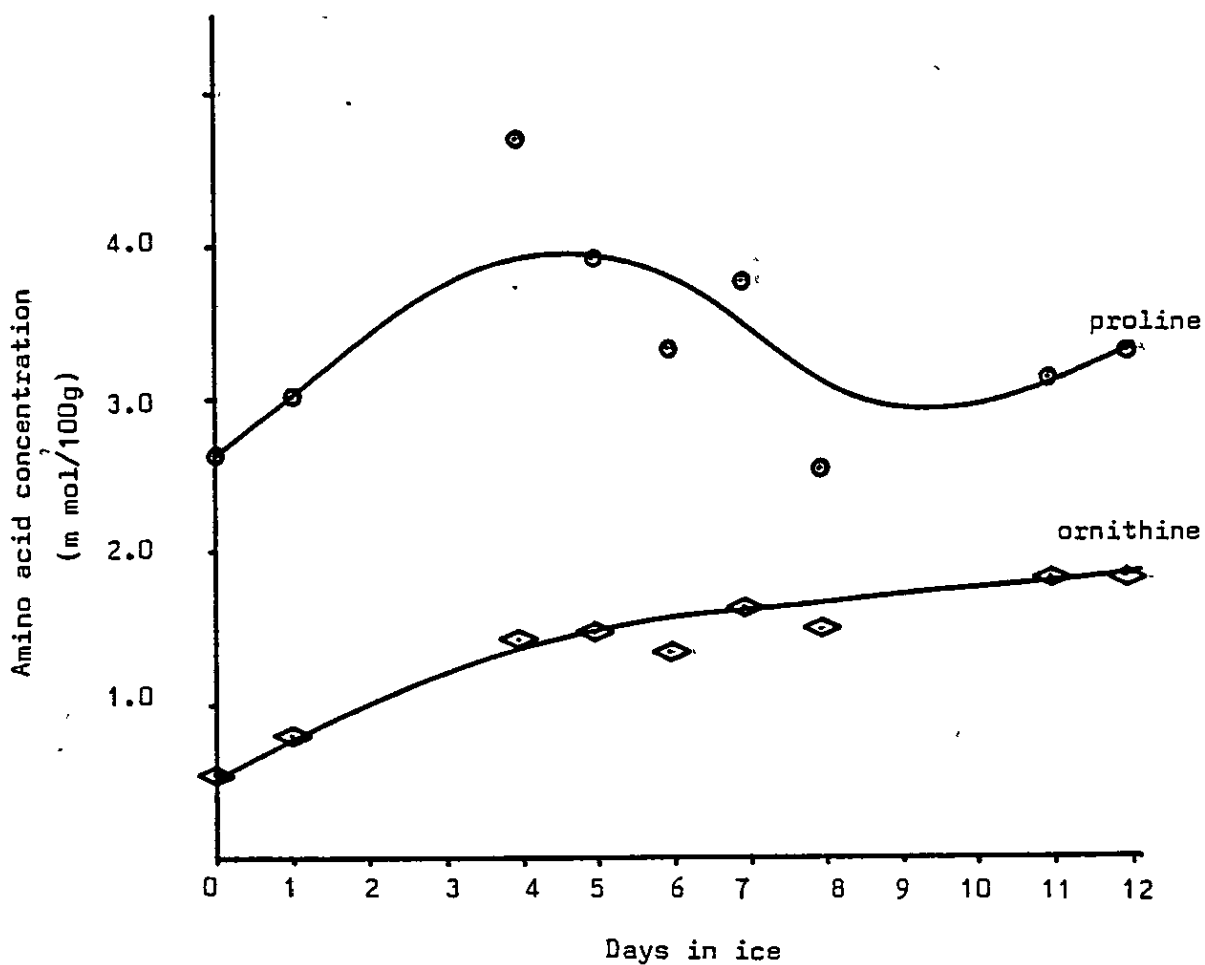


Table 20(a) Proline and ornithine changes of whole, fresh, unpeeled brown shrimp (Crangon crangon) "bag stored" during storage in ice

