THE QUANTIFICATION OF BIOGENIC AMINES IN LOW-TEMPERATURE STORED VACUUM-PACKAGED AND CARBON DIOXIDE MODIFIED ATMOSPHERE-PACKAGED FRESH PORK

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Celine Andree Nadon

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Food Science Department

September 1998

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

0-612-32945-3

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

THE QUANTIFICATION OF BIOGENIC AMINES IN LOW-TEMPERATURE STORED VACUUM-PACKAGED AND CARBON DIOXIDE MODIFIED ATMOSPHERE-PACKAGED FRESH PORK

BY

CELINE ANDREE NADON

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ACKNOWLEDGEMENTS

Sincere appreciation is extended to Dr. Anne Ismond for advising this research and for her valuable guidance and support throughout the project; to the Natural Sciences and Engineering Research Council of Canada for their financial support; to Jim Rogers for his excellent technical assistance, advice, and humour; to Lorna Gibson and the staff at the Lacombe Meat Research Station for their assistance with packaging and continued support; to Randy Roller, both for his SAS knowledge and humour; to Dr. R. Holley, Head, Food Science Dept, and Dr. R. Bird, Foods and Nutrition Dept, for their critical review of this manuscript; and to the graduate students of the Food Science Department, and my family and friends, for their practical and moral support of this endeavor.

This thesis is dedicated to KellyAnne Ross, for her unlimited friendship, support, technical advice, and laughter. Good times.

ABSTRACT

Biogenic amines are formed in foods as a result of amino acid decarboxylation, catalyzed by bacterial enzymes. When consumed in sufficient quantities, these compounds will cause headache, hypertension, fever, and heart failure. Technologies such as vacuum-packaging and carbon dioxide modified atmosphere-packaging (CO_2-MAP), when combined with low temperature storage (-1.5°C), allow fresh pork to have a storage life long enough for export to overseas markets. During low temperature storage of pork in these packaging systems, the lactic acid bacteria (LAB; which possess the enzymes for biogenic amine formation) dominate the microflora. The objectives of this study were to determine the quantities of biogenic amines in packaged fresh pork, to monitor LAB growth, and determine the storage life by sensory evaluation. Vacuum-packaged and CO_2-MAP pork were stored at -1.5 ± 0.5°C for 9 and 13 weeks, respectively. Phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine were determined weekly by HPLC and capillary gel electrophoresis. LAB and carnobacteria were enumerated weekly. Samples were evaluated for odour and appearance. The CO_2-MAP was successful in delaying bacterial growth and the development of unacceptable off-odours compared to the vacuum-packaging. The storage lives of the vacuum-packaged and CO_2-MAP pork were 5 and 13 weeks, respectively. HPLC was the superior method for biogenic amine quantification. Tyramine and phenylethylamine in pork of both packaging treatments approached levels considered to be potentially toxic. Given Canada's increasing role in the export of fresh meat to foreign markets, it is recommended that the formation of biogenic amines in vacuum-packaged and CO_2-MAP pork be further investigated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ABBREVIATIONS

ATCC	American Type Culture Collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydoxytoluene
CFU	Colony forming units
CGE	Capillary gel electrophoresis
CO_2-MAP	Carbon dioxide modified atmosphere-packaging
CTAS	Cresol red thallium acetate sucrose
DAO	Diamine oxidase
Fig	Figure
HMT	Histamine methyltransferase
HPLC	High-performance liquid chromatography
LD_{50}	Lethal dose for 50% of population
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MRS	DeMan, Rogosa, and Sharpe
NOAEL	No observed adverse effect level
ppm	Parts per million
r^2	Correlation coefficient
RIMA	Reversible inhibitors of monoamine oxidase type A
SD	Standard deviation
SSRI	Selective serotonin reuptake inhibitors
TTC	Triphenyl tetrazolium chloride

UNITS

C	celcius	L	liter
		ml	milliliter
d	day	µl	microliter
h	hour		
min	minute	M	molar
s	second	N	normal
		µV	microvolt
cc	cubic centimeter		
cm	centimeter		
g	gram		
kg	kilogram		
mg	milligram		
µg	microgram		

SECTION I

INTRODUCTION

Advances in packaging technology have led to longer shelf lives for fresh pork. Two different systems, vacuum and carbon dioxide modified atmosphere packaging, are effective in delaying the microbial spoilage of fresh pork stored at low temperatures. However, the dominance of lactic acid bacteria on fresh pork packaged with either of these two systems creates an environment favourable for the production of biogenic amines. The biogenic amines phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine cause mild to severe adverse reactions in animals and humans when ingested in sufficient quantities.

The overall objective of this research was to determine the quantities of biogenic amines in vacuum packaged and carbon dioxide modified atmosphere packaged pork stored for specific periods of time at a low temperature. Cuts of pork loin were packaged in either vacuum or carbon dioxide modified atmosphere packaging and stored at 9 and 13 weeks, respectively, at -1.5°C. At weekly intervals, the pork samples from each type of packaging were evaluated for: (1) lactic acid bacteria – using a standard method of enumeration on MRS agar; (2) carnobacteria – using a new method based on the difference in the growth on 2 types of growth agar; (3) biogenic amines - phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine were determined using two methods, high-performance liquid chromatography and capillary gel electrophoresis; (4) sensory properties – odour and appearance were evaluated by an eight-member panel. The two packaging systems were compared with respect to bacterial growth, biogenic amine concentrations, and sensory properties. Storage lives of the pork in each type of package were determined based on the samples'

odour and appearance. The two methods of biogenic amine determination were compared, and their results compared with bacterial growth and sensory properties. Finally, conclusions were drawn in order to establish the toxic potentials of vacuum and carbon dioxide modified atmosphere packaged pork. This research will be the first to determine the amounts of biogenic amines and therefore the toxicological risk of fresh pork packaged using these systems. Given Canada's increasing role in the international pork trade, information regarding the ability to provide safe, fresh pork to export markets such as the United States and Asia is needed.

SECTION II

REVIEW OF LITERATURE

A. Introduction

Biogenic amines are aliphatic or aromatic amines that have biological activity. Numerous mono-, di-, and polyamines are found in the human body with a wide range of physiological functions. These amines include the catecholamines, dopamine, epinephrine, and norepinephrine, and the non-catecholamines. The non-catecholamines are the monoamines, tyramine, histamine, phenylethylamine, and serotonin, plus the diamines, putrescine and cadaverine, and polyamines, spermine and spermidine. The catecholamines are critical for the function of the sympathetic nervous system and many other systems. The non-catecholamines are also of great importance in the human body with a wide variety of physiological functions (Franzen and Eysell, 1969). The endogenous amines have roles or are involved in the pathology of many disorders including tumours of the sympathetic nervous system, hypertension, muscular dystrophy, inflammation and allergy, renal disorder, Parkinson's disease, phenylketonuria, depression, and schizophrenia (Franzen and Eysell, 1969).

These amines appear naturally in food products simply because many of them are also endogenous to animals, plants, and bacteria (Smith, 1981). However, as endogenous as they may be, ingestion of these amines in substantial amounts causes a toxic response. Given the importance of the amines for normal body function and their involvement in such a wide variety of serious disorders, it is not surprising that consumption of biogenic amines in foods could have possible physiological consequences. As this study will demonstrate, certain conditions of food handling and processing have led to the

production and accumulation of biogenic amines in foods beyond levels tolerable by the human body. The amines of importance with respect to this study are histamine, tyramine, phenylethylamine, putrescine, cadaverine, spermine, and spermidine (Fig. 1).

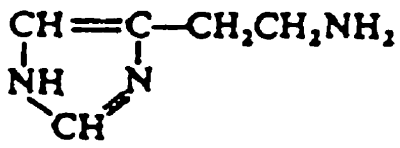
B. The Formation of Biogenic Amines in Food Products

Amino acid decarboxylation by microorganisms is the primary route of biogenic amine synthesis. Their production can typically accompany food spoilage or decomposition; however, they may form in foods before spoilage occurs, possibly to toxic levels (Shalaby, 1996). The availability of free amino acids, the presence of microorganisms capable of amino acid decarboxylation, and environmental conditions suitable for bacterial growth and enzyme production are all factors that govern the production of biogenic amines in foods (Shalaby, 1996).

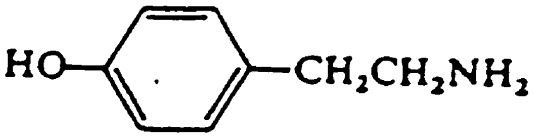
The biological function of the bacterial decarboxylases is a protective one. They can be considered to be secondary metabolites, since they have no critical importance for the cell's survival during the early stages of growth (Gale, 1946; Smith, 1981). The enzymes are produced in response to an acid environment and do not function above pH 6.0 (Gale, 1946). The action of the enzyme effects an increase in the pH of its surroundings toward neutrality as a result of the decarboxylations in attempt to protect the cell from harsh external conditions (Gale, 1946).

Figure 1. The structure of biogenic amines

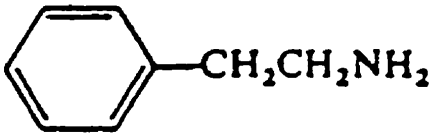
Histamine



Tyramine



Phenylethylamine



Putrescine

$NH_2(CH_2)_4NH_2$

Cadaverine

$NH_2(CH_2)_5NH_2$

Spermidine

$NH_2(CH_2)_3NH(CH_2)_4NH_2$

Spermine

$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

Free amino acids are decarboxylated by bacterial enzymes by removal of the α-carboxyl group to yield the corresponding amine with the liberation of carbon dioxide. The amino acid precursors for biogenic amines are ornithine, lysine, histidine, tyrosine, tryptophan, and phenylalanine. Once decarboxylated, these amino acids give rise to putrescine, cadaverine, histamine, tyramine, tryptamine, and β-phenylethylamine, respectively (Shalaby, 1996). Certain fish have high levels of free amino acids in their muscle tissue. For many other foods, proteolysis (bacterial or autolytic) is important for the release of free amino acids, which are subsequently available for decarboxylation (Shalaby, 1996). The mechanism of decarboxylation is well known and can be either pyridoxal phosphate dependent or non-pyridoxal phosphate dependent. When joined to the enzyme, pyridoxal phosphate creates the active site. The pyridoxal phosphate reacts readily with free amino acids which can be decarboxylated with the elimination of water. In the non-pyridoxal phosphate dependent mechanism, pyruvoyl is required instead and works in a manner similar to pyridoxal phosphate (Recsie and Snell, 1980; Snell *et al.*, 1975).

1. Organisms with Decarboxylase Activity

There are a number of bacteria capable of decarboxylating amino acids by the enzymes they produce. These include *Enterobacteriaceae*, *Clostridium*, and *Lactobacillus* (Shalaby, 1996). Many organisms isolated from meat and meat products, including group D *Streptococci*, the obligately heterofermentative lactic acid bacteria *Lactobacillus brevis*, *L. buchneri*, *L. divergens*, and *L. hilgardii*, the facultatively heterofermentative *L. carnis*, and the homofermentative *L. curvatus* are amine-producing (Shalaby, 1996). It has been shown that the *Lactobacilli*, *L. divergens* (now classified as *Carnobacterium*

divergens) and *L. carnis* (now *C. piscicola*) in particular, are capable of tyrosine decarboxylation to tyramine (Edwards *et al.*, 1987). Contaminant lactic acid bacteria in dry sausage fermentation are histamine, tyramine, and phenylethylamine producing (Eerola *et al.*, 1996); additionally some organisms used as starter cultures in the dairy industry (*Streptococcus lactis*, *Lactobacillus helveticus*) are histamine producing (Stratton *et al.*, 1991).

Lactobacillus and *Carnobacterium*

At this point it is appropriate to briefly distinguish *Carnobacterium* from *Lactobacillus*. From the perspective of this study, the lactic acid bacteria are by far the organisms of greatest importance, to be covered in subsequent sections. The lactic acid bacteria classically include *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacteria*, *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Vagococcus* (Jay, 1992). Lactobacilli can be identified as Gram-positive, non-sporeforming rods, catalase negative, and usually non-motile (Sharpe, 1981). The major classifications of the lactobacilli are homofermentative (producing more than 85% lactic acid from glucose) and heterofermentative (producing lactic acid, CO_2, ethanol, and acetic acid from glucose). In 1987, a new genus, the *Carnobacterium*, was established which previously were considered to be heterofermentative atypical lactobacilli (Collins *et al.*, 1987). Amoung the reclassified species were *Carnobacterium divergens*, *C. mobile*, *C. gallinarum*, and *C. piscicola* (Jay, 1992). The differentiating characteristics of the carnobacteria are that they have meso-diaminopimelic acid in their peptidoglycan, they produce only L-lactic acid,

and they do not grow on acetate (Rogosa) agar (Montel *et al.*, 1991). They are also non-aciduric.

C. Toxicology of Biogenic Amines – Acute Toxicity

1. Histamine

Histamine is a primary aromatic amine. It is found in a wide variety of food products. In most foods that have been handled properly (with respect to time and temperature of storage), the concentrations are usually too low to have any consequence in normal individuals (Taylor, 1986).

Histamine, a potent metabolite, is not foreign to the human body. In fact, endogenous histamine is involved in the allergic response (via tightly regulated release by the mast cells) and is responsible for the immediate symptoms of any allergy (Taylor, 1986). In addition to the allergic response, endogenous histamine is essential for the release of gastric acid.

Symptoms of Histamine Intoxication

Histamine poisoning manifests itself in a wide variety of symptoms. The onset of symptoms is usually quite rapid and will occur within 10 minutes to 2 hours after ingestion (Bartholomew *et al.*, 1987). Histamine can affect the cardiovascular system resulting in dilation of the peripheral blood vessels and capillaries, causing hypotension, flushing, and headache (Taylor, 1986). Additionally, histamine causes an increase in

capillary permeability which leads to the loss of plasma and therefore edema, urticaria, hemoconcentration, and increased blood viscosity (Taylor, 1986). Heart palpitations have also been recorded during histamine poisoning and may be due to the stimulatory effect of histamine on the heart (increasing the rate and strength of contractions).

Histamine affects smooth muscles by causing contractions, particularly in the bronchi and the intestinal tract. The contraction of the smooth muscles in the gastrointestinal tract leads to abdominal cramping, diarrhea, and vomiting (Taylor, 1986).

Finally, histamine is a potent stimulator of the sensory and motor neurons which results in the pain and itching associated with urticarial lesions in histamine poisoning (Taylor, 1986).

Histamine Metabolism

There is an apparent paradox with histamine poisoning. Oral doses of 300 mg pure histamine fail to produce a toxic response (Clifford *et al.*, 1989); however, histamine taken with food often produces symptoms which can be alleviated through antihistamine therapy (Taylor, 1986). This phenomenon can be explained by the presence of potentiating factors, which are present in foods but are lacking when histamine is administered as a pure compound. The potentiating factors (to be discussed in a subsequent section) effectively lower the threshold dose of histamine required to elicit a toxic response by altering histamine metabolism in the body (Taylor, 1986).

Under normal metabolic circumstances, the detoxifying enzymes diamine oxidase (DAO), monoamine oxidase (MAO), and histamine-N-methyltransferase (HMT), present in the liver, intestines, colon, spleen, and stomach, prevent the absorption of histamine from the gastrointestinal tract into the circulatory system. In humans, histamine is methylated by HMT and then undergoes oxidative deamination by MAO. The resulting acetaldehyde is further oxidized and excreted in the urine (Taylor, 1986). An alternate pathway for histamine metabolism involving DAO is favoured by some species including rats and guinea pigs, but not humans. In this pathway, DAO catalyzes the oxidative deamination of histamine, which can then be conjugated with ribose prior to excretion.

If an extremely high dose of histamine is administered that overwhelms these detoxification pathways, some histamine may be absorbed into the bloodstream and cause an adverse response. Greater than 100 mg of histamine per 100 g of food is considered probably toxic (Bartholomew *et al.*, 1987). Alternately, histamine may enter the bloodstream when it is accompanied by a potentiating factor. These potentiators include many pharmaceutical drugs ('monoamine oxidase inhibitors') and other amines present in foods (putrescine, cadaverine, tyramine, phenylethylamine, spermine, and spermidine) (Taylor, 1986). These compounds inhibit the activity of HMT, MAO, and DAO, allowing histamine to remain intact and available for uptake into the circulatory system.

The threshold toxic dose for histamine is not known (Taylor, 1986). Approximately 100 mg may need to be ingested with food before a toxic response is seen (Joosten, 1988). The types and amounts of potentiators in foods are extremely variable; thus

accurate predictions for the dose of histamine required for poisoning are difficult to make. There have been reports of histamine poisoning occurring with foods having histamine concentrations in the range of 70 mg to 1000 mg (Edwards and Sandine, 1981). Less than 5 mg histamine per 100 g of food is considered normal and safe for consumption (Bartholomew *et al.*, 1987).

2. Tyramine

Tyramine, another aromatic amine, is a pressor amine. This means it is a potently vasoactive compound. The toxicity of tyramine is quite complex and has been extensively studied.

Symptoms of Tyramine Intoxication

Tyramine poisoning results in a hypertensive crisis. This crisis is brought on indirectly by tyramine, which stimulates the release of noradrenaline (neurotransmitter) from the sympathetic nervous system (Joosten, 1988). The sympathetic nervous system controls many body functions, so ingestion of tyramine leads to a wide variety of physiological responses (Joosten, 1988). These include peripheral vasoconstriction and an increase in cardiac output resulting in elevated blood pressure. Tyramine also causes pupil and palpebral tissue dilation, lacrimation, salivation, increased respiration, and increased blood sugar (Joosten, 1988). Commonly reported symptoms include hypertension and headache. When ingested in high enough quantities, the hypertensive attack may result in severe headache, brain haemorrhage, and heart failure (Smith, 1981).

Tyramine has been investigated for its role in causing migraines. It was first observed that migraine sufferers recorded many of the same foods causing their attacks as the foods causing hypertensive crises in patients receiving monoamine oxidase inhibiting drugs (Joosten, 1988). It was believed that individuals susceptible to migraines had a reduced capacity for tyramine degradation. Initial research demonstrated that the ingestion of tyramine by migraine sufferers increased the rate of migraine attack compared to a placebo (Hanington, 1967). However, the importance of these results have since been disputed (Anon, 1968). Many of the foods claimed to cause migraines contain no tyramine. Additionally, there is no similarity between headache caused by high blood pressure and migraines. It is now generally accepted that tyramine probably is not an etiological factor in migraine (Joosten, 1988).

Tyramine Metabolism and Monoamine Oxidase Inhibitors

Tyramine metabolism is catalyzed by the same monoamine oxidase system as histamine. The enzyme catalyzes the oxidative deamination of tyramine to *p*-hydroxyphenylacetic acid which is excreted in the urine (Joosten, 1988). Many tyramine intoxications are a result of interactions with monoamine oxidase inhibiting (MAOI) drugs. These drugs, used for the treatment of mental depression, inhibit the action of monoamine oxidase thereby eliminating the detoxification system in the digestive tract and allowing orally administered tyramine to enter the circulatory system (Shalaby, 1996). Although selective serotonin reuptake inhibitors are currently more popular for the treatment of depression, MAOI anti-depressants are also still used (Kennedy, 1997).

The drugs classified as MAOI inhibit monoamine oxidase in the mitochondria of sympathetic neurons (Joosten, 1988). As a result, individuals taking these drugs accumulate large amounts of noradrenaline in their neurons, leaving them with an increased susceptibility to the actions of tyramine. Originally, iproniazid (or isoniazid) prescribed in the treatment of tuberculosis was noted for is euphoric effect. This prompted the use of similar drugs such as tranylcypromine and phenelzine for the treatment of depression (Blackwell, 1963). Currently, in addition to antidepressants, monoamine oxidase inhibitors involve a wide variety of drug classes including cytotoxic agents and antibacterial drugs. Individuals who are taking monoamine oxidase inhibitors are advised by their physicians to avoid foods rich in monoamines to reduce the risk of hypertensive attacks. Dietary restrictions have been in place since 1963; the most frequently restricted foods include wine, beer, aged cheese, non-fresh fish, broad bean pods, yeast extracts, fermented sausage, aged meats, chocolate, soy sauce, and many others (Blackwell, 1963; Lejonc *et al.*, 1979; Sullivan and Shulman, 1984). However, a review of prescribed MAOI diets revealed large discrepancies in the monoamine contents of the foods considered to be potentially hazardous for people on MAOI drug therapy (Sullivan and Shulman, 1984). It has been suggested that complete prohibition of cheese and pickled fish with the recommendation for eating a simplified diet in moderation should be sufficient in preventing hypertensive crises in MAOI patients (Sullivan and Shulman, 1984).

The toxic threshold dose for tyramine is not precisely known but is reported to vary from 20 – 400 mg (Joosten, 1988). Under normal metabolic conditions, (no interaction with

MAOI drugs), large quantities of foods extremely rich in tyramine must be ingested to elicit a toxic response (Joosten, 1988). Individuals taking MAOI drugs are extremely sensitive to tyramine and as little as 6 mg can trigger a hypertensive attack. As with histamine, other foodborne factors such as other amines may potentiate tyramine toxicity by inhibiting amine oxidases (Joosten, 1988).

3. Phenylethylamine

Phenylethylamine is another primary aromatic pressor amine that acts in a manner similar to tyramine, stimulating the release of noradrenaline from sympathetic neurons (Joosten, 1988). However, it is not as important or as potent as tyramine in causing hypertension (Rice *et al.*, 1976). Despite this, there is an abundance of evidence that suggests phenylethylamine may be a very important endogenous metabolite, and that dietary ingestion of phenylethylamine could have serious physiological consequences. During conditions of stress, urinary excretion of phenylethylamine increases (Paulos and Tessel, 1982). Due to its structural and pharmacological similarities with amphetamine, phenylethylamine has been associated with schizophrenia as a possible etiological factor. Paranoid schizophrenics have high concentrations of urinary phenylethylamine whereas non-paranoid schizophrenics do not (Potkin *et al.*, 1979). Additionally, phenylethylamine has been associated with migraine. Oral administrations as low as 3 mmol can trigger an attack (Sandler *et al.*, 1974). Phenylethylamine is proposed to be the link between chocolate and migraine headache (Joosten, 1988; Krikler and Lewis, 1965; Sandler *et al.*, 1974). These physiological and psychiatric effects of phenylethylamine suggest that its presence in foods may present a significant risk.

Phenylethylamine Metabolism

The monoamine oxidase system is also responsible for the metabolism of phenylethylamine (Joosten, 1988).

4. Putrescine and Cadaverine

The short-chain aliphatic diamines, putrescine and cadaverine, have less biological activity than histamine, tyramine, and phenylethylamine. Very large amounts of these amines must be ingested before any reactions are seen, which may include hypotension, bradycardia, dyspnoea, lockjaw, and paresis of the extremities (Franzen and Eysell, 1969). Putrescine is found naturally in almost all bacteria, plants, and animals and plays an important role in nucleic acid function, protein synthesis, and membrane stabilization (Smith, 1981).

