THE COMBINED USE OF MODIFIED ATMOSPHERE PACKAGING (MAP) AND GLUCOSE OXIDASE (GOX) DIPPING SOLUTIONS TO CONTROL MELANOSIS IN SHRIMP

by

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ABSTRACT

Black spot development or "melanosis" is a common defect in fresh shrimp which results in product being devalued and rejected by consumers. Currently, sulfiting agents are used to control melanosis in shrimp. However, with increasing regulatory and consumer concerns about the safety of sulphites as a method of melanosis control, the shrimp processing industry is actively seeking alternative methods to control melanosis on, and extend the shelf life of, fresh shrimp. One method which has the potential to fulfil both objectives are glucose oxidase (GOX)/glucose dipping solutions in conjunction with Modified Atmosphere Packaging (MAP).

Preliminary studies have shown that black spot development can be controlled for 14 days at 4°C in white shrimp (*Pandalus occidentalis*) and pink shrimp (*Pandalus borealis*) using GOX/glucose or GOX/glucose/ascorbic acid in conjunction with gas packaging (60% CO_2 : 40% N_2). This dipping/packaging treatment also improves the physical, chemical and microbiological changes in white shrimp compared to samples dipped only in water and air packaged. This study has shown that the combined use of two or more "barriers" can be used to extend the shelf life of, and control melanosis on, fresh shrimp. This novel process of "dipping" shrimp in GOX/glucose solutions in conjunction with MAP will have a significant effect in the area of shrimp hygiene and will have the potential to minimize shrimp spoilage incurred through melanosis.

RESUME

L'apparition de taches noires ou"mélanose" est un phénomène commun observé sur les crevettes fraîches qui cause une dévaluation et un rejet du produit par le consummateur. Actuellement, des solutions de trempage à base de sulphites sont utilisées pour contrôler la mélanose. Toutefois, avec l'augmentation croissante de l'inquiétude des consummateurs vis-à-vis l'utilisation des solutions de sulfites comme méthode de contrôle de la mélanose, l'industrie de transformation de la crevette recherche activement une méthode alternative permettant de contrôler ce phénomène et de prolonger la durée de conservation de la crevette fraîche. Pour remplir ces deux objectifs, une méthode potentielles sont proposées ici, soit une solution de trempage à base de glucose-oxidase (GOX) / glucose en combinaison avec l'emballage sous atmosphère contrôlée (MAP).

Des études préliminaires ont démontré que le développement des taches noires peut être contrôlé durant 14 jours à 4°C pour la crevette blanche et la crevette rose en utilisant une solution de GOX/glucose ou une solution de GOX/glucose/acide ascorbique en combinaison avec une atmosphère modifiée (60% CO_2 : 40% N_2). Ce traitment de trempage et d'emballage relentit aussi les changements physiques, chimiques et microbiologiques chez la crevette blanche en comparaison avec des échantillons trempés seulement dans l'eau et emballés sous air. Cette étude a démontré que l'utilisation combinée de deux ou plusieurs barriéres peut être utilisées pour prolonger la conservation du produit frais et contrôler l'apparition de la mélanose chez la crevette fraîche. Ce nouveau procédé de trempage dans des solutions de GOX/glucose combiné à l'emballage sous atmosphère contrôlée devrait avoir aussi un effet significatif dans le domaine de l'hygiéne et devrait minimiser la détérioration de la crevette causée par la mélanose.

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LIST OF ABBREVIATIONS

APC:	Aerobic Plate Counte
CA:	Controlled Atmosphere
CAS:	Controlled Atmosphere Storage
CFU:	Colony Forming Unit
CORPAQ:	Conseil des recherches en pêche et agro-alimentaire du Québec
DOPA:	Dihydroxyphenylalanine
EDTA:	Ethylenediaminetetraacetic Acid
EVA:	Ethylene Vinyl Acetate
EVOH:	Ethylene Vinyl Alcohol
FAO:	Food and Agriculture Organization
FDA:	Food and Drug Administration
GDL:	Glucono-delta-Lactone
GLM:	General Linear Model
GOX:	Glucose Oxidase
GRAS:	Generally Recognized As Safe
Hx:	Hypoxanthine
IAEA:	International Atomic Energy Agency
LAB:	Lactic Acid Bacteria
MA:	Modified Atmosphere
MAP:	Modified Atmosphere Packaging
O.D.:	Optical Density

PCA:	Plate Count Agar
PO:	Phenol Oxidase
PPO:	Polyphenoloxidase
PSY:	Psychrotrophic
PVDC:	Polyvinylidene Chloride
RH:	Relative Humidity
SAS:	Statistical Analysis System
STPP:	Sodium Tripolyphosphate
TCD:	Thermal Conductivity Detector
TMA:	Trimethylamine
TVB:	Total Volatile Base
U.V.:	Ultraviolet

SECTION 1: LITERATURE REVIEW

1. INTRODUCTION

1.1 Importance of crustacean fishing industry

Crustaceans, such as shrimp, crab and lobster, are an economically important fishery resource and in many parts of the world they are considered luxury items. In Quebec, which is a major supplier of shellfish, landings of shellfish from 1985 to 1987 were approximately 73,000 metric tonnes with a market value of $ 158.3 million dollars. This represents about 64% of the total value of fish landed in Quebec for those years (Anon, 1988).

Because of their unique biological and biochemical characteristics, crustaceans deteriorate rapidly due to various post-mortem spoilage processes. In shrimps, the initial spoilage is usually due to black spot development, also called melanosis or enzymatic browning. Melanosis starts in the head of shrimps and then spreads to the tail forming zebra like black bands between the sections of the shell. The blackening may occur within a few hours after the death of the shrimp if they are not stored on ice immediately (Faulkner et al., 1954). Melanosis has been observed in all species of shrimp harvested from waters in North America. Although melanosis does not affect the eating quality of crustaceans, it is not appealing to consumers who associate the dark color with spoilage and thus it reduces the market value of shellfish products.

For long term storage, shrimps must be frozen. However, frozen storage only slows down, but does not prevent melanosis (Simpson et al., 1987, 1988). Other disadvantages of freezing for shelf life extension of shrimp are: (a) high energy costs during storage and handling; (b) textural deterioration due to protein insolubilization;

(c) drip loss during thawing; (d) production of non-protein nitrogen and other spoilage related substances (Simpson and Haard, 1987).

Due to the relatively higher energy cost of frozen storage and the superior quality of fresh shrimp to their frozen counterparts, methods to prolong the shelf life of fresh shrimp are of great economic importance to the seafood processing industry. However, to understand the mechanism by which melanosis could be controlled, an understanding of the biochemistry of melanosis is required.

2. BIOCHEMISTRY OF MELANOSIS

2.1 Deterioration and spoilage of crustaceans

Chemically, fish and shellfish are composed of four major components - protein, moisture, fat and carbohydrate (Table 1) and other minor components such as vitamins, enzymes, pigments and flavor components. The relative proportion of all these constituents give fish and shellfish its particular structure and texture, flavor, color and nutritional value. Shrimps are high in moisture and in protein and low in fat and are an ideal substrate for microbial growth. Because of their unique biochemical and biological composition, fish and shellfish are subject to deterioration and spoilage by physical, microbiological and chemical means. Deterioration of shellfish quality is due to naturally present enzymes, indigenous microorganisms or through microbial contamination during catching, handling and processing. Spoilage of fish and shellfish is mainly due to microbial activity particularly by aerobic, psychrotrophic, *Pseudomonas*, *Flavobacterium* and *Achromabacter* species, resulting in changes in odor, flavor and color of crustaceans (Smith et al., 1991). With respect to chemical changes, enzymatic browning or melanosis is the most serious spoilage problem of fresh shrimp.

Table 1. Approximate chemical composition of some fish and shellfish species (%)

Species	Water	Carbohydrate	Protein	Fat	Ash
Blue fish	74.6	0	20.5	4.0	1.2
Cod	82.6	0	16.5	0.4	1.2
Haddock	80.7	0	18.2	0.1	1.4
Halibut	75.4	0	18.6	5.2	1.0
Herring	67.2	0	18.3	12.5	2.7
Mackerel	68.1	0	18.7	12.0	1.2
Salmon	63.4	0	17.4	16.5	1.0
Shrimp flesh	77.4	1.5	18.1	0.8	-

(Converted from Watt and Merrill, 1963)

Usually, the shrimp trawler stays at sea for as long as 3 weeks before returning to port. During this time shrimps are stored in crushed ice and kept at or near 0°C. The progressive changes in ice-stored shrimps can be divided into three phases: During the first phase of ice storage (0 - 7 days), shrimps gradually lose their fresh, sweet flavor. This if followed by phase two (8 -14 days) which is characterized by a flat, tasteless product. In the last phase (> 14 days of storage), rapid deterioration, accompanied by off-odors and off-flavors is evident (Banks et al., 1977). Whereas microbial and biochemical changes resulting in the loss of quality can be slowed down by storage in ice, melanosis development cannot be prevented (Bailey et al., 1960b). Indeed, melanosis is still the major quality problem limiting the shelf life of fresh shrimp stored in ice or under refrigeration.

2.2 Nature of Melanosis -- enzymatic browning

There are four types of browning reactions in food: (1) The Maillard reaction, (2) Caramelization, (3) Ascorbic acid oxidation and (4) Enzymic browning. The first three are examples of non-enzymatic browning reactions in foods whereas melanosis is classified as enzymatic browning (Richardson and Hyslop, 1985).

Enzymatic browning is most commonly observed in fruits and vegetables such as apples, pears, bananas, potatoes, and mushrooms when they are sliced or injured (Mathew and Parpia, 1971). Fruits and vegetables when sliced or injured turn brown very quickly whereas stored mushrooms become black.

Although enzymatic browning has been observed in fruits and vegetables for many years (Lindet, 1895), melanosis in crustaceans was first reported by Fieger (1950). Initially, it was suggested that mold growth caused melanosis. However, subsequent research dismissed this theory and it has been shown that melanosis in crustaceans is also due to enzymatic activity (Alford and Fieger, 1952).

In the frozen storage of crab and lobster meat, the development of blue or black discolorations (usually simply called "blueing"), which is biochemically similar to enzymatic browning in fruits, is one of the most troublesome quality problems. This discoloration may develop to a moderate degree during or shortly after cooking or it may appear after freezing and during storage. One type of blueing, which appears occasionally on king crab, does not develop until the meat is thawed and allowed to stand exposed to air (Babbitt, 1982).

Although excessive browning is undesirable in most products, advantage is sometimes taken of the browning reaction to produce a slightly yellow product e.g. pasteurised apple purée. Enzymatic browning is also responsible for the desirable colour of cocoa, coffee, tea products and dried fruits such as dates and figs. Whereas the dark

discolorations of fruits, vegetables, shrimps, lobster and crab meat may differ slightly in color intensity, their mechanisms of formation are all due to phenolase activity (Mathew and Parpia, 1971; Savagaon and Sreenivasan, 1978; Babbitt, 1982).

2.3 Phenolase: Introduction

The various enzymes which catalyse the oxidation of phenols causing enzymatic browning are commonly known as phenolase. These enzymes are widely distributed in both the plant and the animal kingdoms. This group of enzymes are characterized as being copper dependent and have been extensively studied in fruits and vegetables and as part of the moulting/scelerotization mechanism in insects and crustaceans (Fye, 1974; Ferrer et al.,1989). As early as 1930, Pinkey reported the presence of the enzyme tyrosinase in Crustacea. The importance of this enzyme in the hardening and darkening of the cuticles of certain insects and of crayfish and lobster was reported by Dennell (1947). Bailey et al., (1960a,b) located phenol oxidase (PO) in the exoskeleton and adhering epicuticle, antenna and blood of shrimp (*Penaeus aztecus & Penaeus setiferus*). They reported that the most active shrimp phenol oxidase was found in blood. However, very little information exists about the role these enzymes play in postmortem crustaceans. To date, it is known that PO is unstable in acid pH (<4) and that it is not heat stable. In certain crustaceans, PO exists in an inert form as pro-PO which can be activated by physiological and environmental factors to its active form PO (Yan et al., 1990). Protease (e.g.trypsin) was also reported to activate PO (Simpson et al., 1987).

Damage to plant tissues results in the rapid mixing of substrate and phenolase (monophenol oxygenases) and the enzymes may be activated by release from organelles and loss of inhibitors. In the moulting processes, PO activity is controlled through activation and affinity to substrates, but in postmortem tissue, this control no longer exists and this may result in the development of black spot (Yan et al., 1990).

2.4 Phenolase & enzymatic browning

The formation of the brown pigment melanin is shown below:

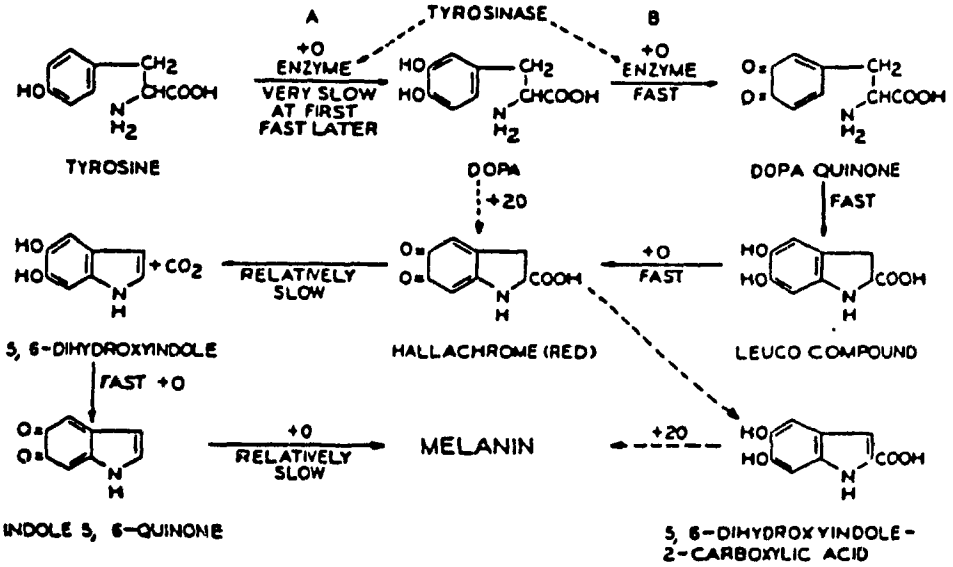


Figure 1. Reactions resulting in melanin formation (Schwimmer, 1981)

The complex phenomenon of melanosis involves both enzymatic and non-enzymatic steps. In animals, phenolase is responsible for melanin formation by first catalysing the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA) and subsequently catalysing its oxidation to form DOPA quinone. The remaining reaction sequence involves non-enzymatic oxidation and polymerization of the quinone to the brown pigment, melanin. Furthermore, melanin can interact with proteins to form complexes. Hydroxylation of monophenols is the slow or rate determining step. Consequently, monophenols undergo a slow hydroxylation reaction before oxidation to orthoquinone.

The mechanism of melanosis in a crustacean species may be quite different because it involves biochemical changes in a postmortem animal.

2.5 Reactions catalyzed by phenolase

The action of phenolase is unusual in so far as two sequential and interdependent chemical reactions are catalysed by the same enzyme. Firstly, an o-diphenone is formed by hydroxylation of monophenol and secondly, an o-quinone is formed by dehydroxygenation of the diphenol. The overall reaction is a simplified representation of the phenolase-catalysed reaction. For hydroxylation, a reducing agent is required to provide two electrons to reduce one atom of oxygen to water:

$$AH + O_2 + 2H^+ + 2e <=======> H_2O + AOH$$

This is a typical reaction of monoxygenases where a separate reducing agent, which is the o-diphenol, is required. The reaction is overall thermodynamically favourable due to the energy released by the formation of water. The incorporation of the oxygen atoms into both water and the o-diphenol has been verified by the use of isotopic oxygen. The requirement of ortho-diphenol as the reducing agent has also been demonstrated by its ability to reduce the lag phase of the reaction time (Robinson, 1987).

2.6 Nomenclature of phenolase

Phenolase belong to the group of enzymes known as oxidoreductases, i.e. they oxidise phenolic substances in the presence of atmospheric oxygen. However, their nomenclature is somewhat confusing and even controversial. Since phenolase catalyze two sequential and interdependent reactions -- hydroxygenation of monophenone to ortho-diphenone and then oxidation of ortho-diphenone to ortho-quinone, the first activity was referred to tyrosinase (monophenol oxidase) activity, e.g. catalyzing the hydroxygenation of tyrosine to DOPA; The latter activity was referred to as catechol oxidase (polyphenol oxidase) activity, e.g. the oxidation of DOPA to DOPA-quinone.

The systematic name of tyrosinase and catechol oxidase are o-diphenol: oxygen oxidoreductase (EC 1.10.3.1). Another enzyme (which exists mainly in fungi and the lac tree) was formerly named p-diphenol oxidoreductase (EC 1.10.3.2) since it had the ability to oxidize p-diphenols, a property not possessed by tyrosinase or polyphenol oxidase. Recently, the two names tyrosinase (1.10.3.1) and laccase (1.10.3.2) were combined in the new systematic name: monophenol monooxygenase (EC 1.14.18.1). Other names for phenolase are cresolase, ortho-diphenol oxidase, diphenol oxidase or catecholase (EC 1.10.3.2) (Eskin, 1990).

2.7 Substrates of phenolase

The substrates for enzymatic browning are simple, substituted phenols or the more complex chlorogenic acid catechin and flavonyl glycosides. Examples of substrates for phenolase are shown in Table 2. The most abundant substrate in animals is tyrosine and in plants chlorogenic acid. In tea, cocoa and coffee beans, where enzymatic browning is beneficial, the concentration of substrate is likely to vary between cultivars and on growing conditions. Whereas phenolase from animals are relatively specific for DOPA and tyrosine, plant phenolase act on a wider range of mono- and ortho-diphenols. Although tyrosine is the main substrate of certain phenolase, other phenolic compounds in fruit, such as caffeic acid, and chlorogenic acid also serve as substrates. These compounds are diphenols and are readily attacked by the catecholase component of phenolase.

Table 2. Substrates of phenolase

Source	Substrates
Apple	Chlorogenic acid
	Catechol
	Caffeic acid
Mango	L-DOPA
	L-Tyrosine
Potato	Chlorogenic acid
	Caffeic acid
	L-DOPA
Sweet potato	Cholorogenic acid
	Caffeic acid
Parsnips	Catechol
	Tyrosine
Lobster and shrimps	Tyrosine

2.8 Structure of phenolase

Phenolase from different sources show a wide range of molecular sizes; some exist as monomers and others as dimers and tetramers. Multiple forms of the enzyme exist with varying specificities from the same source. For example, seventeen multiple forms of potato phenolase were detected using conventional gel electrophoresis. All utilized o-diphenol as substrate and five also utilized monophenols as substrates (Matheis and Belitz, 1975). They separated a monomer (30,000 daltons), several oligomers and isozymes of the polymer size by gel chromatography. These observations agree with the hypothesis that some phenolase monomers are disulfide-linked whereas some isozymes may also arise from interchange of subunits (Fling et

al., 1963). A characteristic of phenolase is the presence of two atoms of copper per active site which exists as a binuclear copper complex, $E(Cu^{2+})_2$. The distance between the copper atoms is approximately 0.6nm. Spectral measurements indicate that the unpaired electrons of the two copper atoms interact in an antiferromagnetically coupled state and that a peroxy complex is formed in the presence of oxygen (Robb, 1984). Both atoms of Cu^{2+} are reduced by the transfer of electrons from o-diphenol to Cu^{+} which are then used to reduce oxygen to water with coupled acceptance of $2H^{+}$ from the oxidised substrate. Cyanide acts as a competitive inhibitor to oxygen. For hydroxylation reactions with monophenol substrates, the o-o bond must undergo distortion and cleavage--a reaction which is thought to be influenced by the substituent ligands of the enzyme and the electrophilic nature of the substrate.

2.9 Factors influencing phenolase activity

Several factors influence phenolase activity, including pH, storage temperature, reducing agent, atmosphere oxygen, etc. Each of the physical and chemical factors shown in Table 3 will be briefly reviewed.

Table 3. Factors influencing phenolase activity

A. Physical:
(i) pH;
(ii) Temperature;
B. Chemical:
(i) Oxygen;
(ii) Substrates;
(iii) Reducing agents;
(iv) Chelating agents;

2.9.1 pH.

The pH optima of phenolase activity from different plant and animal sources is shown in Table 4.

The optimum pH of phenolase activity varies with both the source of enzyme and its substrate. In general, phenolase are active between pH 5-7 and they do not have a sharp pH optimum. For crustacean species, optimum activity of "tyrosinase" were observed at neutral pH. Generally speaking, lowering pH below 4 inhibits phenolase activity. At lower pH values (pH 3), phenolase are irreversibly inactivated. Furthermore, food acidulants, which could be used to reduce pH and inactivate enzyme activity, can also complex or remove copper which is required for enzyme activity.

Table 4. pH optima of phenolase activity

Source	pH Optimum
Apple	4.8*
Pear	6.2*
Apricot	5.0-6.0*
Banana	7.0*
Peach	5.9-6.3*
Beet	6.5-7.0*
Sugar cane	7.0*
Shrimps	7.3-7.9#

(#: Faulkner et al.,1954; *: Mathew and Parpia,1971)

2.9.2 Temperature

The temperature optimum of phenolase activity has been less investigated than the pH optimum. However, the data available indicates that the temperature optimum of the enzyme depends on similar factors as pH optimum i.e. enzyme source and substrate. The activity of peach phenolase was found to increase from 3°C to 37°C and then decline at 45°C. At 3°C, activity was about 50% of the maximum value. In apricots, the enzyme reached its maximum activity at 25°C whereas phenolase from apples and potatoes exhibited maximum activity at 22°C and 25°C respectively. The phenolase activity of Dungeness crab was completely destroyed after heating at 100°C for 20 minutes whereas there was only a slight loss in enzymatic activity after 2 minutes at 85°C (Babbitt, 1982).

Phenolase are not heat stable enzymes. Brief exposure of the enzyme, in *vitro* or in *vivo*, to temperatures of 70-90°C, result in its partial or complete inactivation. Freezing may also affect phenolase activity. Phenolase in fruits and vegetables tissues are generally inactivated by heat (blanching) or chemicals prior freezing to prevent enzymatic browning, which otherwise would take place rapidly upon thawing as a result of cell rupture during freezing and access of the enzyme to its endogenous substrate.

2.9.3 Oxygen

Molecular oxygen is necessary reactant for the melanosis reaction to occur. It can be used to measure phenolase activity by measuring oxygen uptake (nmol/ml reaction mixture) using an oxygen electrode. Elimination of oxygen will therefore reduce the rate and extent of the development of melanosis.

2.9.4 Substrates

The availability of substrate directly affects enzyme activity. The quantity of substrate

in crustaceans determines the extent of melanosis. Babbitt, (1982) reported that in Dungeness crab, the longer the holding time prior to slaughter, the higher the phenolic content and the extent of blue discoloration of the crab meat. The availability of substrate for phenolase can be affected by substrate analogues e.g. methyl-substituted derivatives such as guaiacol and m-diphenols, such as resorcinol (Eskin, 1990). Crabs and lobster should be kept alive and in a healthy condition before processing. If they are stressed too much before processing, the formation and accumulation of some compounds will result in irreversible changes in the flesh and affect quality. The accumulation of phenolic compounds in shrimps and crabs due to a delay in icing prior to processing, will eventually result in the black spot or blue discoloration.

2.9.5 Reducing agents

The presence of reducing agents such as ascorbic acid, thiol (-SH), and possibly NADH, NADPH can inhibit melanosis. The most typical reducing agent is ascorbic acid which acts by reducing the enzymatically formed quinone to o-diphenol. Under such conditions, the reaction cycle continues until all of the reducing agent has been oxidised and only then does the quinone product accumulate to form brown polymeric products.

Some compounds, such as L-cysteine, can combine chemically with the o-quinone to form a stable, colorless product and prevent further reactions leading to melanin formation.

2.9.6 Copper

Copper acts as a prosthetic group in phenolase. Simpson et al., (1987) reported the addition of copper stimulated the phenolase-DOPA reaction in white shrimp. Addition of copper acetate at a molar ratio at 1:1.25 (Cu to PO) increased PO

activity by approximately 37% whereas the same salt at a molar ratio of 1:4 (Cu to PO) only stimulated the reaction by approximately 17%. Therefore, chelation of copper can be used to control phenolase activity and hence melanosis.

3. PREVENTION OF MELANOSIS

In general, phenolase activity is undesirable because the consumer associates the brown or black color with spoilage. Since food is a complex system, it is very difficult to suggest general methods for the prevention of melanosis. Consequently, a variety of methods have been developed to inhibit enzymatic browning.

The methods used to prevent melanosis can be classified into four groups, based on their mode of action:

1. Denaturation of enzyme protein;
2. Interaction with the copper prosthetic group of the enzyme;
3. Interaction with phenolic substrates or quinones;
4. Exclusion of reactants, e.g. oxygen.

Methods already used or potential methods which could be used to control melanosis include:

1. Heat treatments;
2. pH reduction, using the following acidulants;
    a. Ascorbic acid;
    b. Sulfiting agents;
    c. Citric acid;
    d. Phosphoric acid;
    e. Glucono-delta-lactone, (GDL).
3. Chelating agents (e.g. EDTA);
4. Protease;
5. Glucose oxidase/glucose dipping solutions;

6. Modified atmosphere package, (MAP);
7. Ionizing irradiation.

Each of these control methods will be briefly discussed.

3.1 Heat treatments

Heating of food products susceptible to melanosis is a very efficient method of preventing melanosis since phenolase are fairly susceptible to heat and are easily denatured. Heating is mainly used commercially to blanch fruits and vegetables prior to canning. The disadvantage of heating is that it softens the texture of fruit and it results in an undesirable cooked flavour. To prevent excessive enzymatic browning in products such as apple juice, the phenolase enzymes are denatured by heat-treatment at approximately 80°C.

The effect of heating has been studied recently on peeled bananas (Cano et al., 1990). When bananas were blanched in boiling water for 11 minutes and then cooled in ice water for 5 minutes, phenolase activity was inactivated by 96 to 100%. When peeled banana slices were heated in a microwave oven (650 watts for two minutes), followed by cooling in ice water for 5 minutes, blanching was less effective. Furthermore, the efficiency of the treatment was proportional to the maturity of the bananas. The more mature the banana at the time of treatment, the more effective the heat treatment. Bananas stored for 14 days showed losses of 0 to 20% phenolase activity compared to 60% for bananas which had reached senescence (23 days of storage) prior to microwaving. Although both types of heat treatment can reduce the degree of enzymatic browning, both methods have some disadvantages. Microwave heating has been shown to favour non-enzymatic Maillard browning, due to the high moisture of fruit (Cano et al., 1990) and some browning will still be observed. For bananas submitted to blanching by immersion in boiling water, the most obvious disadvantage was over-cooking of the final product. The rate of decrease of phenolase activity, at constant temperature, appears to follow a logarithmic law, as

shown in Figure 2.

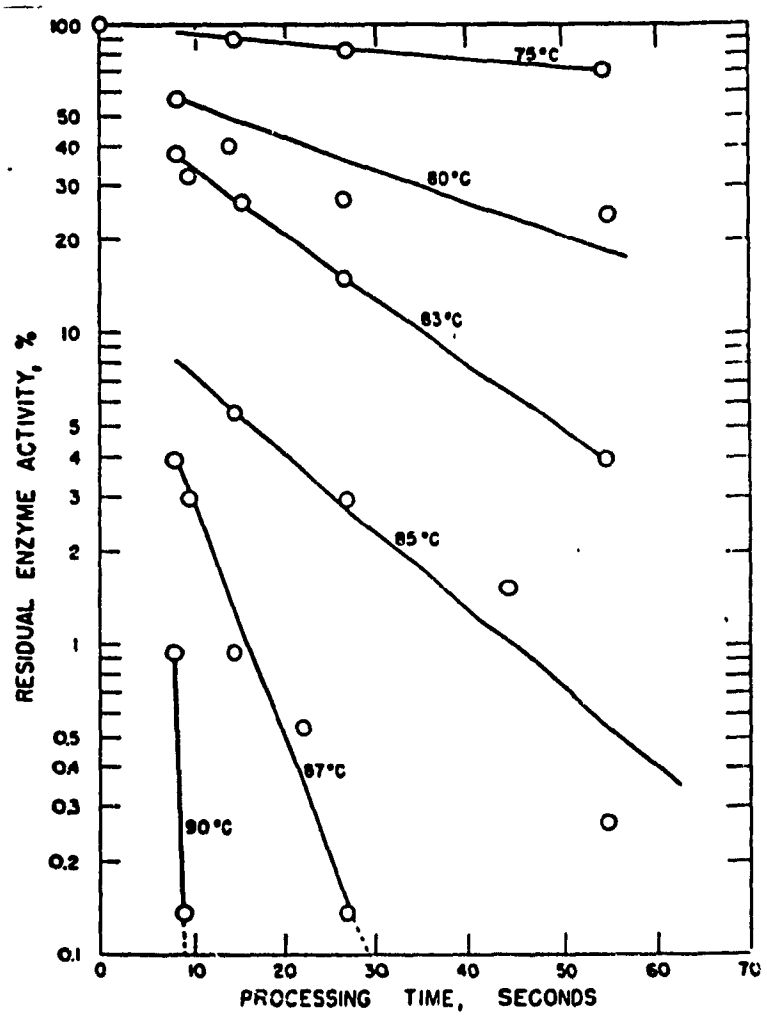


Figure 2. Level of inactivation of polyphenolase activity in pear puree at various temperatures (Dimick et al., 1951).