Storage period (days)	Whole unpeeled shrimp in bag m mol/100g					
	Proline			Ornithine		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	2.069	3.171	2.620	0.558	0.490	0.524
1	3.744	2.285	3.015	0.659	0.868	0.763
4	4.419	4.966	4.693	1.369	1.434	1.395
5	4.537	3.301	3.919	1.546	1.336	1.441
6	2.815	3.802	3.309	1.293	1.353	1.324
7	4.217	3.289	3.753	1.707	1.553	1.630
8	2.135	2.924	2.530	1.416	1.534	1.475
11	2.504	3.750	3.127	1.630	1.990	1.810
12	3.305	3.275	3.290	1.827	1.811	1.819

3.11.2 The results and discussion of ornithine changes in pink and brown shrimps during storage in ice

(b) "open stored" samples

The changes in the ornithine content for "open stored" pink and brown shrimps are shown in graphs 6(b) and 7(b) respectively. Results of the graphs are shown in Tables 18(b) and 20(b).

As with the proline content, the ornithine values in the "open stored" pink shrimps declined throughout the 13 days of ice storage. The probable reasons are given in the previous section 3.10.2. The decline was 95.8% on the 6th day and 99.5% on the 13th day (see Table 19(B)). However, the ornithine content of the brown shrimp by comparison, increased only by 24.1% on the 6th day and then decreased to 56.5% of its initial value. As the brown shrimps are fresh, raw and not processed, compared to the commercial frozen cooked pink shrimps, the bacterial load could be higher. The rate of formation of ornithine could therefore be higher than that in the pink shrimp, and greater than the leaching of the free amino acid, this could account for the slight ornithine content increase in the initial 7 days of ice storage. When the arginine content reached a minimum level, the production of ornithine could be less than the rate of ornithine loss by the leaching effect, hence resulting in an overall decrease ornithine content during the 8th to the 13th day of ice storage.

Graph 7(b) Proline and ornithine changes of whole, fresh, unpeeled brown shrimp "open stored", during storage in ice