The significance of both putrescine and cadaverine lies in their ability to potentiate the toxic responses caused by histamine, tyramine, and phenylethylamine. This potentiation has been shown in animal models. The administration of putrescine to animals increases the lethality of orally administered histamine. The proposed mechanism of action for the potentiation effect of both putrescine and cadaverine on histamine is that they induce increases in the rate of histamine absorption (Jung and Bjeldanes, 1979). Mucin creates a barrier to histamine absorption into the gut. Putrescine or cadaverine were shown to bind with mucin, compromising the effectiveness of the barrier (Jung and Bjeldanes, 1979). Cadaverine increases the transport of histamine across the gut wall by 29% (Jung and Bjeldanes, 1979). The greatest potentiation effect is seen when the ratio of cadaverine to

histamine is 1:2. Additionally, putrescine and cadaverine are inhibitors of MAO (Joosten, 1988)

Metabolism of Putrescine and Cadaverine

The degradation of both putrescine and cadaverine is catalyzed by diamine oxidase (Franzen and Eysell, 1969).

5. Spermine and Spermidine

Although histamine, tyramine, phenylethylamine, putrescine, and cadaverine are considered to be the most important biogenic amines in food products, spermine and spermidine also deserve recognition. This is based on their role in potentiation of monoamine toxicity. Spermine and spermidine are formed from putrescine by the donation of aminopropyl groups (Smith, 1981). These polyamines are found ubiquitously in plants, animals, and bacteria and have many physiological functions (Bachrach, 1973). The potentiating effect of spermine and spermidine may result due to their effect of increasing the transport of histamine across the gastrointestinal wall in the manner described for putrescine and cadaverine (Jung and Bjeldanes, 1979). The polyamines themselves are not toxic; and although they are present in many foods, the effect of exogenous polyamines on the body has not been studied.

Metabolism of Spermine and Spermidine

There are a wide variety of amine oxidases that metabolize polyamines. These include plasma amine oxidase, semen amine oxidase, and brain amine oxidase (Bachrach, 1973). These oxidases catabolize spermine and spermidine into diamines which presumably could be further acted upon by diamine oxidase (Bachrach, 1973).

D. Toxicology of Biogenic Amines - Carcinogenicity

The acute toxicity of the biogenic amines found in foods is the focus of this study. However, there is another aspect of amine toxicity that deserves mention at this point. Amines, including the biogenic amines, are potentially mutagenic precursors that may form carcinogenic nitrosamines (Shalaby, 1996). Upon heating, the diamines, putrescine and cadaverine, are converted to pyrrolidine and piperidine, respectively (Warthesen *et al.*, 1975). In the presence of nitrites and heat, the formation of *N*-nitrosopyrrolidine and *N*-nitrosopiperidine will result. Additionally, tyramine, spermine and spermidine are also nitrosatable *in vivo* (stomach) (Shalaby, 1996). Nitrosamines have been identified as important factors in the etiology of several cancers such as liver, bladder, kidney, pancreas, and gastrointestinal tract (Tannenbaum, 1980). Further, the formation of nitrosamines in the stomach have been found to be highly significant in the development of nitroso-induced cancers (Lijinsky, 1980).

E. The Occurrence of Biogenic Amines in Foods

1. Fish

Fish and fish products have by far received the most attention regarding biogenic amines, with histamine in particular (Shalaby, 1996). Until recently, 'histamine poisoning' has been synonymous with 'scombroid poisoning'. The majority of histamine poisoning outbreaks have been associated with fish of the Scomberesocidae and Scombridae families, which include all species of tuna and some mackerel. Some non-scombroid fish including herring, sardine, bluefish, mahi-mahi, and anchovy, have also been implicated (Taylor, 1986). Cadaverine, also found in fish, is said to be responsible for potentiating the toxic action of the large amounts of histamine (Bjeldanes *et al.*, 1978). Fish such as tuna can contain between 2040- 5000 μg/g of histamine (Rice, *et al.*, 1976, Soares and Gloria, 1994). In fish, the amount of histamine produced is related to the large amounts of free histidine in their tissue (Taylor, 1986). Histidine decarboxylase is an inducible enzyme; thus high levels of free histidine favours enzyme activity (Rice *et al.*, 1976). Tunafish has up to 15 g histidine per kilogram and herring, approximately 1g/kg (Shalaby, 1996). Additionally, both microbial activity and storage temperature have positive correlation with histamine production from histidine (Rodriguez-Jerez, *et al.*, 1994; Wei *et al.*, 1990). Since histidine decarboxylation usually is a part of the spoilage process as a result of bacterial growth, it would be logical to expect unacceptable sensory properties of fishery products to be a good predictor of histamine content; however, this may not be the case since fish with large amounts of histamine may have normal odour and appearance (Shalaby, 1996). More recent work has demonstrated a strong correlation

between the acceptance or rejection of canned tuna and the levels of putrescine and cadaverine, which increase with time and temperature (Sims *et al.*, 1992). A level of 0.4-0.5 μg/g of the total putrefactive amines was suggested as a cut-off point for acceptance.

2. Cheese

Cheese has also been implicated as a source of histamine in poisoning outbreaks and is second to fish (Stratton *et al.*, 1991). During 1967 – 1983, many outbreaks of confirmed histamine poisoning occurred in the Netherlands, United States, Canada, and France. These outbreaks have been reviewed by Stratton *et al.* (1991). The implicated cheeses included Gouda (85 mg histamine per 100 g of cheese) , Swiss (>100 mg/100g), Cheddar (40mg/100g), and Gruyere (30 mg/100g). There were also reports of 4mg/100g causing histamine intoxication in Washington DC (Edwards and Sandine, 1981). This led to the need for evaluating foods for potentiating diamines; namely putrescine and cadaverine.

The Cheese Reaction

Although histamine formation in cheese has been demonstrated and foodborne illness has occurred as a result, tyramine is beyond a doubt the most important biogenic amine in cheese. In initial studies, many patients receiving MAOI drugs found cheese caused a severe hypertensive attack, which was subsequently called the 'cheese reaction'. This term is now a misnomer since it is known that any food rich in tyramine will induce a 'cheese reaction' or hypertensive attack.

Amine production in cheese has been related to certain microorganisms involved with the aging process that produce large amounts of histamine during ripening (Stratton *et al.*, 1991). The length of the aging process is related to amine production in cheese (Blackwell and Mabbitt, 1965). Old cheeses contain more biogenic amines due to more extensive proteolysis leading to more available free amino acids (Joosten, 1988). Joosten (1988) found that cheeses aged for 9 months had up to 880 mg/kg of tyramine and 640 mg/kg of histamine, cheeses aged for 23 months had 1070 mg/kg tyramine and 1020 mg/kg histamine, whereas good quality cheeses aged for only 3 months had 374 mg/kg tyramine and 40 mg/kg histamine. Bacterial quality is critical in producing cheese with a low risk for amine accumulation. High quality cheese made from pasteurized milk contains fewer biogenic amines than cheese made from raw milk; and cheese with excessive gas production due to the growth of contaminating non-starter culture organisms has more biogenic amines (Joosten, 1988). Additionally, the tyramine content of cheese is not related to either appearance or flavour (Blackwell and Mabbitt, 1965).

In recent years there has been interest in using bacteriocin-producing starter cultures in the dairy industry. The theory is that the bacteriocin-producing starter cultures will reduce the prevalence of *Listeria monocytogenes* in unpasteurized dairy products (Joosten *et al.*, 1995). However, suitable bacteriocin-producing starter cultures (*Enterococcus faecalis*) possess tyrosine decarboxylase activity, which leads to tyramine formation during ripening, up to 195 mg/kg (Joosten *et al.*, 1995). Research with *E. faecalis* is ongoing only in Europe. Canada does not use this organism, a suspected pathogen, as a starter culture. Work isolating mutant strains of *E. facaelis* without tyrosine

decarboxylase is under way; thus far mutant strains have been found to be effective in controlling both *Listeria* and tyramine formation in cheese (Joosten *et al.*, 1995).

3. Sausages

Sausages are additional food products that have received much biogenic amine attention. During the fermentation of semi-dry and dry sausages, biogenic amines are formed due to bacterial activity (Smith, 1981). The effect of microorganisms on amine formation in sausages is directly related to starter culture. Semi-dry sausages are usually fermented for short periods of time using a lactic acid starter culture, whereas dry sausages are allowed to ferment for longer periods of time, usually by the action of natural microflora instead of a starter culture (Shalaby, 1996). Canada now uses started cultures for dry sausage fermentation. The use of a starter culture can reduce the levels of all amines in sausages (Hernandez-Jover *et al.*, 1997a; Maijala *et al.*, 1995a). Maijala and colleagues (1995b) also found that high quality raw materials could result in sausages of a wide range in quality (ie. a wide range of biogenic amine levels). The final quality depends on factors such as the starter culture, the temperature of fermentation, and also the quality or freshness of raw materials (Maijala *et al.*, 1995a;1995b).

As with cheese, there is interest in using starter cultures for sausage manufacture that have no amine-producing capabilities. Amine-negative starter cultures (*Pediococcus pentosaceus* and *Staphylococcus carnosus*) reduce the amount of tyramine and phenylethylamine in fermented sausages (Eerola *et al.*, 1996). However, the use of an amine-negative starter culture is futile if the sausage manufacturing process is not carefully controlled (Hernandez-Jover *et al.*, 1997b). Contamination by amine-positive

lactic acid bacteria results in a final product with higher levels of biogenic amines, up to 177 mg/kg tyramine and 67 mg/kg phenylethylamine (Eerola *et al.*, 1996). Similar results have been found with histamine. The use of histidine-decarboxylating starter cultures such as *Lactobacillus hilgardii*, *Morganella morganii*, and *Klebsiella oxytoca*, together with a fermentation temperature of 18°C results in a sausage with 1140 mg/kg of histamine; whereas the same amine-positive cultures and a fermentation temperature of 7°C reduces histamine levels to just over 100 mg/kg (Kranner *et al.*, 1991). Thus the use of amine-negative starter cultures and lower fermentation temperatures are needed in order to reduce the risk of food poisoning from amines in fermented sausages.

4. Fermented Beverages

Fermented beverages such as beer and wine can, on occasion, contain histamine and other biogenic amines (Stratton *et al.*, 1991). It is suspected that it is the biogenic amines that are responsible for the headache and flushing commonly noted with alcoholic beverage consumption (Stratton *et al.*, 1991). During the fermentation of beer, it has been found that only tyramine and tryptamine increase. The other amines (histamine, phenylethylamine, cadaverine, putrescine, spermine, and spermidine) come from raw materials (hops and malt) and the mashing process (Izquierdo-Pulido *et al.*, 1994). A survey of Canadian, American, and European beers revealed ranges of 4.50-7.27 mg/L of histamine and 10.0-15.0 mg/L of tyramine; equivalent to 1.60-2.58 mg and 3.55-5.32 mg in a 12 oz bottle of beer, respectively (Zee *et al.*, 1981). Levels of amines detected in a single final product have usually been below any levels to induce toxic responses, however considering the large quantity of beer consumed there may still be a hazard

(Izquierdo-Pulido *et al.*, 1994; Stratton *et al.*, 1991). Red and white wine have been found to contain an average of 2.39 mg histamine per liter of wine (Ough, 1971). Baucom *et al.* (1986) found that white wine had higher putrescine, cadaverine, and tyramine levels than red wines in New York State. In both red and white wines, histamine, putrescine, and tyramine levels were all less than 4 mg/L and cadaverine was 29.4 mg/L in the white wine and 19.5 mg/L in the red (Baucom *et al.*, 1986).

5. Fresh Meat

With the exception of fish, the majority of foods likely to contain biogenic amines are fermented products. This simply demonstrates the necessity for bacteria-induced decarboxylation in amine production. Thus it may appear surprising that fresh products such as meat that do not undergo a bacterial fermentation or ripening are considered as having potential for biogenic amine accumulation. Nonetheless, these products have been shown to contain many biogenic amines at various levels.

Spermine and spermidine are found in almost all fresh beef and pork. Spermine averages 33.5 mg/kg in fresh pork and 39.8 mg/kg in fresh beef; and spermidine 3.0 mg/kg in fresh pork and 3.1 mg/kg in fresh beef (Hernandez-Jover *et al.*, 1997b). Spermine and spermidine appear to be naturally occurring components of fresh meat (Hernandez-Jover *et al.*, 1997b). The presence of the other amines in fresh meat is considered rare and a result of bacterial growth, perhaps due to temperature abuse (Hernandez-Jover *et al.*, 1997b). Tyramine, putrescine, and cadaverine formation have been shown during meat storage over time (Hernandez-Jover *et al.*, 1996b). While tyramine, histamine,

putrescine, and cadaverine all increased during storage, spermidine remained constant and spermine decreased (Shalaby, 1996). An index has been suggested to indicate meat freshness. The sum of cadaverine, putrescine, histamine, and tyramine is a predictor of meat hygiene. A product with a sum of 5 mg/kg or less is said to be of high hygienic quality (Hernandez-Jover *et al.*, 1996a).

F. Meat Packaging Technology

1. Introduction to Vacuum Packaging

Vacuum packaging is one of the most important packaging technologies. Foods with longer shelf lives are becoming increasingly necessary as large-scale food production and wide-range distribution become more popular (Smith *et al.*, 1993). The prolonged shelf life created by vacuum packaging creates greater marketing potential; fresh products can be in transit for longer periods and reach new and distant markets (Krizek *et al.*, 1995). To create a vacuum package, meat is placed in plastic bags with low gas permeability (polyvinylidene chloride or nylon/polyethylene). The packages are then evacuated and immediately heat-sealed (Jay, 1992).

Microbiology of Aerobically Stored Meats

Fresh cuts of meat such as beef, pork, and lamb initially frequently contain organisms including *Acinetobacter*, *Aeromonas*, *Enterococcus*, *Moraxella*, *Pseudomonas*, and *Psychrobacter* (Jay, 1992). Additionally, yeast and mold species of *Cladosporium*,

Geotrichum, *Mucor*, *Rhizopus*, and *Candida* can be isolated (Jay, 1992). Meat is usually stored under refrigeration, hence temperature is the major determinant of which organisms will survive and proliferate in muscle tissue. The spoilage of refrigerated meat is usually a surface phenomenon, which reflects external sources of microorganisms (Ingram and Dainty, 1971). The 'surface' is defined as the top few millimeters of the meat (Gill, 1983). Since fresh meats have relatively high available moisture, even at the surface (42-70% water), gram-negative bacterial spoilage is favoured over mold spoilage. During spoilage, bacterial numbers will increase and the pH will increase (Jay, 1992). The pH can change from approximately 5.6 in fresh meat to 8.5 in putrid meat (Shelef and Jay, 1970). Spoiled meat will typically develop surface slime due to the coalescence of the surface colonies, causing a tacky consistency (Jay, 1992). The psychrotrophic pseudomonads usually dominate the spoilage flora. Among the fresh meat organisms, pseudomonads grow the fastest, especially at high available surface O_2 (Gill and Newton, 1977). These organisms are the quickest to utilize available low-molecular weight soluble components such as simple carbohydrates (Gill, 1983). When the carbohydrates are exhausted, the pseudomonads (and other gram-negative organisms, *Moraxella*, *Alcaligenes*, and *Aeromonas*) will begin to utilize free amino acids and other nitrogenous compounds (Jay, 1992). When the surface bacterial count reaches log 7.0 to 7.5 /cm^2, odours can be detected, and at approximately log 8.0/cm^2 a slime layer becomes evident (Ayres, 1960). At this point the organisms will begin producing proteolytic enzymes and penetrate further into the muscle tissue (Gill, 1983). The odours usually are due to the production of ammonia, hydrogen sulfide, indole, and amines (Jay, 1992).

Microbiology of Vacuum Packaged Meats

The success of vacuum packaging in extending the storage life of fresh meats is directly due to the effect the packaging technology has on microbial growth. Currently, at least 80% of fresh beef in the US is vacuum packaged (Jay, 1992). The distinguishing feature of a vacuum package is the shift from an aerobic to an anaerobic environment. Generally *Lactobacillus* spp. and *Brochothrix thermosphacta* will dominate refrigerator-spoilage of vacuum-packaged meat (Jay, 1992). However, *B. thermosphacta* will not grow below pH 5.8 so the lactic acid bacteria usually dominate (Gill, 1983). The spoilage of vacuum-packaged meat dominated by lactic acid bacteria will develop slowly as short-chain fatty acids accumulate (Gill, 1983).

Vacuum packaging has been extensively applied to beef with great success. With fresh beef, a shelf life of up to 45 days at refrigeration temperatures (4-5°C) has been reported (Krizek *et al.*, 1995). Dropping the temperature down to 0°C increases the storage life to 10-12 weeks (Shay and Egan, 1986). With fresh pork; however, vacuum packaging has not enjoyed the same success. At refrigeration temperatures vacuum-packaged fresh pork with high initial hygienic quality will last for up to 21 days, and 6 weeks at 0° (Shay and Egan, 1986). The pH of normal pork is approximately 5.4-5.8, which is usually slightly higher than the normal pH of 5.3-5.6 in post-rigor beef (Jay, 1992; Shay and Egan, 1986). The lower pH of beef inhibits bacterial growth more than the higher pH of pork. This, of course, leads to accelerated spoilage (Shay and Egan, 1986).

Biogenic Amines and Vacuum Packaging

The anaerobic environment created by the lack of atmosphere in a vacuum package that achieves the extended storage life also, unfortunately, creates an environment suitable for biogenic amine production. The dominance of lactic acid bacteria in this condition is responsible. As was discussed in a previous section, many of the *Lactobacillus* and *Carnobacterium* sps. have decarboxylating capabilities. Of the bacteria found in vacuum packaged beef, *Carnobacterium divergens*, *C. piscicola*, and other *Lactobacillus* spp. were capable of decarboxylation to produce putrescine, cadaverine, histamine, tyramine, spermidine, and spermine. *Leuconostoc* spp., *B. thermosphacta*, *Pseudomonas* spp., *Enterobacter agglomerans*, and *Citrobacter freundii* were unable to produce cadaverine and tyramine; and *Hafnia alvei* and *Serratia liquifaciens* were unable to produce tyramine (Edwards *et al.*, 1987).

The effects of vacuum packaging on biogenic amine production in fresh beef have been investigated. There are now a few studies that have examined tyramine, histamine, and phenylethylamine; most previous studies on fresh or vacuum packaged meat considered only the putrefactive diamines, putrescine and cadaverine. Because of their offensive odours and flavours, these compounds have been obvious candidates for research (Dainty *et al.*, 1986; Smith *et al.*, 1993). It is now known that the aromatic monoamines also accumulate in vacuum packaged meat. In fresh, vacuum packaged beef stored at 1°C for 120 days, tyramine was undetected until around day 20, and then increased to 286 μg/g (Smith *et al.*, 1993). The production of tyramine correlated with the growth of lactic acid bacteria; tyramine production began when lactic acid bacteria approached log 4 colony

forming units (CFU)/cm^2. At 90 days of storage (after which the meat was considered inedible), the meat contained almost 150 μg/g. Treating the meat with either a sanitizing spray (200 ppm chlorine) or a 3% lactic acid spray had no effect on either lactic acid bacteria growth or tyramine production. They concluded that enough tyramine was formed after 60 days (60 μg/g) to be potentially toxic. A 200 g serving of steak would contain 12 mg of tyramine, capable of producing a mild to moderate reaction particularly in susceptible individuals (Smith *et al.*, 1993).

In a similar study, Krizek *et al.* (1995) investigated biogenic amine accumulation in vacuum packaged beef stored at -2°C and 2°C for 100 days and found comparable results. When stored at 2°C, significant tyramine production began after 20 days, whereas putrescine and cadaverine production did not begin until after 40 days. By 100 days, tyramine accumulated to 150 μg/g, putrescine to 40 μg/g, and cadaverine to 50 μg/g. Decreasing the storage temperature to -2°C was successful in delaying the production of significant tyramine to after 40 days, and putrescine and cadaverine at 60 days. The diamines reached final concentrations of 100 μg/g putrescine and 25 μg/g cadaverine. However, by the end of the storage period, the vacuum packaged beef stored at -2°C still accumulated 140 μg/g of tyramine, which was not significantly different from that at 2°C. Lactic acid bacteria in these samples reached the stationary stage of growth at times that coincided perfectly with the time that significant biogenic amine production began; after 20 days at 2°C and 40 days at –2°C. At these times, lactic acid bacteria were present at log 6-7 CFU/g at both temperatures, and tyramine was almost 20μg/g at –2°C and 60 μg/g at 2°C. These results are in agreement with the findings of

Smith *et al.* (1993) and clearly present a toxic hazard. The particular significance of the work of Krizek *et al.* (1995) lies in the demonstration that toxic levels of tyramine were reached before the meat was spoiled, regardless of storage temperature. Putrescine and cadaverine levels have been used as indicators of meat spoilage. However, we now see that there is enough tyramine forming in vacuum packaged beef to be toxic even before the production of the putrefactive amines begins. Hence after 20 days at 2°C and 40 days at –2°C the meat has normal odour, appearance, and flavour, but is potentially toxic.

The effect of vacuum packaging on biogenic amine production in fresh pork has not been studied. However, it is logical to assume that since these amines have been shown to accumulate in vacuum packaged fresh beef, they may also be produced in pork packaged and stored under similar conditions.

2. Introduction to Carbon Dioxide Modified-Atmosphere Packaging

Carbon dioxide modified-atmosphere packaging (CO_2-MAP) is another recently developed packaging technology that extends the shelf life of fresh meats. Newer than vacuum packaging, this technology is not currently as extensively applied to the storage and transport of fresh meat. However, the effects of this system have already been extensively studied. The processing technology is merely a modification and extension of the vacuum packaging process. Meat is placed in gas-impermeable aluminum foil laminate pouches. The pouches are evacuated, and then immediately filled with carbon dioxide prior to heat sealing. Carbon dioxide at a rate of 2 L/kg is considered optimum (Greer *et al.*, 1993). The combination of the gas impermeable film, optimal storage

temperature (-1.5°C), high levels of CO_2, and low residual oxygen (≤ 500 ppm) create the most effective packaging system for preserving fresh meat (Jeremiah *et al.*, 1995).

Unlike vacuum packaging, CO_2-MAP is very successful in delaying the spoilage of fresh pork. In particular, this system is a form of modified atmosphere packaging known as controlled atmosphere packaging. The atmosphere of CO_2 remains unchanged during storage and is therefore considered to be 'controlled'. With proper preparation and packaging, a storage life of up to 15 weeks is feasible according to appearance and odour (Jeremiah *et al.*, 1992b); however, 9 weeks is the average storage life after which spoilage is evident as off-flavours (Jeremiah *et al.*, 1992b). CO_2-MAP is particularly suitable for normal pork since this meat is relatively insensitive to oxidative deterioration at low oxygen concentrations (Jeremiah *et al.*, 1992a). This results in a stable, desirable colour throughout the storage period. There is usually some residual oxygen in the package, but in very minute quantities. Other red meats such as beef and lamb do not have a stable colour and will rapidly turn brown when there is as little as 0.05% oxygen present in the headspace (Jeremiah *et al.*, 1992a). The undesirable brown colour is a result of the oxidation of red myoglobin to brown metmyoglobin (Ordonez and Ledward, 1977). Even if the residual oxygen in the modified atmosphere packages of red meat is extremely low or negligible, the meat may retain its red colour; however, once the meat is exposed to air (for retail display, for example) the colour becomes unstable (Seideman *et al.*, 1980).