It is evident from Figure 2 that the processing time required to inactivate the enzyme

is short (in the order of seconds), compared to 11 minutes required for bananas. This indicates that the heating time required for phenolase inactivation is dependent on the physical nature of the food subjected to heating. For example, heat transfer is greater in a puree compared to the intact fruit. Furthermore, product pH is different and low pH facilitates inactivation of the enzyme.

Heating studies have shown that phenolase remained fully active when shrimps were exposed for 10 minutes at temperatures ranging between 25°C and 35°C. For complete inactivation of phenolase activity in shrimp, a temperature of 70°C for 2 hours was required (Figure 3).

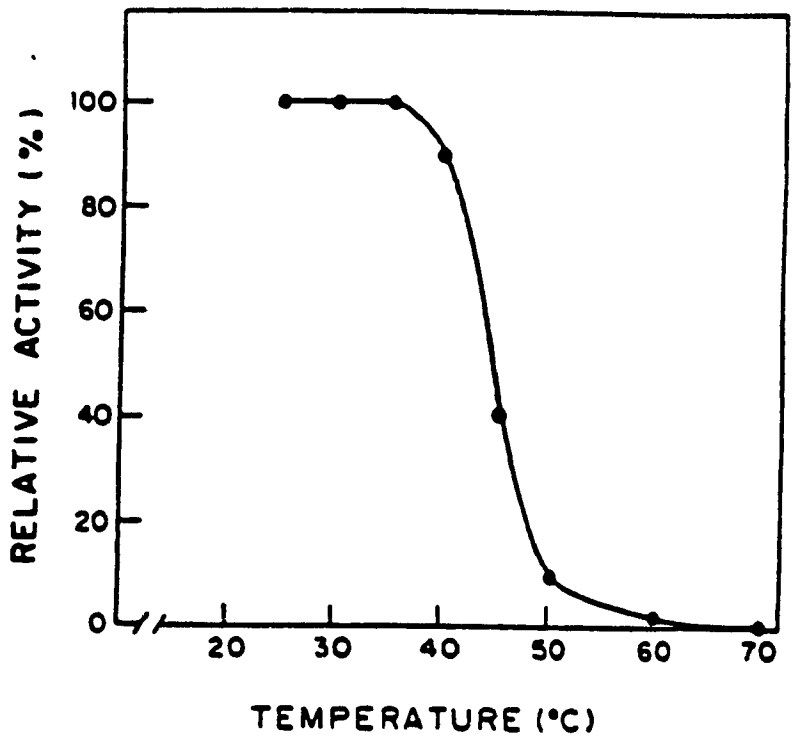


Figure 3. Effect of temperature on shrimp PPO activity (Al-Jassir, 1987).

When freshly caught crabs, in excellent condition, were cooked for 30 minutes at 100°C, phenolase were inactivated and blueing was prevented. However, reducing the time to 15 minutes at 100°C had no effect on phenolase activity or discoloration. However, if the crabs were in poor condition prior to cooking, heating at 100°C for 30 minutes could not prevent blueing.

Although heating is a simple, inexpensive, efficient and a non-toxic method to control melanosis, it is not a practical solution to prevent melanosis in crustaceans which are sold fresh.

3.2 pH reduction treatments

The application of acids is used extensively to control melanosis. Acids employed are those found naturally in plant tissues, such as ascorbic, citric, malic and phosphoric acids. Sulphites can also be used to control melanosis. The use of sulphites to control melanosis is under review by regulatory authorities in the USA and in Canada.

The use of acidulants is based on the fact that lowering pH will reduce or retard the development of enzymatic browning. The optimum pH for most phenolase is between pH 4.0 and 7.0, with little activity below pH 3.0 (Eskin, 1990). In their study on apple juice, Zemel et al., (1990) showed that there was no residual enzyme activity at pH 2.0 (Figure 4). Certain acidulants can also serve as chelating agents of copper, which is essential for phenolase activity.

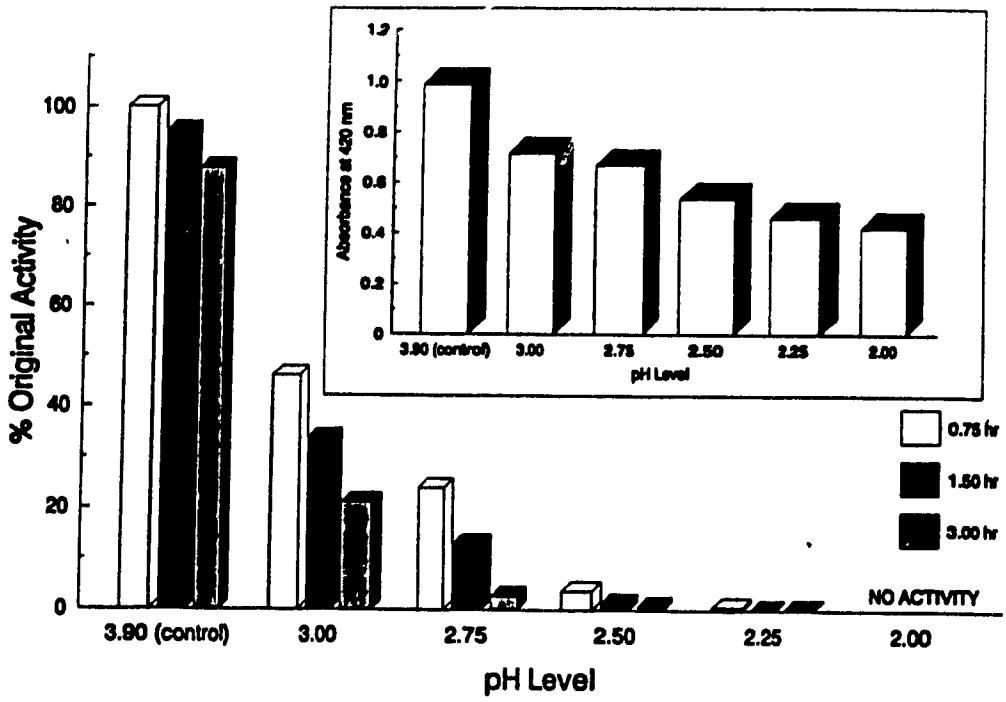


Figure 4. Residual PPO activity in apple juice after exposure to various pH values and times and the effect on color (Zemel et al., 1990).

3.2.1 Ascorbic acid

Ascorbic acid is widely used to prevent oxidative browning of fruit juices (Walker, 1975). Ascorbic acid functions by: 1). Reducing the polymerization of o-quinone; i.e. it reduces the o-quinone back to the phenones. However, when ascorbic acid is consumed, browning will appear (Robinson, 1987). For example, when apples are cut, ascorbic acid is oxidized rapidly and browning results. Ascorbic acid also competes

for available O_2 and lowers pH. It has also been used to delay melanosis in shrimp, but it is not as effective as sulfiting agents (Schwimmer, 1981).

The use of ascorbic acid to inhibit melanosis has several advantages:

1. It has no detectable flavor at the concentration used i.e. 300 mg per 500 gram of fruit;
2. It does not have a corrosive action on metals;
3. It has nutritional value (vitamin C).

The main disadvantage associated with ascorbic acid treatment is its instability. To overcome this limitation, derivatives of ascorbic acid have been studied. Two ascorbic acid derivatives, ascorbic acid-2-phosphate and ascorbic acid-2-sulphate can be used to slowly produce ascorbic acid (Hsu et al., 1988). However, ascorbic acid-2-phosphate and ascorbic acid-2-sulphate were found ineffective in preventing melanosis in mushrooms.

3.2.2 Sulfiting agents

Sulphiting agents have been used extensively to prevent discolorations in food products. The application of sulphur dioxide or sulphites is commonly used to inhibit phenolase activity in fruits and vegetables. They act by reducing product pH and also chelate copper which is essential for the enzymatic reaction. The forms employed include sulphur dioxide and the sodium or potassium salts of sulphide, bisuphite or metabisulphite. In aqueous solutions, sulphur dioxide and sulphite salts form sulphurous acid and ions of bisulphite and sulphite. Sulphur dioxide is the most effective inhibitor of enzymatic browning and is used widely by the food processing industry. At levels of 1 p.p.m., SO_2 causes a significant drop in phenolase activity (approximately 20 percent) whereas at 10 p.p.m. the enzyme is inactivated almost completely. A sodium bisulphite dipping solution (1.25% for 1 min.) is currently used

to control melanosis in shrimp in the U.S.A. (Finne et al., 1986). To date, this treatment is the most effective method of contr lling melanosis in fresh shrimp (Otwell and Marshall, 1986). However, this application is under review by regulatory agencies due to its potential health hazards e.g. allergic reactions, asthma attacks in some consumers of the treated products. The residue of sulphites in shrimps are strictly controlled to <100 ppm for the raw edible portion and <30 ppm in cooked products (Marshall and Otwell, 1986). The mode of action of sulphites is similar to that of ascorbic acid, i.e. competition for oxygen and reduction of o-quinone to phenones (Walker, 1975).

3.2.3 Citric acid

Citric acid (GRAS) is perhaps the most widely used organic acid in the food industry. It is used as a dipping solution to inhibit enzymatic browning in pre-sliced potatoes. It acts by lowering pH and also acts as a chelator of copper thus inhibiting the activity of phenolase (Anonymous, 1990). However, it has not been used to control melanosis in shrimp.

3.2.4 Phosphates

By chelating a wide variety of metal ions and reacting directly with proteins, phosphates can be used in vegetables and sea foods to control pH and reduce the activity of phenolase and delay melanosis. Their addition to fresh sea foods also increases the water holding capacity and reduces drip loss and textural changes upon thawing from frozen storage (Ho, 1989). Al-Jassir, (1987) reported that shrimp dipped in a 9% sodium tripolyphosphate (STPP) solution and then refrigerated delayed melanosis for 2 days compared to control samples. Melanosis could be controlled for a further 6 days when a dipping solution of 9% STPP was used in conjunction with glucose oxidase/catalase (GOX/CAT) at a concentration of 6 units/ml (Al-Jassir, 1987).

3.2.5 Glucono delta-lactone

Glucono-delta-lactone (GDL) is also used as an acidulant in food products. GDL is converted by slow hydrolysis into gluconic acid which lowers product pH to less than 4. Gluconic acid also complexes with trace metals which would otherwise promote oxidation (Anonymous, 1990).

3.2.6 Other acids

Cinnamic acid, coumaric acid and ferulic acid have all been reported to be potential inhibitors of enzymatic browning in fruit juices (Walker, 1976) and in potato tubers (Macrae and Diggleby, 1968). The effect of these three acids on enzymatic browning is shown in Table 5. Cinnamic acid was found to be the most potent inhibitor, requiring less than 0.01% to inhibit enzymatic browning. This low concentration of cinnamic acid used, in conjunction with its low cost, makes it a viable alternative to control enzymatic browning, compared to ascorbic acid.

Table 5. Effect of cinnamic, coumaric and ferulic acid on the browning of apple juice (Walker, 1976).

Final concn of inhibitor (mM)	Degree of browning			
	Granny Smith		Sturmer Pippin	
	Colour developed	Absorbance	Colour developed	Absorbance
Nil (control)	Brown	0·48	Dark brown	0·69
Cinnamic acid				
0·1	Light brown	0·21	Dark brown	0 52
0·25	None	0·05	Light brown	0·41
0·5	None	0·05	None	0·06
0·75	None	0·02	None	0·04
1·0	None	0·02	None	0·04
p-Coumaric acid (4-Hydroxycinnamic)				
0·1	Light brown	0·26	Dark brown	0·57
0·25	Light brown	0·19	Brown	0·37
0·5	None	0·07	None	0·04
0·75	None	0·06	None	0·03
1·0	None	0·05	None	0·02
Ferulic acid (4-Hydroxy-3-methoxycinnamic)				
0·1	Light brown	0·25	Dark brown	0·66
0·25	Light brown	0·21	Dark brown	0·50
0·5	Pale brown	0·17	Light brown	0·28
0·75	Pale brown	0·11	Pale brown	0·10
1·0	None	0·03	None	0·03

Cinnamic acid, coumaric acid and ferulic acid all occur naturally in many edible fruits plants. It is therefore unlikely that there would be any health hazard associated with their use. Therefore, the addition of cinnamic or coumaric acid may offer the food industry an inexpensive and long-lasting method to control enzymatic browning in many foods (Walker, 1976).

3.3 Interaction with the copper prosthetic group

3.3.1 Chelating agents -- Introduction

Any molecule or ion with an unshared electron pair can coordinate or form complexes with metal ions. Therefore, compounds containing two or more functional groups, such as OH, SH, COOH, PO_3, H_2, C=O, S and O, in proper geometric relation to each other, can chelate metals in a favorable physical environment (Lindsay, 1985). EDTA, cyanide, carbon monoxide, diethylthiocarbamate and mercaptobenzothiole are all effective chelating agents of copper (Al-Jassir, 1987).

Chelating agents, such as EDTA, play a significant role in food stabilization through reactions with metallic ions to form complexes that alter the properties of the ion and their effects on food. For the enzymatic browning reaction to occur, the prosthetic group of phenolase (copper) must be present. Chelating agents act by chelating the copper in the phenolase enzyme. Chelating agents, such as EDTA, are not antioxidants but they bind metal ions that catalyse oxidation. Whereas chelating agents can be used to slow down the browning reaction by sequestering the copper ion, they do not completely eliminate melanosis (Langdon, 1987).

3.3.2 Ethylenediaminetetraacetic acid (EDTA)

EDTA is one of the most commonly used chelating agents in foods. It forms chelates of high stability with calcium, through coordination of electron pairs on its nitrogen atom of two of the four carboxyl groups. The spatial configuration of the calcium-EDTA complex is such that it allows additional coordination of calcium with free electron pairs of the anionic oxygen atoms of the remaining two carboxyl groups, resulting in an extremely stable complex utilizing all six electron donor groups (Lindsay, 1985).

3.3.3 Mercaptobenzothiazole

2-Mercaptobenzothiazole has been shown to inhibit banana phenolase and can delay the onset of melanosis at a concentration of 20 uM. The use of

mercaptobenzothiazole could be advantageous compared to cysteine, mercaptoethanol or ascorbic acid since a lower concentration can be used to control melanosis (Palmer and Roberts, 1967).

3.3.4 Potassium ethyl xanthate

Potassium ethyl xanthate is a compound which chelates copper and other metals. It has been used to inhibit copper-containing oxidases. When added to phenolase, it decreased the initial rate of oxidation (Figure 5). Melanosis formation was prevented and oxygen uptake was restricted. It is believed that xanthate combines with quinones formed during oxidation (Pierpoint, 1966).

3.3.5 Diethylthiocarbamate

Diethylthiocarbamate has also been used as a chelating agent for phenolase, although it chelates copper less specifically than potassium ethyl xanthate does. Despite its lower specificity, diethylthiocarbamate is more effective and has been shown to decrease the initial rate of oxidation more effectively than potassium ethyl xanthate (Figure 5).

3.4 Proteases

Taoukis et al., (1990) reported that melanosis in shrimp (*Penaeus duorarum*) could be prevented with the addition of protease ficin (0.5%) and refrigeration (4°C). Under such conditions melanosis was delayed for at least 8 days. The mode of action of ficin is presumed to involve inactivation of the phenolase through the amino acid cysteine which combines with quinone to form a stable compound thereby preventing melanin formation (Schwimmer, 1981).

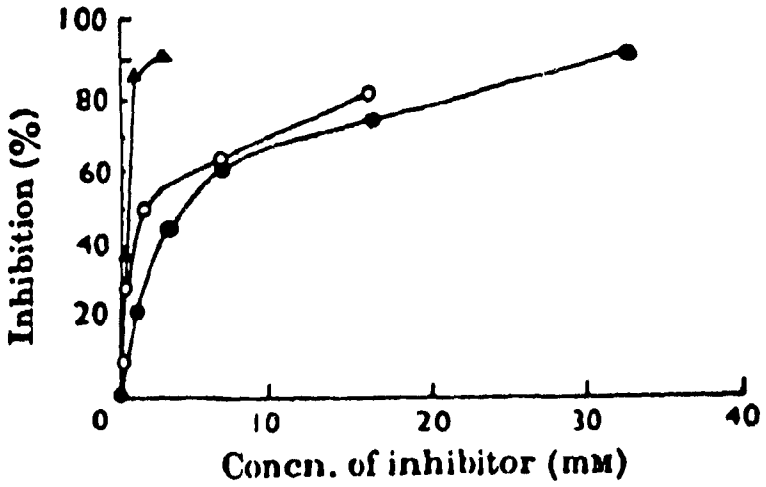


Figure 5. Inhibition of o-diphenol oxidase by potassium ethyl xanthate (●), potassium ethyl xanthate plus 33 mM benzenesulphinic acid (o), diethyl-thiocarbamate (▲).(Pierpoint, 1966).

3.5 Glucose oxidase (GOX)/glucose dipping solutions

Glucose oxidase (E.C. 1.1.3.4 β-D-glucose: oxygen oxidoreductase) is an oxidoreductase which catalyzes the oxidation of glucose to gluconic acid with molecular oxygen being reduced to hydrogen peroxide. Glucose oxidase represents one method of controlling spoilage problems caused by dissolved or headspace oxygen. It works as an oxygen scavenger by converting glucose to gluconic acid. The enzyme is produced by molds, especially *Penicillium notatum* and *Aspergillus niger* (Underkolfer, 1957). Glucose oxidase is used in the food industry primarily to prevent changes in the color and flavour of food products both during processing and in storage.

The glucose oxidase enzyme catalyses a reaction between oxygen and glucose yielding D-gluconic acid and hydrogen peroxide. Catalase, which is often a constituent of

commercial glucose oxidase systems, hydrolyzes hydrogen peroxide to water. The reaction proceeds until the glucose or oxygen substrates are exhausted. The overall reactions catalyzed by these enzymes are shown in Figure 6 (Schwimmer, 1981).

Glucose oxidase was first identified of potential commercial interest in 1943 by Dwight Baker. He developed the first commercial application of glucose oxidase to prevent oxidative changes in bottled beer which has a high residual headspace air content (Scott, 1975).

$$2C_6H_{12}O_6 + 2H_2O + 2O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} 2C_6H_{12}O_7 + 2H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$2C_6H_{12}O_6 + O_2 \xrightarrow{\text{glucose oxidase}} 2C_6H_{12}O_7$$

Figure 6. Reactions catalyzed by glucose oxidase/catalase.

The treatment generates gluconic acid which lowers the product's pH. Furthermore, gluconic acid also acts as a chelating agent. Gluconic acid is colorless, odorless and tasteless and does not affect the organoleptic quality of the food. The hydrogen peroxide generated has antimicrobial properties and has been shown to inhibit *Staphylococcus aureus* when glucose oxidase was added without catalase (Kato et al., 1986). However, the antimicrobial role of hydrogen peroxide in our study is minimal due to the presence of catalase which instantly removes any hydrogen peroxide formed.

One of the best known and earliest successful applications of glucose oxidase/catalase in food is desugarization of eggs prior to spray drying (Baldwin et al., 1953). This enzymatic treatment catalyses the oxidation of the glucose naturally present in eggs thereby minimizing non-enzymatic browning by the Maillard reaction in spray dried

products. Glucose oxidase has also been used to prevent rancidity problems in mayonnaise and inhibit phenolase activity in fruit juices. More recently the use of glucose oxidase/catalase has been receiving attention as a preservative system for fresh fish and shrimp. Treatment of fish with this commercial enzyme system doubled the shelf life of fresh fish compared to untreated fish, at a cost of only a few cents per pound (Field et al., 1986). The fish (whole or fillet, winter flounder) were treated with glucose oxidase/catalase as a dipping solution, packaged in GOX/glucose ice, or algin blankets layered between the fish. Fish treated with a glucose/1 unit GOX dipping solution were organoleptically acceptable after 21 days of refrigerated storage compared to control samples which were spoiled within 7 - 10 days. Judokusumo (1985) also reported a shelf life extension of 28-33% for shrimp by dipping in a solution of 2 units/ml GOX/CAT and 4% glucose.

GOX/glucose dipping solution has the following advantages as an oxygen scavenger/chelating agent:

(a) It is safe. It is fully approved for food use, i.e. it is GRAS additive.
(b) It will not affect the organoleptic quality of fish or shell fish, since the gluconic acid is odorless and tasteless.
(c) It is easy to use. Its application can be accomplished by means of a dip, by storage in specially prepared ice, or by immobilizing the enzyme in algin blankets which are used to cover foods.
(d) It reduces drip loss during storage resulting in better weight retention.
(e) It extends the shelf life of product and reduces the generation of ammonia, which is associated with the putrefactive spoilage of seafood.

Whereas various dipping solutions have been suggested as surface acidifying agents, glucose oxidase/catalase/glucose solutions would appear to fulfil the need for a safe, "natural", cost effective, enzymatic oxygen scavenging dipping system for seafood.

3.6 Modified Atmosphere Packaging

Storage or packaging of product under modified atmospheres (MA) is one technique which can be employed to answer the need for improved product shelf life of fish. A modified atmosphere may be defined as packaging or storage of a perishable product in an atmosphere other than that of air. The normal composition of air is approximately 20% oxygen (O_2), 79% nitrogen (N_2), and 1% carbon dioxide (CO_2). A modified atmosphere, as the name implies, is one in which the normal composition of air is changed or "modified". Whereas some modified atmospheres are employed simply to inhibit oxidation, others are bactericidal or bacteriostatic. Two approaches to this technique are Controlled Atmospheres (CA) and Modified Atmospheres (MA). A controlled atmosphere involves maintaining a precisely defined atmosphere within a sealed storage chamber. This control is achieved by means of system which continuously compensates for atmospheric changes caused by product or microbial respiration or container permeability. Controlled atmosphere storage (CAS) is generally applied for bulk storage of perishable products, specifically fruits and vegetable.

A modified atmosphere (MA) applies to food packaged in small convenient retail units in which the gaseous atmosphere in the packaged products changes continuously throughout the storage period. Thus, whereas both CA and MA methodologies mean that the gaseous atmosphere around the product differs from air, CA has more precise gas compositional control than MA (Kadar, 1980).

Despite the increasing commercial interest in the use of MAP to extend the shelf life of fishery products, the concern about the potential growth of *Clostridium botulinum* in fish stored under MA remains the limiting factor for commercial application of the method. This concern is justified in that (a) non proteolytic *Clostridium botulinum* types B, E and F, all of which occur in aquatic environments (Eyles and Warth, 1981), are capable of growth and toxin production at temperatures as low as 3.3°C

(Eklund et al., 1967a,b), **(b)** *Clostridium botulinum* is a highly prevalent natural contaminant of seafood, and **(c)** the restricted growth of normal aerobic spoilage bacteria of fish flesh by MA may enhance growth and toxin production by *C. Botulinum* before spoilage is detected by the processor or the consumer (Eyles and Warth, 1981). Enfors and Molin (1978) have shown that an atmosphere containing lower CO_2 concentration can stimulate germination and growth of *Clostridium botulinum*. However, when the partial pressure of CO_2 is increased, this gas may not only inhibit germination, but can also delay toxigenesis and promote the death of *Clostridium botulinum* (Doyle, 1983).

3.6.1 Methods of atmosphere modification within a packaged product

Several methods can be used to modify the atmosphere within a package. These include **(i)** Vacuum packaging, **(ii)** Oxygen absorbents, and **(iii)** Gas packaging.

3.6.1.1 Vacuum packaging

Vacuum packaging may be defined as the packaging of a product in a high-barrier package from which air is removed to prevent growth of aerobic spoilage organisms, shrinkage, oxidation, and color deterioration. Vacuum packaging is actually a form of MAP because the removal of air is in itself a modification of the atmosphere. Vacuum packaging is often also regarded as MAP in the sense that elevated levels (10-20%) of carbon dioxide are produced within vacuum packages by microorganisms in the food as they consume residual oxygen (Silliker and Wolfe, 1980), or by respiring. This carbon dioxide can inhibit the growth of undesirable microorganisms. Vacuum packaging is used extensively by the food industry to extend shelf life and preserve the quality of fresh meat and fish. The product is placed in a film of low oxygen permeability, air is evacuated and the package sealed. The shelf life of vacuum packaged meat and fish depends on a number of interrelated factors,

specifically the microbiological quality of product at time of packaging, the pH of meat and fish at time of packaging, packaging film permeability, package integrity and storage temperature. The main disadvantage of vacuum packaging of meat from a commercial viewpoint is that the depletion of oxygen, coupled with the low oxygen permeability of the packaging film, results in a change of meat color from red to brown. Since consumers associate color with freshness, vacuum packaged meat is not normally sold at the retail level. However, this is not a problem with fish due to the lower myoglobin content of fish. The major concern of vacuum packaging of fish is the growth of *Clostridium botulinum* type E in both fresh and smoked products. As a result of this concern, vacuum packaged smoked salmon is only sold in a frozen form due to the ability of this pathogen to grow at 4°C.

3.6.1.2 Oxygen absorbents

A novel method of atmosphere modification within a package is the incorporation of oxygen absorbents to completely remove all traces of residual oxygen in the package headspace. One such product, trade name Ageless, is widely used in Japan. The active component of Ageless is iron oxide which becomes iron hydroxide after absorption of oxygen. It is packed in a small sachet, like a desiccant, and is placed in the package alongside the food. Oxygen absorbents come in a variety of sizes that can absorb 20-200cc of oxygen. As it involves a chemical reaction, and not the physical displacement of oxygen as in gas packaging, it completely removes oxygen from the package headspace throughout storage as long as package integrity is maintained. When used in conjunction with a film of low oxygen permeability, headspace oxygen is reduced to less than 0.05% within hours in the packaged product and remains at this level for the duration of the storage period. Oxygen absorbents have been widely used in Japan to extend the mold-free shelf life of bread, pizza crusts and cakes as well as prevent oxidation of fats in potato chips, dried fish, beef jerky, semi-moist cookies and chocolates. It was shown that oxygen absorbents were three times more effective than gas packaging for increasing the mold-free shelf life of crusty rolls

(Smith et al., 1986).

3.6.1.3 Gas packaging

Gas packaging is simply an extension of vacuum packaging technology. The gas packaging technique involves packaging of product in an impermeable film, evacuation of the air followed by the injection of appropriate gas mixtures and heat sealing of package. Nitrogen, carbon dioxide and oxygen are the three gases commonly used in gas packaging.

Nitrogen is an inert gas, it replaces oxygen and prevents oxidation. It has low solubility in water and fats and prevents the package from collapsing as a result of CO_2 absorbtion in the aqueous phase of the food product. Nitrogen has a negligible effect on bacterial growth and on the shelf life of muscle foods (Coyne, 1932).

Oxygen is generally avoided in gas packaging mixtures unless it is used to fulfil one of the three functions. First, it might be used with gas packaging of red meats in order to retain color or "bloom". Second, it is used in low concentrations in packaging of products which respire, such as fruits and vegetables. Third, and perhaps the most important function, is that it may prevent anaerobic conditions and the growth of potentially harmful anaerobes, specifically *Clostridium botulinum*. High levels of oxygen have been used to extend the shelf life and keeping quality of food. Savell et al.,(1981) used levels of 75% oxygen to successfully extend the shelf life of beef. However, Newton et al.,(1977) reported contrary findings in their studies with beef. Bacterial growth rates were increased and the storage life of lamb was considerably shorter in 80% oxygen, than in low O_2 or O_2 free atmospheres.

Carbon dioxide is the most crucial gas used in gas packaging. Carbon dioxide is both bacteriostatic and fungistatic and it can also kill insects. It is highly soluble in fats and water, where it forms carbonic acid. Its high solubility may lead to the collapse of the

package, whereas the carbonic acid may alter the pH and organoleptic qualities of the product. Examples of some food products currently gas packaged, as well as the composition of gas mixtures used to extend the shelf life of each product, are shown in the following table (Table 6). The optimum blend of gases for a specific product cannot simply be determined by trial and error but only through a detailed, systematic study of the variables influencing product shelf life. Although microbial quality is usually the primary concern the processor, there are many chemical changes which affect color, freshness, flavour and texture (Daniels et al., 1985).

Table 6. Examples of gas mixtures for selected food products

Product	Temp. °C	% O_2	% CO_2	% N_2
Fresh meat	0-2	70	20	10
Cured meat	1-3	0	50	50
Poultry	0-2	60-80	20-40	0
Wet fish				
Fatty	0-2	0	60	40
White	0-2	30	40	30
Cheese	1-3	0	60	40
Baked products	R.T.	0	60	40
Pizza	R.T.	0	60	40
Dry snacks	R.T.	0	20-30	70-80

R.T.: Room temperature (Smith et al., 1988)

For example, with meat, O_2 is necessary for the bright red color or "bloom" which is associated with good quality meat. However, O_2 also promotes microbial growth. Carbon dioxide is a bacteriostatic agent, i.e. it inhibits microbial growth but it will discolor fresh meat. The problem of balancing these two separate effects can be

overcome by using a gas mixture incorporating CO_2, O_2 and N_2. When fresh beef and pork are packed in an atmosphere of 70% O_2, 20% CO_2 and 10% N_2 and kept under refrigerated storage conditions, a shelf life of 10-12 days can be expected providing the meat was of good microbial condition at the time of packaging (Adams and Huffman, 1972). For cured meat products, where O_2 is not necessary, and even detrimental to product color, it is necessary to package in an O_2 free mixture of CO_2:N_2, or 100% of either gas. With fish, autoxidative changes lead to the formation of low molecular weight aldehydes, ketones, alcohols and carboxylic acids. Here, the gas mix employed depends on fat content, which varies from 1% to a maximum of 20% with mackerel. Fish low in fat can be packaged in 60% CO_2:40% O_2 whereas high fat fish, such as mackerel and herring, need to be packaged in an oxygen free environment to prevent rancidity problems (Banks et al., 1980). For meat, fish and poultry products, the main areas affected by packaging of product under a modified gas atmosphere are summarised in Table 7.