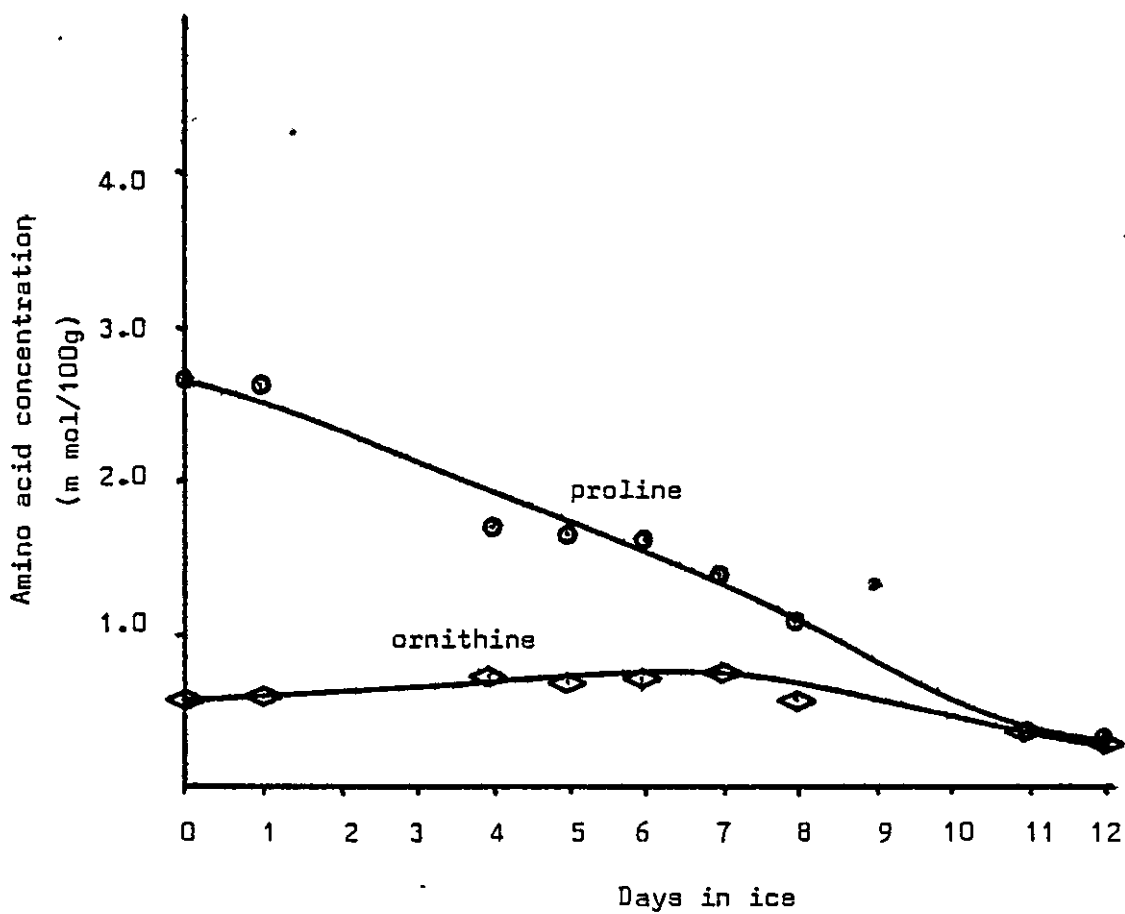


Table 20(b) Proline and ornithine changes of whole, unpeeled fresh brown shrimp (Crangon crangon) "open stored" during storage in ice

Storage period (days)	Whole unpeeled shrimp in open (m mol/100g)					
	Proline			Ornithine		
	X_1	X_2	mean \bar{X}	X_1	X_2	mean \bar{X}
0	2.069	3.171	2.620	0.490	0.528	0.524
1	2.553	2.644	2.599	0.501	0.587	0.544
4	1.158	2.132	1.645	0.460	0.914	0.687
5	1.872	1.309	1.591	0.650	0.639	0.645
6	1.715	1.424	1.570	0.701	0.598	0.650
7	1.308	1.379	1.344	0.612	0.807	0.710
8	1.037	1.069	1.053	0.490	0.577	0.534
11	0.259	0.356	0.308	0.255	0.350	0.302
12	0.265	0.262	0.264	0.214	0.240	0.228

The ornithine content ~~was~~ during ice storage of "open stored" samples generally decreased and probably would not be suitable as a quality index. However results of Cobb et al.,⁽¹⁹⁾ and Vanderzant et al.,⁽²¹⁾ showed significant increases (see Table 19(B)).

3.12.0 The results and discussion of the trimethylamino-nitrogen (TMA-N) changes of brown shrimp (Cranqon cranqon) "bag stored" during storage in ice

The TMA-N results of the brown shrimps (Table 21) are shown in graph 9(B). The TMA-N (initial value of 1.026 mg/100g) content of unpeeled shrimp increased slightly within 24 hours to 1.386 mg/100g and was then stable until the shrimp was spoiled (1.770 mg/100g at the 8th day) thereafter the TMA-N increased sharply until the 10th day (2.739 mg/100g). The TMA-N content of about 1.4 mg/100g sample (from graph 9(B)) at 7th day (36.5% increase) could perhaps be used as the spoilage index for the brown shrimp. As the TMA-N content showed no significant change during the initial days of ice storage, it is not useful as a "freshness" index during this period but only at the late period of ice storage. The sharp increase in TMA-N at the late stage of storage could probably be due to the increase in the bacterial load.

Campbell and William⁽¹²⁾ found that the TMA-N of headed iced shrimp (measured by Dyer's (1945) method)⁽⁸⁸⁾ reached 2.33mg/100g at the 16th day of storage in ice and the shrimp was still acceptable. Bethea and Ambrose⁽¹⁰⁾ showed that the TMA-N content (measured by a modified Dyer (1945) method) of brown, headless shrimp remained at about 1 mg/100g until the 8th day, when the TMA-N started to increase rapidly until the 12th to 16th day of ice storage. These

TMA-N value changes were comparable to the values obtained with the brown shrimp (Crangon crangon) in the experiment here. A value of 1.5 mg TMA-N/100g shrimp has been suggested as indicative of spoilage of Gulf Coast shrimp, by Fieger and Friloux⁽⁷⁾. The Japanese and Australian market has set the TMA-N limit at 5.0 mg TMA-N/100g shrimp muscle (quoted from Montgomery et al.⁽⁸⁹⁾) Flores and Crawford⁽¹⁶⁾ found that the level of TMA-N of intact shrimp (Pandalus jordani) increased from 1.4 to 15.3 mg/16 mg N (0.24 to 1.6 mg/100g) during 8 days of ice storage. Collins et al.,⁽⁹⁰⁾ reported similar findings for Pacific shrimp (Pandalus species) of 0.24 to 1.72 TMA-N/100g during 8 days of ice storage but Collins et al.,⁽⁹⁰⁾ concluded that the TMA-N content would not be suitable as an index of quality, Cobb et al.⁽⁸⁾ found that the TMA-N content correlated with the deterioration of the white shrimp (P. setiferus) and the value obtained was very low due to (as they suggested) the low TMAO content in the shrimp and the effect of salinity. They concluded that the TMA-N value was not a reliable indicator of spoilage. Iyengar et al.,⁽⁸⁴⁾ found that the TMA-N value of shrimp (P. indicus and metapencreus monoceris) leached out during open ice storage and they concluded that the TMA-N content was of limited use as a quality index of iced stored shrimp.