The Effect of Carbon Dioxide on Microorganisms

The secret of carbon dioxide's success in extending the shelf life of fresh meat is its effect on microorganisms. As with vacuum packaging, the anaerobic, high carbon dioxide atmosphere inhibits the growth and/or survival of many organisms present on meat, allowing the lactic acid bacteria to dominate.

The inhibitory effects of CO_2 increase with decreasing temperatures (due to the improved solubility of CO_2) (Jay, 1992). Carbon dioxide decreases generation time and increases the lag phase of bacterial growth (Dixon and Kell, 1989).

The mechanism of CO_2 inhibition of bacterial growth is not completely understood. The various theories regarding this mechanism have been outlined by Dixon and Kell (1989). The ability of CO_2 to alter membrane properties is proposed to be responsible. Carbon dioxide interacts with the lipids of the cell membrane, thus changing their permeability. The effect of carbon dioxide on microorganisms has been called anaesthetic. The interaction of CO_2 and lipid results in an expanded hydrophobic region. This expansion and resultant alteration in the lipid bilayer are thought to affect the function of membrane proteins, which then in turn is responsible for the anaesthetic effect. The CO_2-lipid interactions fall under the *lipid theories of narcosis*. In the contrasting *protein theories of narcosis*, the CO_2 is thought to exert its anaesthetic effects via direct alteration of membrane proteins. Carbon dioxide may also have an effect on the cytoplasm, converting it to a partially gelled condition due to cytoplasmic protein interactions.

During long term storage (24 weeks) of fresh pork in CO_2-MAP at −1.5°C, the lactic acid bacteria, which are CO_2-resistant, were the only organisms able to grow (Greer *et al.*, 1993). The pseudomonads, enterics, and *B. thermosphacta* were either undetectable or declined to undetectable after the first week of storage. The microbial population of CO_2-MAP pork is fairly well defined (McMullen and Stiles, 1993). At −1°C, the majority of the lactics were *L alimentarius*, *L. farciminis*, and *L. sake* (homofermentative). The heterofermentative lactic acid bacteria were *L. viridescens* (now *Weissella viridescens*) and *L. minor*. *C. divergens* was also isolated. The importance of the carnobacteria is difficult to determine. Using a plate count agar (PCA), 85% of the isolates were principally carnobacteria with some homofermentative lactobacilli, but using MRS medium 62% of the isolates were homofermentative and carnobacteria were rarely isolated (McMullen and Stiles, 1993). The different media used for their isolation explains this apparent discrepancy. The MRS lactic acid bacteria medium may be selective for certain types of lactobacilli, and acetate in the medium may have slowed the growth of carnobacteria (Schillinger and Holzapfel, 1990).

CO_2-MAP and Biogenic Amines

The fact that the lactic acid bacteria and perhaps the carnobacteria in particular are extremely important in CO_2-MAP meats is significant due to their known ability to decarboxylate amino acids. The effects of this packaging technology and long term, low temperature storage on the formation of biogenic amines in either beef or pork have not yet been studied. However, with the current information available regarding vacuum

packaging and biogenic amines, lactic acid bacteria, and the microbial ecology of CO_2-MAP pork, it is reasonable to assume it is likely that some biogenic amines will form.

G. The Canadian Pork Industry

The safety of vacuum and modified atmosphere packaged pork is of interest to Canada. Canada is significantly increasing its pork exports and has the advantage of providing fresh pork instead of frozen. This has enabled the country to export large amounts of fresh pork to Asian markets. Ensuring the effectiveness of vacuum and controlled atmosphere packaging is critical in maintaining Canada's reputation and ability to supply high quality fresh pork to its export markets.

H. Risk Assessment

The concept of risk assessment is an important consideration for any hazardous component in food or the environment. A hazard is a biological, chemical, or physical agent or property of food that may have an adverse health effect; whereas risk is the function of the probability of an adverse effect and the magnitude of that effect consequential to the hazard in food (Huggett *et al.*, 1996). In general, the term 'risk assessment' is usually applied to determining risk of cancer, but nonetheless can be applied to any hazard (Hathcock and Rader, 1994). Assessing a risk usually is through a quantitative measurement by a mathematical model. For some types of hazards, this

model is relatively straightforward and would involve dose-response studies (usually in animals), estimation of human exposure, and then calculating the correlation between animals and humans in a given situation to arrive at a human risk value (Hathcock and Rader, 1994). Although straightforward, there are problems inherent in this method such as the difficulties encountered when extrapolating experimental animal data to humans (Hugget *et al.*, 1996). Ultimately; however, a decision must be reached regarding the acceptability or tolerance of the calculated risk.

A factor that becomes extremely important when making that decision is a risk/benefit analysis. In addition to the risks, a hazard may be accompanied by many benefits and these must also be carefully considered (Brown, 1980). For example, the addition of preservatives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitrite, and sulfite to foods do present certain risks to human health. However, the benefit of decreased microbial growth, longer storage lives, plus stable colours and flavours are perceived by many to outweigh the risk of adverse effects (Hathcock and Rader, 1994).

1. The Problem with Amines

With respect to biogenic amines in foods, risk assessment becomes an uncertain endeavor. While there is no dispute that biogenic amines are a hazard, assessing the level of the hazard and therefore the risk is extremely difficult. An accurate dose-response relationship is almost impossible to create due to the confounding variables (monoamine oxidase inhibiting drugs and potentiating amines). While the severity of symptoms are

dose-related, a dose of 100 mg of a biogenic amine may cause no response in one individual whereas 6 mg can cause a severe hypertensive attack in another. Additionally, calculations of LD_{50}'s and NOAEL's are difficult and would be essentially futile in the case of biogenic amines, since the ultimate threshold doses of these amines are highly dependent on the confounding variables, thus rendering the LD_{50}'s (lethal dose) and NOAEL's (no observed adverse effect level) of the pure compounds inapplicable.

Some have suggested that there has been a gross over-reaction to the potential hazards of biogenic amines in foods (Sullivan and Shulman, 1984). As described in a previous section, immediately after data regarding the amine content of various foods were published, extremely strict dietary guidelines were put forth in order for patients on MOAI drug therapy to avoid the risk (McCabe, 1986; Sullivan and Shulman, 1984). It would appear this extreme approach – to avoid any and all risks with little regard for complete clinical evidence – was done without consideration of the benefits, such as the enjoyment of a varied diet. Newer drugs have been developed for the treatment of depression, selective serotonin reuptake inhibitors (SSRI), which do not compromise the patients' ability to metabolize amines. An even newer class of anti-depressants, selective reversible inhibitors of monoamine oxidase type A (RIMA), also do not interact with tyramine (Davidson, 1997). However, even with the development of SSRI and RIMA, many psychiatrists maintain that the classical MAOI's are an extremely effective anti-depressant therapy and should not be neglected (Kennedy, 1997). The excessive restrictions that have accompanied MAOI drug therapy have prevented many individuals from the benefits of such drug treatment (Sullivan and Shulman, 1984). Also, these

dietary restrictions lead clinicians to avoid prescribing what may be a highly effective treatment for depression (Gardner *et al.*, 1996). MAOI diets that are over-restrictive can be dangerous, since after eating a supposedly 'restricted' food and experiencing no adverse reactions, patients undergoing therapy may develop indifferent attitudes about the recommended diet. This certainly could lead to general non-compliance, and consumption of other restricted but **definitely** dangerous foods such as aged cheese (Gardner *et al.*, 1996).

The Risk of New Technology

Nevertheless, biogenic amines can be extremely potent biological compounds with severe physiological consequences; hence their accumulation in our food supply must be closely monitored. We are now at the point where new technologies (vacuum packaging and modified atmosphere packaging) may be creating ideal environments for the accumulation of biogenic amines in products that previously were not even considered for this hazard. Perhaps the 'risk/benefit' concept will be our most useful tool when making new toxicological conclusions. Economically, Canada is now able to compete in a global market with the increases in pork exports to other countries. Additionally, the dominance of innocuous lactic acid bacteria precludes the growth of pathogenic organisms in the meat, and the longer storage lives allow people around the world to enjoy the health benefits of readily available fresh meat. The benefits of these packaging technologies are numerous and already well known; however, the risks are yet to be determined.

SECTION III

MATERIALS AND METHODS

A. Preliminary Experiments

1. Low Temperature Storage Chamber

Prior to beginning this research, it was necessary to verify that the refrigeration unit (Econaire Systems, Foster Refrigerator Co., Hudson NY) could maintain the desired storage temperature, -1.5°C. Four type T thermocouples (copper/copper–nickel) were inserted into the centres of 4 individual pork chops (obtained from a local retail market). The pork chops were placed inside the chamber. The thermocouples were attached to a personal computer equipped with Quicklog PC version 2.4.0, Analog Connection Development System version 2.3.1 (Omega Engineering Inc., CT). A program was set up to take analog readings from each of the four thermocouples at 10 min intervals over 8d. Results showed the unit maintained a temperature of -1.54 ± 0.18°C.

2. Selective Medium for Carnobacteria

An additional preliminary experiment was an attempt to utilize a selective medium for the enumeration of *Carnobacterium* spp. from the pork. Cresol Red Thallium Acetate Sucrose Agar (CTAS; Agriculture Canada, 1993; Baird *et al.*, 1989) was selected. The medium was not commercially available. The recommended composition is shown in Table 1.

Peptone, agar, Tween 80, and yeast extract were purchased from Difco Laboratories (Detroit, MI). Sucrose, manganese sulphate, sodium nitrate and di-potassium

Table 1. Recommended composition of Cresol Red Thallium Acetate Sucrose agar. (From Baird *et al.*, 1989)

Component	g/L
Peptone	10
Yeast extract	10
Sucrose	20
Tween 80	1
Sodium nitrate	15
Manganese sulphate $\cdot 4H_20$	4
Di-potassium hydrogen phosphate	2
Thallium acetate	1
Nalidixic acid	0.04
Cresol red	0.004
Triphenyltetrazolium chloride	0.01
Agar	15

biphosphate were from Fisher Scientific (NJ). Thallium acetate, nalidixic acid, cresol red, and triphenyltetrazolium chloride were obtained from Sigma Chemical Co. (MO)

CTAS Trial 1

In the first attempt with CTAS medium, potassium hydrogen sulphate was used in place of dipotassium hydrogen phosphate. This was due to an error in the method published by Agriculture Canada (1993). Additionally, manganese sulphate anhydrous was substituted for manganese sulphate•$4H_2O$. All the ingredients except triphenyl-tetrazolium chloride (TTC) were suspended in 1 L of distilled water and brought to a boil to dissolve. The solution was then cooled to 55°C in a water bath. The pH was subsequently adjusted to 9.1 with 1 N NaOH. The medium was autoclaved at 121°C for 15 min and then cooled to 55°C again in a water bath. TTC (0.01g) was dissolved in 10 ml distilled H_20. The TTC solution was sterilized through a 0.22 micron filter (Cameo 25ES Nitrocellulose, Micron Separations Inc., Westborough, MA) with a 10 cc syringe and then was added to the cooled medium. The completed medium was swirled to ensure complete mixing and then poured into plastic petri dishes, allowed to dry, and inverted for storage at 2°C. Using the spread – plate technique (See Section B.1., Bacterial Analyses), a fresh pork loin sample (obtained from a local retailer) was evaluated for carnobacteria.

Trial 1 Results

The results in quadruplicate showed excessive growth (> 300 colonies per plate) after 3 d aerobic incubation at 25°C. Typical colonies were small, white, and opaque. Other colonies were bronze-purple metallic. Many colonies produced a change in the colour of

the medium from red to yellow. No attempts were made to further identify the organisms.

CTAS Trials 2 & 3

In the second and third attempts with this medium the method was followed as described by Baird *et al.* (1989) with the proper ingredients and was autoclaved for 10 min instead of 15. Immediately after autoclaving, the mixture contained a white precipitate and also large chunks of what appeared to be solidified agar. However, these 'agar' lumps would not melt and remained unidentified. Due to the clumping and the precipitate, the media was extremely difficult to pour into plates and could not be used. At approximately the same time in a personal communication with Agriculture and Agrifood Canada (Dr. L. Jeremiah, Lacombe AB) it was discovered that the general consensus on CTAS was that it did not work. The method was not pursued any further.

3. Cultures for Positive Controls

Cultures of *Lactobacillus hilgardii* (ATCC # 8290) and *Carnobacterium divergens* (ATCC #35677) were obtained from American Type Culture Collection (Rockville, MD) as freeze-dried pellets. The organisms were resuscitated in deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit MI) and then streaked onto MRS slants. Cultures were kept at 4-5°C. At 4 week intervals a colony of each organism was picked from a control plate and streaked onto new slants.

B. Experiments *Phase 1* – Packaging

1. Pork Samples

The pork for all experiments was purchased from a federally inspected commercial abattoir, Fletchers Fine Foods (Red Deer, AB). Twenty pork loins (bone in, totaling 340 pounds) were selected off the line in the packing plant. A representative from Agriculture and Agrifood Canada (Lacombe, AB)and a quality manager from Fletchers Fine Foods were present. These individuals (with expertise in meat science and packing) ensured that all loins selected were of normal muscle quality based on muscle colour (not pale, soft and exudative or dark, firm and dry). The loins were packaged in plastic–lined cardboard boxes and transported to the Meat Research Station at the Lacombe Research Centre (Agriculture and Agrifood Canada, Lacombe, AB). At the research station the loins were sliced into 1 inch chops. The chops were placed into plastic tubs in a refrigerator (1°C) until they were packaged, a time period of approximately 2-3 h.

2. Packaging

Vacuum Packaging

One hundred pork loin chops were vacuum-packaged. Each chop was scraped with a pork scraper in order to remove fat and debris from the surface. Each chop's bone was covered with bone wrap in order to prevent puncturing of the package. Chops were placed in 12 by 18 inch plastic bags (polyvinylidine chloride, low gas permeability), 4 per

bag. The bags were evacuated and sealed in a Multivac AG3 vacuum packager (Geprüfte, Sicherheit). The vacuum packages were stored at 1°C until transport.

CO_2 Modified Atmosphere Packaging

Foil Laminate Bag Preparation

The bags for CO_2-MAP were composed of sheets of aluminum foil laminate (gas impermeable). Two sheets were superimposed and then heat–sealed on 3 sides to create a pouch approximately 14 by 22 inches. In total 38 bags were assembled.

Packaging

One hundred sixteen pork loin chops were packaged in a modified atmosphere. Again, each chop was scraped with a pork scraper in order to remove surface debris. Chops were placed on plastic trays, 4 per tray. Each tray had approximately 1.2 kg of pork. The trays were slid into the foil laminate pouches. Using the Captron III® packaging system (RMF, Grandview, MO) the bags were evacuated, inflated with 3 L of carbon dioxide, and heat-sealed. Three packages were checked for gas composition: one from the beginning of the packaging run, one from the middle, and one from the end. Adhesive nickel pads were placed on the corner of the pouches and the needle of a glass syringe was inserted into the packages through the centre of the nickel pad. Ten cc of gas was drawn out of the bag and into the syringe. Once the needle was removed, the nickel pad was immediately sealed to the bag with a hot iron. The gas was analyzed in a Mocon Oxygen Headspace Analyzer (Modem Controls Inc., Minneapolis, MN). Residual

oxygen was 220±30 ppm. The CO_2-MAP pouches were placed in a refrigerator (1°C) until transport.

Extra Samples

The remaining pork loin chops were maintained at the Lacombe Research Centre in frozen storage. There were 9 CO_2-modified atmosphere packaged trays and 12 vacuum packages frozen in cardboard boxes. The remaining pork was stored in plastic-lined cardboard boxes.

Transport

The 29 CO_2-MAP packages and 25 vacuum packages were transported to the University of Manitoba approximately 24 h after packaging. The samples were transported in coolers with ice. Total time for the transportation was approximately 17 h. Upon arrival at the University of Manitoba all samples were placed on shelves in the environmental chamber set at −1.5°C.

C. Experiments *Phase 2* – Storage and Sampling

All samples remained in the low temperature chamber until they were selected. The chamber door was opened once per week when two packages of each type were randomly selected for analysis. One of each was immediately place into a freezer (-22°C). The other 2 were removed and analyzed within a 48 h period for the following parameters:

1. Bacterial Analyses

All techniques were aseptic. All analyses were performed in duplicate. A sample was removed from one location (the eye of the loin) on each of the four chops using a 3.57 cm diameter coring device, avoiding fat and connective tissue. The surfaces (approximately 5 mm depth) were removed from the cored sample using a knife, and the two lateral surfaces were pooled to obtain a total of 20 cm^2 per pork chop. Samples were stomached for 2 minutes (Stomacher, Lab-Blender 400 Seward Laboratory, London UK) in 90 ml 0.1% peptone water. After serial tenfold dilutions of each homogenate in 0.1% peptone water, lactic acid bacteria and carnobacteria were determined by the spread plate technique. Each week a negative control, a negative buffer control, and positive control plates streaked with *L. hilgardii* and *C. divergens* were incubated with the sample plates.

After the samples were extracted from the pork chops, the chops were transferred to individual plastic Ziploc® bags (DowBrands, USA) and stored at 4°C until analysis for biogenic amines (approximately 24 h later).

Lactic Acid Bacteria

Lactic acid bacteria were enumerated on MRS Agar (Difco Laboratories, Detroit, MI). After appropriate dilutions in 0.1% peptone water, the samples were delivered to the surface of the pre-poured MRS plates and spread with a bent glass rod. Enumeration followed 3 d of incubation at 25°C using a BBL anaerobic system (Becton and Dickenson Co., Cockeysville, MD). The selectivity of the MRS medium has been

published (Baird *et al.*, 1989). Plates with between 30 and 300 colonies were counted and averaged for each week.

Carnobacteria

Due to the failure of the CTAS medium (see Preliminary Experiments), two additional methods for the enumeration of carnobacteria in the pork were attempted.

MRS Differential Method

The MRS differential method was based on the theory that the acetate in MRS medium may slow the growth of carnobacteria, which are sensitive to acetate (Schillinger and Holzapfel, 1990). After the MRS plates for the lactic acid bacteria were counted after 3 d of incubation, the plates were returned to the BBL incubator for an additional 2 d for a total incubation time of 5 d. The results from the first count (lactic acid bacteria) were to be subtracted from the count after the 5^{th} day (lactic acid + carnobacteria). The difference was presumed to be *Carnobacterium*. However, after several weeks the method was abandoned. The small amount of acetate (5%, w/v) in the MRS was not hindering the growth of *Carnobacterium*. *C. divergens* on a positive control plate grew well on MRS after only 3 d.

Rogosa Method

After week four of the study another method for enumerating carnobacteria was tried. The new Rogosa method was again based on the sensitivity of the carnobacteria to acetate. After appropriate dilutions in 0.1% peptone water, homogenates were delivered

to the surface of pre-poured Rogosa SL Agar plates (Difco Laboratories, Detroit, MI). Enumeration followed 3 d of incubation using a BBL anaerobic system. Plates with between 30 and 300 colonies were counted and averaged for each week. The high amount of acetate in the Rogosa medium (15%, w/v) was hypothesized to be enough to preclude the growth of carnobacteria. This was proven by the inability of the *C. divergens* (streaked on the positive Rogosa control plate) to grow at all. The results from the Rogosa medium were subtracted from the results of the MRS plates (lactic acid bacteria + carnobacteria). The difference was presumed to be carnobacteria.

2. Biogenic Amine Analyses

HPLC Method

Extraction

The pork samples were removed from the refrigerator 24 hours after the extraction for bacterial analyses. Using a 3.57 cm diameter coring device, a sample was excised from one location on each chop (from the eye of the loin) taking care to avoid fat and connective tissue. The lateral surfaces (5 mm depth) were cut from the cored sample using a knife and the 2 surfaces were pooled and their total mass recorded. The method used from that point on was as described by Eerola *et al.* (1993). Initially amines were extracted with perchloric acid. The pork samples were cut into small pieces and placed in stainless steel mixing tubes with 15 ml of 0.4 M perchloric acid (Fisher Chemical Co., MD). Samples were homogenized with a Sorvall Omni-Mixer (Ivan Sorvall, Inc., CT) for 2 min. Homogenates were centrifuged at 7800 g for 10 min (Sorvall Superspeed

RC2-B; Ivan Sorvall, Inc., CT). The supernatants were poured through Whatman filter paper #4 – lined plastic funnels into 50 ml volumetric flasks. The pellets were re-homogenized for 2 min with 15 ml of 0.4 M perchloric acid and then centrifuged for 10 min again at 7800 g. The supernatants were filtered and added to their respective supernatants from the first extraction. The volumetric flasks were then made up to 50 ml with 0.4 M perchloric acid.

Derivatization

The next step of the procedure involved the dansylation of the amines. One ml of the extracted samples was placed in screw-cap test tubes. Three ml of each of the remaining extracts were placed into snap-top vials and stored at 4-5°C. To the extract in the screw-cap test tubes, 200 µl 2N NaOH (Fisher Scientific, NJ) and 300 µl saturated $NaHCO_3$ (Fisher Scientific, NJ) were added and then vortexed. Two ml of a dansyl chloride solution [2 mg dansyl chloride (Sigma Chemical Co., MO) per ml acetone] were added and the solutions were again vortexed. The tubes were protected from light and incubated in a water bath for 45 min at 40°C. The dansylation was then stopped by the addition of 100 µl ammonia. The tubes were incubated for 30 min at room temperature. The derivatization was complete upon addition of acetonitrile (Fisher Scientific, NJ) to make up the tubes to a volume of 5 ml.

Chromatographic Separations

a) Mobile Phases

Mobile phase #1: 0.1 M ammonium acetate was prepared by dissolving 7.7g ammonium acetate (Fisher Scientific, NJ) in 1L purified water (Millipore Corp., MA). The solution was vacuum filtered using a 0.45 μm filter and vacuum degassed for 5 min. Mobile phase #2: acetonitrile, was vacuum filtered through a 0.45 μm filter and degassed for approximately 20 s.

b) HPLC Specifications

The samples and standards were analyzed using a Maxima 820 HPLC program (Dynamic Solutions, Millipore, MA) equipped with 2 Model 501 HPLC Pumps, a 484 Tunable Absorbance Detector (set at 254 nm), a 410 Differential Refractometer with temperature control (set at 37°C), a manual injector (model U6K) and a standard reversed-phase C18 μBondapak column, 300 x 3.3 mm (all Waters, Division of Millipore, MA). A Waters System Interface Module integrated the system to a 386 PC.

The flow rate was 1.0 ml/min. The gradient elution program began with 50% #1 and 50% #2 and reached 10% #1 and 90% #2 after 15 min. From 25 min to 28 min the phases were returned to 50% #1 and 50% #2. The system was equilibrated for an additional 7 min. Samples and standards were injected at 20 μl each, in duplicate. Peak heights (μV) and solution concentrations (mg/ml) were calculated using the computer program.

Preparation of Standards

Approximately 5 mg each of β-phenylethylamine, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tyramine hydrochloride, spermidine phosphate salt hexahydrate, and spermine diphosphate salt (Sigma Chemical Co., MO) were dissolved together in 50 ml 0.4 M $HClO_4$; and their exact masses were recorded. This standard solution was diluted in order to obtain concentrations of 0.1 mg/ml, 0.05 mg/ml, and 0.01 mg/ml. Additionally, 1 mg of each of the amines were dissolved in 1 L of 0.4 M $HClO_4$, their exact masses recorded, and then were further diluted to obtain concentrations of 0.001 mg/ml, 0.005 mg/ml, 0.0001 mg/ml, and 0.00001 mg/ml. The standards were derivatized in the same manner as previously described.