Table 7. Changes in meat, fish and poultry as by MAP

Enzymatic aging processes :	Unaffected
Microbial spoilage :	Increased CO_2 reduces growth of aerobic spoilage psychrotrophs by penetration memberance and lowering intracellular pH
Fat oxidation :	Reduced O_2 reduces oxidation of fats, although oxidation can still occur at low O_2 tensions
Oxidation of myoglobin :	Increased CO_2 promotes metmyoglobin formation and color darkening

(Smith et al., 1988)

For most muscle foods, gas packaging is only effective when used in conjunction with refrigerated storage. However, gas packaging has been successfully applied to extend the mold free shelf life of baked products stored at ambient temperature (Smith et al., 1983). Using a CO_2:N_2 (60:40) gas mix, these authors reported a mold free shelf life of baked products for upwards of 1-3 months at room temperature. Presently, more than 200 bakery firms in Europe use gas packaging technology to extend the mold free shelf life and keeping quality of rolls, cakes, pizza, baguettes and sliced bread. In addition to products shown in the table, gas packaging is now being used for shelf life extension of fresh pasta, cheese, rice, peanuts and pecans, prepared salads, sandwiches and gourmet foods. The main benefits associated with gas packaging are extended product shelf life and associated increases in market area, improved product presentation and a reduction in freezer storage costs (Table 8).

Table 8. Advantages of gas packaging of food

Increased shelf life
Increased market area
Reduction in production and storage costs
Reduction in use of inhibitors
Improved presentation
Fresh appearance
Clear view of product
Easy separation of slices

(Smith et al., 1988)

Some of the disadvantages of the technique (Table 9) include initial cost of packaging equipment, secondary fermentation problems and the potential growth of microorganisms of public health concern. Whereas this latter topic has been the subject of several investigations, there is little conclusive evidence that gas packaging

represents a significantly greater hazard than packaging in air, particularly under conditions of mild temperature abuse i.e. 5-20°C where CO_2 is less effective (Hintlian and Hotchkiss, 1986).

Table 9. Disadvantages of gas packaging of food

Initial high cost of packaging equipment, film, etc.
Discoloration of meat pigments
Leakage
Fermentation and swelling
Potential growth of organisms of public health significance

(Smith et al., 1988)

3.6.2 Mechanism of bacterial action of CO_2

Although the preservative action of CO_2 in foodstuffs has been known for many years, its mechanism of antibacterial action has not been fully determined. However, several theories have been postulated. One theory is that the displacement of O_2 was the main reason for the antimicrobial properties of CO_2. However, this theory was refuted by Coyne (1932), who showed that aerobic spoilage organisms of fish grew well in 100% N_2 but not in 100% CO_2, indicating that displacement of O_2 was not the only reason for the antimicrobial effect of CO_2. Valley and Rettger, (1927) suggested that CO_2 acted by lowering substrate pH. However, Coyne (1932), demonstrated that pH alone does not account for the inhibitory effect of CO_2 in studies in buffered and unbuffered media. Two recent hypotheses have been postulated for the inhibitory effect of CO_2. The first considers the effect of CO_2 on specific enzymes necessary for cell metabolism. King and Nagel (1967) demonstrated that a 50% CO_2 atmosphere had a mass action effect on certain decarboxylation reactions. They found *Pseudomonas aerugenesis* to have decreased levels of isocitrate dehydrogenases and

malate dehydrogenases. The second theory suggests that CO_2 acts on the cell membrane, affecting its permeability characteristic and its external environment by redistributing lipids at the surface. This has been demonstrated using a model system by Sears and Eisenberg (1961), and has been proposed as the mechanism by which CO_2 inhibits spore germination (Enfors and Molin, 1980). In conclusion, whereas there have been many studies on the effect of CO_2 on microorganisms, there is little conclusive evidence of its mechanism of action. The following appears to be the salient points of all previous investigations:

1. Carbon dioxide lowers the *intra-* and *extra*cellular pH of tissues and possibly that of microorganisms.
2. The carbon dioxide/bicarbonate rate has an observed effect on permeability of cell membranes.
3. Carbon dioxide may affect the membrane potential of organisms.
4. Carbon dioxide may have a direct influence on the equilibrium of decarboxylation enzymes of microorganisms.

3.6.3 Physical and chemical effects of CO_2 on fish muscle

3.6.3.1 pH

In general, the dissociation of carbonic acid in fish flesh results in a slight drop in pH. Both the buffering capacity of the fish proteins and the composition of the spoilage flora determine the magnitude of pH change (Cutting, 1953). However, Barnett et al., (1978) found no significant change in the pH of salmon flesh stored in 90% CO_2. The extent to which pH decreases is proportional to the concentration of CO_2 in the atmosphere (Lannelongue et al., 1982). Studies have shown that CO_2 is absorbed rapidly and pH drops over the first two days of storage. Parkin et al., (1981) noted a drop on pH of rock fish from pH 6.7 to pH 6.3 which was maintained throughout the storage period. However, Fey and Regenstein (1982), found that after an initial decrease, fish pH increased and after 27 days storage in a CO_2 enriched environment

had reached a level similar to its initial pH.

3.6.3.2 Color

One of the major problems with using elevated levels of CO_2 for storage of red meat, is that discoloration occurs partly due to the formation of metmyoglobin. In general, fish muscle contains lower levels of myoglobin and discoloration is a less significant problem. Color changes in fish include greying of the cornea, bleaching of the skin and damage to bloom at high CO_2 concentrations (>60%) (Stansby and Griffiths, 1935). Goodfellow (1982), found it necessary to restrict CO_2 levels to 25% or less to prevent discoloration problems.

3.6.3.3 Drip loss

A further problem caused by elevated CO_2 levels and associated pH change, is a reduction in water binding capacity and increase in drip loss of fish. Fey and Regenstein (1982), found increased drip losses for red hake, chinook salmon and to a lesser extent, sockeye salmon stored in 60% CO_2, 21% O_2, 19% N_2 compared to air packaged products. Tiffiney and Mills, (1982) found that packaging in 100% CO_2 increased the rate and quantity of drip loss. In all instances the quantity of drip loss of fish stored in high CO_2 concentrations was higher at O°C than 5°C.

3.6.3.4 Organoleptic changes

Organoleptic changes may also be induced by storage of fish in high CO_2 atmospheres. These include acidic-type odours noticed when packs are first opened, but which dissipate with time (Stier et al., 1981). Textural changes, such as powdery, dry and tough, and grainy flesh (Wang and Brown, 1983) have also been reported. Flavor may also be affected. Jensen et al., (1980) noted an acidic taste in cod packed in high CO_2 or in vacuum, whereas Haard and Lee (1982) found salmon steaks to

have a carbonated, bland taste after storage in 100% CO_2.

3.6.3.5 Pack collapse

As previously discussed, CO_2 is highly soluble in water and readily dissolves in fish tissues. This solubility can lead to collapse of rigid retail packs if a high CO_2 level has been used (Jensen et al., 1980). This problem can be prevented by reducing the proportion of CO_2 in the atmosphere and using N_2. When using flexible barrier films, the collapse of the pack is unacceptable as it gives the appearance of a vacuum pack.

3.6.4 Packaging film permeability

Modified atmospheres packaging can only be successful if used in conjunction with packaging materials of correct O_2/CO_2 permeability characteristics. It is no use having the correct atmosphere if the film allows the atmosphere to change too rapidly. The properties required for a suitable packaging film are seldom formed in one polymer, therefore individual polymers are laminated to produce films of superior barrier properties. Examples of polymers used in construction of barrier films are:

(a) Polyethylene
(b) Polypropylene
(c) Polystyrene
(d) Polyvinylidene chloride (PVDC)
(e) Ethylene vinyl acetate (EVA)
(f) Ethylene vinyl alcohol (EVOH)
(g) Metallyed polyesters

Examples of high, medium and low barrier film are shown in Table 10.

Table 10. Oxygen permeation rates for packaging films* expressed in different units

Film	cc /100 in^2 day ATM	cc / m^2 day mmHg	cc / m^2 day ATM
PP/EVOH/PP	<0.001	<0.00001	0.01-0.02
Foil laminate [mylar/AL/poly]	<0.01	<0.00001	<0.01-0.1
PVDC	1	0.02	15
Acrylonitrile polymer	1	0.02	15
Brickpak flat	2-3	0.04-0.05	30-40
PET (polyester)	4-6	0.08-0.13	60-100
PVC	10	0.2	150
Brickpat (folded and scored)	100	2	1500
HDPE	130	2.6	1980
PP	150	3.0	2280
LDPE	400-500	8-10	6000-7000

* For 1 mil flat film at 30°C and 50% RH.

3.6.5 Storage temperature

Modified atmosphere packaging (MAP) is not a substitute for proper storage temperature. The effectiveness of MAP decreases as the storage temperature increases due to the fact the solubility of CO_2 also decreases at higher temperatures. For respiring products, increasing the temperature also increases the rate of respiration, resulting in a decease in shelf life. The effects of temperature abuse are

particulary important from the standpoint of safety. Temperature abuse of MAP muscle food may result in the rapid growth of both spoilage and pathogenic bacteria. The minimum reported temperature for the growth of *Salmonella* and *Escherichia coli* inoculated in ground meat was 12.5°C when the meat was packaged in low and high permeability film (Goepfert and Kim, 1975). *Styphylococus aureus* can grow and produce enterotoxin under anaerobic conditions at 10°C or less. The minimum recorded temperature for enterotoxin production under anaerobic conditions is 10°C Whereas *Yersinia enterocolitica* has been reported to grow at temperatures as low as -2°C. Of major concern with respect to safety of MAP fish, is the growth of and toxin production by *Clostridium botulinum* type E, which is capable of growth at 3.3°C (Palumbo, 1986). Proper refrigeration is therefore essential in order to assure the effectiveness of CO_2 as an antimicrobial agent and to prevent potential growth of pathogenic organisms.

3.6.6 Levels of microbial contamination

The shelf life extension of fish under modified atmosphere is dependent on the initial microbial load and the types of organisms. Higher initial counts will accelerate spoilage and the shelf life of fish products. Furthermore, high numbers of aerobic bacteria will also consume rapidly headspace O_2 and may change the electronegative potential of product and enhance the growth of anaerobic organisms such as *Clostridium botulinum* type E. Decadt and Debevere (1990), reported an improvement in the shelf-life of North Sea Shrimps (*Crangon crangon*) by using a combination of lysozyme with modified atmosphere packaging (35% CO_2, 65% N_2 stored at 6°C). Decadt and Debevere recommended this treatment as a highly effective method for improvement of shelf-life of fresh shrimps.

Although there are concerns about the growth and toxin production of *Clostridium botulinum*, type E, in MAP fish, this hazard may be eliminated by the maintenance

of low temperatures ($\sim 0°C$), the presence of some O_2, and the use of additional agents such as potassium sorbate.

3.7 Irradiation

The major disadvantage of chemical dipping solutions is that they can only be used to reduce the microbial load of fish prior to packaging. Possibly the only method which could be used to extend the shelf life of MAP packaged food after packaging is irradiation.

Research on preservation of foods by ionizing radiation dates back to the 1940's, the process has not yet been approved by the FDA as a food preservation method. In 1977 the joint FAO/IAEA/WHO/Expert committee on wholesomeness of irradiated food gave provisional acceptance for irradiation of cod and redfish (Ocean perch) up to a maximum dose of 220 Krad, and in the fall of 1980 that committee recommended unconditional acceptance of irradiated foods up to a dose of one million rads (10 KGy) (WHO, 1981). More recently an internal task force created by the FDA to provide recommendations for irradiation preservation based on recent findings and current knowledge in toxicology and radiation chemistry, recommended that food irradiated at doses up to, and including a maximum of 100 Krad (1KGy), will be deemed wholesome and safe for human consumption (FDA, 1981).

The shelf life extension of irradiated fish, like MAP fish, is a function of many variables including dose level, pre-irradiation quality, numbers and types of bacteria present, product environment and storage temperature. Some shelf life extensions for various fish species after treatments with different dose levels under varying conditions are given in a recent comprehensive review on preservation of seafood with ionizing radiation (Nickerson et al., 1983). Miyauchi (1970) reported that the shelf life of cod fillets could be extended by treating with 100 Krad of irradiation whereas Hannesson and Dagbjartsson (1970) recommended an irradiation dose of

200 Krad for maximum shelf life extension of cod. The maximum acceptable dose level for cod has been reported to range from 150-300 Krad (Rhodes, 1964). Vacuum packaging has been used in conjunction with irradiation to inhibit oxidative rancidity problems. Irradiation and CO_2 atmosphere storage has also been used by Graikoski et al., (1968) to extend the shelf life of yellow perch fillets. Irradiation of cod fillets with 100 Krad under various packaging conditions extended the period of acceptability, but not as much as when stored under MAP condition. Combinations of sorbate-irradiation treatments have been shown to give long extension to fish shelf life (Licciardello et al.,1984).

Spoilage of seafoods at temperatures above freezing is mainly due to bacterial action particulary *Pseudomonas* species. Fortunately, of the various species normally found in fish from temperate waters, *Pseudomonas* species are the most radiosensitive and their numbers are reduced to a very low level after treatment with low doses of ionizing radiation (Thronley, 1963). However, a major concern about irradiation is the growth of *C. botulinum*, particularly under temperature abuse storage conditions. Since an irradiation dose of 100 Krad would not have a significant effect on the reduction of *Clostridium botulinum* type E spores in fish fillets, it is presumed that the botulism risk, particularly in CO_2-irradiated cod fillets would be greater or similar to that in non-irradiated CO_2-packed fish. Reduction of other sensitive spoilage microorganisms are also of concern to regulatory authorities since a reduction in the number of spoilage indicator organism also reduces the safety of product (Maxcy and Tiwari, 1973). Public acceptance of irradiation has been hindered by fear and controversy, which involves the erroneous definition of radiation as a food additive and the widespread reputing of some ill-conceived and ill-interpreted research. In general, all research has indicated radiation to be a safe and effective method of reducing microbial load and extending the shelf life of food.

4. RESEARCH OBJECTIVES

Of the various physical, chemical and thermal processing methods available for shelf life extension of fresh shrimp, refrigeration is by far the most important method of short term preservation for the fish industry. However, it has been shown that even under refrigerated storage conditions (0-3°C), shrimp continues to spoil due to the activities of aerobic, psychrotrophic strains of bacteria, predominantly *Pseudomonas* spcies. However, it is possible to modify the storage conditions and thus the environment to which spoilage bacteria are exposed at refrigerated temperatures. With regard to further spoilage control, it is widely accepted that two or more simultaneous suboptimal environmental conditions will be far more inhibitory than each component considered separately. The combined use of several preservation methods can be explained by the "hurdle concept" (Leistner and Rodel, 1976). This states that several hurdles (barriers) or "inhibitory factors", even if one of them individually cannot inhibit microorganisms, will nevertheless reduce or inhibit microbial growth if the hurdles are incorporated into a substrate in sufficient number and height. With respect to fresh shrimp, there are several methods of spoilage control which employ one or more barriers in conjunction with refrigerated temperatures to extend the shelf life, thereby preserving the quality of fish. They include Modified Atmosphere Packaging (MAP) and enzymatic dipping solutions (Glucose oxidase/glucose). MAP has been used for the shelf life extension of beef, pork, fruits, vegetables, and bakery products. GOX, an oxygen scavenging enzyme, generates gluconic acid from glucose which lowers the pH, thus inhibiting the growth of spoilage microorganisms and activity of enzymes. However, since the combined use of MAP and glucose oxidase dipping solutions has not been investigated as a means of controlling melanosis and extending the shelf life of shrimp, this thesis addresses the potential for extended shelf life of shrimp through the combined use of these "hurdles" or "barriers", The specific objectives of this research are:

(i) To monitor the combined effect of GOX/glucose dipping solutions and MAP to

extend the shelf life of and control melanosis on shrimp;

(ii) To determine the physical/chemical/microbiological changes in the GOX/MAP treated shrimp;

(iii) To determine if there are any significant statistical differences among sample treatments and, based on this statistical analysis, to recommend the best dipping/packaging treatment to control melanosis and extend shelf life of shrimp.

SECTION II: MATERIALS AND METHODS

1. Preliminary studies with glucose/glucose oxidase (GOX)

1.1 Preparation of gluconic acid standard curve

A 1% (w/v) stock solution of gluconic acid was made by dissolving 1 gram of gluconic acid in 100 ml double distilled water. Working solutions were made by diluting the 1% stock solution with double distilled water to give gluconic acid solutions of 500, 1000, 1500, 2000, 2500 and 3000 ug/ml. Approximately 3 ml of each working gluconic acid solution was transferred to a U.V. glass cuvette and the O.D. measured spectrophotometrically at 340 nm using a Beckman DU-65 spectrophotometer (Beckman Ltd.). The gluconic acid standard curve is shown in Figure 7.

1.2 Preparation of 4% glucose/GOX (1 unit/ml) enzyme solution

A preliminary study was carried out to determine the concentration of gluconic acid produced from a 4% glucose/GOX(1 unit/ml) dipping solution. The 4% Glucose solution used in this study was prepared by dissolving 60 grams of α-D-Glucose in double distilled water in a 100 ml volumetric flask. The solution was covered and kept at room temperature for 24 hours to undergo mutarotation to the α and β glucose forms. The 4% (w/v) glucose working solution was prepared by pipetting 100 ml of 60% (w/v) glucose solution into a 1500 ml volumetric flask and making up to the mark with double distilled water.

Dried commercial glucose oxidase/catalase enzyme was obtained from Genencor International Inc (IL 60008, USA). Twenty seven ml of double distilled water was added to each bottle to give an enzyme stock solution containing 1500 units of enzyme/ml. One ml of this enzyme solution was transferred to 1499 ml 4% (w/v) glucose solution to give a 4% glucose/GOX (1 unit/ml) solution. 3 ml of this solution was transferred to a U.V. glass cuvette and changes in O.D., (Figure 8) were

measured at 340 nm at 1 minute intervals up to 2 hours at room temperature. The concentration of gluconic acid (% w/v) equivalent to the maximum O.D. was obtained from the previously prepared gluconic acid standard curve (Figure 7).

1.3 Effect of various concentrations of glucose with the concentration of GOX held constant (1 unit/ml) on gluconic acid production

A preliminary study was carried out to determine the effect of different concentrations of glucose solutions (0-10% w/v) with a standard concentration of GOX (1 unit/ml) on gluconic acid production. Standard glucose solutions were prepared from a 40% (w/v) stock solution and the appropriate amount of GOX added to give a final concentration of 1 unit/ml as shown in Table 11.

Table 11. Preparation of various concentration of glucose/GOX (1 unit/ml)

Glucose sol. (%)	40% Glucose (ml)	Dist.water (ml)	GOX (1500 u/ml) (ml)
0	0	149.9	0.1
1	3.75	146.15	0.1
2	7.5	142.4	0.1
3	11.25	138.65	0.1
4	15.0	134.9	0.1
5	18.75	131.15	0.1
6	22.5	127.4	0.1
7	26.25	123.65	0.1
8	30.0	119.9	0.1
9	33.75	116.15	0.1
10	37.5	112.4	0.1

A 3 ml amount of each working solution was measured spectrophotometrically at 340 nm at 10 minute intervals for 1 hour at room temperature (Figure 9).

1.4 Effect of various GOX concentrations (0-10 units/ml) with the concentration of glucose held constant (4% w/v) on gluconic acid production

To study the effect of different concentrations of GOX with 4% glucose on gluconic acid production, the following solutions were prepared from a 40% stock solution of glucose as shown in Table 12.

Table 12. Preparation of various concentrations of GOX with 4% glucose

unit of GOX (u/ml)	40% Glucose (ml)	Dist.water (ml)	GOX (1500 u/ml) (ml)
0	15	135.0	0
1	15	134.9	0.1
2	15	134.8	0.2
3	15	134.7	0.3
4	15	134.6	0.4
5	15	134.5	0.5
6	15	134.4	0.6
7	15	134.3	0.7
8	15	134.2	0.8
9	15	134.1	0.9
10	15	134.0	1.0

A 3 ml amount of each solution was measured spectrophotometrically at 340 nm at 10 minute intervals for 1 hour at room temperature (Figure 10).

1.5 Effect of pH on GOX activity

The effect of pH on enzyme activity was investigated at 5 pH levels - pH 2,4,6,8 and 10. The buffer solutions (pH 2-10) were prepared using the method as described by Christian and Purdv (1962). Buffered working solution were prepared by pipetting 20 ml of 40% of glucose stock solution into a 200 ml volumetric flask and making up to the mark with appropriate pH buffer solution. A 0.1 ml of GOX enzyme solution (1500 u/ml) was added to 149.9 ml of bufferred working solution to give a final enzyme concentration of 1 unit/ml as described previously. The pH of each working solution was checked using a previously calibrated pH meter. A 3 ml amount of each solution was transferred to a standard cuvette and changes in O.D. were measured at 340 nm at 10 minutes intervals for 1 hour at room temperature (Figure 11).

1.6 Production of gluconic acid from 4% β-D-glucose/GOX (1 unit/ml)

To compare the effect of gluconic acid production directly from 4% β-D-glucose and from 4% (w/v) glucose solution prepared from 40% (w/v) dextrose stock solution, a 4% β-D-glucose solution was prepared by dissolving 4 gram of β-D-glucose (BDH chemicals) in 100 ml double distilled water in 100 ml volumetric flask. A 0.1 ml amount of GOX enzyme solution (1500 u/ml) was added to 149.9 ml of 4% β-D-glucose solution as described previously. A 3 ml amount of this solution was measured spectrophotometrically at 340 nm at 20 minute intervals at room temperature over a 1 hour period (Figure 12).

2. Studies to control melanosis and extend shelf life of shrimp

2.1 Experimental design

Two factorial experimental designs were used in this study. In the first study, a 2x3 factorial design with 3 replicate experiments was used. Each replicate consisted of 2

dipping solutions (water, glucose oxidase/glucose) and 3 packaging treatments (air, CO_2:N_2/60:40 and vacuum packaging). The experimental treatments in the first design are summerized below:

Air/Distilled water (Control);
Air/Glucose (4% w/v)/GOX (1 unit/ml);
CO_2:N_2 (60:40)/Distilled water;
CO_2:N_2 (60:40)/Glucose (4% w/v)/GOX (1 unit/ml);
Vacuum Packaging/Distilled water;
Vacuum Packaging/Glucose (4% w/v)/GOX (1 unit/ml).

In the second study, a 4x2 factorial design with 3 replicate experiments was used. Each replicate consisted of 4 dipping solutions (water, GOX/glucose, ascorbic acid and a mixture of ascorbic acid and GOX/glucose solution) and 2 packaging treatments (air, CO_2:N_2/60:40). All replicated experiments were done in a randomized order. The experimental treatments in this design were:

Air/Distilled water (Control);
Air/Glucose (4% w/v)/GOX (1 unit/ml);
Air/Ascorbic acid (1% w/v);
Air/Glucose (4% w/v)/GOX (1 unit/ml)/Ascorbic acid (1% w/v);
CO_2:N_2 (60:40)/Distilled water;
CO_2:N_2 (60:40)/Glucose (4% w/v)/GOX (1 unit/ml);
CO_2:N_2 (60:40)/Ascorbic acid (1% w/v);
CO_2:N_2(60:40)/Glucose(4%w/v)/GOX(1 unit/ml)/Ascorbic acid(1% w/v).

All treatments were done in triplicate and in a randomized order.

2.2 Solutions and sample preparation

2.2.1 Shrimp samples

Due to the short shrimp fishing processing season, two types of shrimp were used in this study. In the first study, frozen white shrimp (*Pandalus occidentalis*) were used due to the unavailability of fresh shrimp at the onset of this research project. The shrimp were obtained frozen and deheaded from a commercial supplier (Club Price, Montreal). The shrimp samples were kept frozen at -80°C until required for the various dipping/packaging treatments. Prior to each treatment, the shrimp samples were defrosted by running tap water over the package for about 1 hour until completely thawed.

In the second study, which took place during the shrimp processing season, fresh pink shrimp (*Pandalus borealis*) were used. The shrimp were obtained within 24 hours of harvesting from Fruits des Mers, Matane, Quebec. The shrimp were transported in refrigerated trucks (0-2°C) and dipped/packaged immediately upon receipt in the laboratory. At each specified sampling day 2 bags from each replicated treatment were analysed for physical, chemical and microbiological changes during storage.

2.2.2 Preparation of dipping solutions

The four dipping solutions used in this study consisted of:

a. Double distilled water;

b. Glucose (4% w/v)/glucose oxidase/catalase(1 unit/ml) solution;

c. Ascorbic acid (1% w/v);

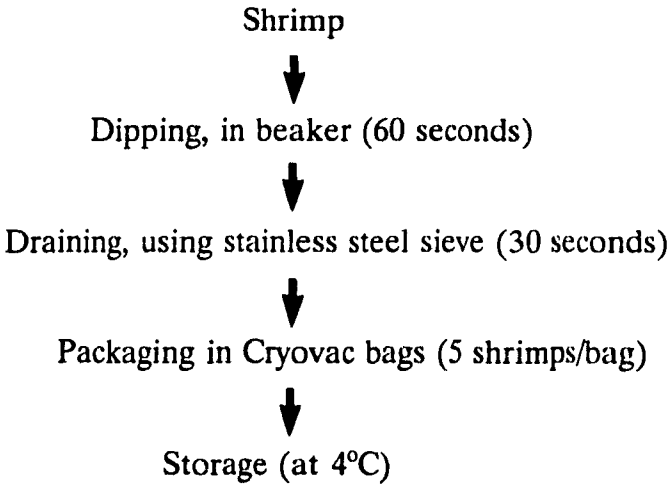
d. Ascorbic acid (1% w/v)/glucose (4% w/v)/glucose oxidase/catalase (1 unit/ml).

The double distilled water was obtained from our laboratory. The 4% (w/v) glucose solution was made by adding 15 ml of 40% glucose solution to 1485 ml of distilled water. To 1499 ml of this solution, 1 ml of GOX/CAT (1500 unit/ml) was added to give a final working solution of 4% glucose/GOX(1 unit/ml). The ascorbic acid dipping solution was made by dissolving 15 grams of ascorbic acid in 1500 ml distilled water. Solution (d) was made by adding 15 grams of ascorbic acid to 1500 ml of the

prepared GOX/glucose solution (solution b).

2.2.3 Sample preparation

Shrimps were dipped in the various dipping solutions, drained, packaged as outlined in the flow diagram below:



2.2.4 Packaging

The dipped and drained shrimps were packaged in 20 x 20 cm Cryovac bags (Cryovac Ltd, Quebec, Canada) i.e. 4/5 shrimp (approximately 50 grams) per bag. Cryovac bags were made of Polyethylene (PE)/Polyvinylidene (PVDC)/Polythene polymers. The average oxygen permeability of the packaging material used was <5 cc per m^2/day atm. at 25°C. 50% R.H.

Shrimp samples were packaged under 3 gaseous conditions (i) Air (ii) CO_2/N_2 (60:40) and (iii) Vacuum packaging. Packaging was done using a Multivac type A 300/42 packaging machine (Multivac Co. Company. Sepp Haggenmuller KG, W.Germany). Gas packaging was achieved using a Smith's proportional Gas mixer, model 299-028 (Tescom Corporation, Minneapolis, Minnesota 55441, USA). Gases (CO_2 and N_2)

were obtained from Medigas Ltd. (Medigas Ltd. Quebec, Canada).

3. Physical, chemical, microbiological and sensory analysis

In the first study, all packaged/dipped white shrimp were analyzed for (1) physical changes e.g. pH, changes in headspace gas composition; (2) chemical changes e.g. total volatile base (TVB), hypoxanthine (Hx); (3) microbiological changes e.g. aerobic plate count (APC), lactic acid bacteria (LAB) count and psychrotrophic plate count and (4) sensory changes e.g. black spot development/melanosis score, odor, overall acceptability after 0, 3, 7, 14 days at 4°C.

In the second packaging/dipping study, the pink shrimp were monitored for sensory changes e.g. black spot development/melanosis score, color/odor only, after 0, 3, 6, 9, 12, 15 days at 4°C.

3.1 Physical analysis

Physical analysis comprised of measurement of changes in headspace gas composition and pH throughout storage.

3.1.1 Head space gas analysis

Changes in head space gases (CO_2, O_2 and N_2) were analyzed using a Varian 3300 Chromatography fitted with a Thermal Conductivity Detector (TCD) (Varian Associates, Houston, Texas). A two column system (column A:122 cm x 0.32 cm Porapak Q, stainless steel 80/100; column B: 152 cm x 0.32 cm Molecular Sieve 5A, stainless steel 80/100) was used to obtain a complete chromatogram of all components in the gas mixture from a single injection. Helium was used as the carrier gas at a flow rate of 20 ml/min. The temperatures of initial column, injector and detector were 80°C, 100°C and 100°C respectively.