3.13.0 The results and discussion of pH values changes of pink shrimps (Pandalus montagui, stored in open) and brown shrimp (Crangon crangon, stored in bag) during storage in ice

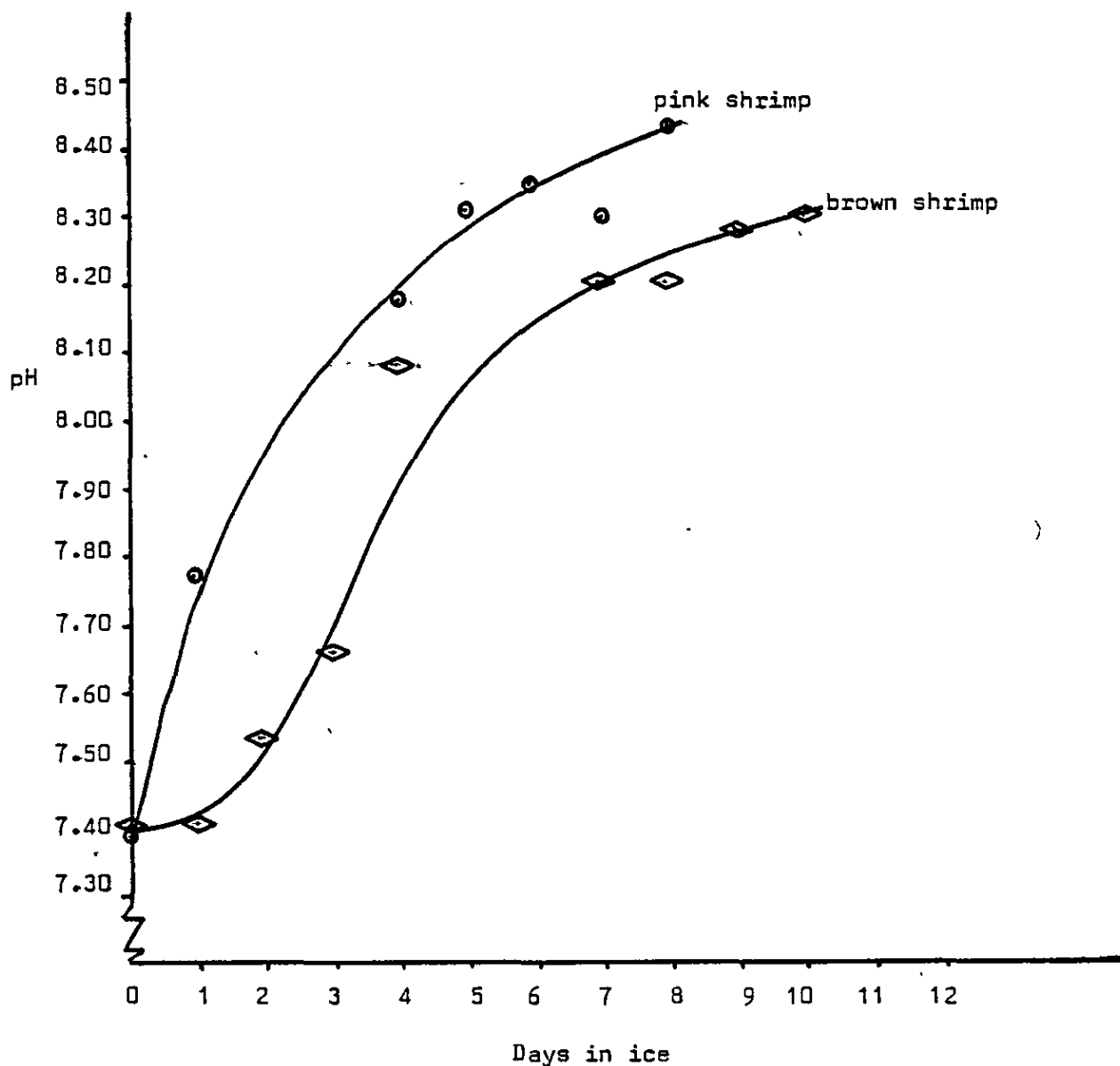
Results of the pH values of the pink (see Table 18(b) and brown shrimps are shown (see Table 21) in graph 8.