Calibration curves were constructed for each of the 7 amines in the prepared standard solutions. Each amine had an r^2 between 0.9988 – 0.9997 (Appendix 1-7). Retention times shifted over time; thus in order to accurately identify the amines from the pork samples, standards, in addition to the samples, were run on the HPLC each week. Chromatograms for a standard mixture as well as a sample are shown in Figs. 2 and 3.

Calculation of Results

Sample results were given by the computer as mg/ml. The results were converted to μg/g of pork by the following formula:

$$\text{Concentration in pork } (\mu g/g) = \frac{\text{solution conc. (mg/ml)} \times \text{50 ml dilution factor} \times 1000\ \mu g/mg}{\text{Sample Weight (g)}}$$

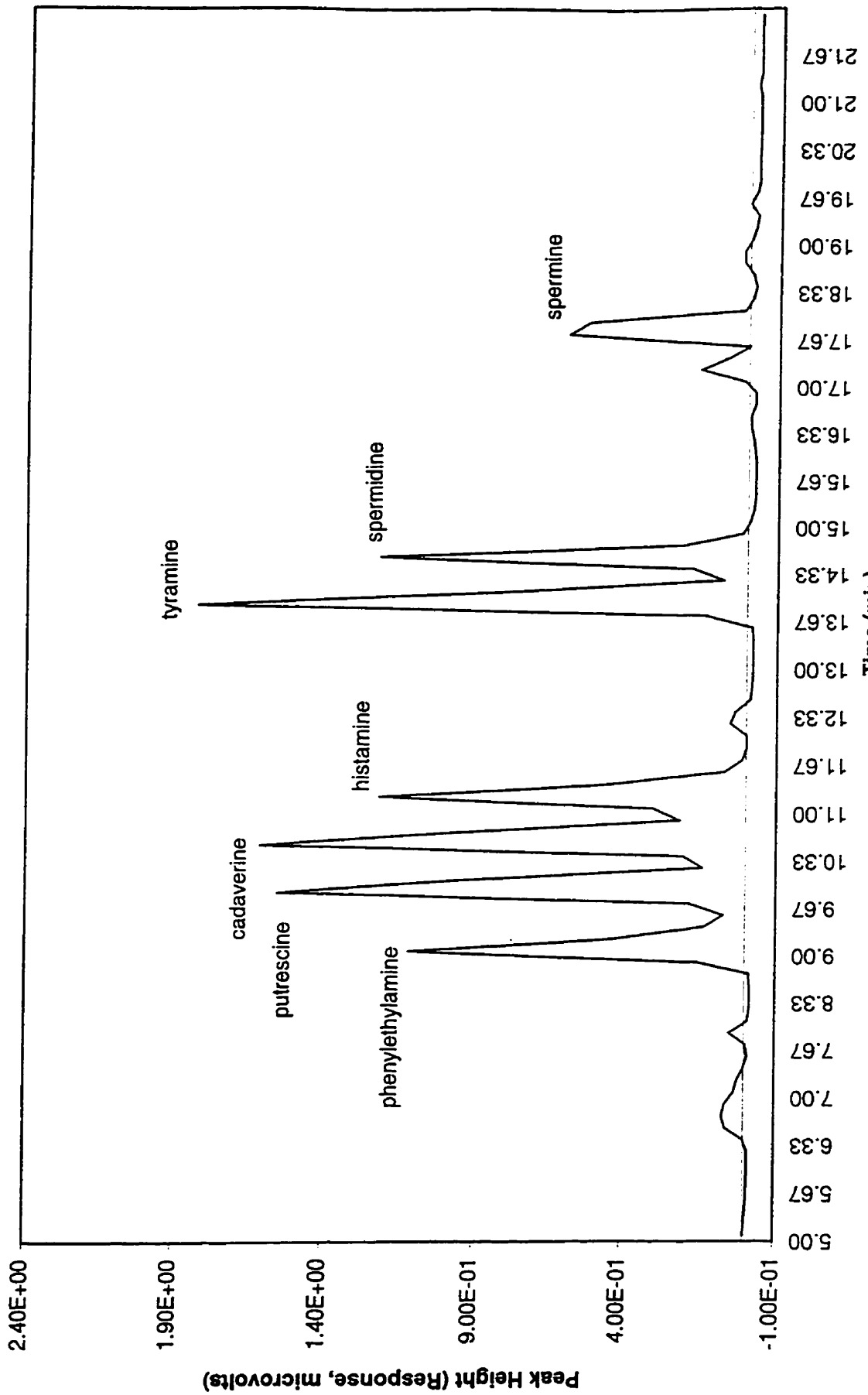


Figure 2. Sample HPLC chromatogram for biogenic amines working standards

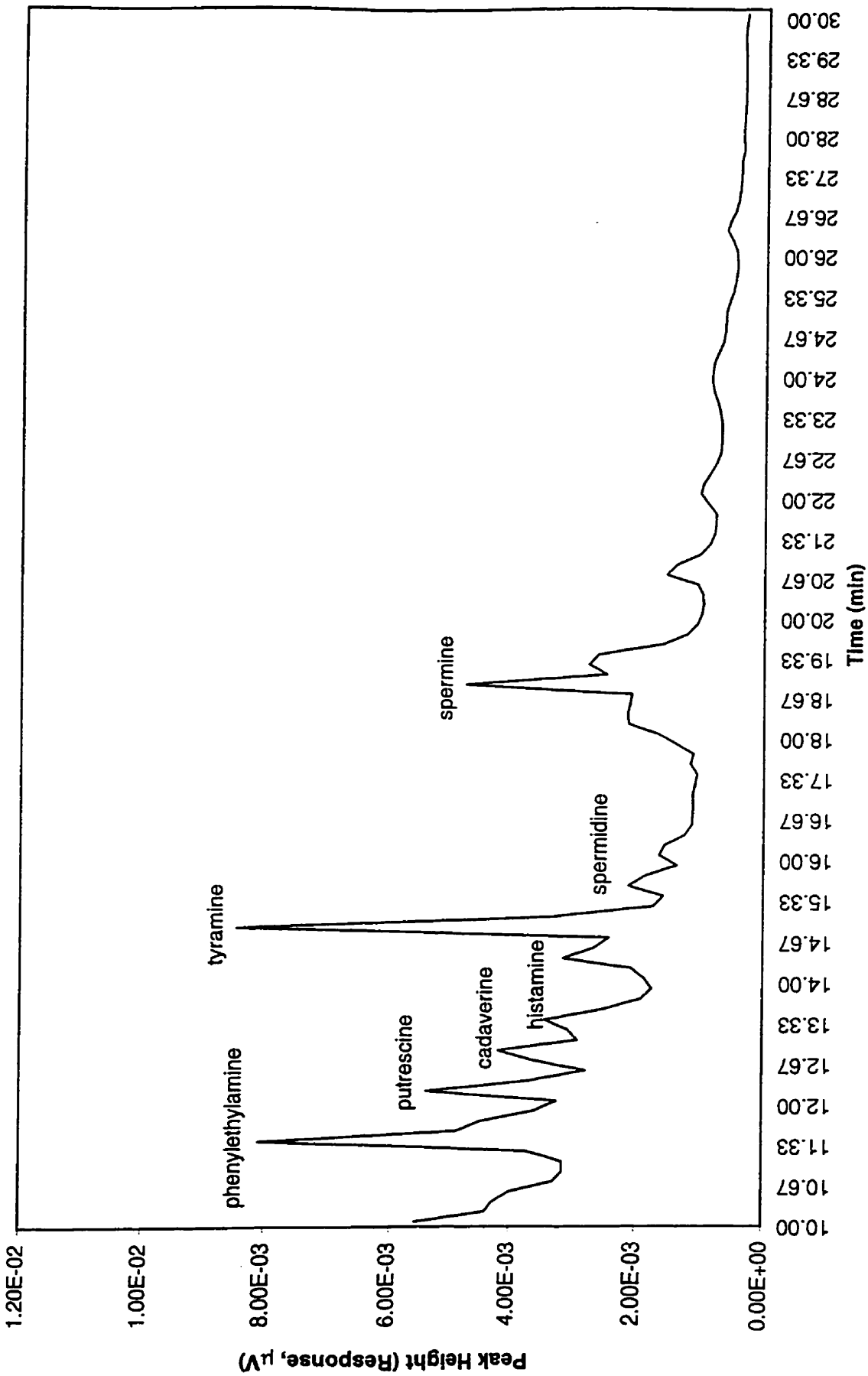


Figure 3. Sample HPLC chromatogram of biogenic amines extracted from fresh pork

Results from each week were averaged.

Capillary Gel Electrophoresis Method

The determination of biogenic amines by capillary gel electrophoresis (CGE) was performed at the Department of Agricultural, Food, and Nutritional Science, University of Alberta (Edmonton, AB). Samples from the extraction process previously described were transported to the University of Alberta in coolers; all further analyses were performed by the University of Alberta. Upon receiving the samples, they were refrigerated overnight and then frozen at –28°C.

Preparation of Extracts

Prior to analysis, samples were thawed at 4°C, centrifuged in a Beckman Microfuge (Beckman Coulter, Fullerton CA) at speed 8 for 4 min. An aliquot was neutralized with KOH and stored at 4°C for 1 h to allow KClO4 crystals to precipitate. After centrifuging again at speed 8 for 4 min, the supernatant was derivatized at 60°C with ATTO-TAG™ [3-(2-furoyl)quinoline-2-carboxaldehyde; Molecular Probes Inc., Eugene OR] and KCN with phosphate buffer in order to render the biogenic amines fluorescent.

Electrophoresis Specifications

The fluorescent compounds were separated on a P/ACE Beckman 2100 Capillary Electrophoresis System (Beckman Coulter, Fullerton CA). The voltage was applied in reverse polarity using a 27 cm (20 cm to detector) x 50 μm uncoated silica capillary. The buffer used was 10 mM sodium phosphate in 1-propanol.

Biogenic Amine Determination

Samples and standards were introduced to the column under pressure and separated with a two step voltage gradient. Both the migration time and the area of the samples were compared with the standards for β-phenylethylamine, putrescine, cadaverine, histamine, and tyramine. The peak areas were integrated on Beckman Software V. 7.01 (Beckman Coulter, Fullerton CA). Compounds detected were reported on a μM basis.

Calculation of Results

The results for each amine were converted to μg/g of pork by the following formulae:

Eq. 1 Extract Concentration (g/ml) =

$$\frac{[\text{result } (\mu M) / 10^6 \ \mu mol/mol] \times \text{molecular weight (g/mol)}}{1000 \text{ ml/L}}$$

Eq. 2 Concentration in pork (μg/g) =

$$\frac{\text{extract conc. (g/ml)} \times 50 \text{ ml dilution factor} \times 10^6 \ \mu g/g}{\text{Sample Weight (g)}}$$

D. Experiments *Phase 3* – Sensory Evaluation

1. Panel Members and Information Session

The sensory evaluation experiments were approved by the Faculty of Human Ecology Ethics Review Committee (University of Manitoba, Winnipeg MB). Eight graduate

students and technicians from the Department of Food Science volunteered for the sensory panel. Prior to the sensory evaluation, the panel members attended a 1 h information session. During this session, the attributes to be evaluated (odour and appearance) were defined and the scales on which they were to be rated were explained. At the end of the session the panel members were provided with the general information recommended by the Ethics Committee and the Standard Consent Form to read and sign (Appendix 8 and 9). Informed consent was obtained from all participants.

2. Sample Preparation and Evaluation

Preparation

Pork loin chops were randomly assigned 3-digit numbers. The samples were removed from the freezer 30 h prior to the time they were scheduled to be evaluated. The samples were transferred into individual Ziploc® bags labeled with the assigned codes.

Evaluation

The participants were randomly assigned 2-digit codes to ensure confidentiality. Panel members evaluated the samples in random order. Each panel member evaluated a total of 96 samples in 2 d, 2 sessions/d. The scores were marked on ballot forms for odour and appearance (Appendix 10 and 11).

E. Statistical Analyses

The statistical analyses of lactic acid bacteria, biogenic amines by HPLC, and the sensory properties, odour and appearance, were evaluated using the General Linear Model Procedure of the SAS Institute (SAS, 1990) and the Duncan's Multiple Range test ($p \leq 0.05$).

SECTION IV

RESULTS AND DISCUSSION

A. BACTERIAL ANALYSES

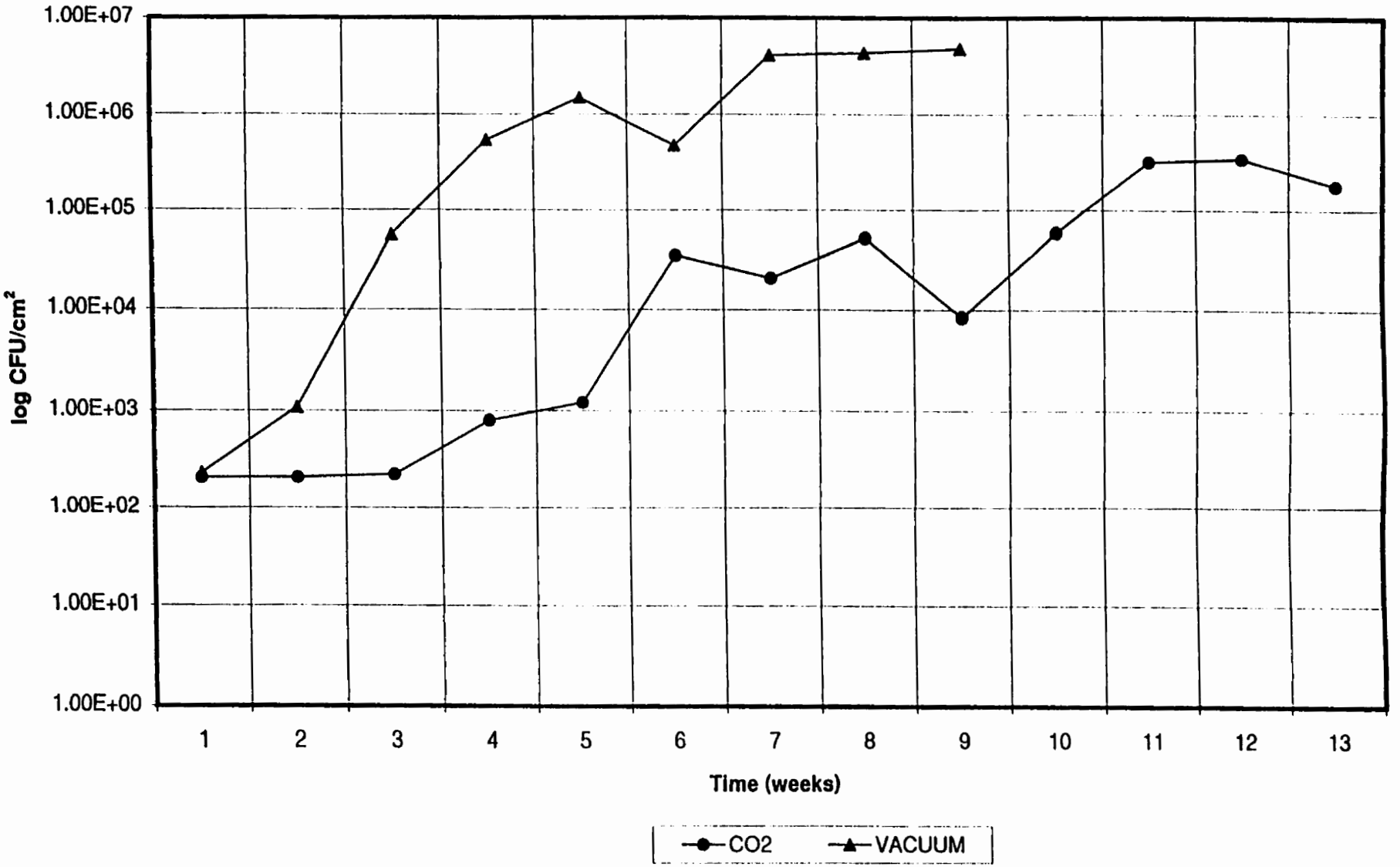
1. Lactic Acid Bacteria

The purpose of these experiments was to monitor the growth of lactic acid bacteria on the surface of the pork packaged in vacuum and carbon dioxide modified atmosphere packaging (CO_2-MAP) during the given storage period.

Vacuum Packaging

The results of the growth of lactic acid bacteria in vacuum packaging are shown in Fig. 4. The population was initially log 2 CFU (colony forming units)/cm^2. The lactic acid bacteria increased steadily for the first 5 weeks of storage until a population of log 6 CFU/cm^2 was reached. For the remaining 4 weeks the population maintained a plateau between log 5 and 7 CFU/cm^2. The population during the last 3 weeks of storage were significantly higher than the first 6 weeks (Appendix 12). Schillinger and Lucke (1987) determined the maximum cell density for lactic acid bacteria growing on porcine tissue to be log 8 cells/cm^2. Other published reports have demonstrated lactic acid bacteria on vacuum-packaged beef as initially log 3 CFU/cm^2 and growing to a population higher than log 7 CFU/cm^2 after 9 weeks of storage at –2 and 1°C (Krizek *et al.*, 1995; Smith *et al.*, 1993). Given the higher pH of pork muscle tissue compared to beef, it was logical to expect the growth of lactic acid bacteria in our pork to be the same as or exceed values obtained from beef stored in equivalent conditions. The lower bacterial populations in this study may be an indication of the strict sanitary and temperature controls adhered to throughout the slicing, packaging, and transport of the primal cuts; which may not be

Figure 4. The growth of lactic acid bacteria in fresh pork stored at -1.5°C in vacuum and carbon dioxide modified atmosphere packaging



possible outside a laboratory setting in typical commercial conditions. In addition to storage temperature and the permeability of the packaging film, initial microbial load has previously been shown to significantly affect the storage life of fresh pork (McMullen and Stiles, 1991; 1993). Reducing the initial microbial population would be successful in enhancing the effectiveness of vacuum packaging for long term storage of fresh pork.

CO_2-MAP

Growth of lactic acid bacteria in modified atmosphere packaged pork samples is also shown in Fig. 4. The initial population was log 2 CFU/ cm^2 and did not begin to increase until the 4^{th} week of storage. Significant changes in the bacterial population occurred at weeks 6 and 11 (Appendix 13). From week 5 to week 13 the lactic acid bacteria increased steadily to reach a final population of log 5 CFU/cm^2. These results were typical for the lactic acid bacteria of pork packaged in modified atmosphere; Greer and colleagues (1993), using the same Captech® process as this study, found that the lactic acid bacteria reached log 7 CFU/cm^2 after 13 weeks of storage at −1.5°C. Their study involving CO_2-MAP continued for an additional 9 weeks. From week 13 to week 24, the lactic acid bacteria population maintained a plateau between log 7 and 8 CFU/cm^2. The Captech® process has been successful in retarding microbial growth as compared to initial carbon dioxide modified atmosphere-packaging processes. Prior to the invention of the Captron, lactic acid bacteria would reach populations of log 6 CFU/cm^2 after only 4 weeks of CO_2-MAP refrigerated storage (Spahl *et al.*, 1981). Using the Captron, we found that after 4 weeks there was less than log 3 lactic acid bacteria CFU/cm^2, which was not a significant increase compared to initial numbers (Appendix 13).

Colony Characteristics and Microbial Ecology

Typical colonies on MRS agar from both vacuum packaged and CO_2-MAP pork samples were small (less than 2 mm in diameter), white, and opaque. These characteristics were consistent with those of the lactic acid bacteria (Agriculture Canada, 1993). The ecology of the lactic acid bacteria (excluding carnobacteria) on chilled vacuum and modified atmosphere packaged pork is well defined (Hitchener *et al.*, 1982; McMullen and Stiles, 1993). After 10 weeks of storage in CO_2-MAP, 76% of the organisms isolated on MRS agar were homofermentative lactobacilli and 24% were heterofermentative (McMullen and Stiles, 1993). The most frequent homofermentative strains were *Lactobacillus alimentarius*, *L. farciminis*, and *L. sake*. Heterofermentative strains most often included *L. viridescens*, and *L. minor* (McMullen and Stiles, 1993).

Comparison of Vacuum and Modified Atmosphere Packaging

The CO_2-MAP was more successful than the vacuum packaging at lengthening the storage life of fresh pork stored at −1.5°C. The CO_2-MAP effectively delayed the growth of lactic acid bacteria; after the first week of storage the population of lactic acid bacteria on the vacuum-packaged samples was significantly higher than the CO_2-MAP (Appendix 14). This was expected since it is generally known that increasing CO_2 leads to a decrease in the bacterial multiplication rate as well as an increase in the length of the lag phase (Dixon and Kell, 1989). By week 5 of the storage period the vacuum-packaged samples had in excess of log 6 CFU/cm^2 whereas the CO_2-MAP samples showed only log 3 CFU/cm^2. Furthermore, CO_2-MAP inhibited the growth of the lactic acid bacteria more than the vacuum packaging; the maximum load on the vacuum-packaged samples was

4.81×10^6 CFU/cm^2 compared to a maximum population of 3.45×10^5 CFU/cm^2 on the modified atmosphere packaged samples. After the initial week, vacuum-packaged pork had significantly higher counts of lactic acid bacteria than CO_2-MAP pork throughout the remainder of the study (Appendix 14). Thus the superiority of carbon dioxide modified atmosphere packaging over vacuum packaging in extending the storage life of fresh pork maintained at low temperature has been demonstrated.

2. Carnobacteria

An additional bacterial analysis of the pork samples was an attempt to enumerate carnobacteria during the storage period. The enumeration of carnobacteria from food products has been difficult ever since the discovery of the genus in 1987 when they were described as typical lactobacilli with the inability to grow on acetate-containing media (Collins *et al.*, 1987). As an increasing amount of lactic acid bacteria from vacuum packaged meats are unable to grow on acetate media, researchers have been attempting to enumerate and identify carnobacteria from these types of products. One of the first studies, in 1982, reported the prevalence of what were later to be discovered as carnobacteria in meat. Out of a total of 177 isolates from vacuum-packaged beef, 159 were the atypical lactobacilli (Hitchener *et al.*, 1982). Currently, we still do not have comprehensive data regarding the prevalence of carnobacteria in vacuum and modified atmosphere packaged meats. This is due to the lack of a selective medium specifically for the carnobacteria. They can be easily identified through a few biochemical characteristics such as the presence of *meso*-diaminopimelic acid in the cell wall, the

lactic acid isomers produced, the production of citrulline from arginine, and some carbohydrate fermentations (Montel *et al.*, 1991). However, even with these biochemical characteristics there still can be difficulty enumerating carnobacteria since widely different results can be obtained depending on the medium used to cultivate the isolates from the meat. When modified atmosphere-packaged pork was evaluated using plate count agar, the majority of the isolates identified through biochemical testing were carnobacteria (all were *C. divergens*); whereas on MRS agar the carnobacteria were rarely isolated (McMullen and Stiles, 1993).

Vacuum Packaging

The growth of carnobacteria by the difference of the growth on MRS and Rogosa (acetate) agar is shown in Fig. 5. Values were obtained from week 5 through week 9 for the vacuum-packaged samples. By this method, almost all of the lactic acid bacteria enumerated on MRS agar for those 5 weeks indicated were carnobacteria (>90%, Fig. 6).