Samples were prepared for head space gas analysis by attaching a small quantity of silicon sealant to the outside of each package. This technique enabled small amounts of head space gas (0.5 ml) to be withdrawn at regular intervals using a Dynatech gas tight syringe (Dynatech Precision Sampling Corporation, Louisiana, USA.) without damaging the integrity of the packaging material. Air was used as the internal standard. Percentage by volume of gases was calculated from relative peak areas integrated with an Hewlett Packard 3390A integrator (Hewlett-Packard Company, Pennsylvania, USA).

3.1.2 pH

Shrimp pH was measured using a Corning 220 pH meter (Corning Ltd.) previously calibrated with pH 4 and 7 buffer solutions (Canlab, Inc. Ontario, Canada). The pH was measured by immersing the electrode directly into a 1:1 homogenate of shrimp. This homogenate prepared by homogenizing 10 gram of shrimp with 10 ml of distilled water in a stomacher bag and stomaching for 2 minutes in a Lab Blender 400 model stomacher (Seward Medical UAC House, London, England).

3.2 Chemical analysis

Chemical analysis performed on shrimp included total volatile base (TVB) test and hypoxanthine (Hx) test.

3.2.1 Total volatile base (TVB) test

The microbial activity and biochemical changes during iced or chilled storage of seafood are responsible for the decline of organoleptic quality, especially the build up of odorous compounds such as ammonia, monomethylamine, trimethylamine (TMA) and other volatile amines.

The TVB test involves distillation of amine compounds into boric acid solution and titration with a standardized acid (H_2SO_4 or HCl). Ten grams of shrimp were mixed with approximately 40 ml distilled water and then stomached for 2 - 4 minutes. The contents were then transferred to a 1000 ml round bottom distillation flask, 2 gram of magnesium oxide and a few anti-bumping granules were added and the mixture boiled for 25 minutes. The distillate was received in a 250 ml Erlenmeyer flask containing 25 ml of 2% boric acid and a few drops of methyl red bromocresol green as indicator. After distillation, the solution was titrated to its original color using standard 0.05 N H_2SO_4 solution. A blank was titrated as described previously.

The total volatile bases (TVB) were expressed as milligrams of nitrogen per 100 gram samples using the following equation:

$$TVB = \frac{(V4 - V5) \times N2 \times 100 \times 14}{W2}$$

Where:

V4 = volume (ml) H_2SO_4 used for sample

V5 = volume (ml) H_2SO_4 used for blank

N2 = normality of H_2SO_4

W2 = weight of sample in grams

3.2.2 Hypoxanthine (Hx) test

The hypoxanthine (Hx) test was used as an indicator of chemical spoilage of shrimp. This test is based on the action of xanthine oxidase on hypoxanthine in the presence of oxygen. Hypoxanthine is converted to xanthine and then to uric acid which is quantified spectrophotometrically at 290 nm.

A 50 g amount of shrimp was blended for 2 minutes with 200 ml of 6% perchloric acid ($HClO_4$) and the extract filtered through a fluted Whatman #1 filter paper. Prior to analysis, 10 ml of filtrate was neutralized to between pH 7.0 and 7.6 by carefully adding an equal volume (10 ml) of potassium hydroxide-phosphate buffer solution. Before the addition of extract, xanthine oxidase (20 units/ml) was diluted in a ratio of 0.5:25 with 0.05 moles phosphate buffer.

Hypoxanthine content, expressed as umoles/g sample was calculated from the formula:

$$Hx = \frac{H \times [\, V1 + (\, 0.01 \times M \times W \,)]}{V4 \times W} \times \frac{V2 + V3}{V3} \times \frac{1}{G}$$

Where,

H = ug Hx from standard curve (Figure 20)

M = moisture content of fish expressed in percent

V1= volume (ml) of perchloric acid used in 1:4 extraction

V2= volume (ml) of KOH/phosphate buffer used for neutralization

V3= volume (ml) of extract neutralized by KOH/phosphate buffer

V4= volume (ml) of sample extract added to test tube

W = weight (g) of sample used in 1:4 extraction

G = gram molecular weight hypoxanthine (Hx), i.e. 136.1

3.2.3 Preparation of hypoxanthine (Hx) standard curve

A hypoxanthine (Hx) standard curve was prepared by dissolving 5.0 mg hypoxanthine in 100 ml distilled water overnight at room temperature. The hypoxanthine standard curve was prepared as shown in Table 13.

Table 13. Preparation of hypoxanthine standard curve

tube no.	Hx Std. (ml)	H_2O (ml)	Buffer (ml)	Enzyme (ml)	Conc.Hx (ug)
1	0.2	2.3	2.0	0.5	10
2	0.4	2.1	2.0	0.5	20
3	0.6	1.9	2.0	0.5	30
4	0.8	1.7	2.0	0.5	40
5	1.0	1.5	2.0	0.5	50
6	0.2	2.8	2.0	0	10
7	0	2.5	2.0	0.5	-
8	0	3.0	2.0	0	-

All test tubes were incubated in a water bath at 37°C for 30 minutes, cooled and measured spectrophotometrically at 290 nm. Standard curve (Figure 20) was obtained statistically by GLM procedure of Statistical Analysis System (SAS) personal computer program.

3.3 Microbiological analysis

Microbiological analyses done on packaged/dipped shrimp were aerobic plate counts (APC), psychrotrophic counts and lactic acid bacteria counts (LAB).

3.3.1 Preparation of dilutions

The packaging film was cut aseptically by swabbing the outside of each package with 95% ethanol. An initial 10^{-1} dilution was prepared by adding 10 g of shrimp to 90 ml of 0.1% sterile peptone water (Difco) in a stomacher (Seward & CO., Stanford St.,

London) for 1 minute. A series of decimal dilutions (10^{-2} to 10^{-5}) were made, again using 0.1% peptone water as the diluent. All diluents prepared by dissolving 1 gram of peptone in 1 litre of distilled water and sterilizing by autoclaving for 15 minutes at 15 psi. (121°C).

3.3.2 Media

The following media were used for each microbiological test. Plate count agar (PCA) (Difco) was used for total aerobic plate count and psychrotrophic bacteria whereas Lactobacilli MRS agar (Difco) was used for lactic acid bacteria. All media were prepared from commercially produced dehydrated media according to the manufacturer's instructions and sterilized in an autoclave at 15 psi for 15 minutes.

3.3.3 Bacterial counts and incubation conditions

All microbiological counts were done using a pour plating technique. A 1.0 ml amount of the appropriate dilution was transferred to petri dishes and covered with molten media pre-cooled to 45°C. For total aerobic plate counts and lactic acid bacteria counts, all plates were incubated aerobically at 35°C for 48 h. For psychrotrophic plate counts, plates were incubated aerobically at 4°C for 10 days.

Plates containing 25-250 colonies/plate were enumerated using a Darkfield Quebec colony counter (Scientific Instruments, Quebec, Canada). Counts were expressed as CFU/gram of sample which was obtained by multiplying the plate count by the reciprocal of the appropriate dilution.

3.4 Sensory evaluation

At day 0 and at each subsequent sampling time, samples were subjectively evaluated by a 6 member untrained panel for odor and overall acceptability and melanosis

score. Odor and overall acceptability was evaluated using a 10 centimeter unstructured scale (0 cm = no off odors, highly acceptable; 10 cm = extreme off odor, highly unacceptable (Larmond, 1979).

For evaluation of melanosis, standard color charts served as a reference for black spot development. For white shrimp, this chart was developed in our laboratory. Shrimp were packaged in air (5 shrimps/bag) stored at 4°C and examined daily for black spot development (melanosis). Shrimp were photographed at the various stages of black spot development depending on the degree of melanosis. For pink shrimp, a standard melanosis chart, developed by McEvily et al., (1991) was used to evaluate the melanosis score for the various packaging/dipping treatments of pink shrimp. In both charts the following scales were used to evaluate melanosis:

0	**Absent (no black spot).**
2	**Slight, noticeable on some shrimp.**
4	**Slight, noticeable on most shrimp.**
6	**Moderate, noticeable on most shrimp.**
8	**Heavy, noticeable on most shrimp.**
10	**Heavy, totally unacceptable.**

For each attribute (melanosis score, odor/overall acceptability), a score of 6 was considered to be the upper limit of acceptability implying that the shelf life was terminated when this score was reached.

3.5 Statistical analysis and graphics

All statistical analysis (GLM, Duncan Multiple Comparison of Means) were done using the Statistical Analysis System (SAS) program on a personal computer. All graphics were done using the Harvard Graphic program.

SECTION III: RESULTS AND DISCUSSION

1. Preliminary studies with glucose/glucose oxidase (GOX) dipping solutions

Glucose/GOX dipping solutions have been used previously to extend the shelf-life and preserve the quality of fish. For example, Field et al., (1986) used a dipping solution of 4% glucose/GOX (1 unit/ml) and 0.125% (w/v) gluconic acid but gave no reasons for the choice of these concentrations. To determine the concentration of gluconic acid produced from various glucose/GOX concentrations, preliminary studies were conducted on glucose/GOX dipping solutions over a range of concentrations of glucose (0-10%) with GOX held constant at 1 unit/ml. Gluconic acid production was determined from the gluconic acid standard curve (Figure 7).

It is evident from Figure 7 that the absorbance by gluconic acid increased in a linear manner over the concentration range tested. Linear regression analyses of the data showed an R^2 value of 0.992, indicating the adequacy of the curve to determine the concentration of gluconic acid over the range tested.

The production of gluconic acid from a 4% glucose/GOX (1 unit/ml) solution over a period of two hours is shown in Figure 8. It is evident from Figure 8 that the concentration of gluconic acid produced from 4% glucose/GOX (1 unit/ml) also increased in a linear manner with time. The concentration of 0.125% (w/v) gluconic acid, used in previous studies by Field et al., (1986) could therefore readily be obtained from a 4% glucose/GOX (1 unit/ml) dipping solution after approximately one and half hours (Figure 8).

The production of gluconic acid from various concentrations of glucose, with GOX held constant at 1 unit/ml is shown in Figure 9. It is evident that this plot follows a simple Michaelis-Menten plot for an enzyme-substrate catalyzed reaction. The absorbance for a 4% glucose/GOX (1 unit/ml) was 1.1 at 340 nm. This was equivalent

to a concentration of 1,250 ug/ml of gluconic acid or 0.125% (w/v). At the maximum reaction rate (approximately 9-10% glucose), the O.D. at 350 nm was 1.5 which was equivalent to a concentration of 0.175% (w/v) gluconic acid (Figure 7). Therefore, whereas a high concentration of glucose would generate a high concentration of gluconic acid, a 10% glucose dipping solution to control melanosis on shrimp would not be practical for two reasons: **(i)** it would be more expensive and **(ii)** it may be impart a sweet taste to shrimp.

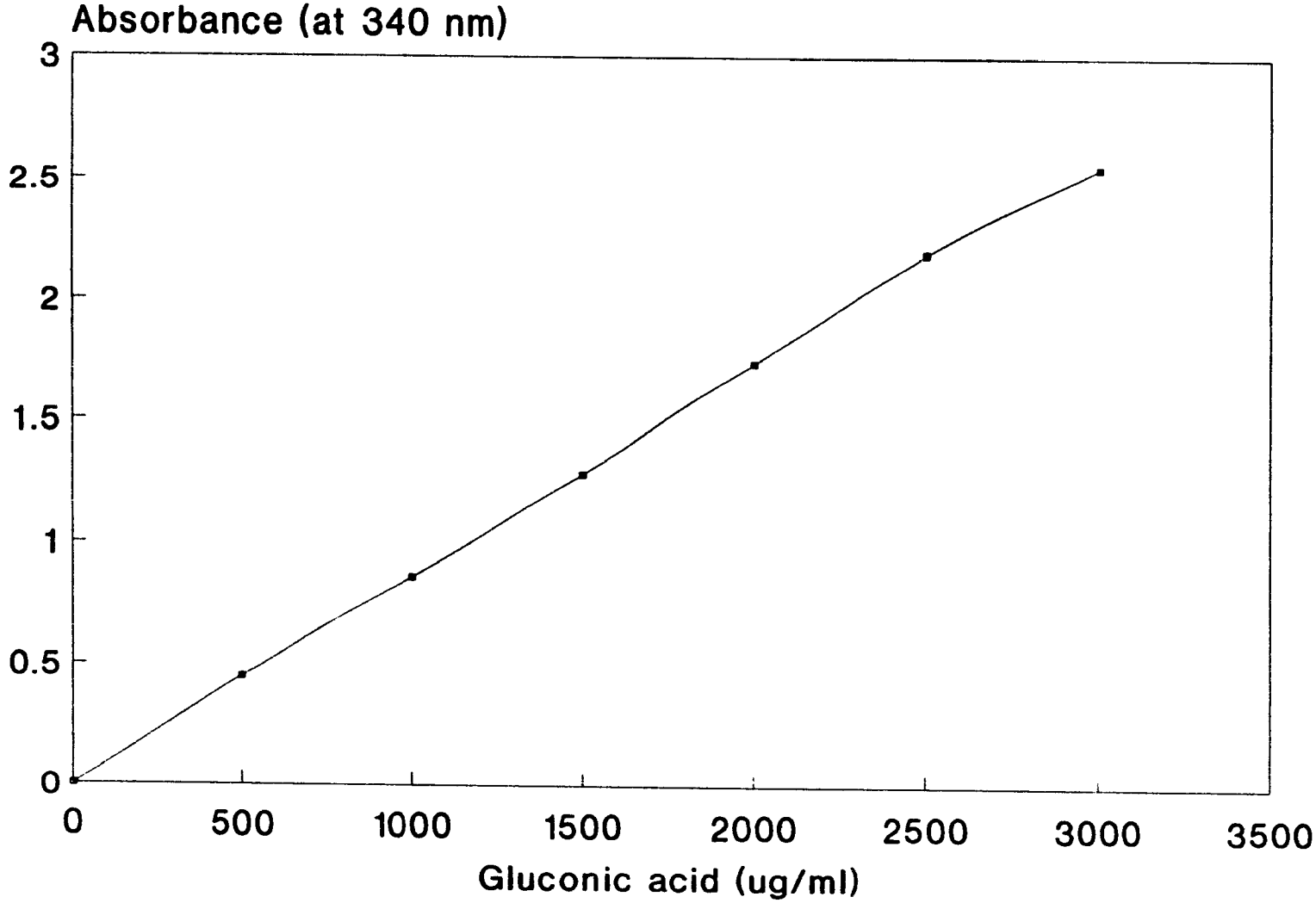
The effect of various concentrations of GOX (1-10 units/ml) with glucose held constant at 4% on gluconic acid production is shown in Figure 10. It is evident that this plot again follows a simple Michaelies-Menten plot with rate of reaction increasing with increasing enzyme concentration. It is evident from this plot that the maximum concentration of enzyme which is required with 4% glucose is 4 units/ml of GOX. This dipping solution i.e. 4% glucose /GOX (4 units/ml) would generate approximately 0.3% gluconic acid (Figure 7). As shown from Figure 10, a concentration of 1 unit/ml GOX with 4% glucose again gave an absorbance of approximately 1.1 at 340 nm which was equivalent to 0.125% (w/v) gluconic acid (Figure 7). Therefore, a high concentration of gluconic acid (0.3% w/v) and possibly a long extension of shelf life of fish could be obtained by increasing the concentration of GOX to 4 units/ml with 4% glucose solution. However, the cost of enzyme and possible flavor changes resulting from high concentration of gluconic acid produced may be two explanations for the limit of the concentration of GOX to 1 unit/ml with 4% glucose solution as used in previous studies (Field et al., 1986).

The effect of pH on the stability/activity of GOX is shown in Figure 11. It is evident that GOX is active over a fairly wide pH range (2-12) with optimum activity being observed at pH 4.0. The optimum activity/stability at this pH range explains why GOX is used as an oxygen scavenging system to inhibit polyphenoloxidase activity in fruit juices, which have pH values of pH 3-5. It is evident from Figure 11 that the enzyme still has approximately 70% activity at neutral-alkaline pH ranges and at

more acid pH range (pH 3).

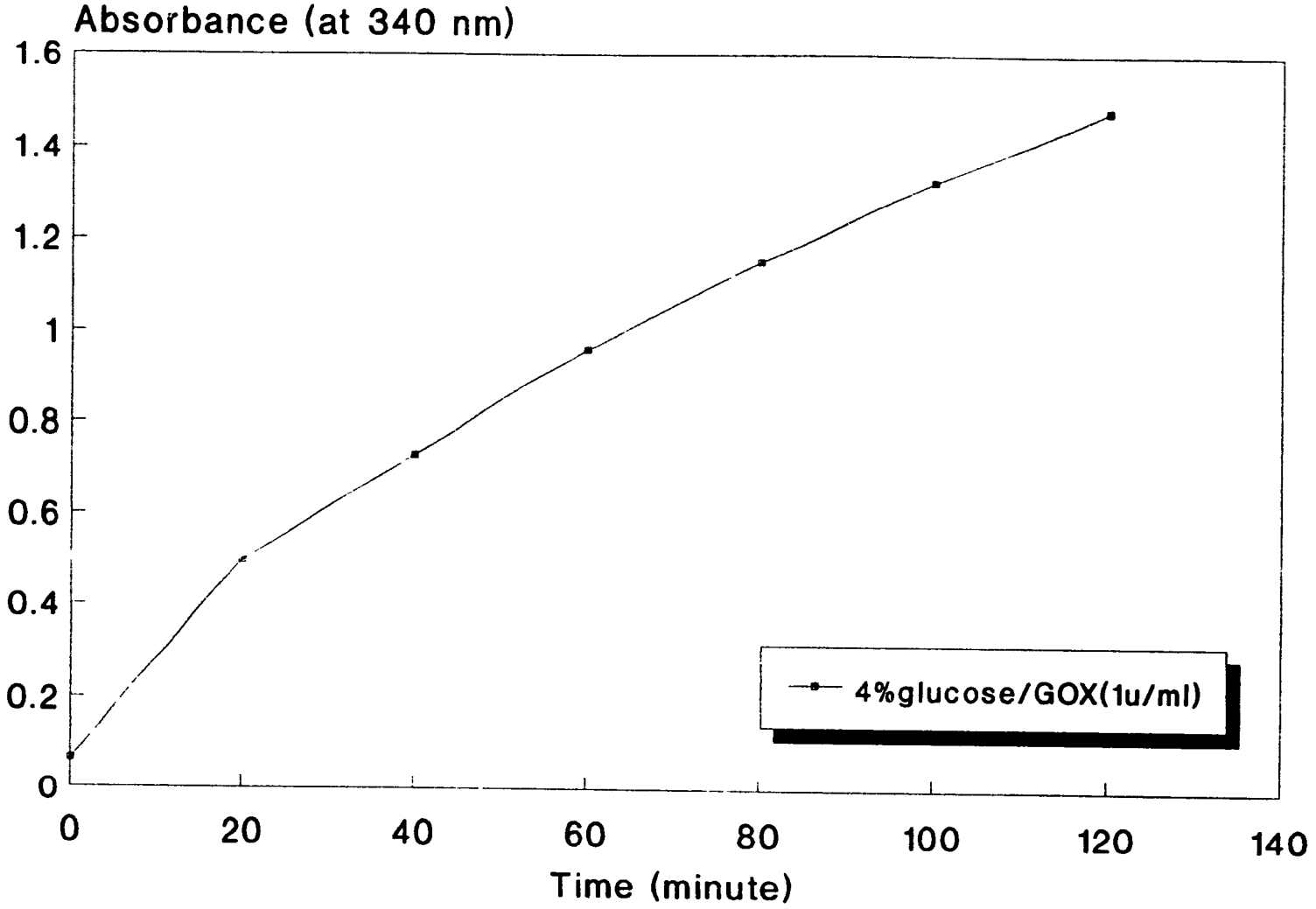
Glucose oxidase produces gluconic acid from β-D-glucose. In this study, β-D-glucose was formed from a 60% (w/v) solution of α-D-glucose which was left overnight to undergo muturotation to equal parts of α and β-D-glucose. A preliminary study was carried out to determine rate/amount of gluconic acid production from a 4% β-D-glucose and from a 4% α-D-glucose produced from a concentration glucose stock solution. It is evident from Figure 12, that there was no difference in the rate/amount of gluconic acid starting initially with a 4% β-D-glucose solution. However, since β-D-glucose is very expensive ($25/100g), it is commercially more viable to use a 4% solution of α-D-glucose produced from a concentrated stock solution in conjunction with GOX as the dipping solution. Based on these preliminary studies, a 4% glucose (w/v)/GOX (1 unit/ml) was used as the dipping solution throughout this study.

Figure7. Gluconic acid standard curve



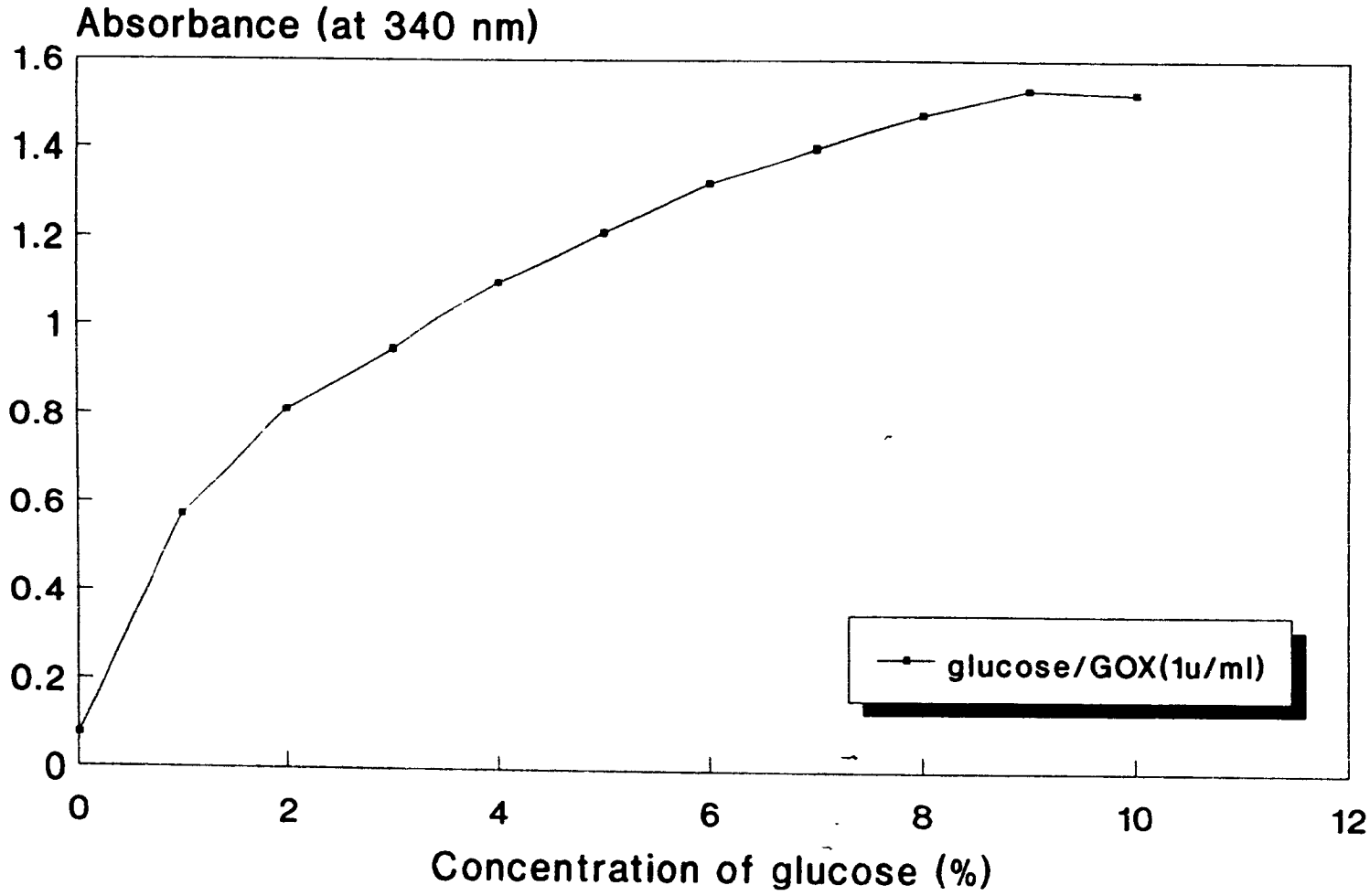
Data used to plot figure are average values of triplicate results for two experiments

Figure8. Gluconic acid from GOX/glucose



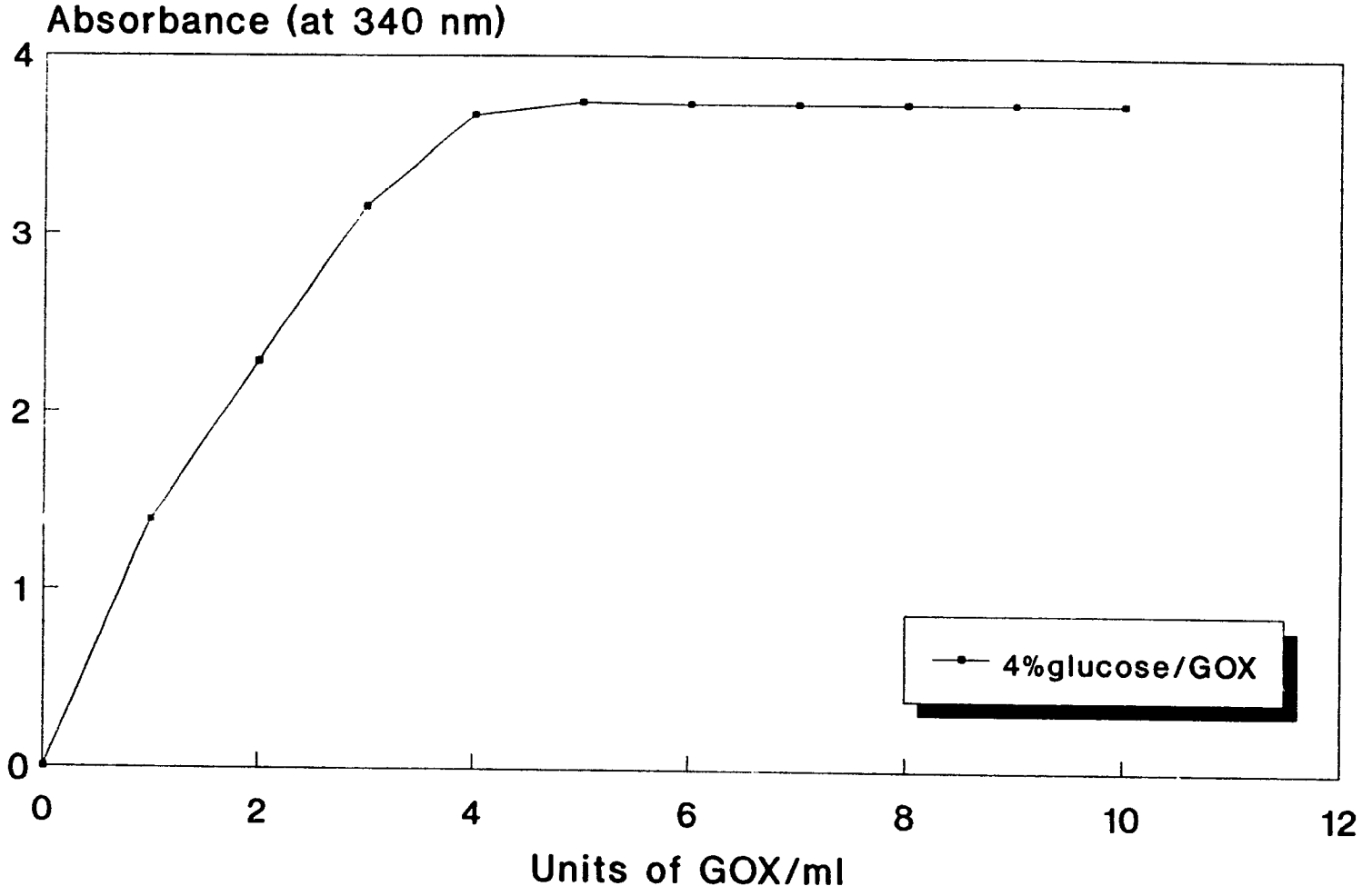
Data used to plot figure are average values of triplicate results for two experiments