The pH changes of the pink shrimp showed a large increase from the initial values of 7.43 to 8.38 on the 8th day of ice storage. The pH initial values of the pink shrimps were similar to those of the "bag stored" brown shrimp which had an initial value of 7.40 and reached pH 8.20 on the 8th day of ice storage. The pH of the fresh brown shrimp was relatively constant for the first 2 days. This could probably be due to:-

- 1) the production of lactic acid, but the pH did not decrease as reported in some of the literature;
- 2) trimethyl amine oxide (TMAO) had a pH near to neutral, hence it had a buffering action on resisting any pH change.

However, the pink shrimp showed an increase in pH from the initial day probably due to the deterioration which had occurred prior to ice storage.

Graph 8 pH values of whole unpeeled pink shrimp ("open stored")
and brown shrimp ("bag stored") during storage in ice



Vanderzant and Nickelson⁽⁹¹⁾ showed that the pH of white shrimp (P. setiferus) increased gradually during ice storage. They found that the pH at which the shrimp spoilage occurred was 7.7 to 8.4 for the white shrimp and 7.8 to 8.0 for the brown shrimp, and in both cases a pH above 8.0 was considered as indicative of spoilage. Baily et al.,⁽⁶⁾ reported that shrimps with pH greater than 7.95 was considered spoiled, but Bethea and Ambrose⁽¹⁰⁾ showed that brown headless shrimp (Penaeus aztecus) had pH of 8.20 on the 8th day and was still acceptable. Cobb et al.,⁽⁸⁾ found that the white shrimp (P. setiferus) were unacceptable in the pH range of 7.5 to 8.0.

In this experiment here, the upper limit of pH at which the brown shrimp was unacceptable was pH 8.20 (on 7-8th day of ice storage) but the pink shrimp was still acceptable at pH 8.43 on the 8th day of ice storage. Flores and Crawford⁽¹⁶⁾ found that the pH of intact Pacific shrimp (Pandalus jordani) increased from 7.6 to 8.8 during the initial days of ice storage and Flick and Lovell⁽⁹⁾ reported an initial pH of 7.4 for Penaeus aztecus.

3.14.0 The results and discussion of the "K values" changes of brown shrimp, "bag stored" during storage in ice

Graph 9(A) (see Table 21 for the results) showed that the "K values" (32.8% at zero day and 53.6% after 24 hours) increased rapidly during the initial period of ice storage, up to 7 days (68.2%). Thereafter the "K values" showed no significant change (63.7% on the 10th day). The increase in the "K values" during the initial storage period could probably be due to the increase in the inosine and hypoxanthin content from the ATP degradation (see section 1.4.0). While the ATP and its related compounds decreased, the inosine would have reached the maximum level and then decreased to produce the hypoxanthine (the Hy production occurred usually at about 48 hours post mortem -(Flick and Lovell⁽⁹⁾)). This could probably explain the very slow rate of change in the "K values" during the later period of ice storage.

The "K values" obtained here showed a similar pattern of changes to that of Ino and Hy contents of brown shrimp (Penaeus aztecus) obtained by Flick and Lovell⁽⁹⁾, and the pattern of "Ino + Hy" values of cod (Gadus morhua) during ice storage, as reported by Jones and Murray.⁽²⁹⁾

The "K values" indicated possible usefulness as a "freshness" index, due to the large increase in the early period but not in the later spoilage period. The Japanese investigators^(92,93,43) showed that the "K values" were of possible use in their fish and shellfish as a quality index.

3.15.0 General discussion for the "bag stored" brown shrimp

Graph 9 and Table 21 show the changes of proline, ornithine, TMA-N and "K values" of brown shrimp, "bag stored" in ice.

It can be seen that the TMA-N value is a good indicator of spoilage. There was a significant change after 6 to 7 days of storage, correlating with organoleptic sign of spoilage.