CO_2-MAP

Values from the Rogosa differential method were obtained from week 6 through week 13 for the CO_2-MAP samples, with the exception of week 12 for which a negative value for the difference was obtained. The results are also shown in Fig. 5. For weeks 6 through 8, the carnobacteria represented the majority of the lactic acid bacteria (>95%, Fig. 6). After week 8, however, the carnobacteria decreased steadily from 69.5% of the total lactic acid bacteria in week 9 to 11.2% in week 13.

Figure 5. The growth of carnobacteria in fresh pork stored at -1.5°C in vacuum and carbon dioxide modified atmosphere packaging.

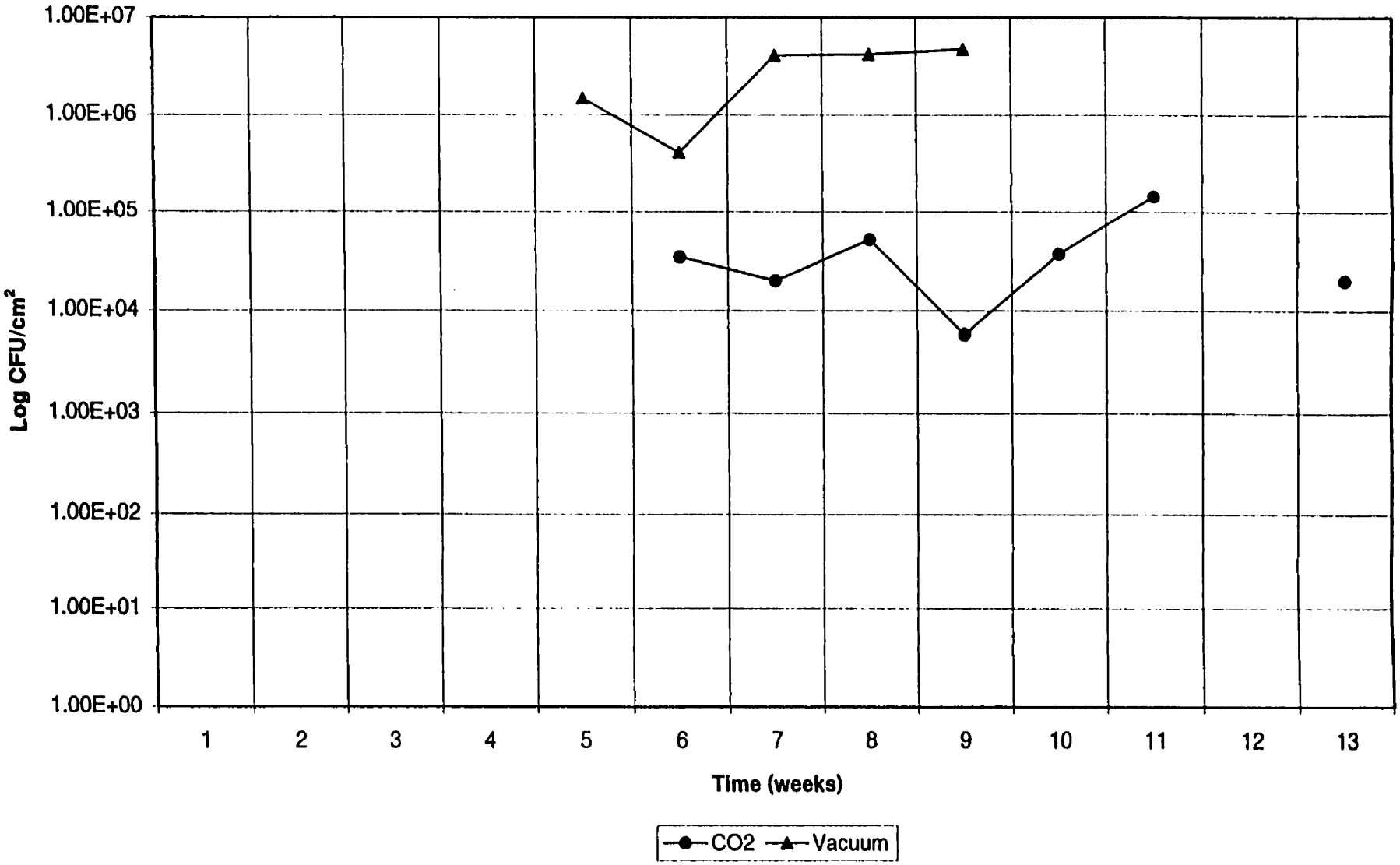
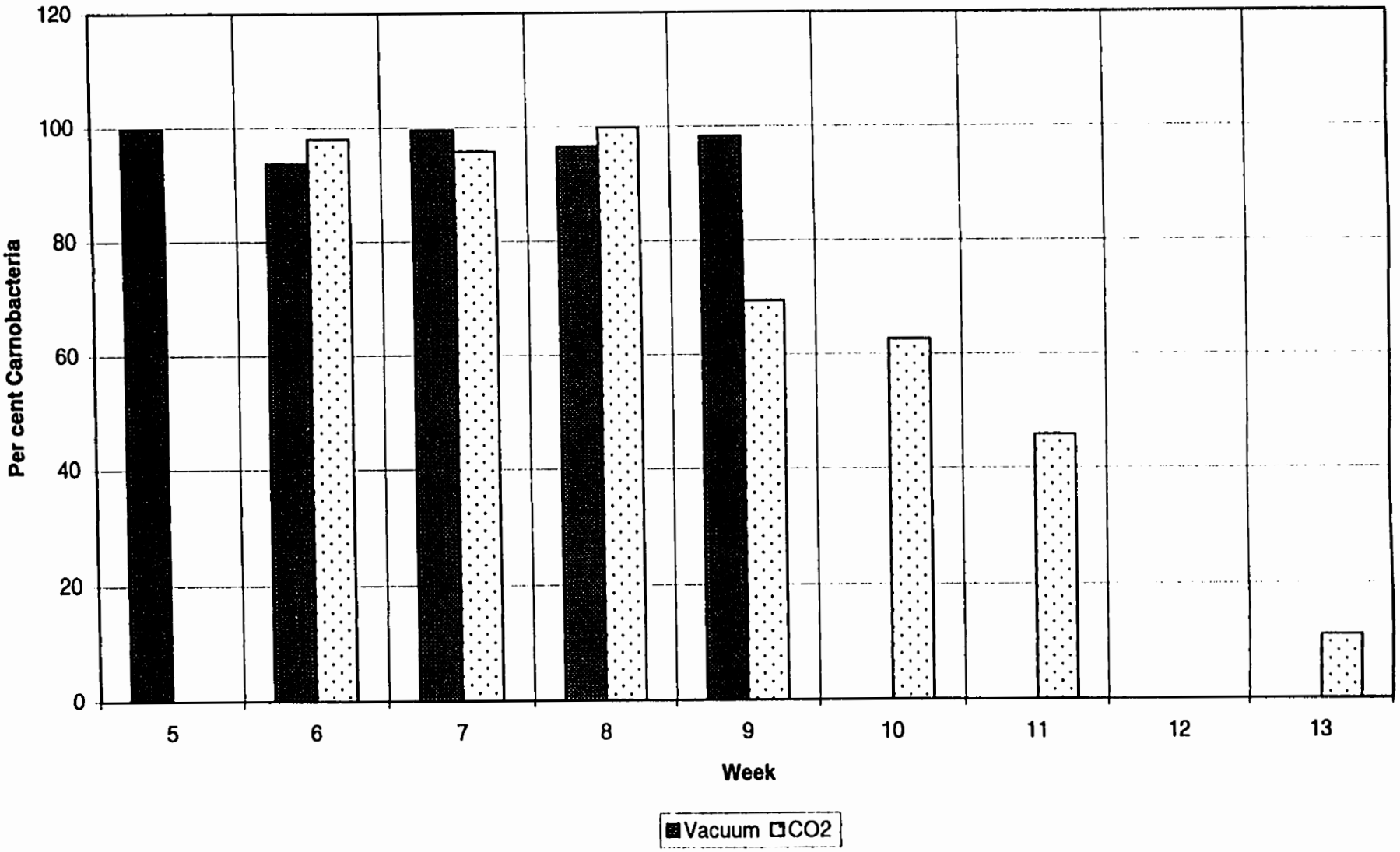


Figure 6. The percentage carnobacteria of total lactic acid bacteria in chilled pork stored at -1.5°C in vacuum and carbon dioxide modified atmosphere packaging.



Comparison of Vacuum and Modified Atmosphere Packaging

For the second half of the storage period that was evaluated for carnobacteria, the vacuum packaged samples had consistently dominant carnobacteria. The CO_2-MAP samples, however; had a high proportion of carnobacteria until week 8 when the population declined sharply. The modified atmosphere was successful in inhibiting carnobacteria for the latter part of the storage period at −1.5°C. This information is valuable since it provides evidence that the inhibition of the growth of carnobacteria may be a contributing factor in obtaining the longer storage lives of CO_2-MAP pork.

B. BIOGENIC AMINE ANALYSIS

1. High-Performance Liquid Chromatographic Method

The initial objectives of this research were to quantify biogenic amines on the pork surface throughout the storage period using reversed-phase HPLC as the analytical method.

Phenylethylamine

Vacuum Packaging

The highest amount of phenylethylamine occurred at week 4 of the storage at -1.5°C when the concentration reached nearly 40 μg/g (Fig. 7). This increase, or spike, at week 4 was the only significant increase in phenylethylamine concentration throughout the 9 week storage period in the vacuum-packaged pork (Appendix 15). For all the other

weeks, the concentrations ranged from approximately 4 μg/g to 22 μg/g; however, these changes were not significant.

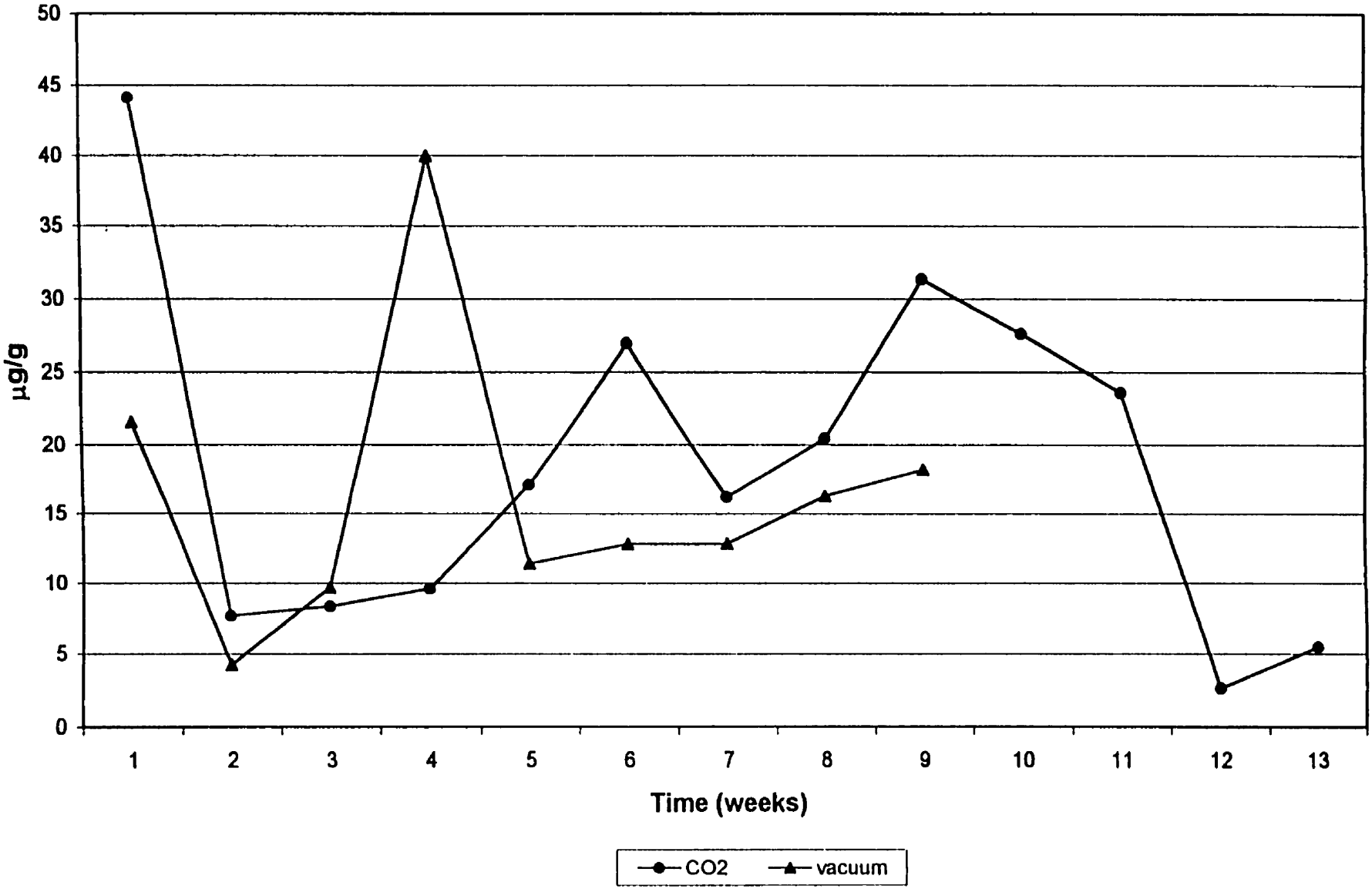
CO_2-MAP

The phenylethylamine concentration on the surface of the CO_2-MAP pork was initially high at nearly 45 μg/g (Fig. 7). At week 2 there was a significant decline to 7 μg/g. After the decline at week 2 there was a significant increase in phenylethylamine at week 6 (Appendix 16).

Comparison – Vacuum Packaging and CO_2-MAP

There were no significant differences at any week between the phenylethylamine levels from the vacuum-packaged and carbon dioxide modified atmosphere-packaged pork (Appendix 17). Literature information regarding the levels of phenylethylamine in meat is scarce. Smith *et al.* (1993) did not detect any phenylethylamine above the normal physiological muscle level (<1 μg/g) in vacuum-packaged fresh beef stored for 120 days at 1°C.

Figure 7. Phenylethylamine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC



Putrescine

Vacuum Packaging

There were no significant changes in putrescine concentration during the first 3 weeks of storage (Fig. 8; Appendix 18). At week 4 there was a significant increase when the concentration spiked at approximately 22 μg/g. After decreasing sharply, the putrescine levels steadily increased during the seventh, eighth, and ninth weeks to reach a final concentration of just over 20 μg/g.

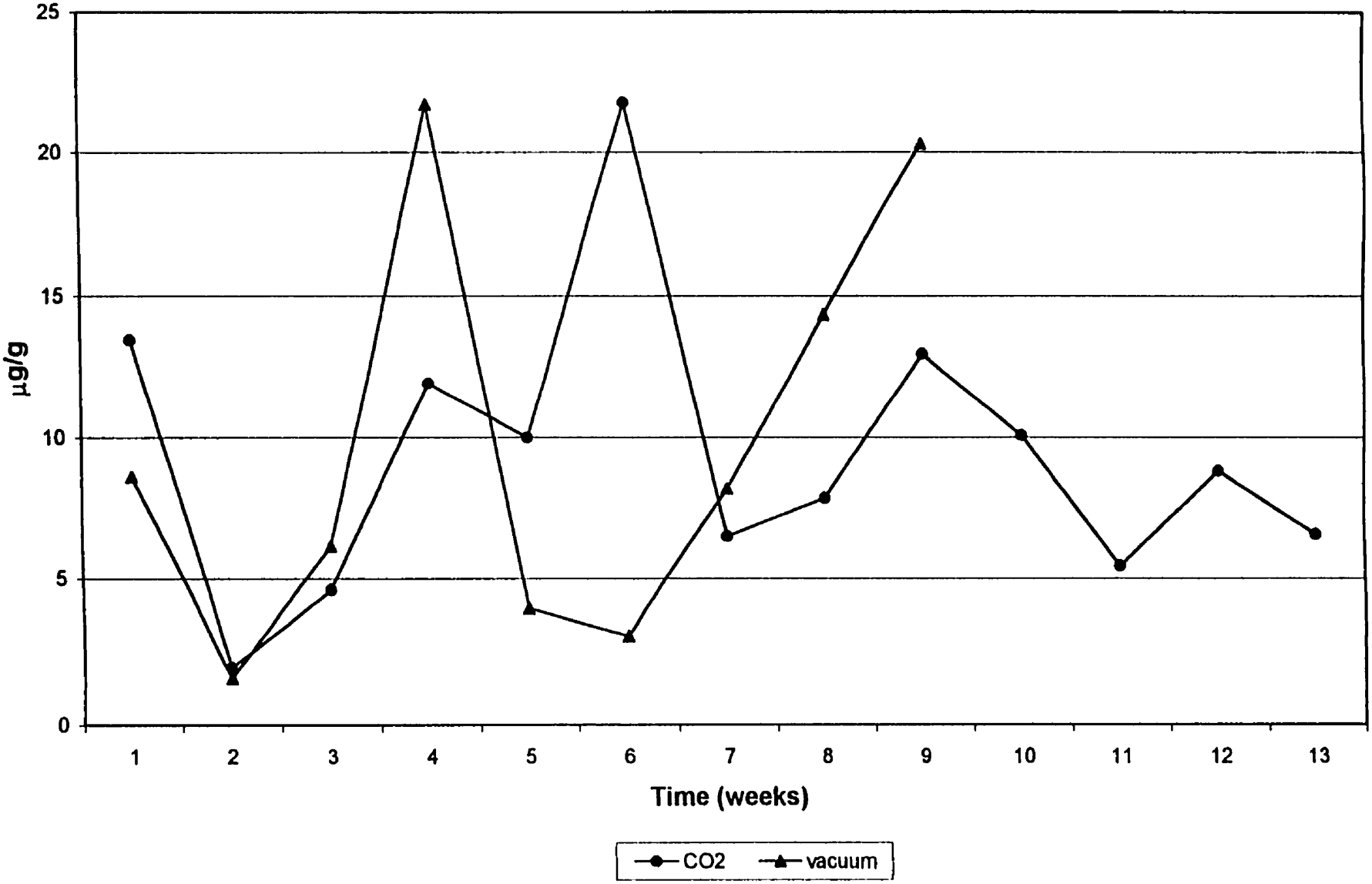
CO_2-MAP

The highest putrescine level in the CO_2-MAP pork was nearly 22 μg/g, recorded at week 6 (Fig. 8). For all other weeks, the levels varied between 2 μg/g and nearly 14 μg/g with no other significant increases or decreases from week to week (Appendix 19).

Comparison – Vacuum Packaging and CO_2-MAP

There were significant differences in putrescine levels of the vacuum and CO_2-MAP pork (Appendix 20). In weeks 5 and 6, the CO_2-MAP pork had significantly higher putrescine than the vacuum-packaged. Conversely, in week 8 the vacuum-packaged pork had higher putrescine than CO_2-MAP pork Additionally, there was a increasing trend observed in the putrescine levels of the vacuum-packaged pork during the final 3 weeks of the storage period. This trend was not observed with the CO_2-MAP pork. Clearly this is related to the significant increase in lactic acid bacterial growth during weeks 7, 8, and 9 in the vacuum-packaging but not in the CO_2-MAP.

Figure 8. Putrescine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC



The initial putrescine levels in pork of both packaging treatments determined in these experiments were slightly higher, but comparable to what is considered typical baseline level for fresh meat (Edwards *et al.*, 1983). The Edwards group (1983) recorded basal putrescine levels in fresh chilled pork at 2.3 μg/g.

Cadaverine

Vacuum Packaging

The concentration of cadaverine in the vacuum-packaged pork did not increase until weeks 8 and 9 of the storage period (Fig. 9; Appendix 21). There was a spike at week 4 to nearly 24 μg/g, however it was not a large enough increase to be significant. Following week 7, the cadaverine levels steadily increased to reach almost 70 μg/g after 9 weeks of storage at −1.5°C.

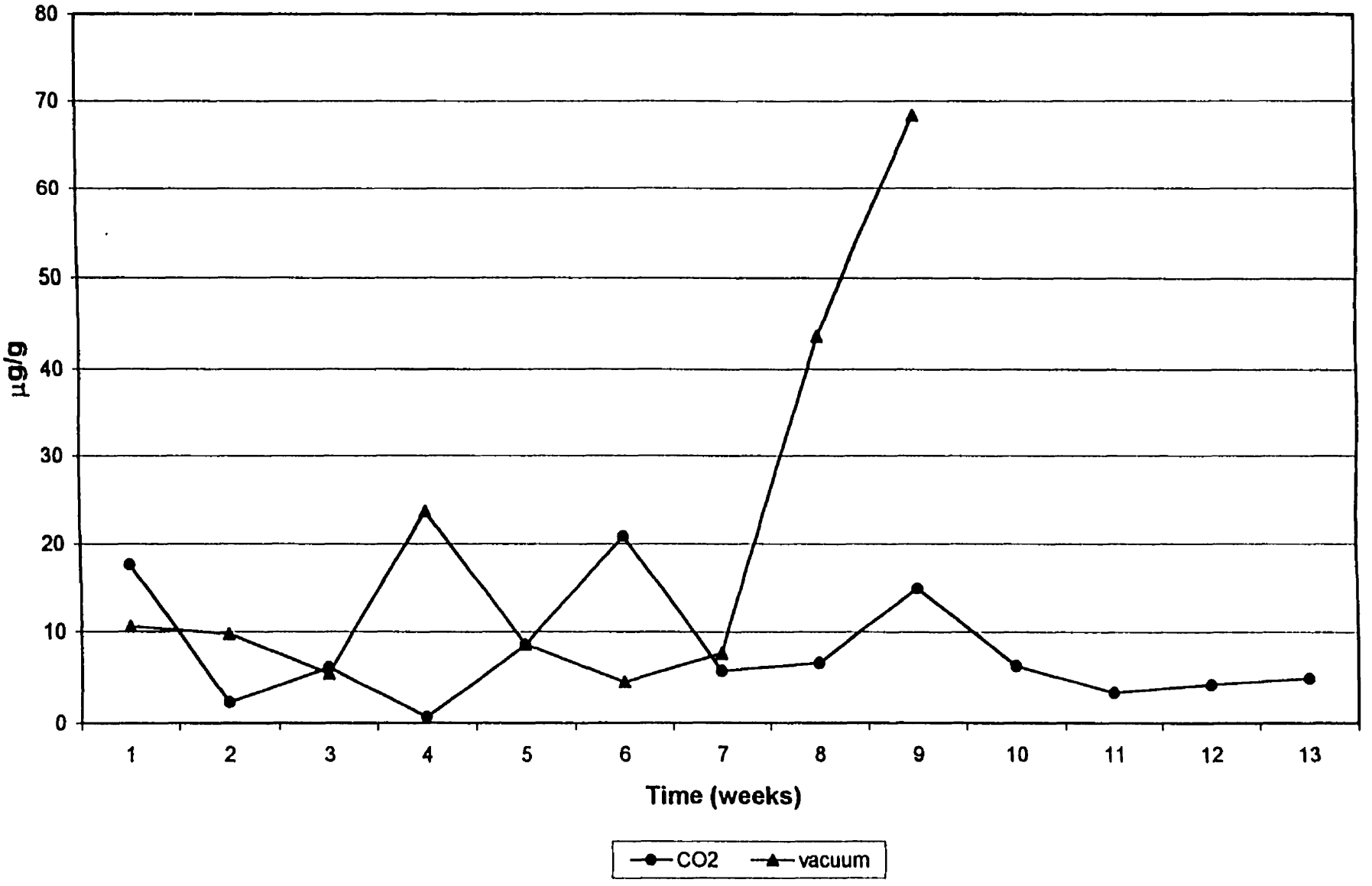
CO_2-MAP

In the carbon dioxide modified atmosphere-packaged pork the cadaverine levels were very similar to the putrescine in both concentrations and trends. The only significant change in cadaverine levels occurred when the level jumped from 8.5 μg/g in week 5 to 21 μg/g in week 6 (Fig. 9; Appendix 22).