Figure9. Gluconic acid from GOX and various concentrations of glucose



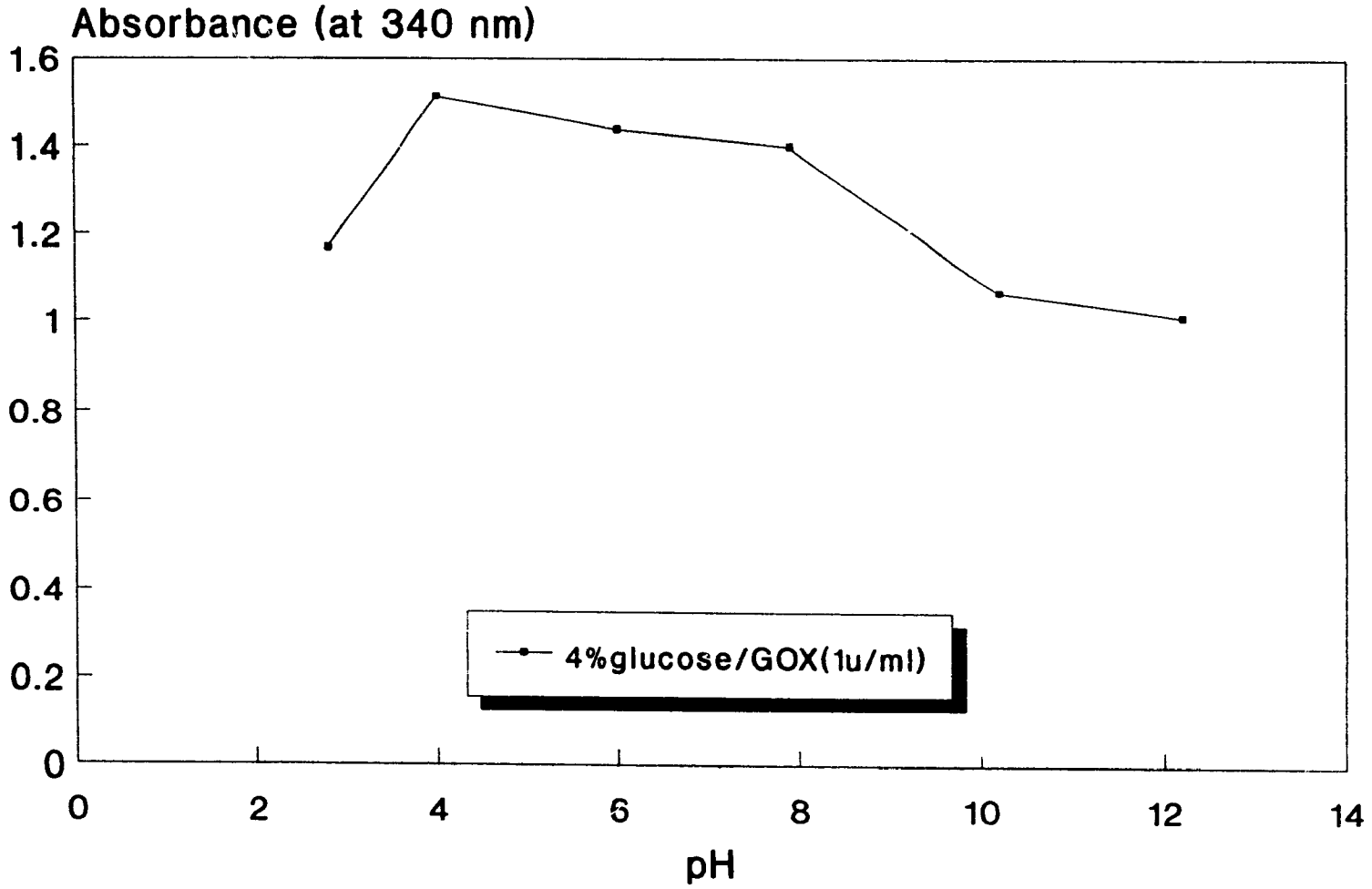
Data used to plot figure are average values of triplicate results for two experiments

Figure10. Gluconic acid from glucose and various units of glucose oxidase



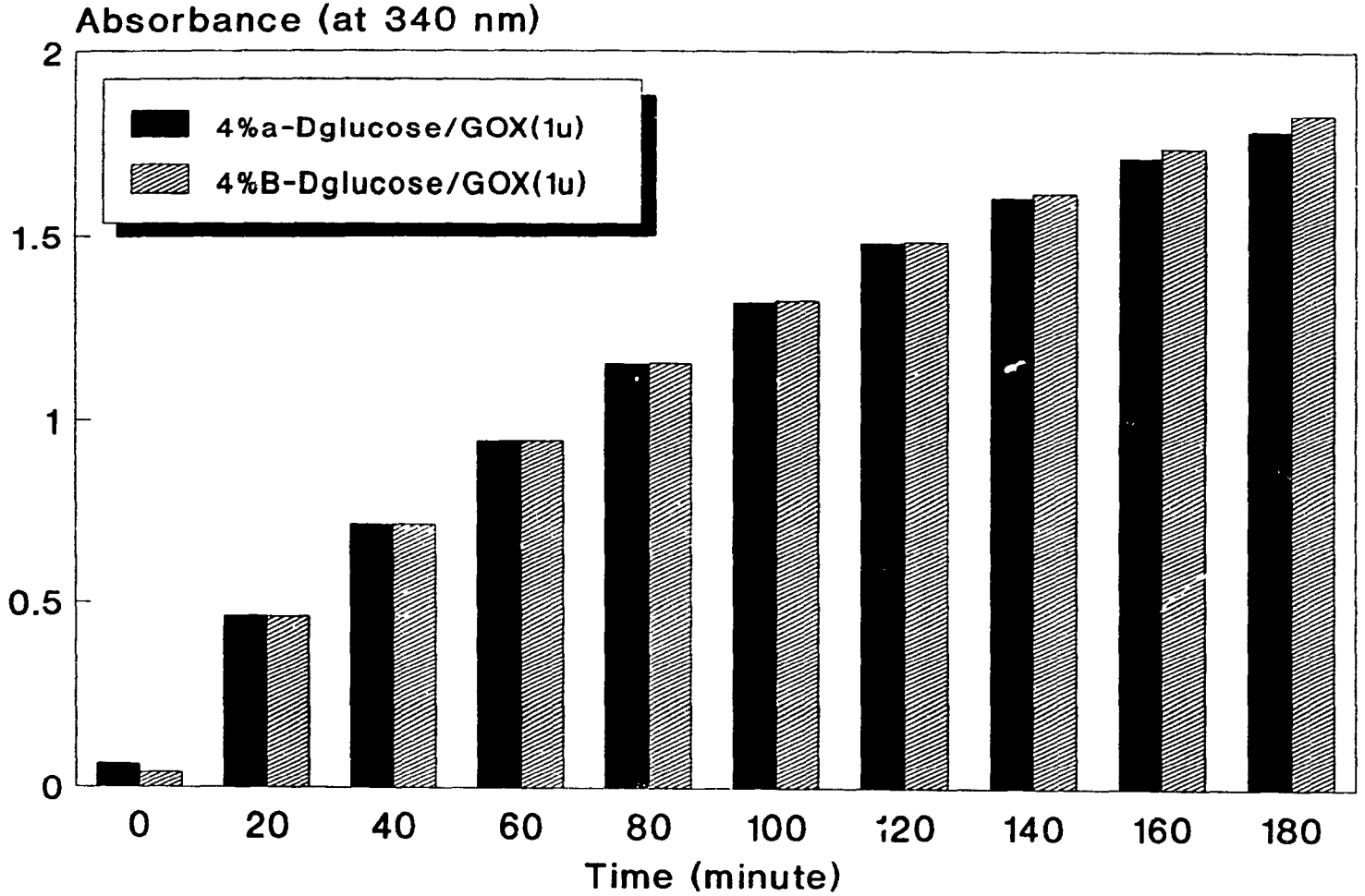
Data used to plot are average values of triplicate results for two experiments

Figure11. Gluconic acid from GOX/glucose at various pH levels



Data used to plot are average values of triplicate results for two experiments

Figure12. Gluconic acid production from a- and B-D-glucose



Data used to plot figure are average values of triplicate results for two experiments

2. Dipping/packaging studies with white shrimp

2.1 Objectives

The objectives of this initial study were to determine the effect of selected packaging/dipping solutions to prevent melanosis in shrimp and to extend the shelf life and keeping quality of the shrimp at 4°C. In this study, defrosted frozen white shrimp were used due to the unavailability of fresh shrimp at the time of this study. The results of the physical, chemical, microbiological and sensorial changes of the various packaging/dipping treatments are discussed below. All results are the average of triplicate samples.

2.2 Headspace gas composition

Changes in headspace gas composition for the various packaging/dipping treatments of shrimp are shown in Figures 13-15. For samples packaged in air (Figure 13) and dipped in water or GOX/glucose dipping solution prior to packaging, headspace O_2 decreased to less than 1% after 14 days with a concomitant increase in headspace CO_2 to approximately 15-20% (Figure 13). This reduction in headspace O_2 and increase in CO_2 is mainly due to the growth and metabolism of aerobic and facultatively aerobic bacteria although GOX and some endogenous enzymes may contribute to a reduction in headspace O_2. It is evident from Figure 13 that dipping shrimp in GOX/glucose solution has little or no effect in controlling microbial growth (and also melanosis) in air packaged samples. In contrast, the headspace gas composition changed only slightly in gas packaged/vacuum packaged samples (Figures 14-15). This is due to the fact that concentrations of CO_2 > 20% or low O_2 concentration (<1%) inhibit the growth and metabolism of aerobic spoilage microorganisms of muscle foods (Smith et al., 1991). For shrimp packaged in CO_2:N_2 (60:40) gas mixture, headspace O_2 never increased beyond 1% throughout storage indicating the accuracy of gas flush and the integrity of the high O_2 barrier film used

in this study. The slight decrease in headspace CO_2 observed initially in gas packaged samples is due to dissolution of CO_2 in the aqueous phase of shrimp at lower storage temperatures (Smith et al., 1991). Changes in the headspace O_2 and CO_2 composition for vacuum packaged samples are shown in Figure 15. Headspace O_2 remained at 1-2% throughout storage whereas headspace CO_2 increased from 2% to approximately 3-5% after 14 days. The presence of headspace CO_2 in the vacuum packaged samples is due to its evacuation from intracellular respiring tissue and possible production by facultative bacteria, particularly lactic acid bacteria during storage. The changes in headspace gas composition observed indicate the effectiveness of GOX/glucose dipping solution and MAP to control microbial spoilage and extend the shelf life of fresh shrimp. The trends observed for changes in headspace gas composition for packaged shrimp are similar to those observed in other muscle foods (Lambert et al., 1991a,b,c).

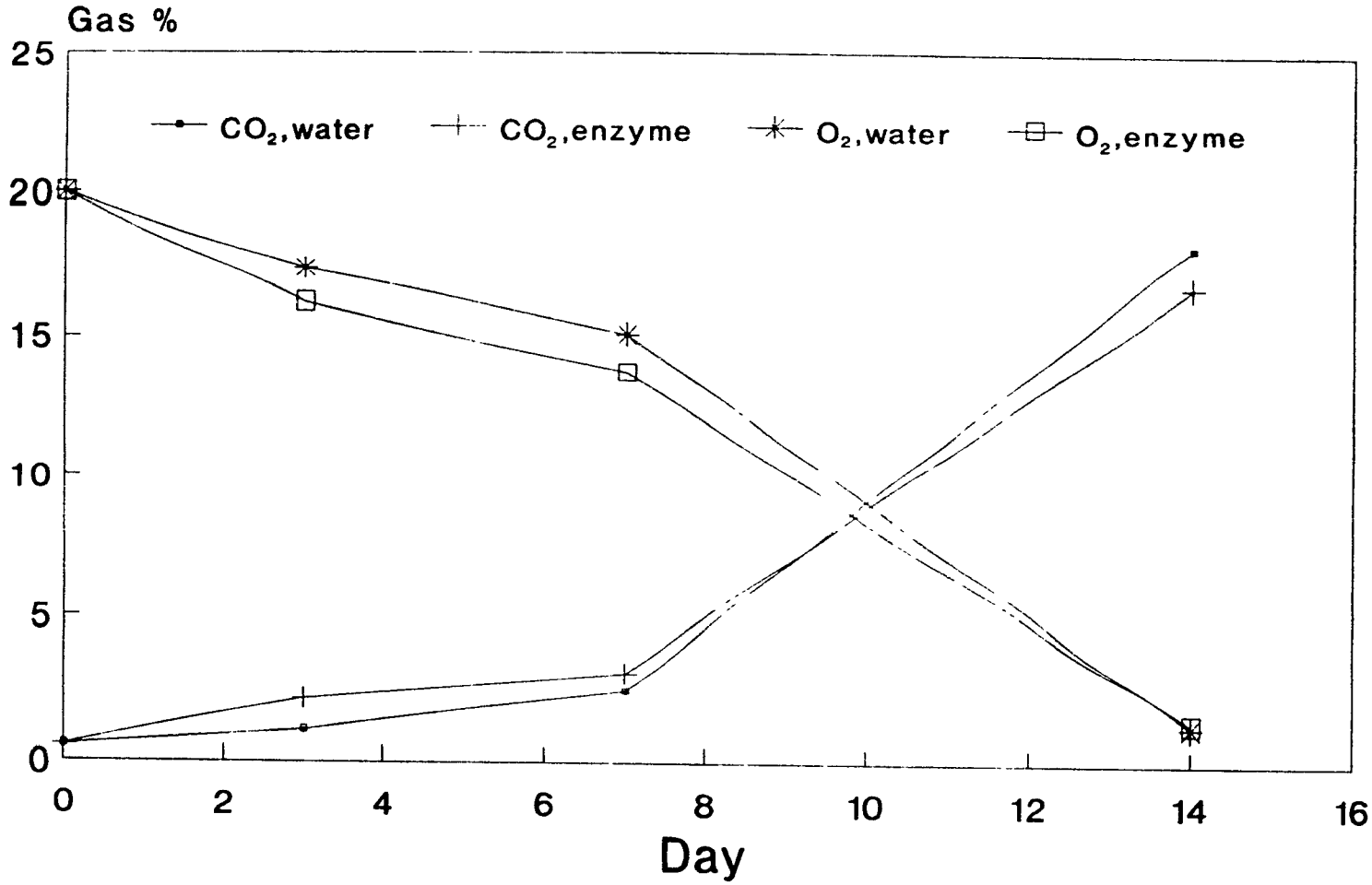
2.3 Changes in pH

Changes in pH for the various dipping/packaging treatments of shrimp are shown in Figures 16-17. pH is a good index of chemical reactions occurring in stored shrimp due to the effects of autolysis and microbial growth. Generally speaking, products of glycolysis such as lactic acid, proteolytic products, such as amino acids, result in a decrease in pH, whereas amines and some low molecular weight volatile compounds, such as ammonia, resulting from bacterial deamination of amino acids and ATP degradation products, will result in an increase in pH (Flick and Lovell, 1972).

In this study, the pH of all samples decreased only slightly from an initial pH of 7.4 to 6.96-6.34 after 14 days storage (Figures 16-17). The slight change of pH during the storage of shrimps can be explained by the buffering effect of muscle proteins and release of amino acids, as a result of proteolytic activity of aerobic and facultatively aerobic spoilage microorganisms. Statistical analysis showed that at the end of 14 days storage at 4°C, pH of the water/vacuum packaged samples and GOX/air packaged

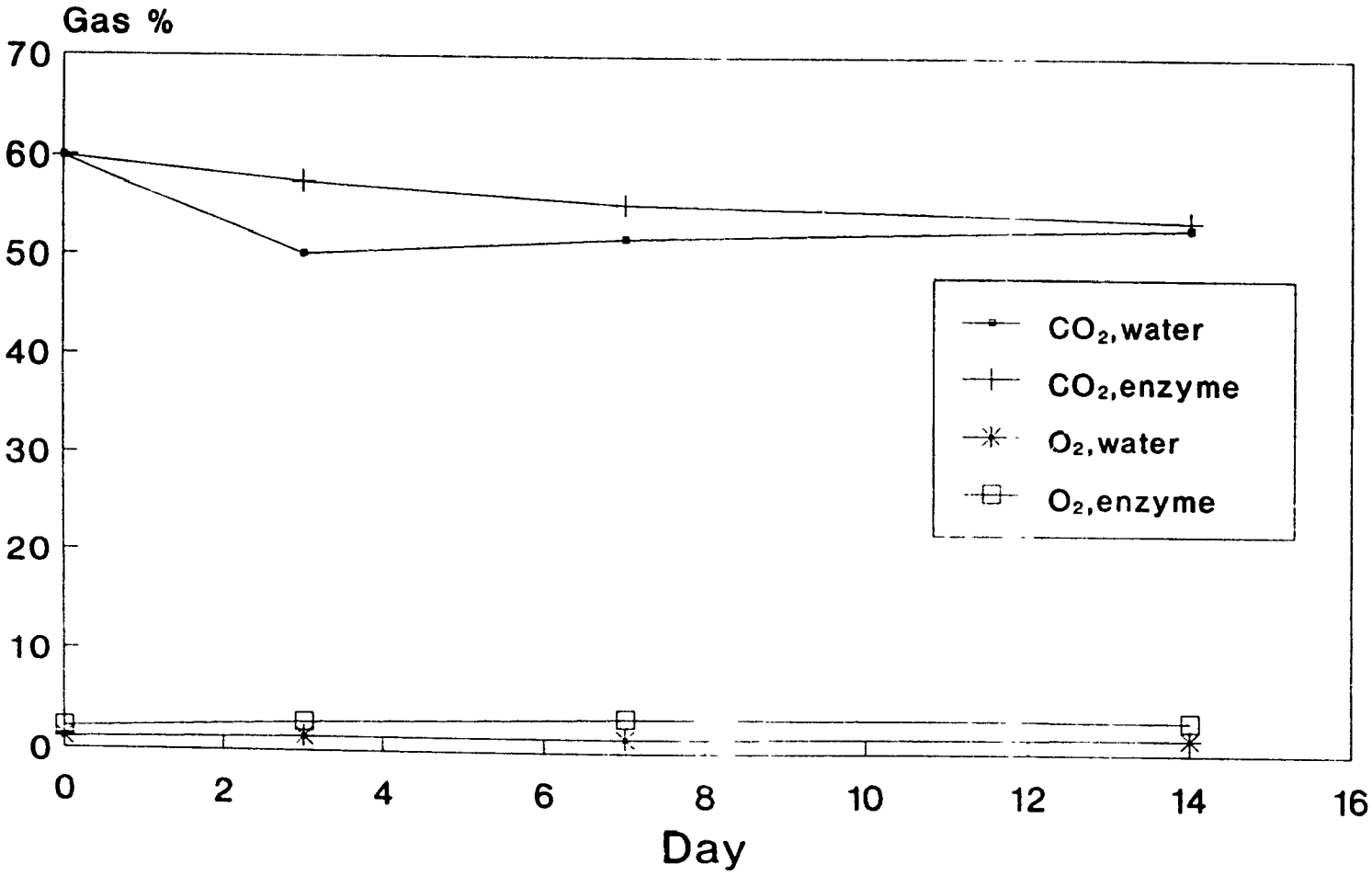
samples were significantly less ($p<0.05$) than all other packaging/dipping treatments. This pH decrease can be attributed to: **(1)** dissolution of CO_2 in the fish tissue, **(2)** production of gluconic acid from GOX/glucose, **(3)** production of lactic acid as a result of growth of aerobic spoilage microorganisms and lactic bacteria particularly in shrimp packaged under vacuum packaging conditions

Figure13. Changes in Headspace Gas of air packaged white shrimp



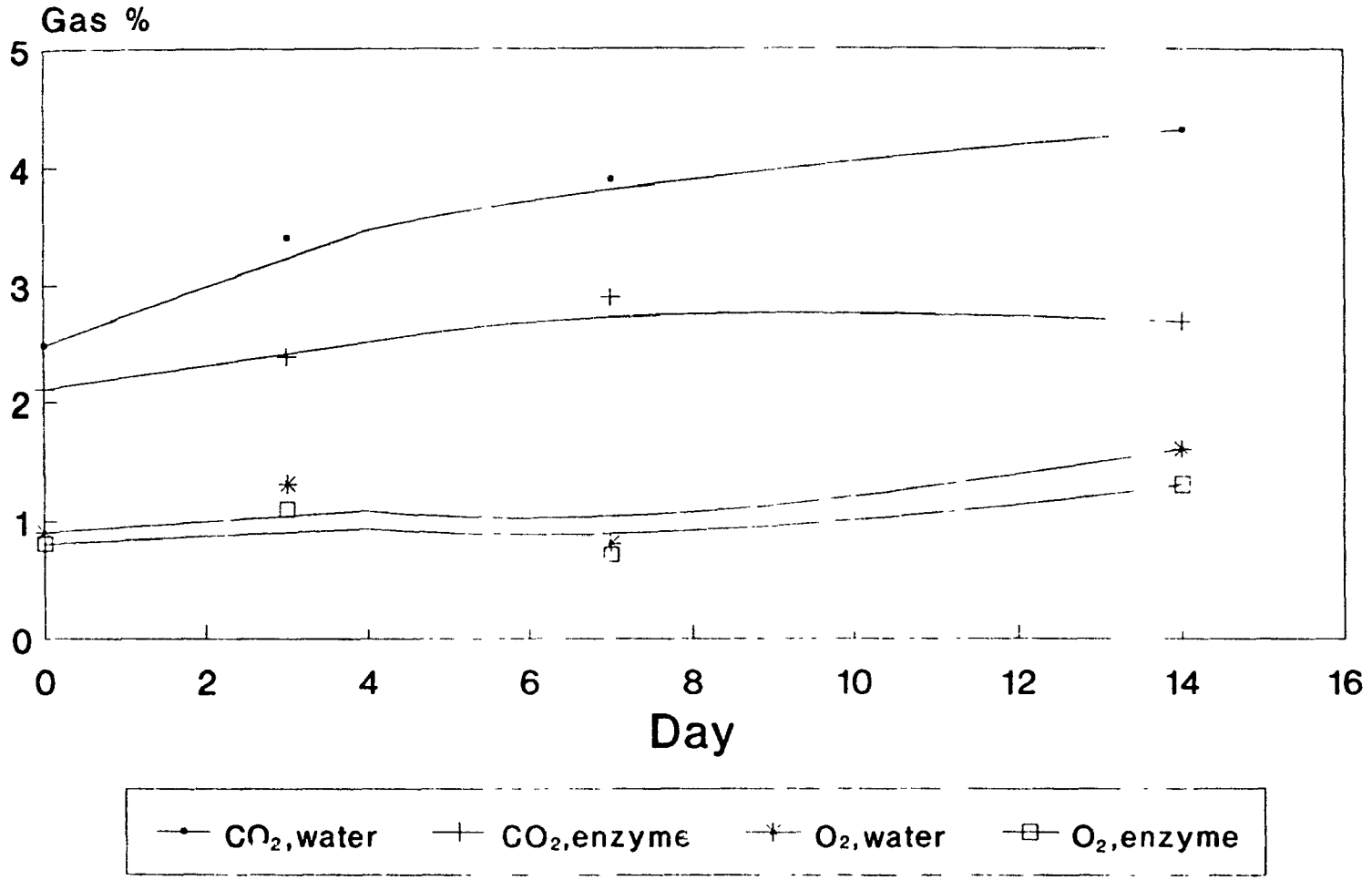
Data used to plot figure are average values of triplicate results for two experiments

Figure14. Changes in Headspace Gas
of white shrimp packaged in gas
(CO_2:N_2/60:40)



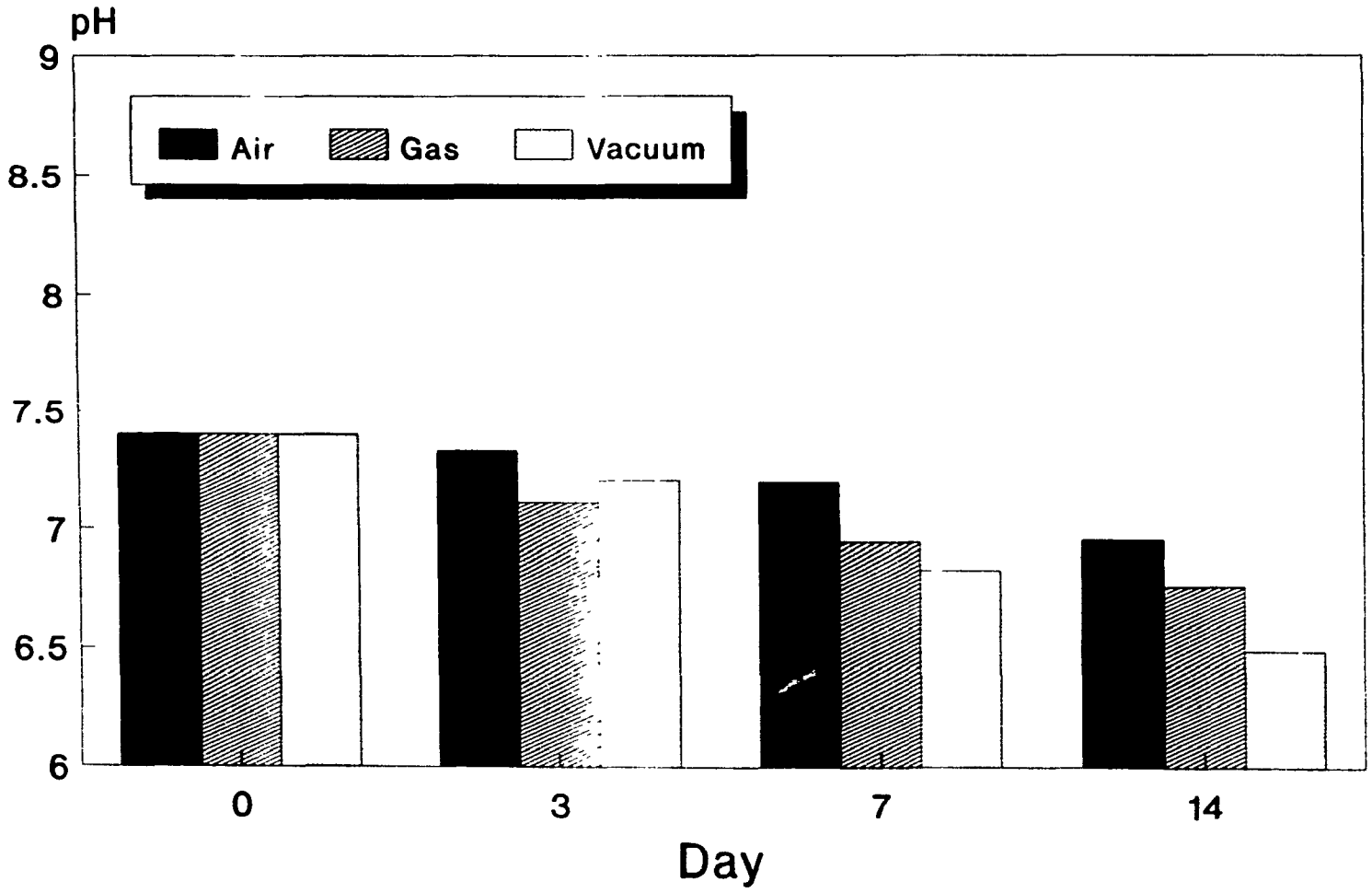
Data used to plot figure are average values of triplicate results for two experiments

Figure15. Changes in Headspace Gas of vacuum packaged white shrimp



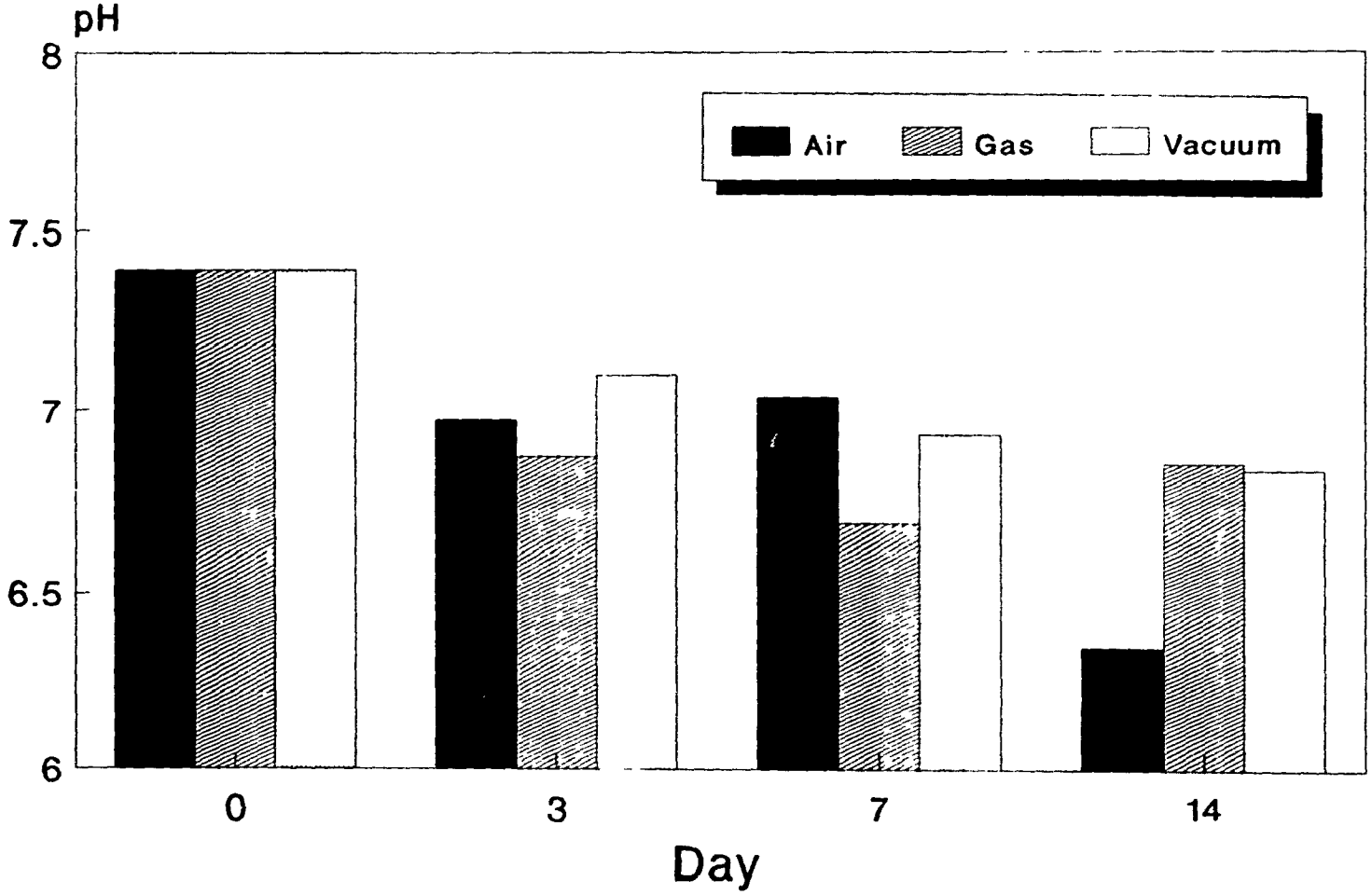
Data used to plot figure are average values of triplicate results for two experiments

Figure16. pH of white shrimp dipped in water



Data used to plot figure are average values of triplicate results for two experiments

Figure17. pH of white shrimp dipped in GOX/glucose



Data used to plot figure are average values of triplicate results for two experiments

2.4 Change in total volatile base (TVB)

The total volatile base test measures low molecular weight volatile bases and amine compounds produced by microbial decarboxylation of amino acids. Changes in the TVB value of the various dipping/packaging treatments of shrimp are shown in Figures 18-19 respectively. The TVB values of shrimp dipped in water and packaged in air, under vacuum and under a CO_2:N_2 (60:40) gas atmosphere increased from an initial value of 26.78 mgN/100g to 75.68, 64.26 and 51.21 mgN/100g respectively (Figure 18). A TVB value of 45 mgN/100g or less is regarded as a reliable indicator of fresh shrimp. This value was reached after approximately 8-9 days for shrimp packaged in air and under vacuum and after 12-13 days for shrimp dipped in water and packaged under a CO_2:N_2 atmosphere (Figure 18). Similar but lower trends in TVB values were observed for shrimp dipped in GOX/glucose solution prior to packaging under similar gaseous conditions. Statistical analysis of the data showed that all packaging treatments were significantly different from each other ($p<0.05$). Only one dipping/packaging treatment, i.e., GOX/glucose/CO_2:N_2 had an acceptable TVB value (42 mgN/100g) after 14 days storage at 4°C. This observation was in agreement with the work of Lannelongue et al., (1982) with brown shrimp stored in retail packages containing a CO_2 enriched atmosphere and can be attributed to the inhibitory effect of CO_2 on bacterial activity, specially gram-negative *Pseudomonas* which are the predominant spoilage bacteria of shrimp and fish. The results observed have shown that the combined use of GOX/glucose dipping solutions in conjunction with gas packaging and vacuum packaging is an effective method of controlling spoilage bacteria of fresh shrimp as shown by the lower TVB values. However, the TVB may not always be good indicator of shrimp quality since the volatile base nitrogen may be reduced through drip loss or permeation through the packaging film (Cobb III et al., 1973).