The ornithine and pH changes (graph 8) showed a similar pattern to that of the "K values" during ice storage. Therefore, these tests could be useful as a measurement of the freshness of "bag stored" brown shrimps during ice storage.

The proline values showed no significant or definite pattern, and therefore could not be used as a quality index, for either freshness or spoilage.

On the 7th day of ice storage when spoilage was evident from both the discolouration and odour noted the TMA-N content was about 1.4 mg/100g (from graph 9(B)), the "K value" reached was about 62% (from graph 9(A)) and the pH was 8.20. It can, therefore, be regarded that below these values, the shrimp quality would be acceptable. The corresponding 7th day ornithine value was about 1.75 mmol/100g (from graph 9(C)) and this ornithine value could perhaps be regarded as a spoilage index limit for the fresh brown shrimp stored in bag.

There seemed in the brown shrimps (bag stored) to be good correlation between the ornithine content and the following

determinations over the period of ice storage:-

- 1) ornithine with "K values", the correlation coefficient (R) obtained was 0.8120 at 95% level of significant, i.e. at $P < 0.05$;
- 2) ornithine with pH values, R obtained was 0.8298 at $P < 0.05$.

Absolute correlation was not expected as the determinations were of different compounds in the shrimp, but as the statistical data showed, one would expect ornithine, "K values" and pH of the shrimps to rise over the storage period.

However, the limitation of this test (as in any test applied to biological materials) is the variation in the raw materials (i.e. pink and brown shrimps) which are affected by seasonal and species variations and by physiological conditions.

Graph 9 Proline, ornithine, trimethylamine-nitrogen (TMA-N) and
"K value" changes of brown shrimp "bag stored" during
storage in ice