Comparison – Vacuum Packaging and CO_2-MAP

The differences in cadaverine concentrations between the two packaging treatments were found in the second half of the storage period. At week 6, the CO_2-MAP pork had significantly higher cadaverine levels than the vacuum-packaged due to the week 6 spike.

Figure 9. Cadaverine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC



However, similar to the putrescine, cadaverine was significantly higher in the vacuum-packaged pork during the final weeks of storage. The vacuum-packaged pork showed a trend of increasing cadaverine accumulation during weeks 7, 8, and 9. This trend was not observed in the CO_2-MAP pork. Again, like the putrescine, the differences between the cadaverine levels of the two packages must be related to the suppression of bacterial growth by CO_2.

The initial levels of cadaverine in the samples of this study, like that of putrescine, were higher yet comparable to the accepted baseline level of this amine in fresh meat, 1.3 μg/g (Edwards *et al.*, 1983).

Histamine

Vacuum Packaging

The histamine concentration on the surface of the vacuum-packaged pork remained low throughout the entire storage period (Fig. 10). There were two spikes in histamine concentration; both were significant (Appendix 24). The first occurred at week 2 when the concentration jumped sharply to about 14 μg/g; the second spike was at week 4, the highest recorded histamine level at just over 15 μg/g.

CO_2-MAP

Histamine levels oscillated throughout the 13 week storage period at −1.5°C although in general, the concentrations were low (Fig. 10; Appendix 25). The highest histamine level, 12 μg/g, was detected as a spike at week 6.

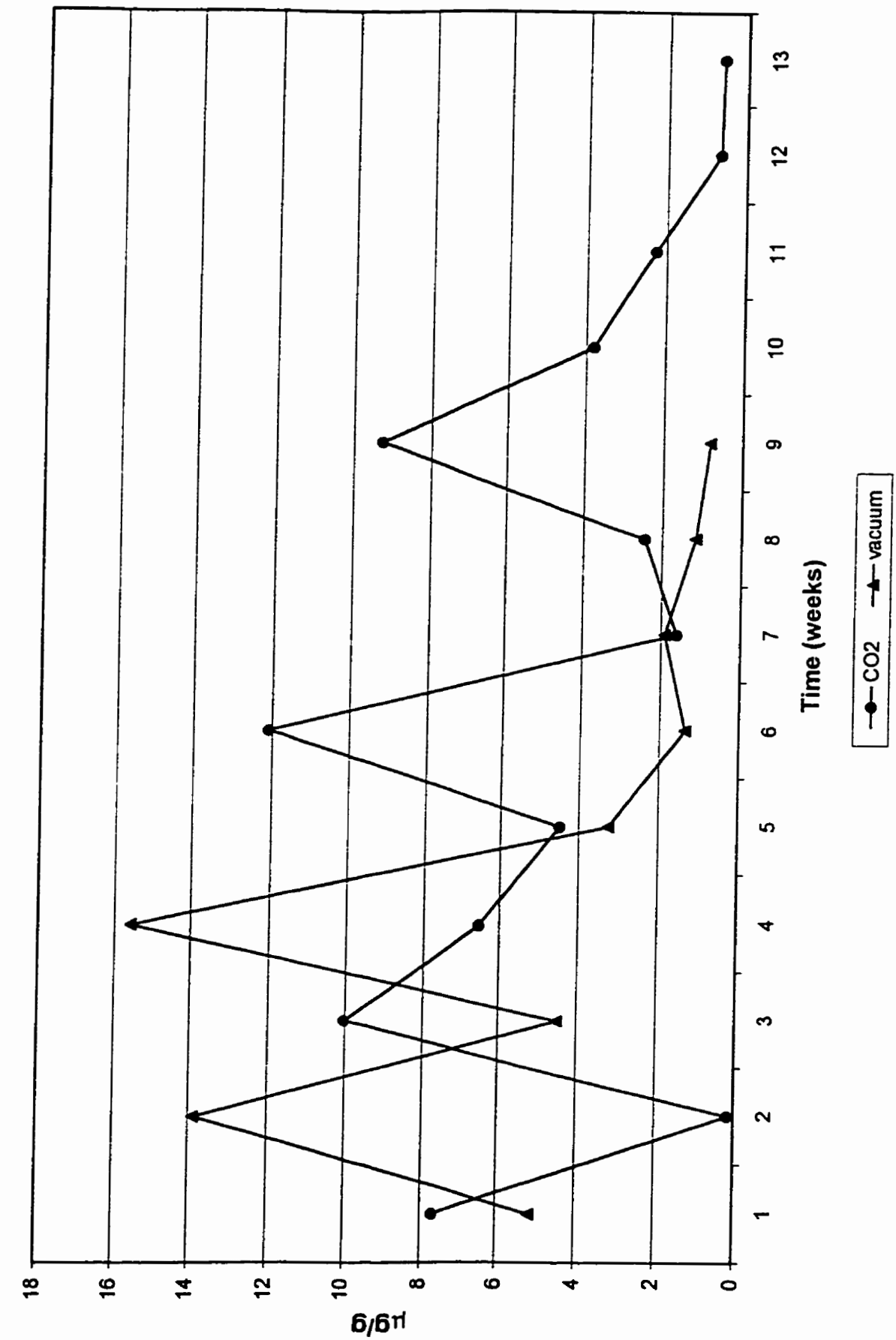


Figure 10. Histamine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC

Comparison – Vacuum Packaging and CO_2-MAP

The histamine levels of the pork of both packaging treatments were low. There were a few differences when comparing the two types of packaging; at week 2 the vacuum-packaged pork had higher histamine, at weeks 6 and 9 the CO_2-MAP histamine levels were higher (Appendix 26). There is little information available on histamine in modified atmosphere-packaged meats. However, our results are in agreement with other investigations regarding histamine accumulation in vacuum-packaged meats; that histamine only increases slightly with time in storage above the normal physiological value of less than 1 μg/g (Rice *et al.*, 1975; Smith *et al.*, 1993).

Tyramine

Vacuum Packaging

Tyramine accumulation on the pork surface was low until week 4 when there was a significant increase to 37 μg/g (Fig. 11; Appendix 27). Tyramine remained high through week 5, decreased slightly to 17 μg/g during weeks 6-7, then increased significantly to 38 μg/g in the eighth week and remained high for week 9. These results are comparable to previously published reports of tyramine in chilled vacuum-packaged beef (Smith *et al.*, 1993). After 9 weeks of storage at 1°C, their vacuum-packaged beef accumulated tyramine to a level of approximately 60 μg/g (Smith *et al.*, 1993). The Smith study continued for an additional 8 weeks; they found that tyramine continued to increase steadily to reach a final concentration of nearly 200 μg/g after 120 days of chilled storage.

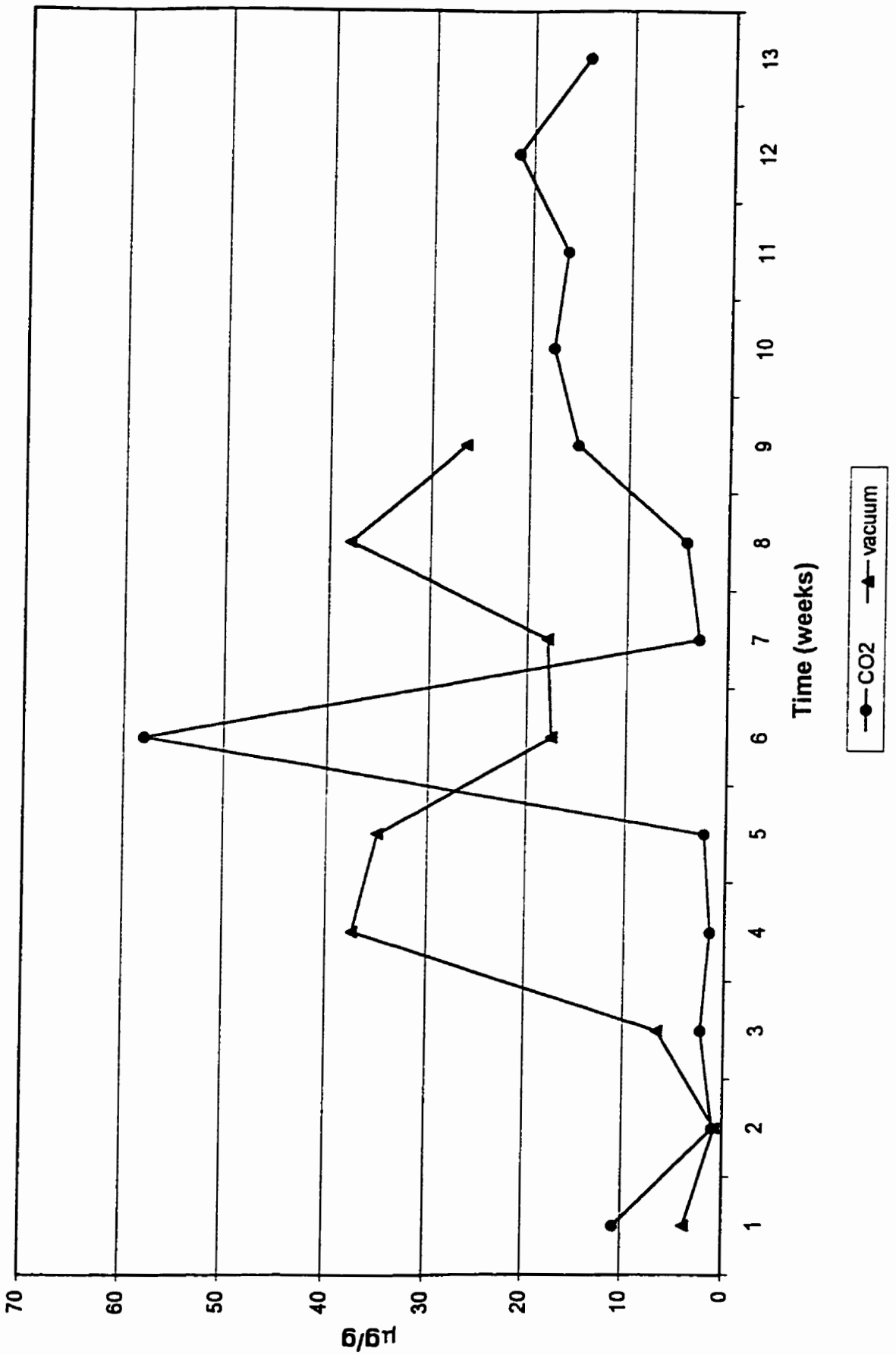


Figure 11. Tyramine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC

CO_2-MAP

The tyramine levels in the CO_2-MAP pork were low with the exception of a large spike at week 6 (Fig. 11; Appendix 28). During this sharp increase, the level of tyramine approached 60 μg/g. Towards the end of the 13 week storage period the levels of tyramine remained in the range of 14 μg/g to 21 μg/g.

Comparison – Vacuum Packaging and CO_2-MAP

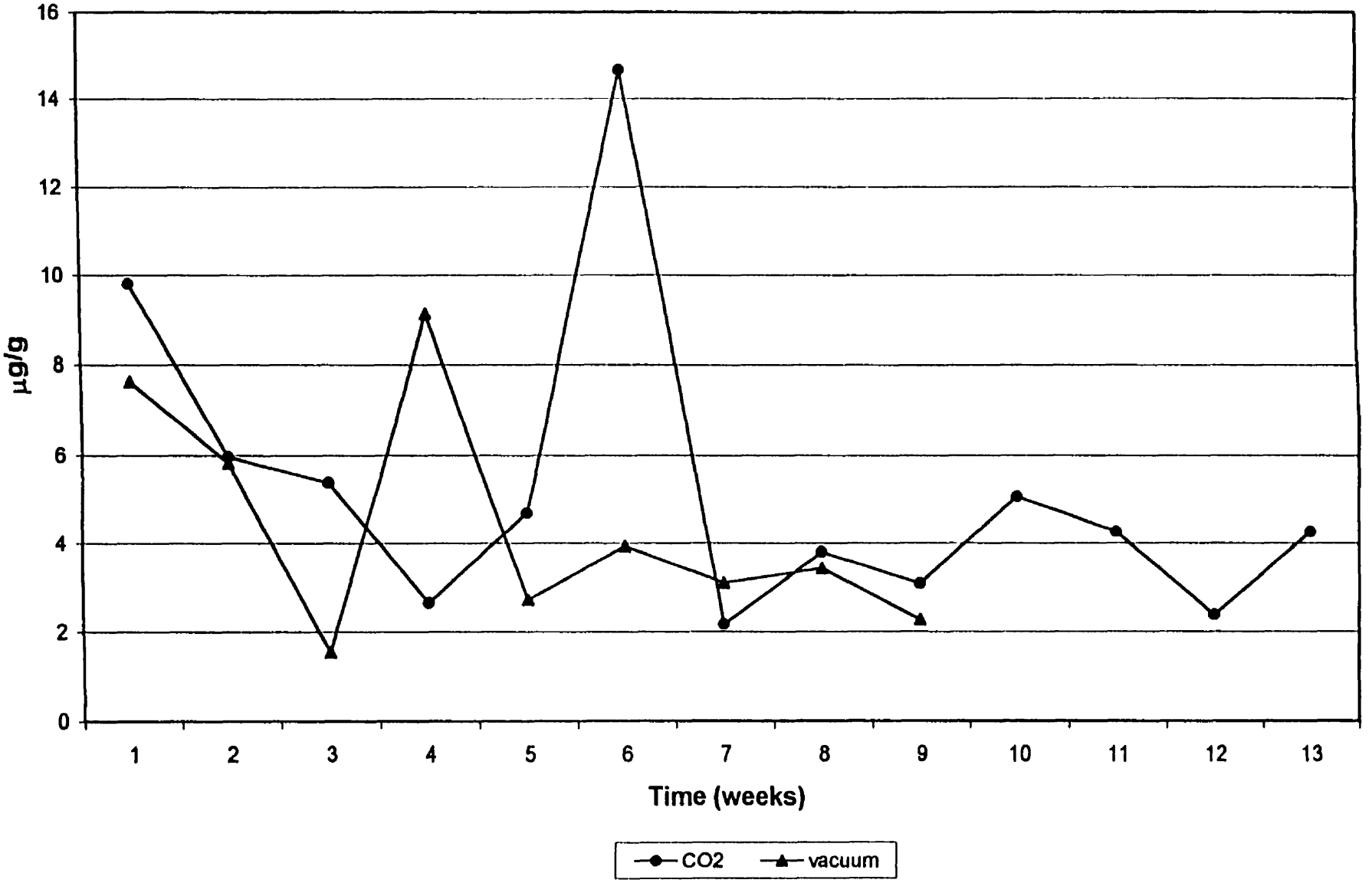
There were many differences in tyramine accumulation between the vacuum and CO_2-MAP pork throughout the storage period. While the CO_2-MAP pork had the highest level of tyramine for only a short period of time around week 6, the vacuum-packaged pork maintained generally higher tyramine levels for most of the storage period after week 3 (Appendix 29).

Spermidine

Vacuum Packaging

Spermidine levels were low throughout the 9 week storage period. With an initial concentration of 7.6 μg/g in the first week, the level of spermidine subsequently decreased to 1.5 μg/g by week 3 (Fig. 12; Appendix 30). As with the other amines, there was a significant spike in spermidine concentration to 9 μg/g at week 4. The concentration then declined and stayed between 2.2 μg/g and 3.9 μg/g for the remaining 4 weeks.

Figure 12. Spermidine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC



CO_2-MAP

As with most of the other biogenic amines in the CO_2-MAP pork, the only significant increase in spermidine occurred at week 6 when the concentration reached nearly 15 μg/g (Fig. 12; Appendix 31). For all other weeks in the 13 week storage period, spermidine levels ranged from 2 μg/g to 9.7 μg/g.

Comparison – Vacuum Packaging and CO_2-MAP

The spermidine levels of the vacuum-packaged and CO_2-MAP pork were quite comparable. On two occasions, week 3 and week 6, the CO_2-MAP pork had higher spermidine accumulations (Appendix 32).

Spermine

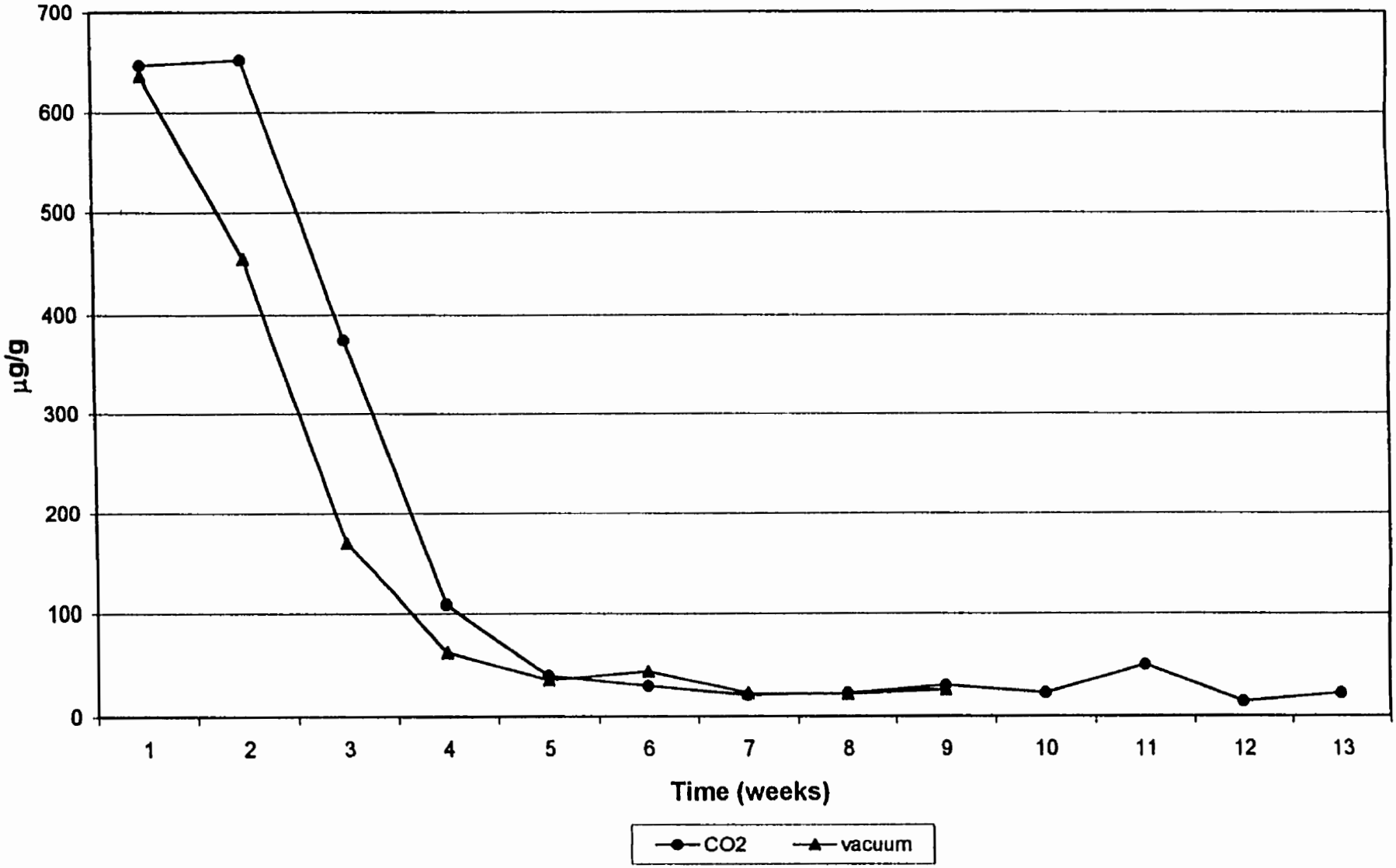
Vacuum Packaging

Spermine concentrations were initially very high, over 600 μg/g (Fig. 13). The level decreased significantly every week for the first 4 weeks down to 63 μg/g (Appendix 33). For the remainder of the storage period the spermine level remained unchanged.

CO_2-MAP

Spermine levels in the CO_2-MAP pork also were initially high, 646 μg/g for both weeks 1 and 2, and then gradually decreased as the time in storage progressed (Fig. 13). After the 4^{th} week, spermine levels were below 40 μg/g and there were no other significant changes (Appendix 34).

Figure 13. Spermine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by HPLC



Comparison – Vacuum Packaging and CO_2-MAP

There was very little difference observed between the two packaging systems with respect to spermine concentrations in the pork. Identical trends were observed in both cases: high initial levels of spermine which declined in the first few weeks of storage and then remained unchanged for the remainder of the storage period. The only difference noted was the sharper decline in spermine for weeks 2 and 3 of the pork which was vacuum-packaged (Appendix 35). The high physiological levels of spermine in pork muscle that decrease in the carcass with time has been reported by other researchers (Shalaby, 1996). This same trend has also been observed in the spermine levels of various fish (Shalaby, 1996).

HPLC Biogenic Amines Determination: General Conclusions

Comparison of Packaging Treatments

There were several outstanding differences detected between the biogenic amines of the vacuum-packaged and the carbon dioxide modified atmosphere-packaged pork. In both types of packaging, there was a spiking phenomenon observed with many of the biogenic amines. These sharp changes in biogenic amine concentrations may be related to the levels of mono and diamine oxidases in the meat responsible for amine catabolism; however, this hypothesis has not been investigated. This theory has been pursued with respect to amine buildup in cheese (Voigt and Eitenmiller, 1978). They found that a wide variety of retail cheeses in the United States contained very low oxidase activity. However, they did conclude that when the oxidases were present, the amine content of the cheeses were lower (Voigt and Eitenmiller, 1978). In the vacuum-packaged samples

these spikes occurred at week 4 of the storage period whereas in the CO_2-MAP they occurred at week 6. Thus the modified atmosphere was successful in delaying the first significant increases in biogenic amines by 2 weeks. This was not surprising since the CO_2 was expected to increase the lag phase, and it was found that the growth of lactic acid bacteria was also delayed in the CO_2-MAP relative to the vacuum-packaged pork.

Another noteworthy difference was the greater accumulation of the putrefactive amines, putrescine and cadaverine, in the final weeks of storage in the vacuum-packaged samples. These results relate well with the odour scores of the sensory evaluation experiments and also with the growth of lactic acid bacteria, as mentioned previously. Odour scores during the final 3 weeks of the vacuum-package pork were unacceptable whereas the CO_2-MAP remained acceptable. Thus in this study, the deterioration of vacuum-packaged sample odour can be attributed to the accumulation of significant amounts of putrescine and cadaverine as a result of high populations of bacteria.