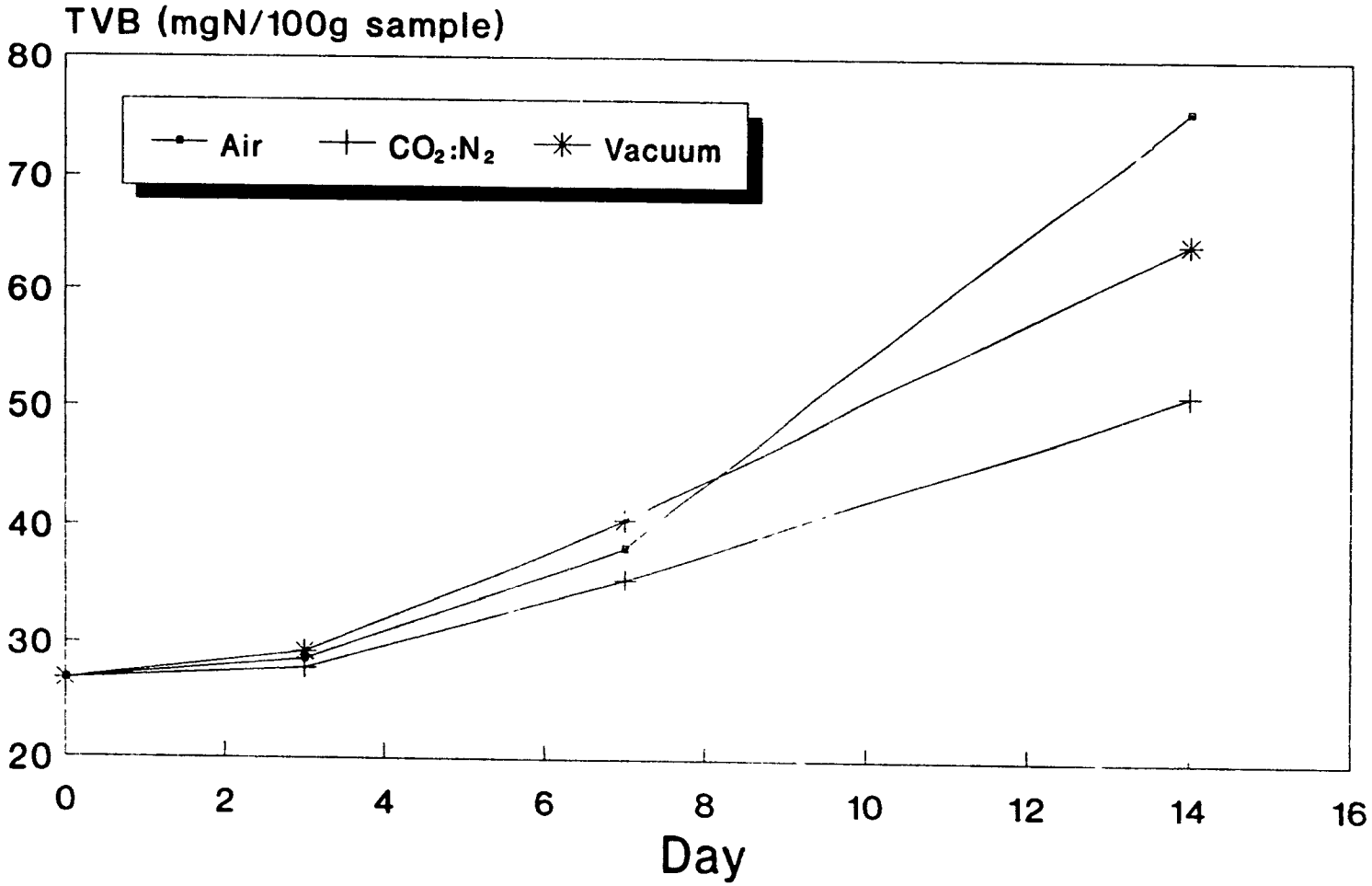
2.5 Changes in Hypoxanthine values

Hypoxanthine (Hx) is an excellent indicator of quality for iced or refrigerated shrimp. Its accumulation reflects an initial phase of autolytic deterioration followed by bacterial spoilage. It is produced at a sufficiently regular rate during refrigerated storage of shrimp and it is a reliable indicator of shrimp quality.

Hypoxanthine results for all dipping/packaging treatments were obtained from the hypoxanthine standard graph (Figure 20). All points on this graph were an average of three replicates. The absorbance increased in a linear manner over the concentration range tested and had a R^2 value of 0.99 indicating the adequacy of the graph to predict the concentration of hypoxanthine.

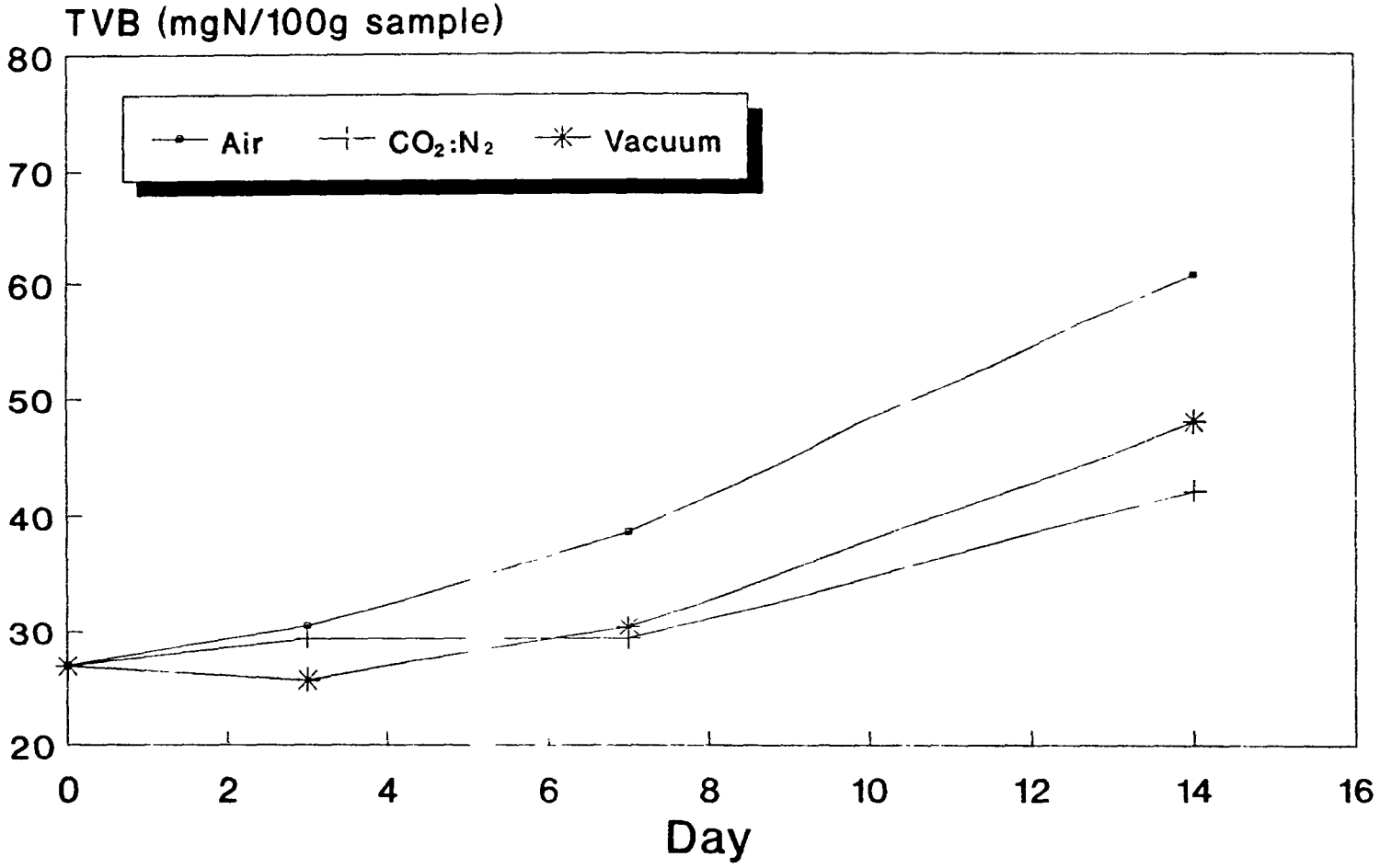
An average value of 4 umoles/g is regarded as the upper level of acceptability for fresh shrimp. Initial hypoxanthine values were similar in all samples after day 1 (Figures 21-22). However, at the end of the 14 day storage period, only shrimp dipped in the GOX/glucose solution and packaged either in a gas atmosphere of CO_2:N_2 (60:40) or vacuum packaged had acceptable hypoxanthine levels (< 4 umoles/g). All other packaging/dipping treatments had concentrations greater than this recommended hypoxanthine level of good quality shrimp. Samples dipped in GOX and vacuum packaged were significantly superior ($p<0.05$) from their gas packaged counterparts. These results can be attributed to the inhibition of the aerobic psychrotrophic bacteria by the combined effect of GOX and packaging under MAP conditions. The results again emphasize the possible extension in shelf life/quality through the combined use of GOX/glucose dipping solutions and MAP packaging.

Figure18. Total Volatile Base of white shrimp dipped in water



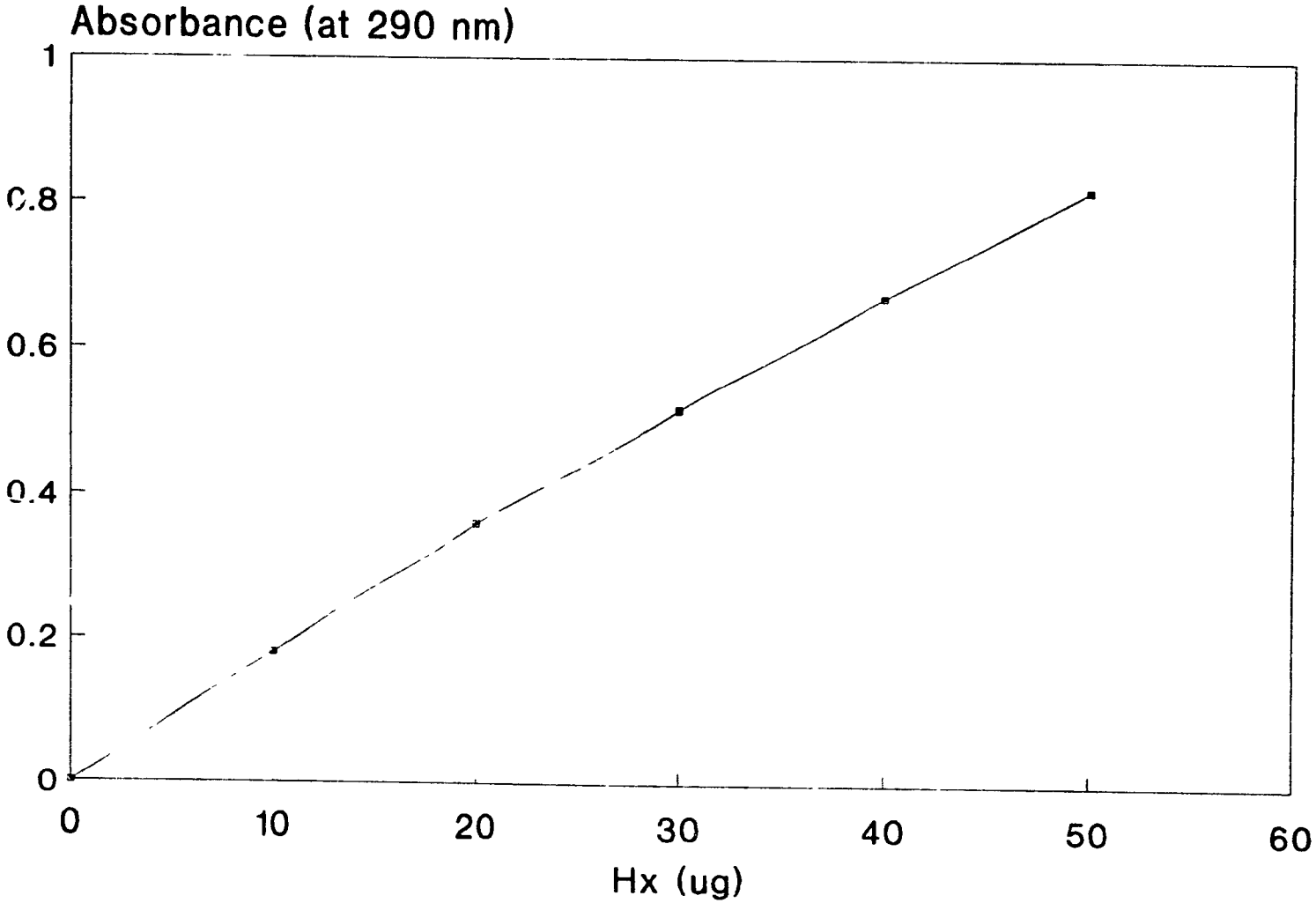
Data used to plot figure are average values of triplicate results for two experiments

Figure19. Total Volatile Base of white shrimp dipped in GOX/glucose



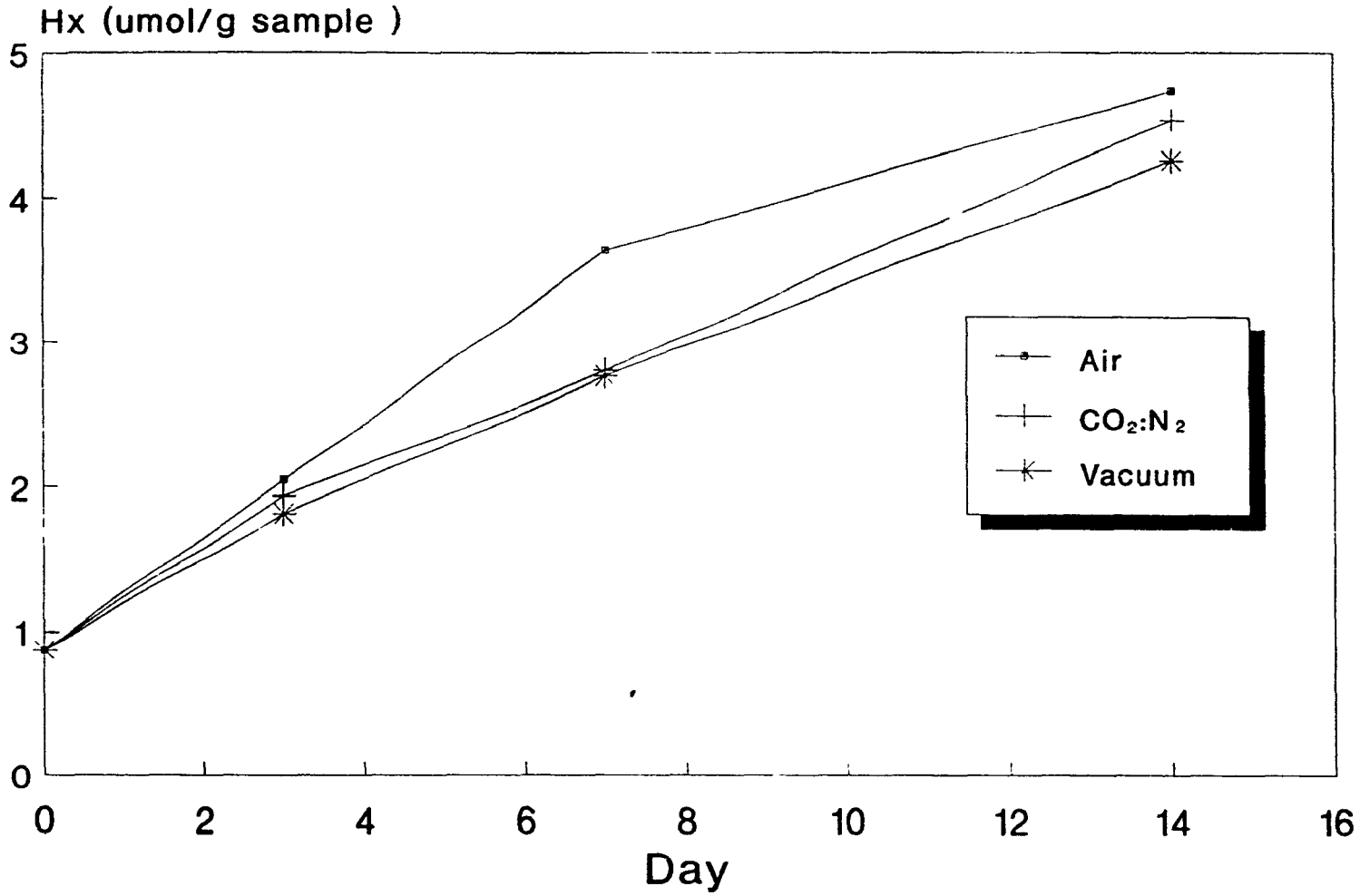
Data used to plot figure are average values of triplicate results for two experiments

Figure20. Hypoxanthine standard graph



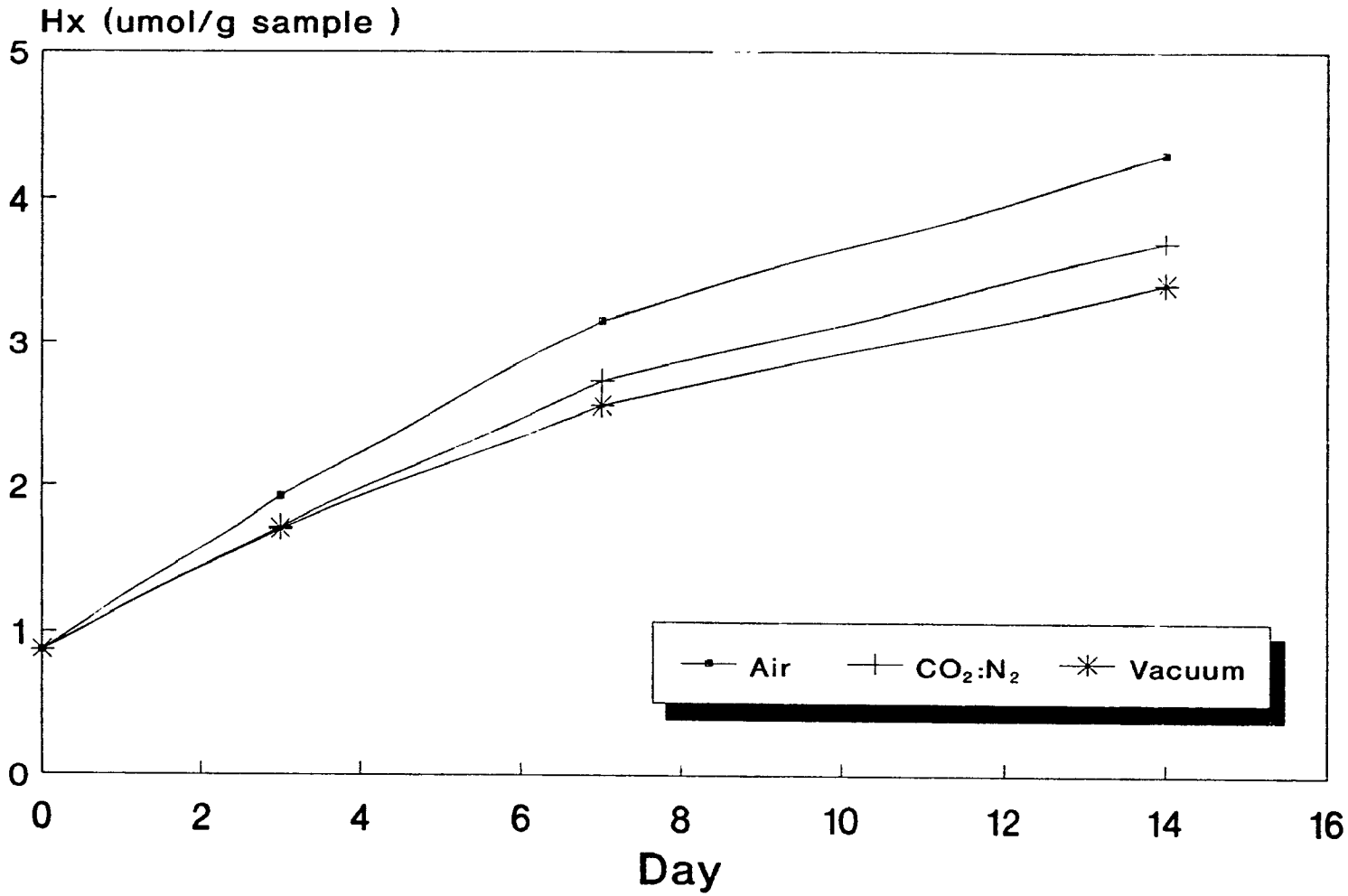
Data used to plot figure are average values of triplicate results for two experiments

Figure21. Hypoxanthine of white shrimp dipped in water



Data used to plot figure are average values of duplicate results for two experiments

Figure22. Hypoxanthine of white shrimp dipped in GOX/glucose



Data used to plot figure are average values of duplicate results for two experiments

2.6 Changes in microbial counts

Changes in the aerobic plate counts (APC), psychrotrophic counts and lactic acid bacteria (LAB) counts for the various dipping/packaging treatments are shown in Figures 23-28 respectively. The number of days to reach a count of 10^6 cfu/g was taken as the standard for spoilage and termination of shelf life.

2.6.1 Aerobic plate counts (APC)

Aerobic plate counts (APC) increased from approximately $2x10^3$/g (Day 0) to unacceptable levels i.e. $>10^6$-10^7 CFU/g after 7-14 days for most dipping/packaging treatments (Figure 23-24). Only shrimp dipped in GOX/glucose solution and gas packaged or vacuum packaged had significantly different ($p<0.05$) and acceptable APC counts, i.e., $<10^6$-10^7 at the end of the storage period (Figure 24). The overall effect of GOX/glucose dipping solution and MAP is an extension in the lag phase of bacterial growth and subsequent extension in shelf life. For example, lag phases of 6-10 days have been reported for brown shrimp, sword fish and fin fish (Lannelongue et al., 1982). In addition to extending the lag phase and inhibiting subsequent bacterial spoilage, high CO_2 levels have residual bactericidal effects on the initial spoilage flora (Lannelongue et al., 1982).

2.6.2 Psychrotrophic bacteria counts

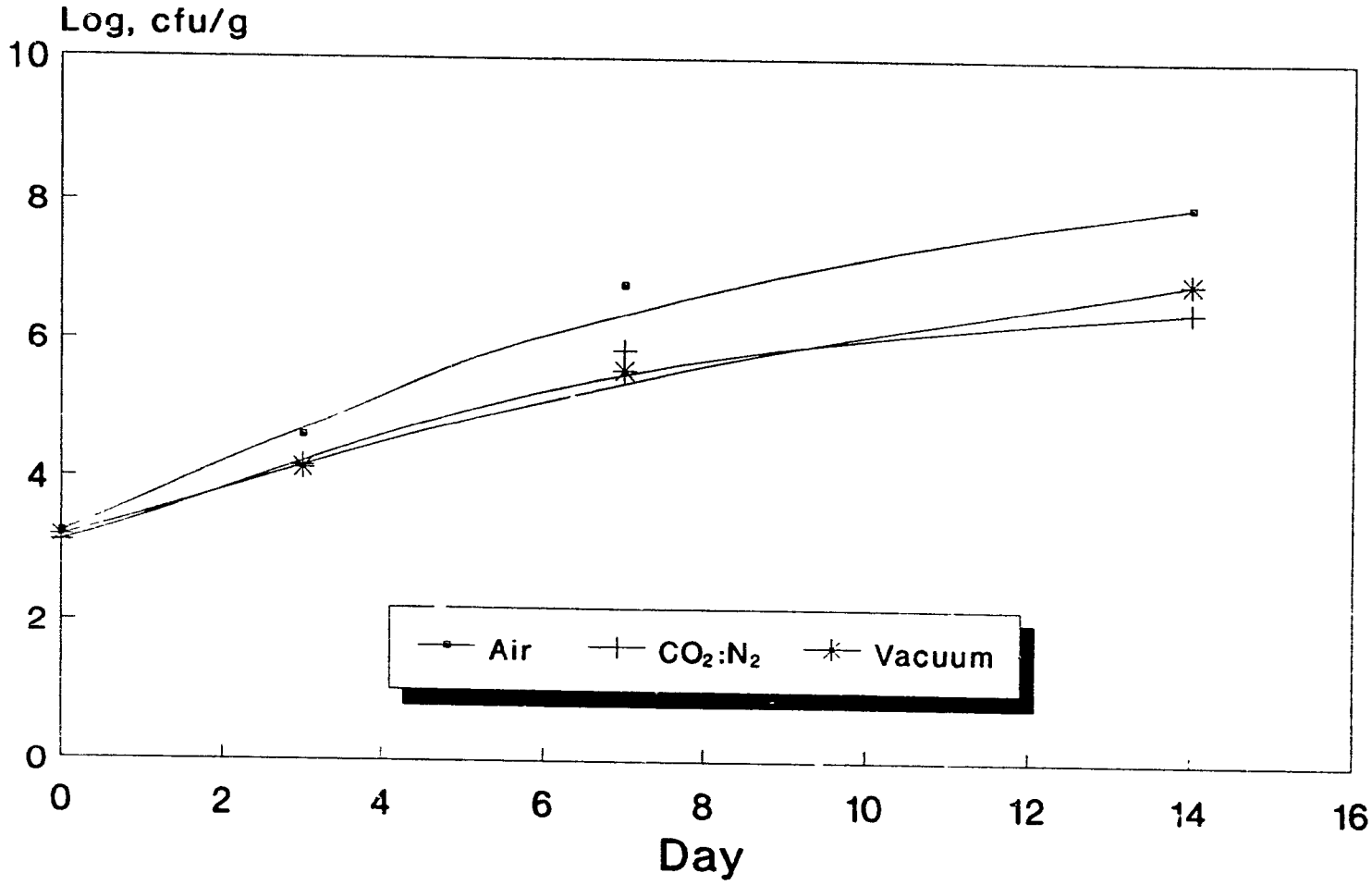
Changes in psychrotrophic bacteria counts for various treatments of shrimp are shown in Figure 25-26. Since low storage temperatures (4°C) and aerobic storage conditions favor the growth of psychrotrophic bacteria, counts were significantly high in shrimps dipped in water or GOX/glucose and air packaged. A 0.5 log reduction in psychrotrophic counts was obtained by dipping shrimp in GOX/glucose prior to packaging in air. However, there was no significant difference in counts between shrimp dipped in GOX/glucose and shrimp dipped in water prior to air packaging.