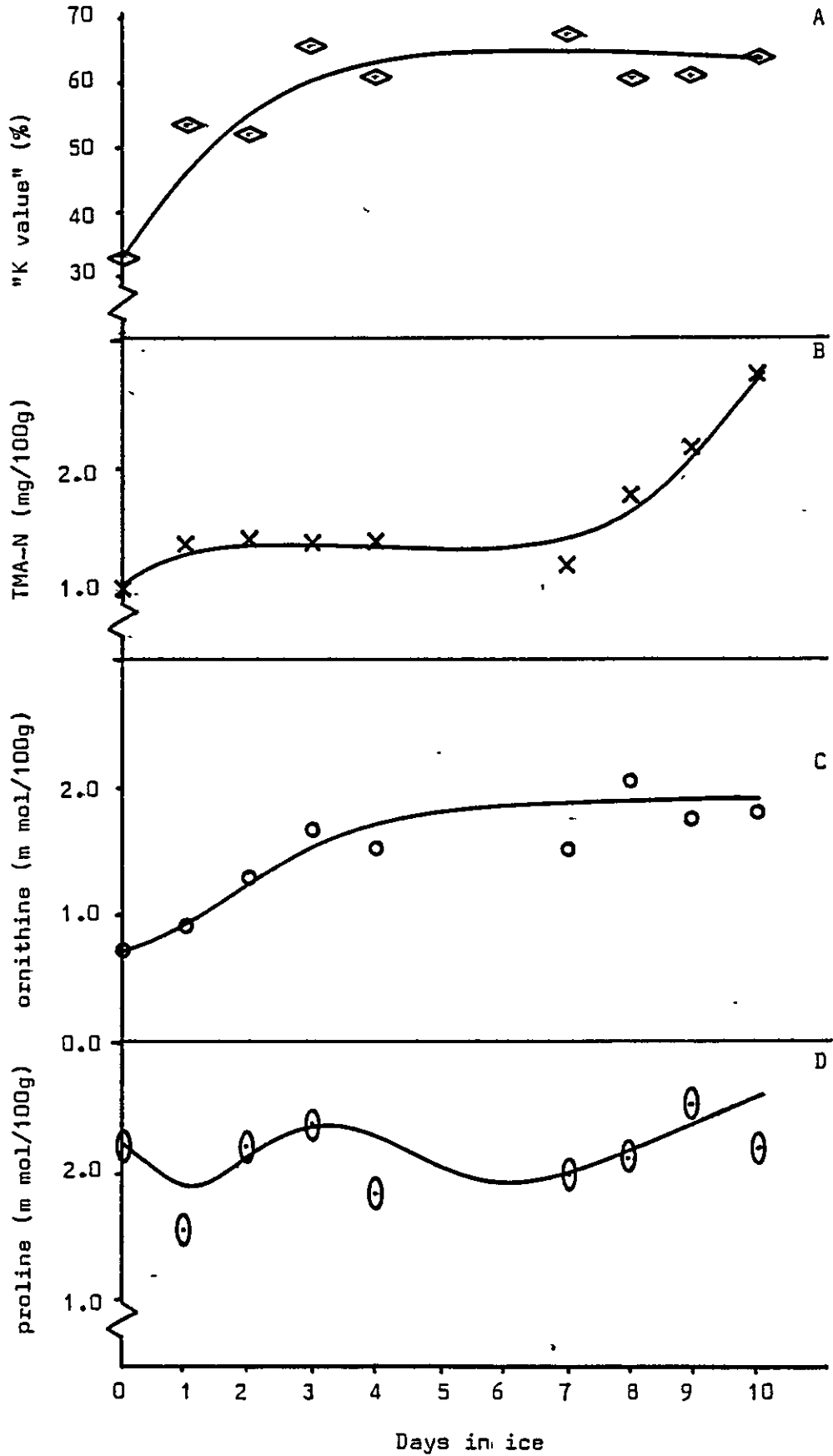


Table 21 Proline, ornithine, trimethyl amine-nitrogen (TMA-N); "K value" and pH changes of brown shrimp (Crangon crangon)
"bag stored", during storage in ice

Storage period (days)	Proline m mol/100g			Ornithine m mol/100g			TMA-N mg/100g			"K value" %			pH		
	X ₁	X ₂	mean \bar{X}	X ₁	X ₂	mean \bar{X}	X ₁	X ₂	mean \bar{X}	X ₁	X ₂	mean \bar{X}	X ₁	X ₂	mean \bar{X}
0	2.420	2.022	2.221	0.832	0.617	0.724	0.876	1.176	1.026	31.1	34.5	32.8	7.35	7.45	7.40
1	1.579	1.588	1.584	0.952	0.902	0.927	1.482	1.290	1.386	52.5	54.6	53.6	7.30	7.50	7.40
2	1.960	2.500	2.230	1.278	1.303	1.291	1.351	1.500	1.426	50.8	53	51.9	7.55	7.50	7.53
3	2.352	2.471	2.412	1.833	1.478	1.656	1.230	1.548	1.389	63.5	65.4	64.5	7.70	7.62	7.66
4	1.518	2.210	1.864	1.479	1.600	1.539	1.454	1.362	1.408	58.8	60.9	59.9	7.95	8.20	8.08
7	1.934	2.100	2.017	1.518	1.527	1.523	1.128	1.272	1.200	68.7	67.7	68.2	8.25	8.15	8.20
8	2.367	1.924	2.144	1.883	2.214	2.049	2.088	1.452	1.770	63.1	60.9	62.0	8.20	8.20	8.20
9	2.313	2.800	2.557	1.840	1.646	1.743	2.208	2.106	2.157	63.0	61.2	62.1	8.30	8.25	8.28
10	2.215	2.202	2.209	1.782	1.739	1.761	2.370	3.108	2.739	63.4	64.0	63.7	8.25	8.35	8.30

4.0.0

CONCLUSION

The following points have been concluded from the results of this work:-

- 1) the application of Chinard's method to standard amino acids solutions was shown to be reproducible for proline and ornithine.
- 2) Application of Chinard's method for the measurement of the changes in proline and ornithine concentrations during storage of shrimps revealed that for open storage, ~~substantial~~ leaching of the amino acids occurred. A combination of bag storage, and separate measurement of the ornithine concentration resulted in ^{detection of} a definite increase in ornithine concentration during ice storage. This change could provide a basis for the quality assessment of shrimps, in contrast to the "Ornithine Equivalent" measurement initially studied.
- 3) Comparison with more standard measurements showed that whilst the TMA-N measurement could serve as a spoilage index, the pH and "K value" resembled the ornithine changes and were more relevant as potential freshness indicators.

5.0.0

SUGGESTION FOR FUTURE WORK

A comparison of the separation of ornithine from proline by a column technique (possible automation using an auto-analyser) and the batch technique using Chinard's method should be investigated.

It would be interesting to investigate the effect of cooking (heating generally), and freezing and subsequent cold storage (with or without preservatives) on the ornithine values and its interpretation.

6.0.0

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