There is opportunity to speculate on the reason for differences found in the levels of the various amines when comparing the two packaging treatments of this research. In addition to the effects of CO_2 on membrane lipids, membrane proteins, and cytoplasm; CO_2 also is known to affect bacterial enzymes (Dixon and Kell, 1989). Carbon dioxide causes alterations in the rate of bacterial enzymatic reactions, in either an inhibitory or stimulatory manner. Carboxylation and decarboxylation reactions are known to be particularly affected by CO_2 (Dixon and Kell, 1989). Additionally, CO_2 may affect bacterial enzyme synthesis. Thus in this study it is clear that the carbon dioxide in the

modified atmosphere was responsible for the differences in amine concentrations when compared to the vacuum condition; most probably a result of the effects of CO_2 on bacterial decarboxylases.

The potential for the use of the diamines as spoilage indicators in fish, meat, and other products stored at chill temperature has been well investigated (Edwards *et al.*, 1983; Mietz and Karmas, 1977). Results have been conflicting. Some have reported that significant changes in either putrescine or cadaverine did not occur until the meat was obviously spoiled, thus rendering their ability to act as a spoilage indicator or predictor useless (Edwards *et al.*, 1983). Conversely, others have reported that the relationship between putrescine, cadaverine, and other amines (histamine, spermidine, and spermine) and the quality of fresh tuna can be an effective chemical index to indicate quality (Mietz and Karmas, 1977).

HPLC Method Evaluation

With HPLC as the analytical method, biogenic amines in this investigation were detected in amounts comparable to meat that was packaged and stored under similar conditions (Krizek, *et al.*, 1995). The investigation by the Krizek group, who also used HPLC as the analytical method, found that vacuum-packaged fresh beef stored at –2°C accumulated a maximum of 140 μg/g of tyramine, 100 μg/g putrescine, and 30 μg/g cadaverine. These values certainly are within the range of the concentrations determined by HPLC in this study. However, they found all three amines increased at a constant rate over time to

reach peak concentrations at the end of the 100 days rather than peak at certain points in the storage period, like some of the amines in this study at weeks 4 and 6.

The HPLC method used in this study is a well-established, previously published method (Eerola *et al.*, 1993). The perchloric acid extraction has been shown to be highly effective, with recoveries of biogenic amines ranging from 90% to 103% (Eerola *et al.*, 1993). The limits of determination for this method were 1 μg/g for tyramine, spermine, spermidine, and cadaverine; and 2 μg/g for phenylethylamine and putrescine (Eerola *et al.*, 1993).

The drawback to the HPLC method used in this study was the extremely large variation amoung samples, standard deviations were very high, and in many cases the coefficient of variation was greater than 100%. Other investigators have not reported such large variations, the coefficient of variation is most often less than 10% (Eerola *et al.*, 1993; Krizek *et al.*, 1995). The biogenic amine standards used for analysis in this study were determined with high precision. Thus, the reason for the high variation must be related to either the natural variation in the biological material (this study was the first to determine biogenic amines in pork tissue) or the extraction procedure, or both.

2. Capillary Gel Electrophoresis Method

Due to the high variability of the HPLC biogenic amine analysis, another method was selected in an attempt to verify the results. It was at this point that the comparison of analytical methods became an additional objective. Capillary gel electrophoresis is a new

method with respect to biogenic amine determination and there is very little literature information available. The particular method used in this study is being developed by researchers at the University of Alberta (Dr. E. LeBlanc, Department of Foods and Nutrition, University of Alberta, Edmonton, Alberta). At the time they were able to determine phenylethylamine, putrescine, cadaverine, histamine, and tyramine, but not spermidine or spermine. All analyses were performed by technicians at the University of Alberta.

According to the electrophoresis results, the amines were below the limit of detection in many samples whereas they were detected in almost every sample by HPLC. For putrescine, cadaverine, histamine, and tyramine the electrophoresis limit of detection was 0.1 μM, for phenylethylamine it was 0.2 μM. Tyramine was detected in 70% of the samples, cadaverine in 64%, putrescine in 61%, histamine in 45%, and phenylethylamine in 23%.

Vacuum-Packaging

The samples in which phenylethylamine was detected contained less than 2 μg/g (Fig. 14). The putrescine was less than 2 μg/g for the first 7 of the 9 weeks, then increased to 16 μg/g by week 9 (Fig. 15). There was a similar trend with the cadaverine, which was present at less than 2 μg/g until week 8 when it increased to 48 μg/g and then ended the storage period with 30 μg/g at week 9 (Fig. 16). For the entire storage period there was less than 1 μg/g of histamine detected (Fig. 17). The tyramine concentration was initially

Figure 14. Phenylethylamine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by capillary gel electrophoresis

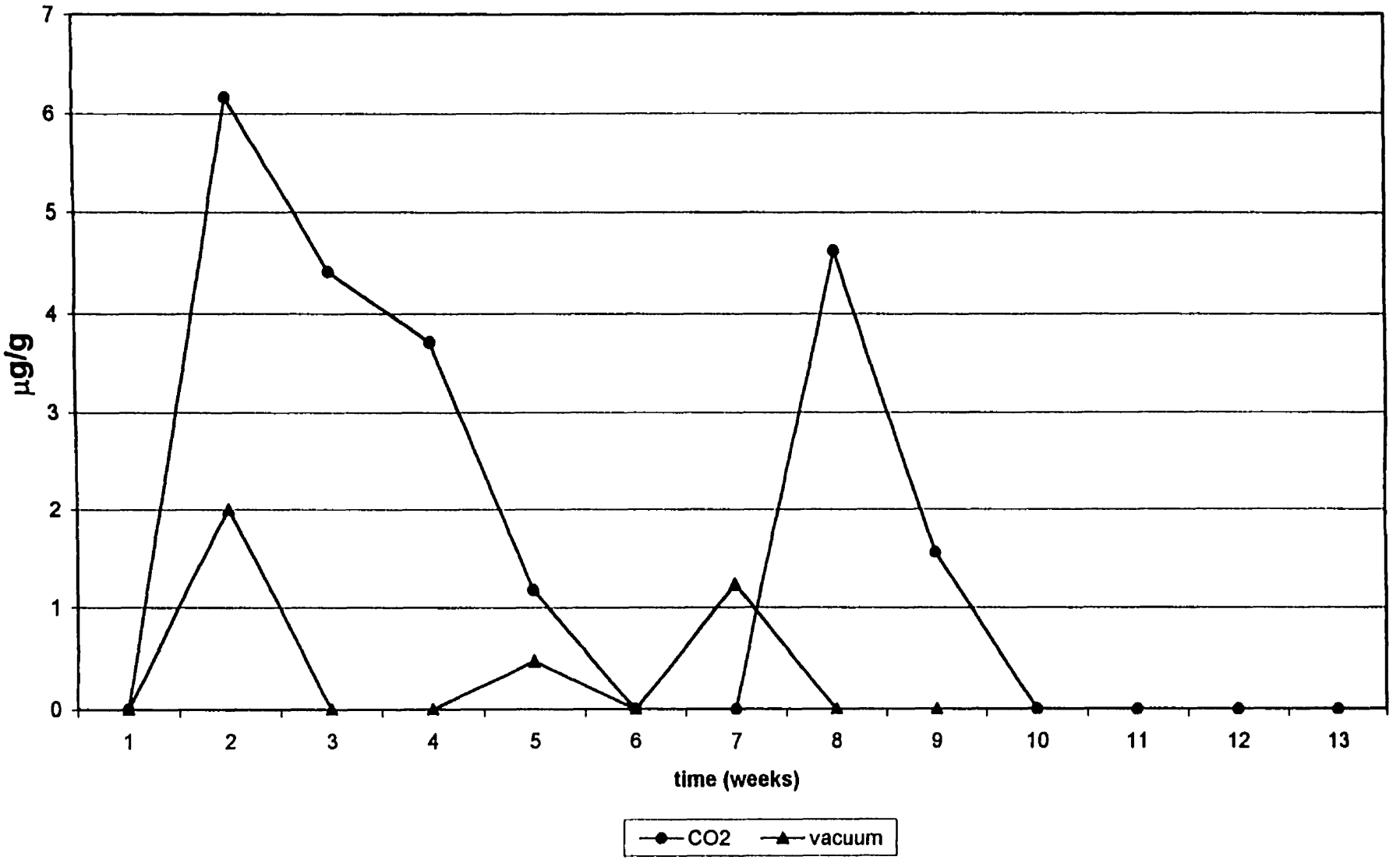


Figure 15. Putrescine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by capillary gel electrophoresis

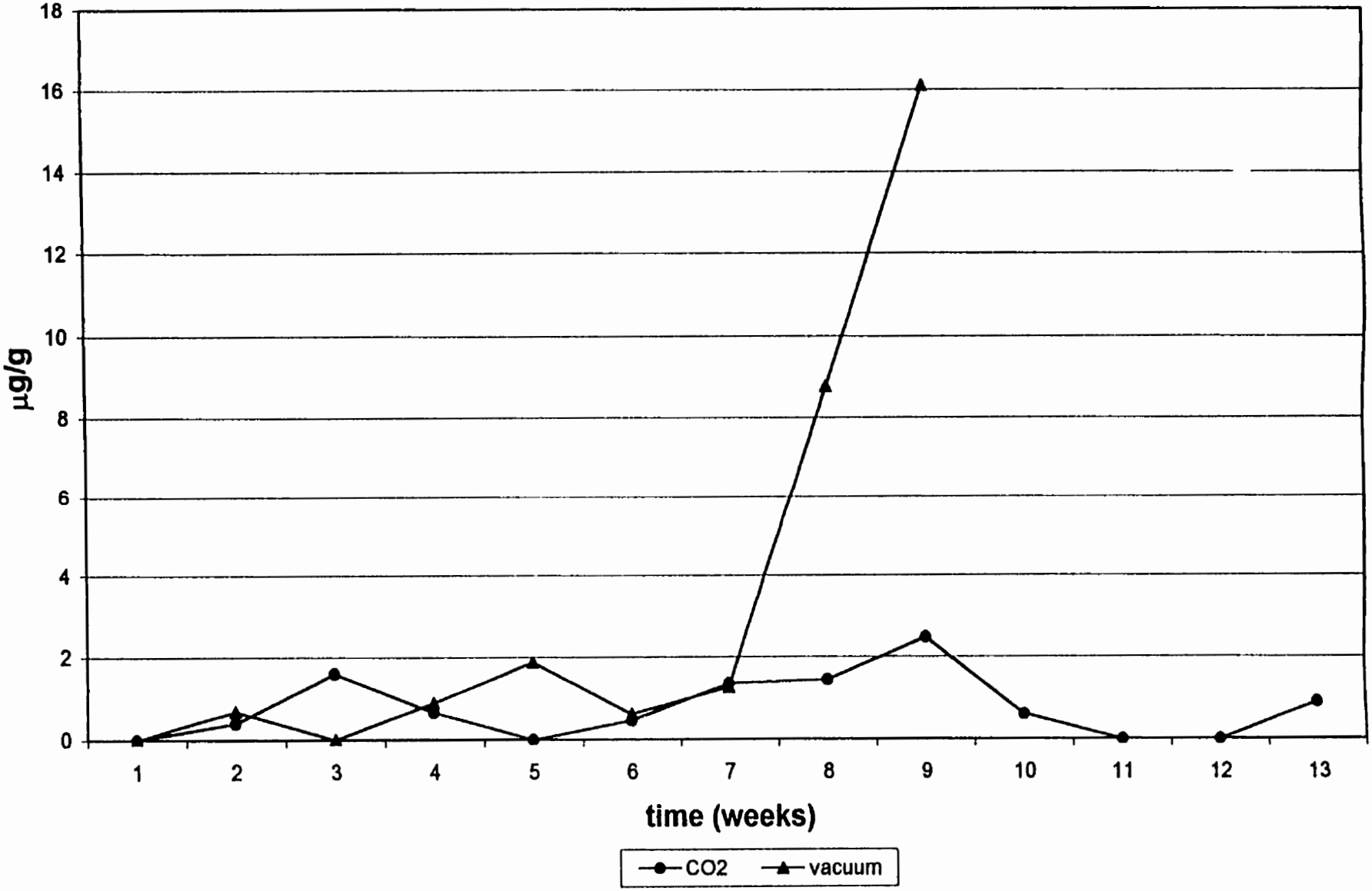


Figure 16. Cadaverine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by capillary gel electrophoresis

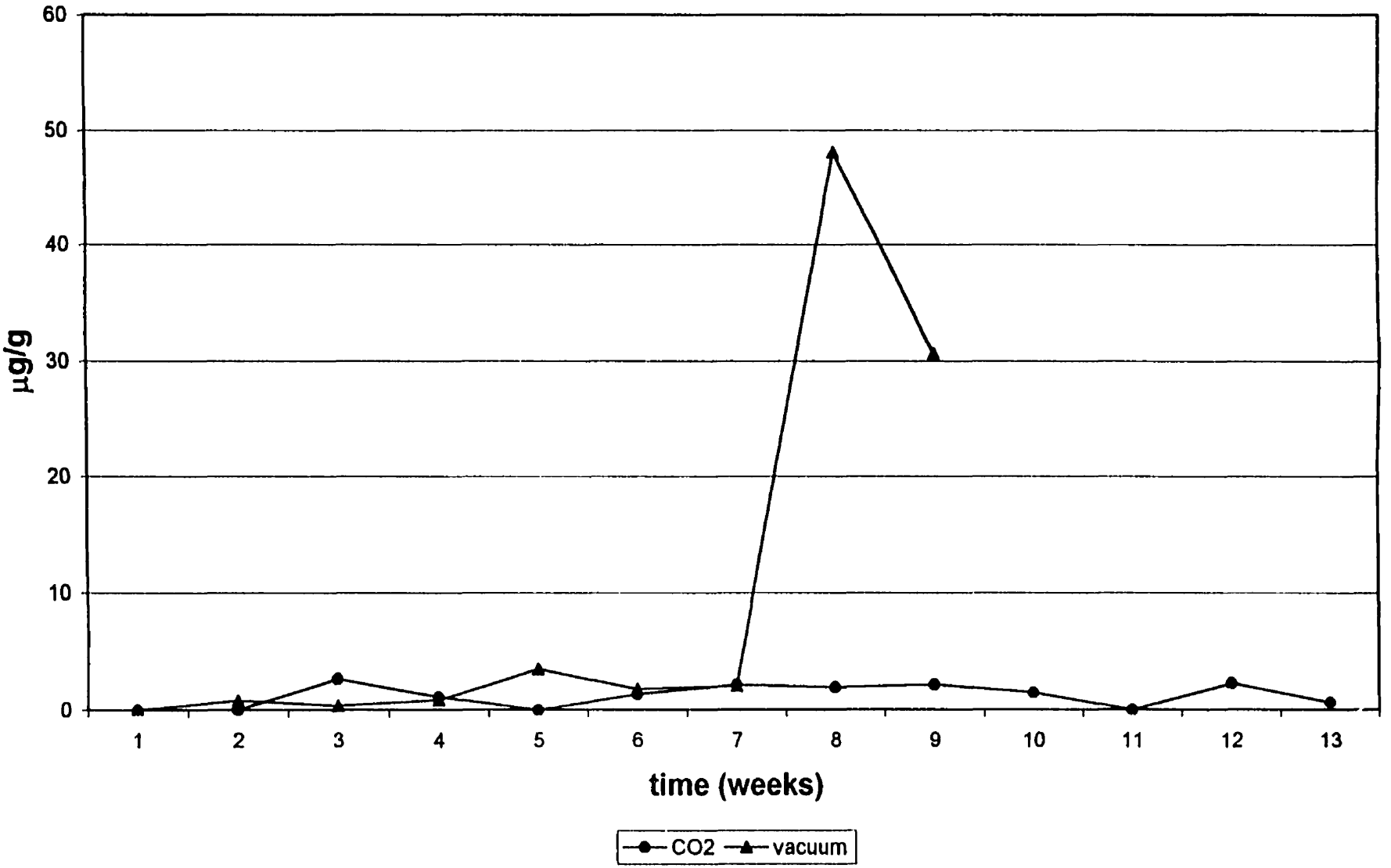


Figure 17. Histamine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by capillary gel electrophoresis

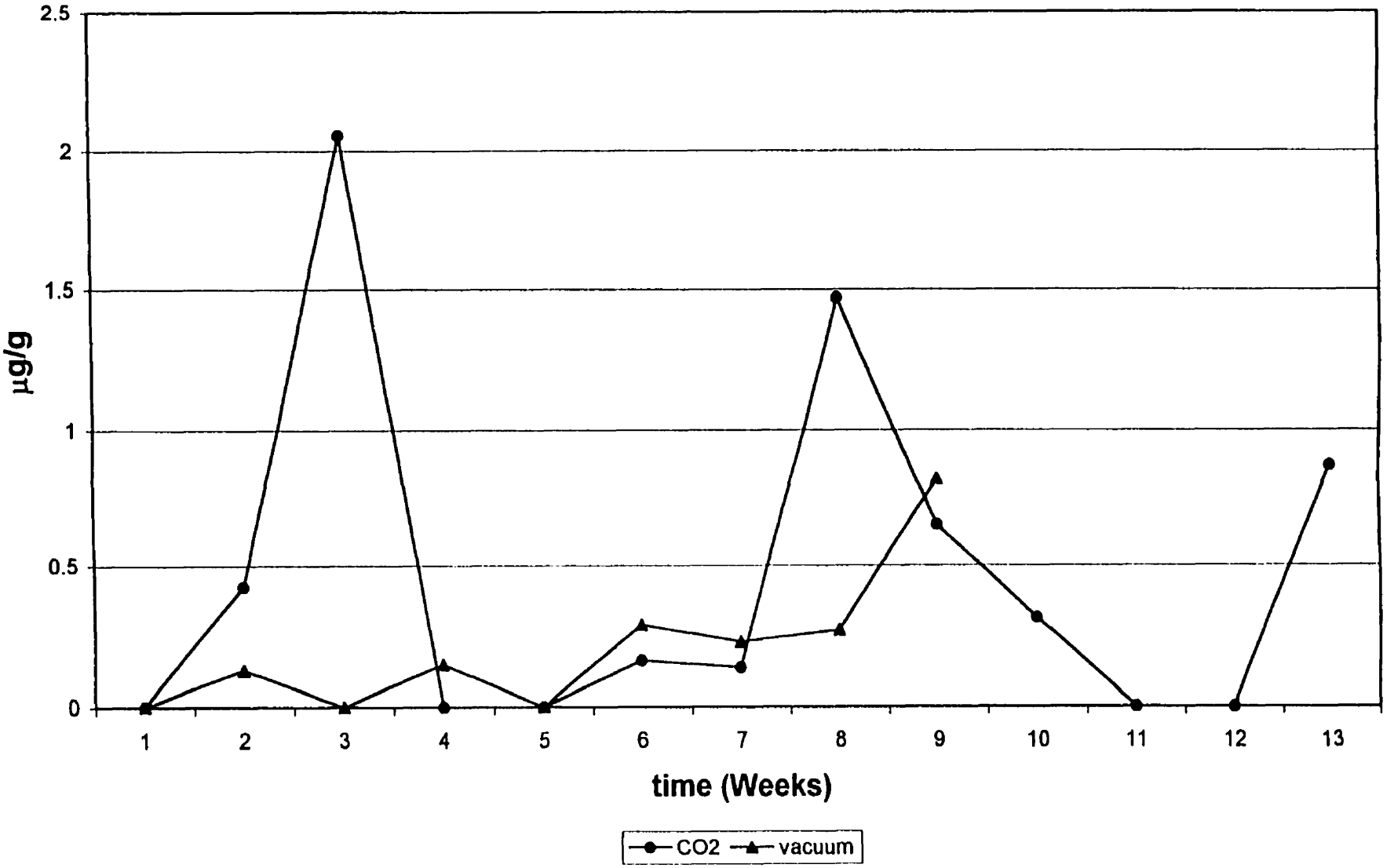
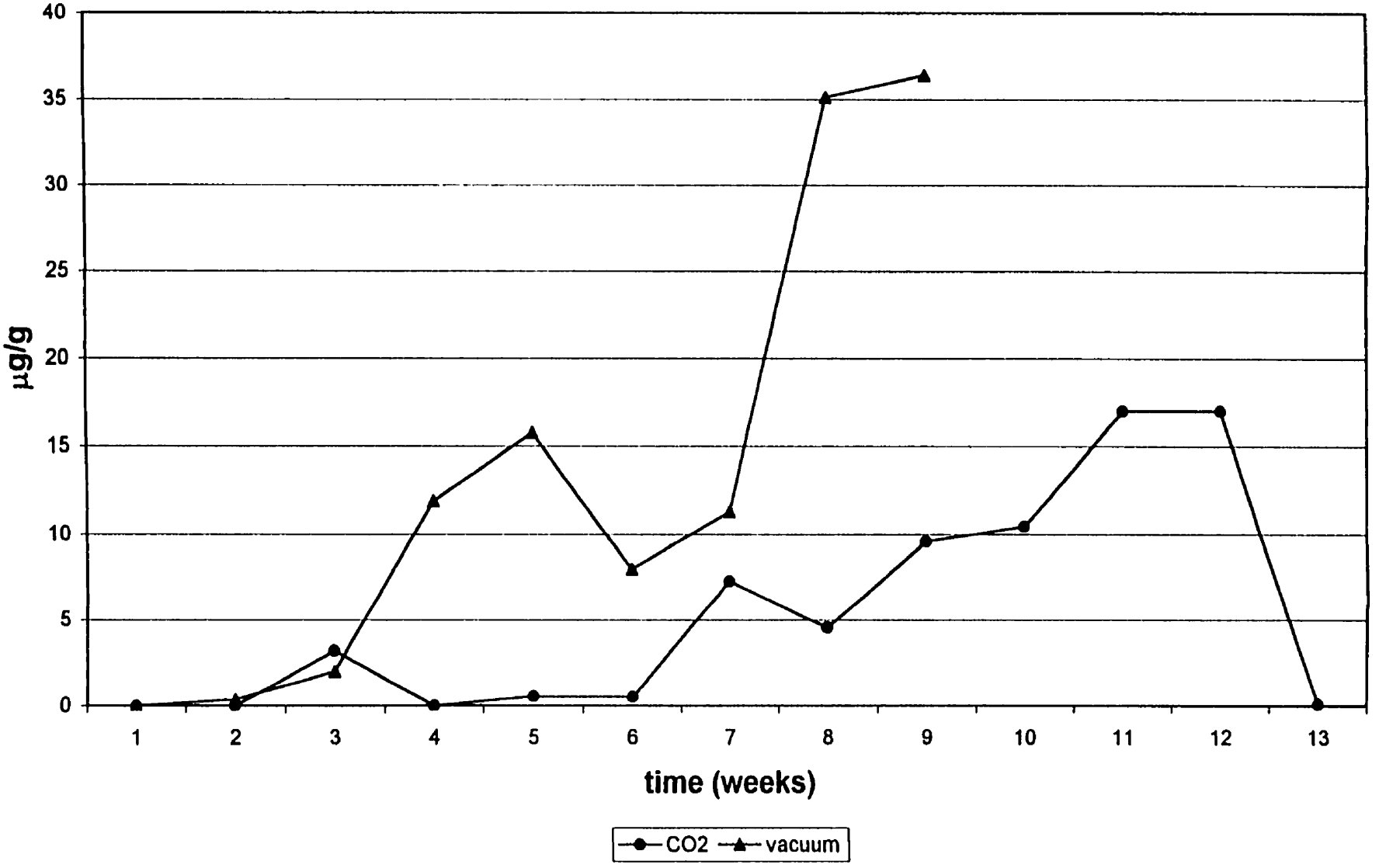


Figure 18. Tyramine in vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C determined by capillary gel electrophoresis



low and then increased after week 7 to a final concentration of 36 μg/g at week 9 (Fig. 18).