However, dipping shrimp in water and packaging under CO_2:N_2 or vacuum packaging resulted in approximately 1 Log reduction in psychrotrophic counts. However, dipping in GOX/glucose solution and packaging under vacuum or CO_2:N_2 atmosphere resulted in approximately further 1 Log reduction in psychrotrophic counts. This indicates that the combined use of GOX/glucose dipping solutions and MAP has the potential to extend the shelf life of fresh shrimp by inhibiting *Pseudomonas* species, the main psychrotrophic spoilage bacteria of shrimp and other muscle foods (Lambert et al., 1991a,b,c).

2.6.3 Lactic acid bacteria (LAB) counts

Similar trends were observed for lactic acid bacteria counts (Figure 27-28). Indeed, approximately 80-90% of the APC/psychrotrophic count comprised of lactic acid bacteria which were identified on the basis of their morphological characteristics on MRS agar and the absence of catalase activity. Lactic acid bacteria have been shown to become the predominant spoilage microorganisms in vacuum packaged/gas packaged muscle foods. They may also be the dominant spoilage microorganism in air packaged fish which becomes anaerobic due to the growth of aerobic spoilage microorganisms (Lambert et al., 1991a,b,c). The low residual O_2 concentration and elevated CO_2 concentration favors the growth of lactic acid bacteria at refrigerated storage temperatures. Although lactic acid bacteria reached spoilage levels (10^6 cfu/g) within 12-14 days, with the exception of GOX/CO_2:N_2 treatments, lactic acid bacteria are of low spoilage concern at these levels.

Figure23. APC of white shrimp
Dipped in water



Data used to plot figure are average values of triplicate results for two experiments

Figure24. APC of white shrimp
Dipped in GOX/glucose

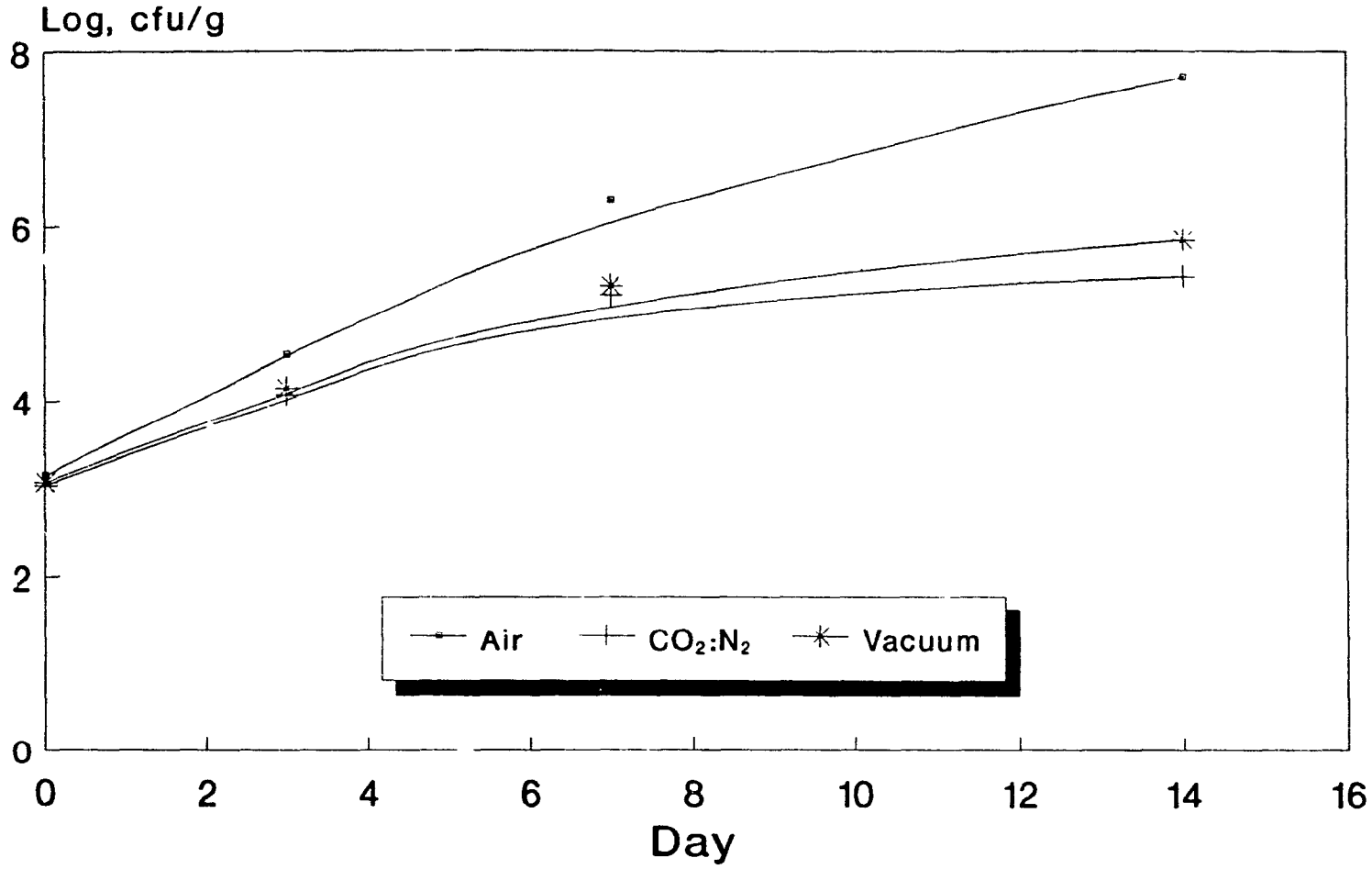


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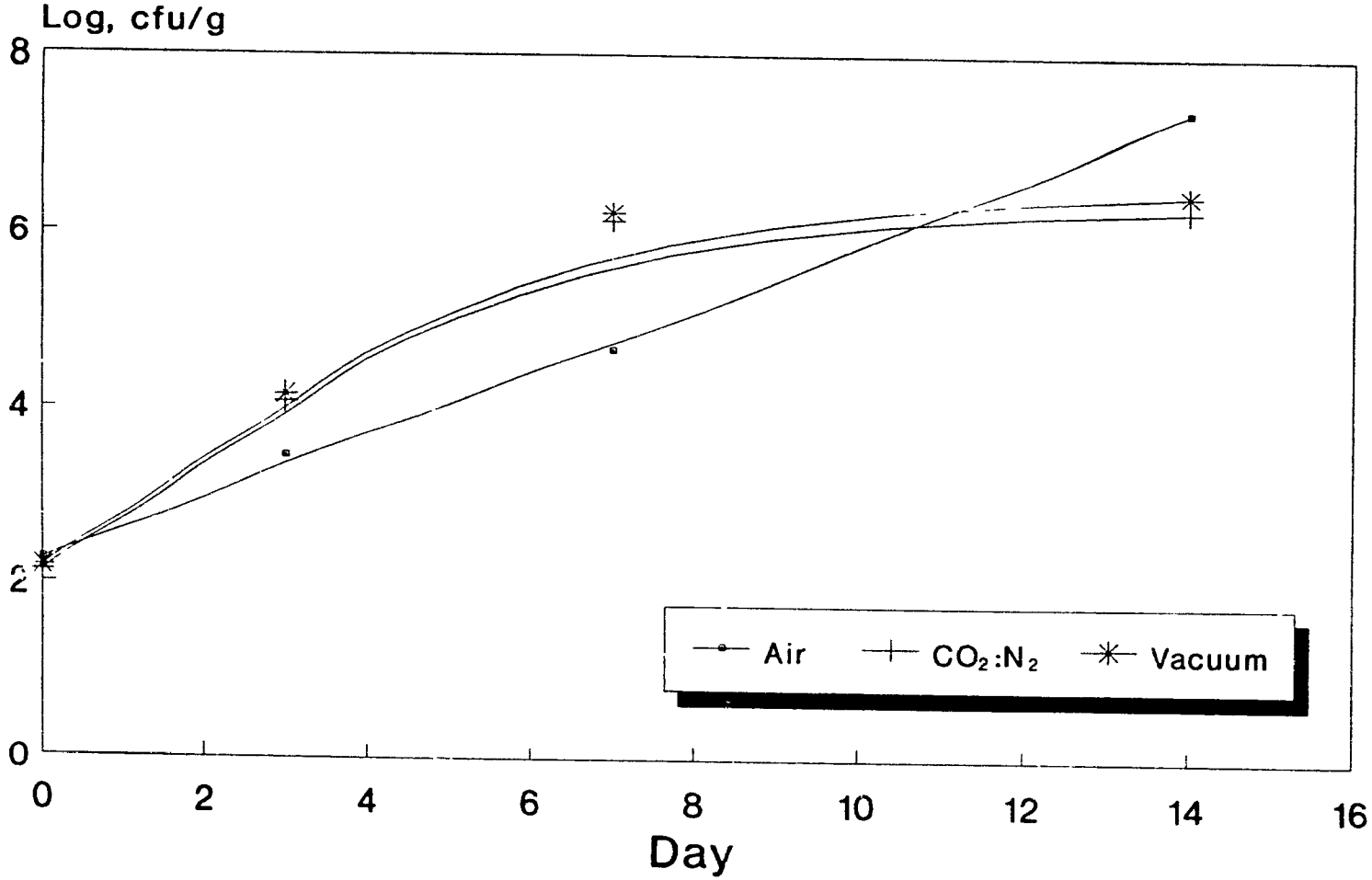
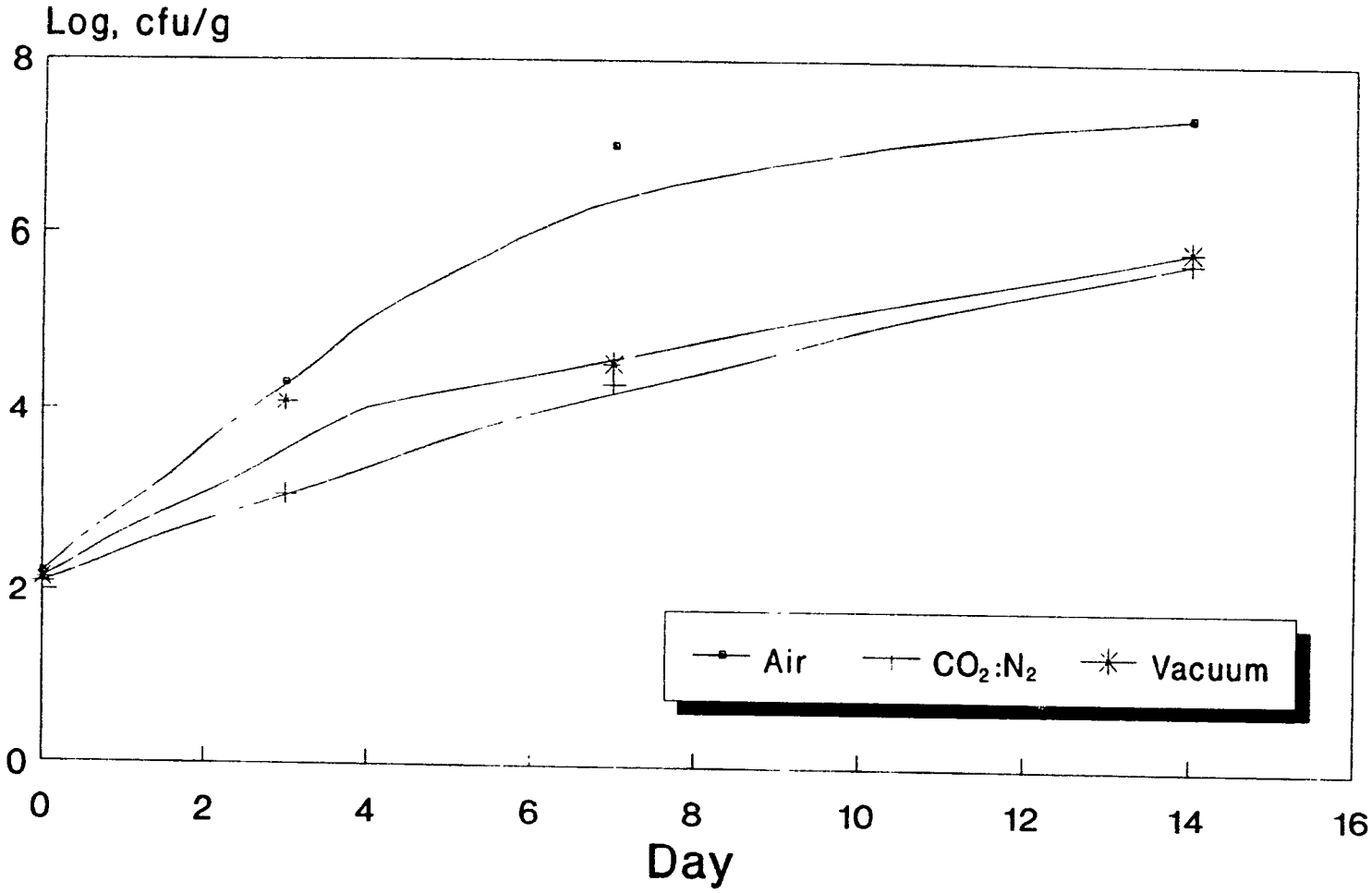
Figure25. Psychrotrophic count of white shrimp dipped in water



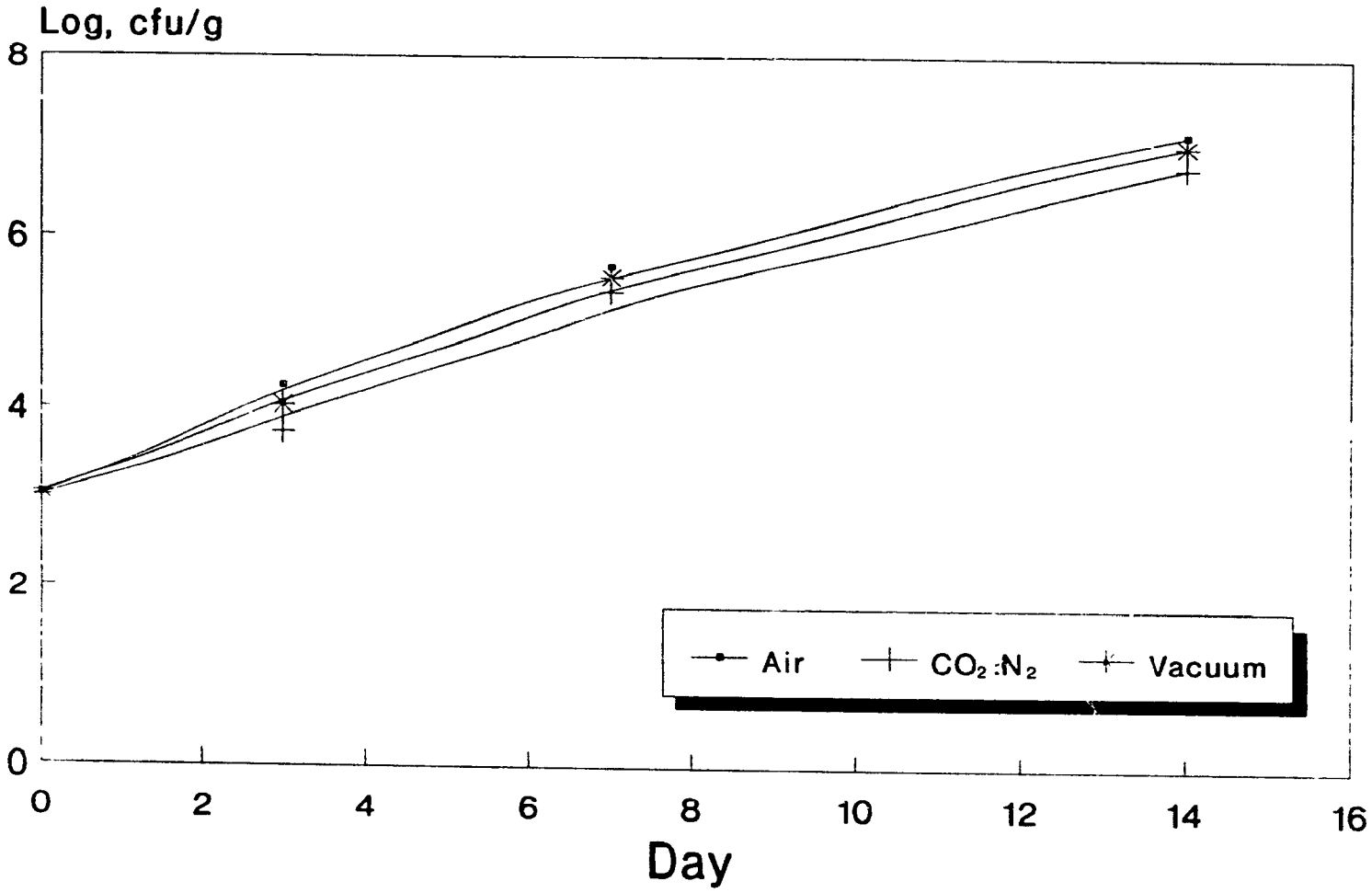
Data used to plot figure are average values of triplicate results for two experiments

Figure26. Psychrotrophic count of white shrimp dipped in GOX/glucose



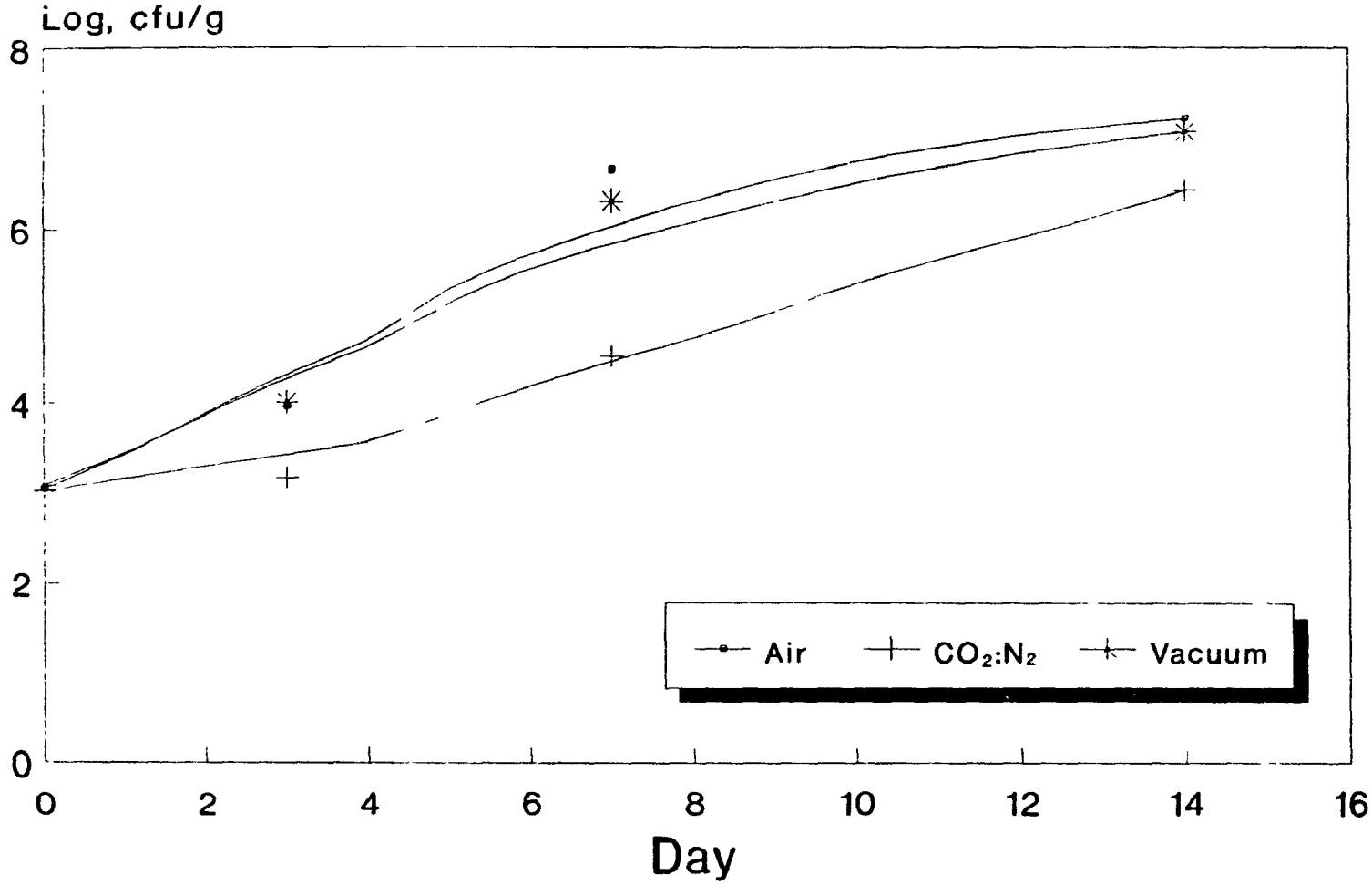
Data used to plot figure are average values of triplicate results for two experiments

Figure27. LAB count of white shrimp dipped in water



Data used to plot figure are average values of triplicate results of two experiments

Figure28. LAB count of white shrimp dipped in GOX/glucose



Data used to plot figure are average values of triplicate results for two experiments

2.7 Melanosis score

As mentioned in the literature review, shrimp melanosis, commonly termed "black spot" is a surface discoloration caused by enzymatic formation of precursor compounds which polymerize with cellular constituents to form insoluble pigments. The endogenous enzyme, polyphenoloxidase (PPO), which catalyses the initial step in black spot formation, remains active throughout post-harvest processing unless the shrimp are frozen or cooked. However, PPO activity can resume in raw shrimp upon thawing The most common method to control melanosis involves sulfites. However, recent regulatory concerns about adverse reactions to sulfites in foods has resulted in research to find alternative methods to control this aesthetic defect. Two methods which could fulfil this objective are GOX/glucose dipping solution and MAP.

The melanosis score of white shrimp was evaluated using a standard melanosis score chart developed in our laboratory (Figure 29). A score of between 4-6 was judged as acceptable by panellists. Shrimps with a score >6 were regarded as having a major defect and unacceptable to consumers. On the basis of this ranking, the melanosis scores for the various dipping/packaging treatments of white shrimp are shown in Figures 30-31. It is evident from Figures 30 & 31 that shrimp dipped in water and GOX/glucose dipping solutions, and air packaged had unacceptable melanosis scores (>6) after 3 days at 4°C. No statistical difference ($p<0.05$) was observed between the dipping/packaging treatments.

However, all other dipping/packaging treatments had acceptable melanosis scores after 14 days storage at 4°C. Statistical analysis showed that shrimp dipped in GOX/glucose solution and gas packaged in a CO_2:N_2 (60:40) mixture had a significantly lower melanosis score (4.7) after 14 days compared to all other dipping/packaging treatments. This shows that the combined use of GOX/glucose dipping solution and MAP is an effective way of controlling black spot development in white shrimp. This inhibition can be attributed to either an inhibition of PPO by

gluconic acid from GOX/glucose. However, it is more likely attributable to the low O_2 tension in the package headspace which is required for PPO activity. Whatever the reason the combined use of GOX/glucose dipping solutions & MAP has the potential to be an effective combination treatment to control black spot development in shrimp and further it offers the shrimp processor an alternative viable method to control melanosis and to replace the current practice of dipping shrimp in bisulphite solutions.

Examples of black spot development for various packaging/dipping treatments of white shrimp are shown in Figure 32.

2.8 Odor and overall acceptability

Shrimp were also evaluated simultaneously for odor and overall acceptability (results not shown). A score of 6 was also regarded as the upper level of acceptability for both these parameters. Odor and overall acceptability scores were similar to melanosis scores, i.e., shrimp dipped in water and air packaged had scores of >6 after 5-7 days. However, all other dipping/packaging treatments had a acceptable odor/overall acceptability scores after 14 days at 4°C. Therefore the combined use of GOX/glucose dipping solutions not only has the potential to retard microbial growth and metabolism as shown by the chemical indicators of spoilage, but also control melanosis for 14 days or longer at 4°C.

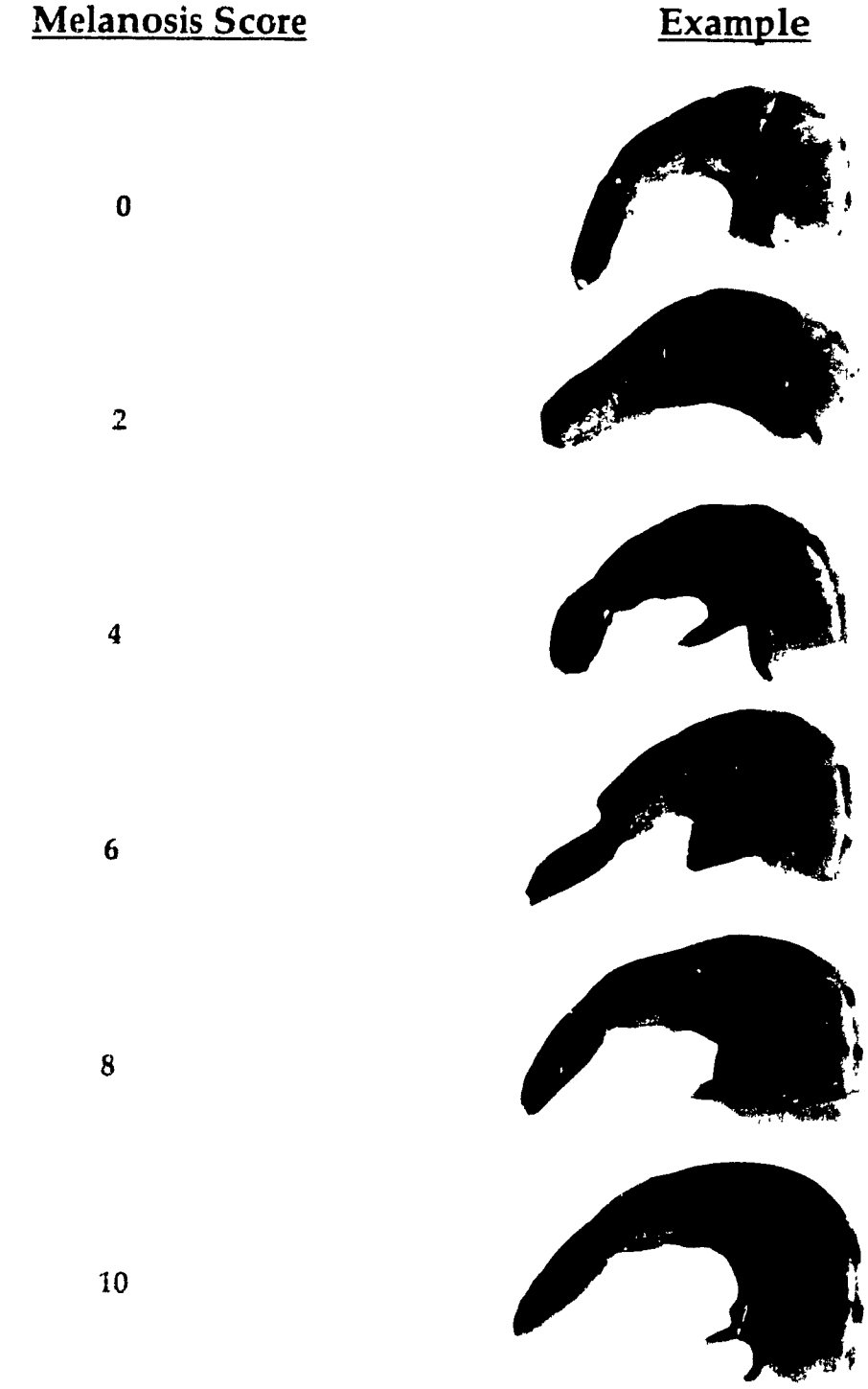
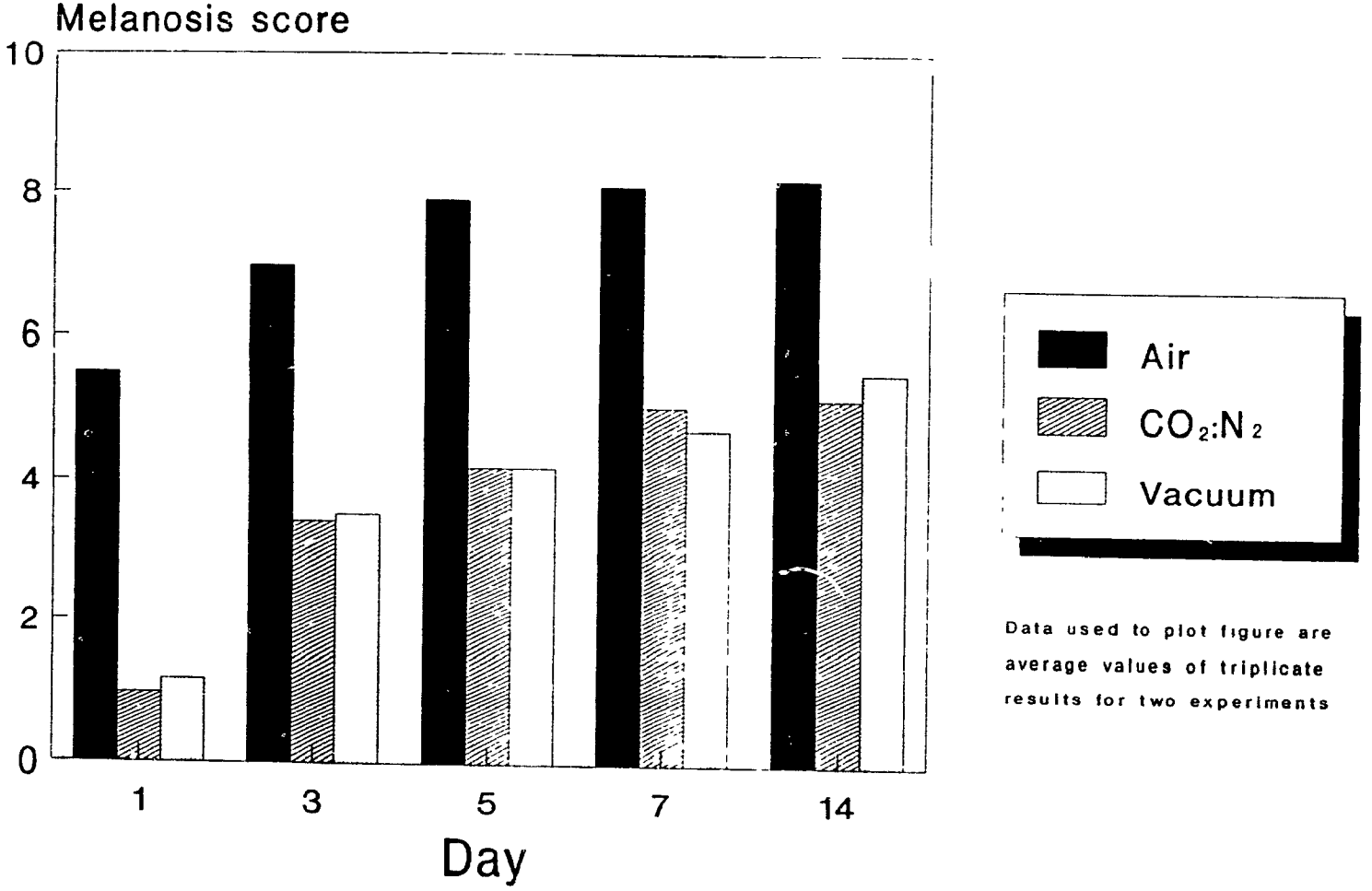


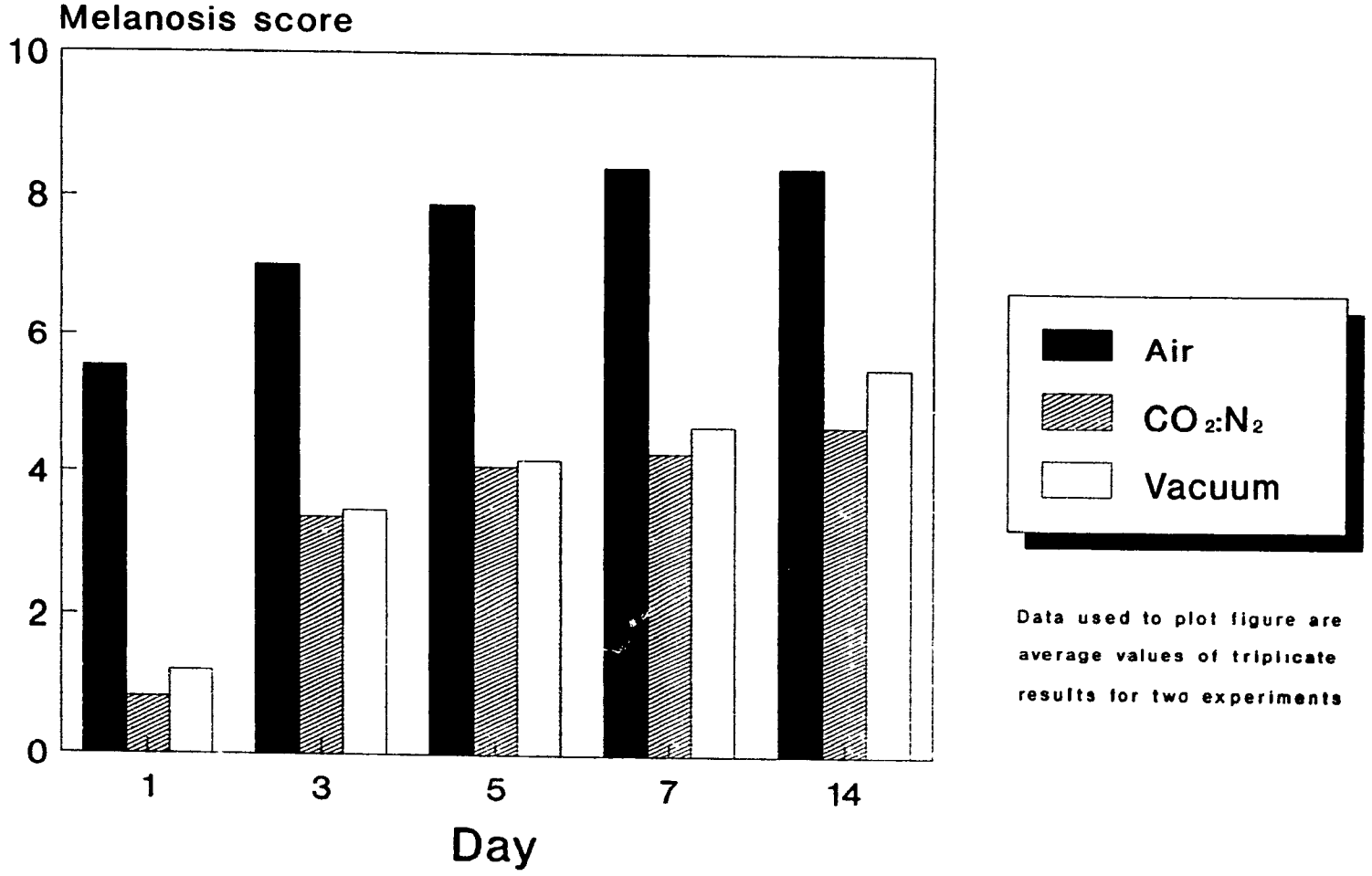
Figure 29. Examples of various degrees of blackspot development on white shrimp (*P. occidentalis*) and the corresponding melanosis scores.

Figure30. Melanosis score of white shrimp dipped in water



Data used to plot figure are average values of triplicate results for two experiments

Figure31. Melanosis score of white shrimp dipped in GOX/glucose



Data used to plot figure are average values of triplicate results for two experiments

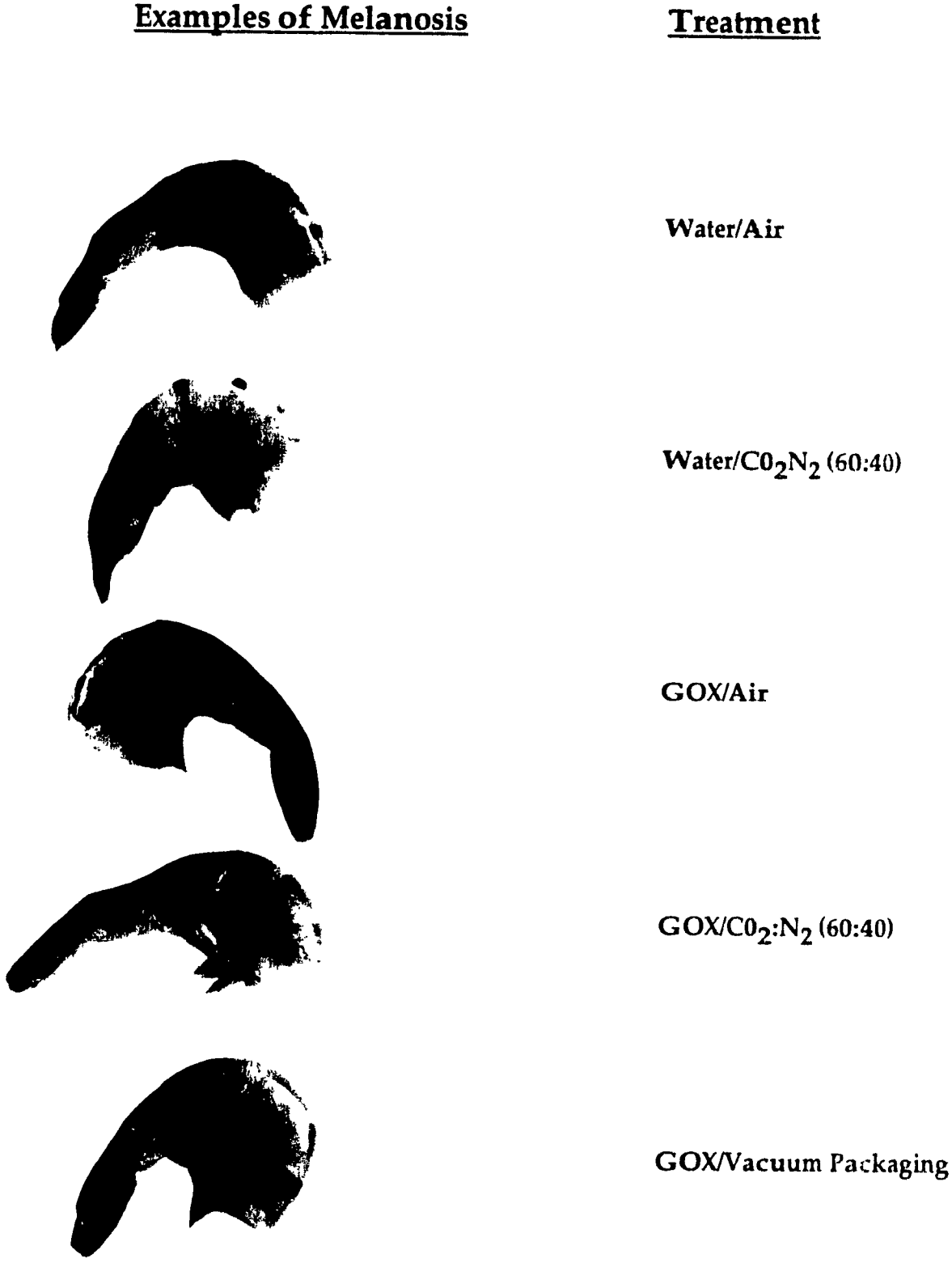


Figure 32. Examples of degrees of blackspot development on white shrimp (*P. occidentalis*) for various packaging/dipping treatments after 14 d at 4°C.

3. Control of melanosis in pink shrimp

In the initial study, defrosted frozen white shrimp were used throughout the study due to the unavailability of fresh shrimp. The results of that initial study indicated that the GOX/glucose dipping solution in combination with gas packaging gave the most favorable results in terms of melanosis control and shelf life extension of white shrimp. In this study, this dipping/packaging treatment was investigated to control melanosis in fresh pink shrimp (*Pandalus borealis*). This variety of shrimp was selected due to its availability at the time of this part of the study, and since melanosis is more troublesome in pink shrimp compared to other shrimp species. In addition to the GOX/glucose dipping solution used previously, two other dipping treatments were investigated to control melanosis - a 1% ascorbic acid dipping solution, alone and in conjunction with GOX/glucose solution. Ascorbic acid is a reducing agent and it has been reported to enhance the oxygen scavenging abilities of glucose oxidase (Genencor International Inc., Personal Communication). In this study, only melanosis score, odor and overall acceptability score were monitored. Melanosis was evaluated using the chart (Figure 33) developed for pink shrimp by McEvily et al.,(1991). A melanosis of 6 or greater was again used as the upper level of consumer acceptability of fresh shrimp.

The melanosis scores for all dipping treatments of air packaged pink shrimp are shown in Figure 34. Similar trends were observed for air packaged pink shrimp compared to white shrimp i.e. a melanosis score of >6 was observed after 3-4 days irrespective of the dipping treatment (Figure 34). However, when shrimp dipped in similar solutions were packaged in a CO_2:N_2 atmosphere, melanosis, odor and overall acceptability scores were greatly improved. For example, at the end of the 14 day storage period, pink shrimp had a melanosis score of 3-4 for the various dipping treatments with the lowest score of ~3.9 being obtained for the GOX/glucose/ascorbate dipping solution and gas packaged (60%CO_2:40%N_2) shrimps (Figure 35). This lower score can perhaps be attributed to (i) the greater oxygen

scavenging capacity of the dipping solutions, (ii) a greater reduction in product pH and inhibition of PPO enzyme. It also shows the potential for other combination treatments as potential dipping solutions to control melanosis. Examples of other dipping treatments in conjunction with gas packaging to control melanosis in pink shrimp are shown in Figure 36.

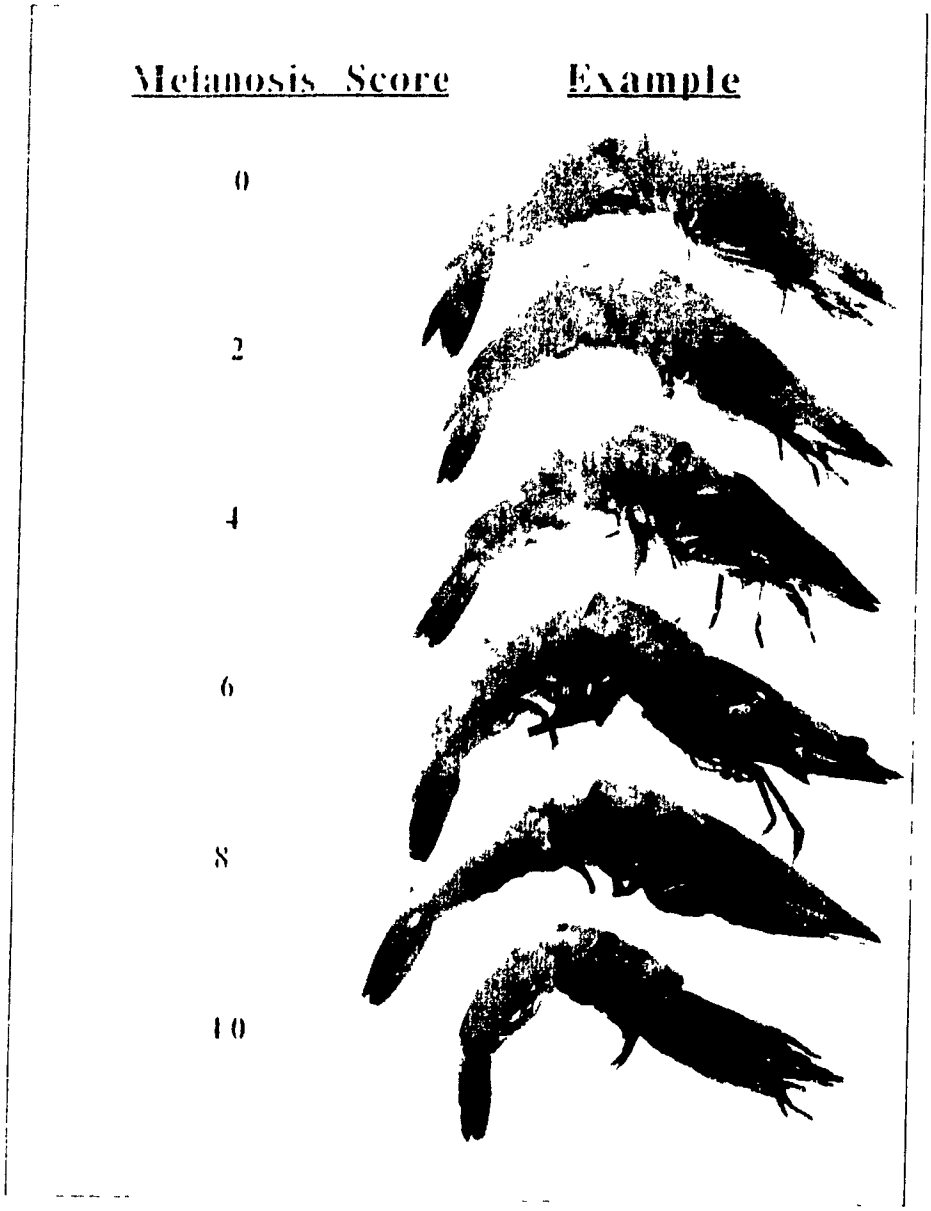
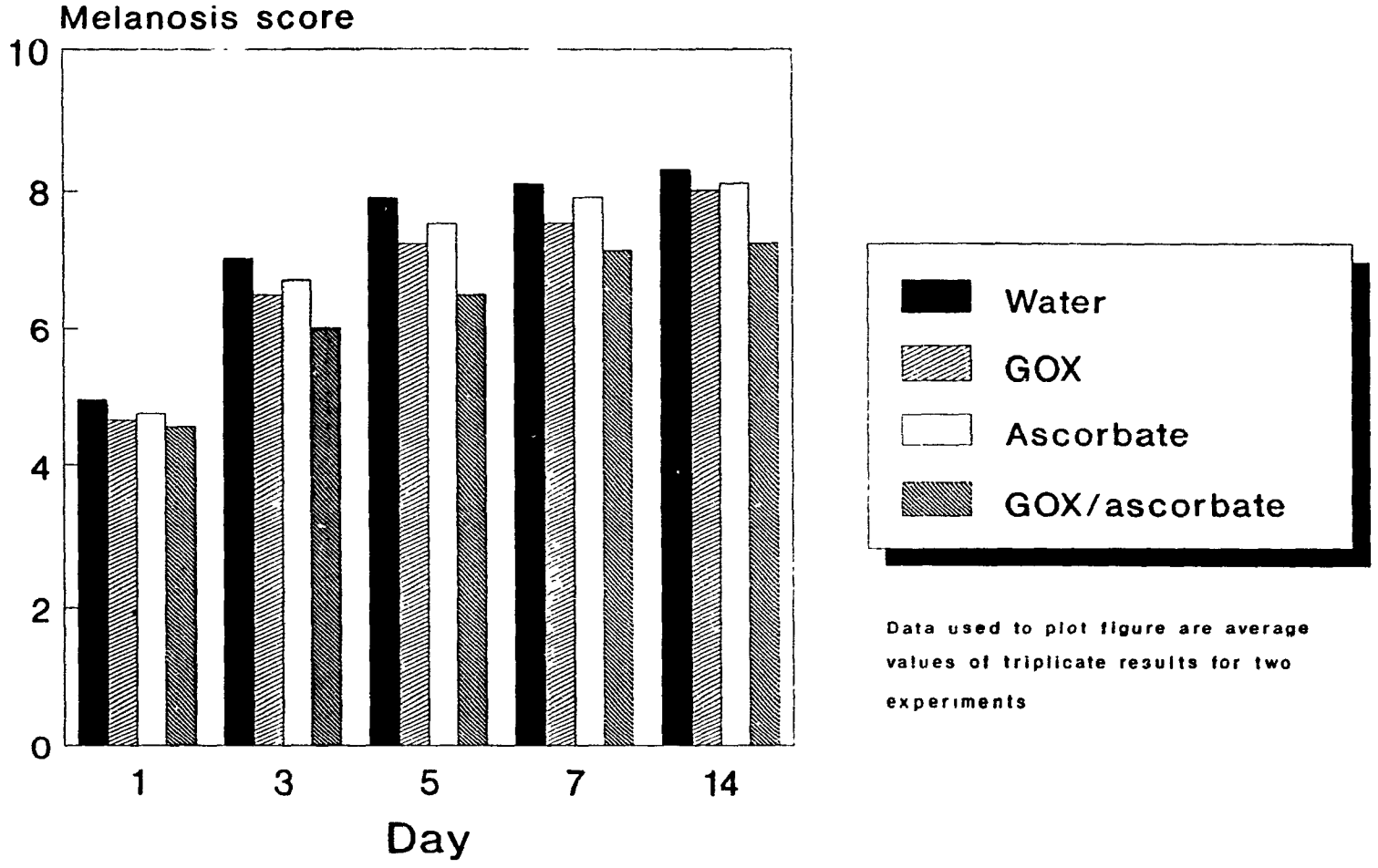


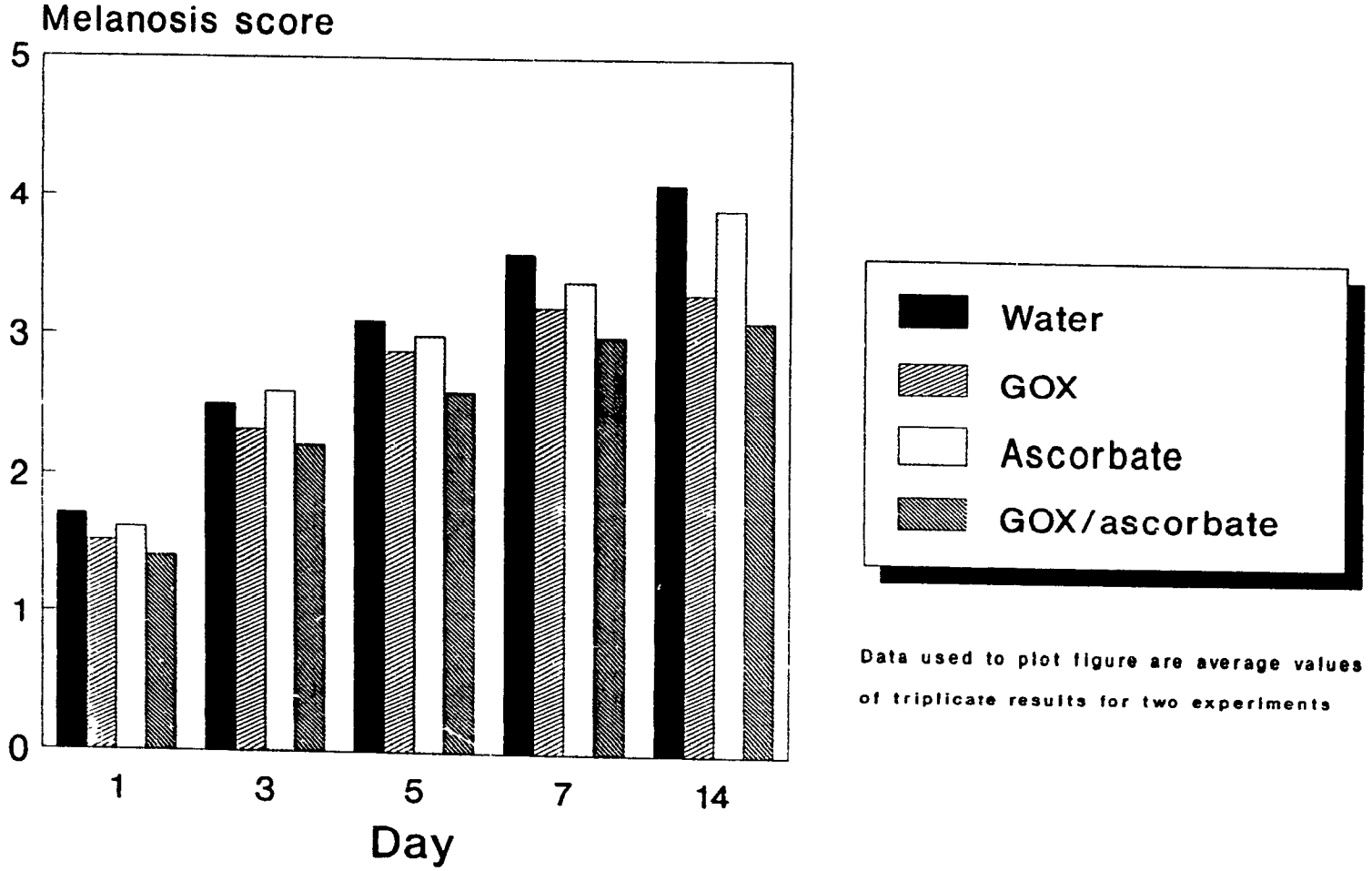
Figure 33. Examples of various degrees of blackspot development on pink shrimp *(P. dourarum)* and the corresponding melanosis scores (McEvily et al., 1991).

Figure 34. Melanosis score of pink shrimp packaged in air



Data used to plot figure are average values of triplicate results for two experiments

Figure35. Melanosis score of pink shrimp packaged in gas (CO_2:N_2/60:40)



Data used to plot figure are average values of triplicate results for two experiments

Figure 36. Examples of degree of blackspot development on pink shrimp (*P. borealis*) dipped in (i) GOX/glucose (ii) Ascorbate and (iii) GOX/glucose/ascorbate solutions and packaged in $C0_2$:N_2 (60:40) gas atmosphere. All samples stored at 4°C for 14d.

4. Summary of results and conclusion

A multiple comparison of means for the selected biochemical data and microbiological data for various dipping/packaging treatments of white shrimp are summarized in Tables 14-15 respectively. Means of each column with the same superscript are not significantly different from one another at the $p<0.05$ level. It is evident form Tables 14-15 that most dipping treatments for air packaged shrimp were significantly different from similar treated shrimp packaged under modified atmospheres. Shrimp dipped in GOX/glucose solutions and packaged in 60%CO_2:40%N_2 had consistently superior biochemical, melanosis scores and microbiological counts compared to other packaging treatments.

The extension in shelf life/melanosis scores for the various dipping/packaging treatments of white shrimp are summarized in Table 16. This shelf life was based on the time for aerobic plate counts to reach 10^6 cfu/gram and a melanosis score of 6. Based on this data the shelf life of white shrimp dipped in water or GOX/glucose dipping solution and packaged in air was terminated after 3-7 days at 4°C. However, shelf life almost be increased by 70% through the dipping shrimp in water prior to gas packaging. Longer extensions in shelf life were possible using GOX/glucose dipping solutions in conjunction with gas packaging and vacuum packaging.

Similar extensions in shelf life were also possible for pink shrimp dipped in various solutions prior to packaging under a CO_2:N_2 (60:40) atmosphere (Table 17). Ascorbic acid has been shown to be an effective dipping solution to control melanosis on cut fruits. However, it has not been reported to be as effective as sulfites to control melanosis on shrimp (Schwimmer, 1981). Nevertheless, in conjunction with a GOX/glucose dipping solution, it appears to enhance the ability of this solution to control melanosis on shrimp. Further studies are need to determine the maximum possible shelf life obtainable using ascorbic acid or its derivatives & GOX/glucose in conjunction with MAP to control melanosis on shrimp.

In conclusion, the combined use of dipping solutions in conjunction with MAP can significantly increase the shelf life of, and inhibit black spot development on, fresh white and pink shrimp. Such an extension in shelf life and keeping quality of fresh shrimp will obviate the need for sulfite dipping solutions to control melanosis. Furthermore, a reduction in frozen storage costs will enhance the marketability of fresh shrimp and accrue economic benefits in the form of energy savings to the Quebec and Canadian shrimp processing industry.

Table 14. Means*of selected biochemical data for white shrimp after 14 days at 4°C

Treatment	pH	Hx (umol/g)	TVB (mgN/100g)	Melanosis score
Water/Air	6.96±0.02a	4.75±0.07a	75.68±2.01a	8.2±0.31a
GOX/Air	6.34±0.01e	4.29±0.06c	60.69±0.74c	8.4±0.29a
Water/CO2:N2	6.77±0.04c	4.54±0.04b	51.21±1.25d	5.1±0.08c
GOX/CO2:N2	6.85±0.03b	3.69±0.09d	42.13±2.10f	4.7±0.11d
Water/Vacuum	6.49±0.03d	4.25±0.05c	64.26±1.13b	5.6±0.17b
GOX/Vacuum	6.83±0.02bc	3.40±0.03e	47.84±0.87e	5.5±0.02b

a - f means in the same column with the same letter superscript are not significantly different ($p<0.05$)

* Mean+standard deviation

Table 15. Means* of microbiological data for white shrimp after 14 days at 4°C

Treatment	APC	PSY	LAB
	(Log no. of cfu/g)		
WaterAir	7.94±0.03a	7.37±0.03a	7.15±0.01b
GOX/Air	7.71±0.04b	7.33±0.02a	7.23±0.02a
Water/CO_2:N_2	6.47±0.07d	6.26±0.04c	6.76±0.06d
GOX/CO_2:N_2	5.43±0.02f	5.76±0.01d	5.44±0.02e
Water/Vacuum	6.89±0.03c	6.46±0.01b	7.03±0.03c
GOX/Vacuum	5.84±0,05e	5.88±0.06d	7.07±0.04c

a - f means in the same column with the same letter superscript are not significantly different ($p<0.05$)

* Mean±standard deviation

Table 16. Shelf life of white shrimp at 4°C

Treatment	(Days)
Water/Air	3 - 6
GOX/Air	3 - 7
Water/CO_2:N_2	10 - 12
GOX/CO_2:N_2	> 14
Water/Vacuum	10 - 12
GOX/Vacuum	> 14

Based on time to reach a melanosis score of 6 & APC count of 10^6cfu/gram

Table 17. Shelf life of pink shrimp at 4°C

Treatment	Days
Water/Air	2
GOX/Air	2 - 3
Ascorbate/Air	2 - 3
GOX/Ascorbate/Air	3 - 4
Water/CO_2:N_2	> 14
GOX/CO_2:N_2	> 14
Ascorbate/CO_2:N_2	> 14
GOX/Ascorbate/CO_2:N_2	> 14

Based on time to reach a melanosis score of 6

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