CO_2-MAP

When detected, the phenylethylamine in the CO_2-MAP samples did not exceed 6 μg/g. There were 2 small increases in phenylethylamine at weeks 2 and 8 (Fig. 14). The putrescine levels fluctuated throughout the 13 weeks with maximum concentrations at weeks 3 and 9; however, the concentration never exceeded 2.5 μg/g (Fig. 15). Cadaverine also fluctuated but did not exceed 3 μg/g in any week (Fig. 16). The results of the histamine determination were the same as the cadaverine with respect to the low level detected, less than 2.5 μg/g (Fig. 17). Tyramine; however, was the only amine detected that increased substantially (Fig. 18). The initial tyramine level was very low but then increased to nearly 17 μg/g in the final weeks of storage.

Capillary Gel Electrophoresis Biogenic Amines Determination: General Conclusions

Comparison of Packaging Treatments

Several factors prevent the meaningful comparison of biogenic amine concentrations of the two packaging treatments determined by the electrophoresis method. Statistical evaluation of the data was not possible. This was because only one half of the total pork extract samples were sent for analysis by electrophoresis, the determinations were not done in duplicate, and the method was not able to detect amines in a substantial number of the samples. This was a surprise since the amines were expected to be well above the limits of detection as they were with the HPLC determination. However, from a

toxicological perspective, fluctuations in biogenic amine concentrations as low as most of the levels detected by capillary gel electrophoresis (below 10 μg/g) would be irrelevant. In general; however, it can be said that the CO_2-MAP pork had fewer biogenic amines than the vacuum-packaged pork, in particular, the putrescine, cadaverine, and tyramine.

Capillary Gel Electrophoresis Method Evaluation

Based on the inability to detect the amines in many samples, capillary gel electrophoresis was not an efficient method for this determination of biogenic amines in fresh pork. The results were inconclusive and the number of samples that had no detectable amines led to an incomplete data set. Many types of capillary electrophoresis have been successfully applied to diverse quantitative food analyses including the determination of analytes such as inorganic ions, nitrates and nitrites, organic acids, amino acids, polyphenols, and proteins from a vast variety of food matrices (Lindeberg, 1996). Thus it had been expected that the determination of amines from pork would be a realistic endeavor.

Unfortunately it was not possible to do a spermine determination with this method, which would have assisted in conclusions regarding the reliability of the method. This is because spermine concentrations in muscle tissue and their performance over time is well established; they are known to be very high in freshly slaughtered muscle (in the hundreds of micrograms) and then decrease over time (Shalaby, 1996).

3. Method Comparison: HPLC and Capillary Gel Electrophoresis

There is one fact to consider when comparing the suitability of these two methods for biogenic amines determination in fresh pork: either the electrophoresis method

underestimated the biogenic amine concentrations, or the HPLC method grossly overestimated them. Evidence strongly points to the former.

The HPLC determinations were done each week on fresh extracts immediately following the removal of the pork from the storage chamber. Conversely, the electrophoresis determinations were performed on perchloric acid extracts of the pork samples many months after extraction. In the time between sample extraction and electrophoresis determination, the extracts were stored at 4°C in sealed plastic vials. During this time there could have been deterioration of the extracts. However, simply the large difference in sample handling procedures alone could quite possibly explain the discrepancy in results. Since the HPLC determinations were performed on the freshest extracts, it seems this method was likely to be the more accurate.

The concentrations of the biogenic amines determined by the HPLC method were quite comparable to literature values for similar products stored long term at the same temperatures (as was discussed in a previous section). Many of the electrophoresis results remain near the natural physiological levels of biogenic amines in muscle. Since we know that the lactic acid bacteria (and possibly carnobacteria) dominate the flora in both of the packaging systems, and that these organisms are indeed capable of amino acid decarboxylation; it therefore seems unlikely that the concentrations of biogenic amines would remain so low during storage.

The use of HPLC for biogenic amine determination has been well established for application to an extremely diverse range of food products, including dairy products, beverages, chocolate, fish products, and meat products (Hurst, 1990). The work-up procedure of the sample leading up to its evaluation is a critical step in any determination, and is ultimately related to the final chromatographic separation (Battaglia, 1982). The preparation procedure for the HPLC determination (perchloric acid extraction, derivatization with dansyl-chloride) was developed together with the HPLC specifications, such as the mobile phases and gradient profile (Eerola *et al.*, 1993). The extraction, derivatization, and HPLC determination of amines from meat samples by this specific method has been established (Eerola *et al.*, 1993). Perhaps this specific extraction procedure was not the ideal method for pork samples and the capillary gel electrophoretic detection of amines.

C. SENSORY EVALUATION

The purpose of the sensory evaluation was to determine the storage life of the pork in each type of packaging based on the odour and overall appearance of the pork loin cuts. Eight untrained panelists participated in the small scale affective (consumer oriented) study.

The sensory evaluations were performed on previously frozen samples (see Materials and Methods). The effects of freezing and thawing on the quality and retail case life of pork have been investigated (Greer and Murray, 1991). In that study, pork loin cuts were

frozen for 90 days (-30°C) and thawed (2°C) for 48 hours prior to analysis. Their research revealed that except for a slight darkening of lean muscle colour, freezing and thawing had no effect on pork quality, in particular odour and subjective appearance (Greer and Murray, 1991). These results implied that the use of frozen/thawed pork instead of fresh was both a valid and convenient method for the evaluation of pork muscle characteristics.

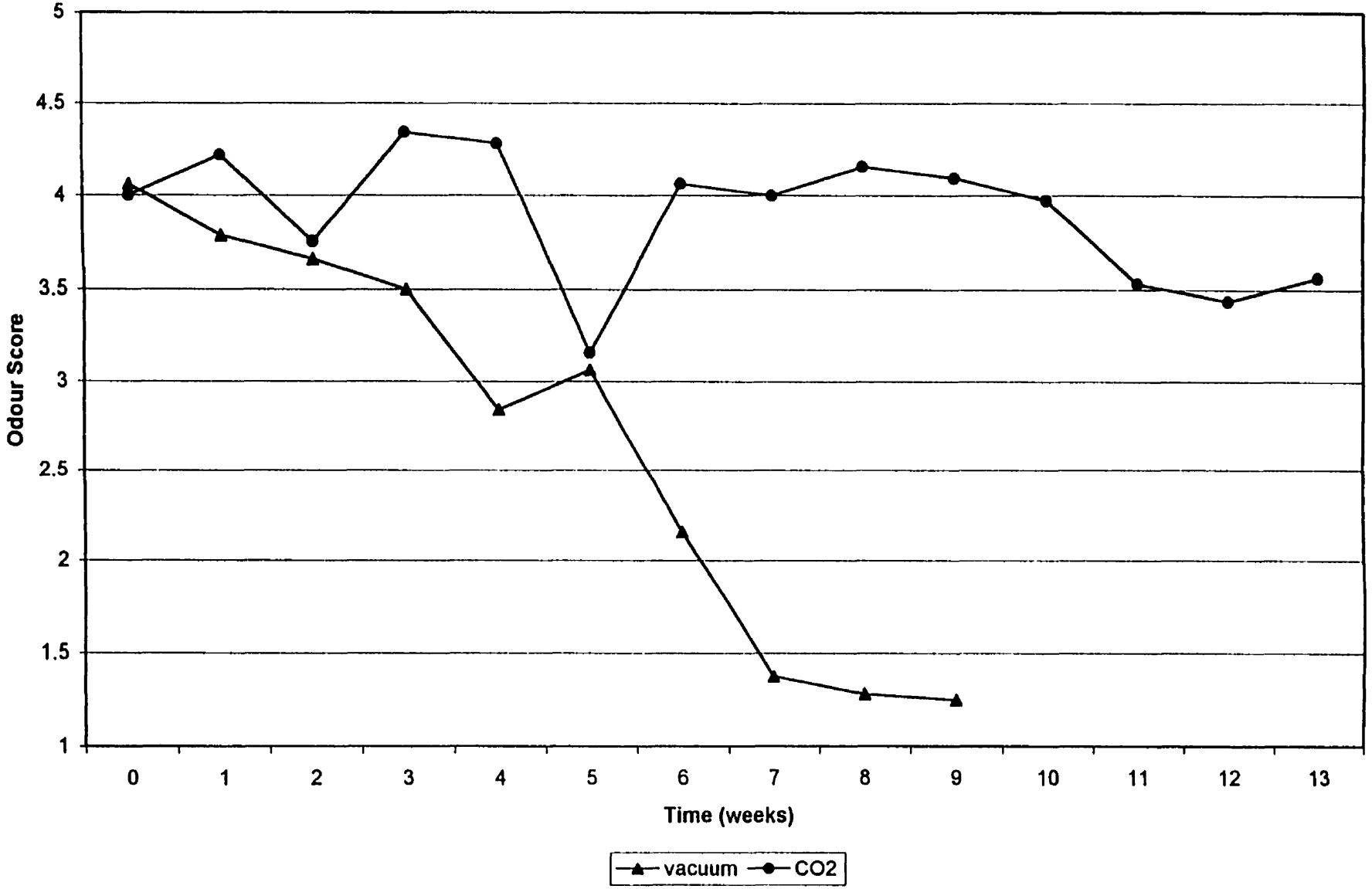
1. Evaluation of Sample Odour

Samples were scored on a 5-point odour scale. A score of 3 was chosen to designate an unacceptable odour or the end of the storage period. The category for a score of 3 was "neither acceptable nor unacceptable". This was assumed to mean that product rejection was probable. Panel members stated that the scent of the plastic Ziploc® bags in which the samples were contained may have interfered with their evaluation of odour.

Vacuum Packaging

The odour of the vacuum-packaged pork was initially acceptable (score = 4.06) then steadily declined to unacceptable (score = 2.84) by week 4 of the storage at −1.5°C (Fig. 19). There was a significant decrease in odour acceptability between week 3 and week 4 (Appendix 36). Except for a marginally acceptable score at week 5 (3.06), the odour thereafter became increasingly unacceptable. Significant decreases in odour scores occurred between weeks 5, 6, and 7. The development of unacceptable odour after only approximately 4-5 weeks of storage was very early relative to comparative studies. Investigations by Jeremiah *et al.* (1992b) revealed that off odours (identified as dairy and

Figure 19. The odour scores of vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C



putrid odours) in vacuum-packaged fresh pork stored at –1.5°C did not occur until week 15. Panelists in our study commented on the extremely objectionable odour of many of the samples from weeks 6, 7, 8, and 9. Edwards and Dainty (1987) identified volatile spoilage compounds in vacuum packaged pork stored at 5°C as 'acid', or 'sour', due to the accumulation of organic end-products from the glucose metabolism of lactic acid bacteria. It would follow that these end-products were also responsible for the off-odours of the pork in our study since the lactic acid bacteria were shown to proliferate. Based on sample odour, the vacuum-packaged pork in this study had a storage life of 4 weeks when kept at –1.5°C.

CO_2-MAP

The pork packaged in a carbon-dioxide modified atmosphere maintained acceptable odour throughout the storage period (Fig. 19). The lowest score received, at week 5 (3.16), was a significantly lower odour score compared to weeks 4 and 6; however, it was still considered acceptable (Appendix 37). These findings are comparable to those of Jeremiah *et al.* (1995), who demonstrated the development of slight off-odours (determined by a trained, experienced panel) after 12 weeks of CO_2-MAP storage at –1.5°C. These slight off-odours (not identified) were still acceptable after 15 weeks of storage. Based on odour, the carbon dioxide modified atmosphere-packaged pork in this study had a storage life of at least 13 weeks at –1.5°C.

Comparison – Vacuum Packaging and CO_2-MAP

The CO_2-MAP was quite successful in increasing the storage life of the fresh pork on the basis of odour by approximately 9 weeks. After week 2, the odour scores for the vacuum-packaged pork were significantly lower than those for the carbon dioxide modified atmosphere- packaged pork, with the exception of week 5 (Appendix 38). The presence of saturating levels of CO_2 in the package were sufficient to suppress the formation of off-odours associated with the rapid bacterial growth in vacuum-packaged pork. This was reasonable due to the significantly higher and faster growth of the lactic acid bacteria in the vacuum-packaged pork.

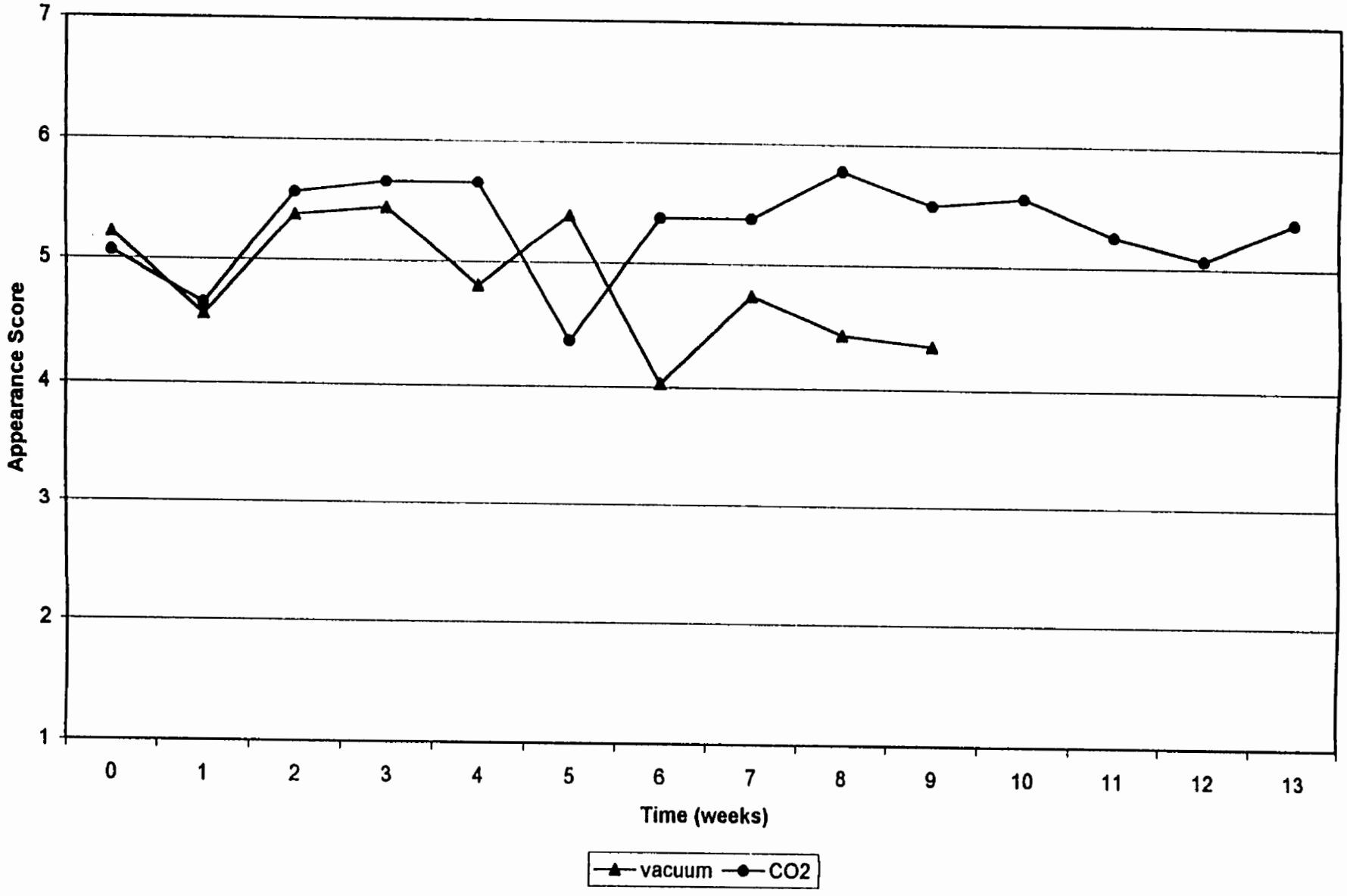
2. Evaluation of Overall Sample Appearance

Samples were scored on a 7-point scale. A score of 4 (neither desirable nor undesirable in appearance) was chosen to indicate an unacceptable overall appearance. Panel members commented that the interaction of the meat and plastic bag (an effect of meat sticking to the plastic) may have interfered with the evaluation of appearance.

Vacuum-Packaging

The appearance of the vacuum-packaged samples remained acceptable throughout the nine-week storage period at −1.5°C (Fig. 20). These results are in agreement with recent investigations which found no decline in overall appearance of vacuum-packaged pork loins until 18 weeks of storage at −1.5°C (Jeremiah *et al.*, 1992b). In that study, it was muscle discolouration due to metmyoglobin (brown) production that was responsible for the eventual decline in appearance. The lowest score of the vacuum-packaged samples in

Figure 20. The appearance scores of vacuum-packaged and CO_2-MAP fresh pork stored at -1.5°C



this study, 4.03, was recorded at week 6. The only significant decline in the overall appearance score occurred between weeks 5 and 6 (Appendix 39).

CO_2-MAP

The appearance of the CO_2-MAP pork also remained acceptable throughout its 13-week storage period at −1.5°C (Fig. 20). There is little information available regarding the effect of CO_2 atmospheres with very low oxygen levels on pork colour. This study agrees with other preliminary work (Jeremiah *et al.*, 1995) in that normal pork is relatively resistant to deterioration in appearance due to low oxygen levels in the package. Jeremiah *et al.* (1995) found that CO_2-MAP pork loins stored at −1.5°C maintained an appearance within the acceptable range after 15 weeks. The scores for the samples in this study only decreased to below 5 on two occasions, weeks 1 and 5. The effect of the meat sticking to the plastic bags may have been responsible for these lower scores. The decline to a score of 4.38 at week 5 was a significant decrease compared to weeks 4 and 6. Overall, the CO_2-MAP was very effective in maintaining acceptable appearance of the pork; there was no significant difference between the appearance scores from week 0 and week 13 (Appendix 40).

Comparison – Vacuum Packaging and CO_2-MAP

According to overall meat appearance, both the vacuum-packaged and CO_2-MAP pork were in the acceptable range throughout their respective storage periods of 9 and 13 weeks. It has been established by Hall *et al.* (1980) that packaging treatment has little effect on the appearance of pork. They found no differences in muscle colour, muscle

surface discolouration, or overall appearance between vacuum-packaged and a variety of CO_2/N_2 modified atmosphere-packaged fresh pork loins after 4 weeks of refrigerated storage. After week 4; however, the scores for the CO_2-MAP pork in this study remained significantly higher than the scores for the vacuum-packaged (Appendix 41). Thus the modified atmosphere was more successful in maintaining desirable pork appearance during the entire 13 week storage period at -1.5°C.

3. Sensory Evaluation – General Conclusions

Based on sensory evaluation, the storage life of the fresh pork in vacuum-packaging stored at –1.5°C was 4-5 weeks. In comparison, the storage life of the CO_2-MAP pork was at least 13 weeks. With respect to the vacuum-packaged pork, odour was the factor responsible for limiting the storage life. While the appearance of the vacuum-packaged pork remained acceptable for the 9 week duration, the development of objectionable odours after approximately 4-5 weeks limited the storage life of pork stored under these conditions. According to our results these odours can be attributed to the rapid growth of bacteria in the vacuum-packaged pork; whereas their growth is slowed in the CO_2-MAP pork.

SECTION V

CONCLUSIONS AND RECOMMENDATIONS

A. RELATIONSHIPS BETWEEN BIOGENIC AMINES, LACTIC ACID BACTERIA, CARNOBACTERIA, ODOUR, AND APPEARANCE

There are many relationships that may be drawn which interconnect biogenic amine production (as determined by HPLC), bacterial growth, and the organoleptic properties of fresh pork with respect to vacuum- and modified atmosphere-packaging. The accelerated growth of lactic acid bacteria in the vacuum-packaged pork led to its hastened spoilage compared to the CO_2-MAP pork. Significant increases in the growth of lactic acid bacteria in the vacuum-packaged pork at weeks 7, 8, and 9 coincided with the significant decrease in odour scores at the same time. The final link was the significant increase in both putrescine and cadaverine which occurred in the final 3 weeks of the vacuum-packaged storage. Correspondingly, neither putrescine nor cadaverine increased significantly in the final weeks of CO_2-MAP storage and this was reflected in the acceptable odour scores during the last weeks of storage.

In the vacuum-packaged pork there was a significant peak in each of phenylethylamine, putrescine, histamine, tyramine, and spermidine at week 4 of the storage at −1.5°C. This was reflected in a significant decrease in odour score at week 4 when the score initially dipped to the unacceptable range. However, there were no significant trends at week 4 with the lactic acid bacteria that would explain the spiking.

A similar phenomenon occurred with the CO_2-MAP pork; there were significant increases (in the form of spikes) in the concentrations of phenylethylamine, putrescine,

cadaverine, histamine, tyramine, and spermidine at week 6. However, at week 6 there were significant *increases* in both odour and appearance scores. Additionally, there was a significant increase in lactic acid bacteria at week 6. This jump in the bacterial population may have been responsible for the short-term increase in biogenic amines but cannot explain the apparent increase in odour and appearance acceptability.

Determining relationships that involve the carnobacteria are slightly more difficult. However, the decline in carnobacteria during the last 5 weeks of CO_2-MAP storage and their near total dominance in the vacuum-packaged samples indicate that they may be at least in part responsible for the putrefactive amine production leading to meat spoilage.

Therefore, the concentrations of putrescine and cadaverine corresponded with pork odour and increased with the growth of lactic acid bacteria, especially in the conditions created by a vacuum package. In either type of package, most of the amines increased sharply at a certain point during the storage. Although the reasons for the spike in amine concentrations remain unknown, it *is* known that the carbon dioxide modified atmosphere packaging delayed the increases by 2 weeks relative to the vacuum.

B. TOXIC POTENTIAL OF VACUUM-PACKAGED AND CARBON DIOXIDE MODIFIED ATMOSPHERE-PACKAGED FRESH PORK

From a toxicological standpoint, the amount of histamine in the pork of either packaging systems is no probable cause for concern. Bartholomew *et al.* (1987) determined that > 5mg/100g (= 50 μg/g) is required for the food to be considered possibly toxic. The highest concentrations of histamine determined in this study were 12 μg/g in the CO_2-MAP pork (week 6) and 15 μg/g in the vacuum-packaged pork (week 4). However, even with these low concentrations there is a possibility of an adverse reaction due to the presence of both putrescine and cadaverine, histamine potentiators.

A concentration of 30 ppm (= 0.03 μg/g) phenylethylamine is regarded as acceptable (Nout, 1994), a level which the phenylethylamine in the pork of both packaging systems of this study were both well above by at least 3 orders of magnitude. The toxicological significance of phenylethylamine is not yet well understood. However, it should be noted that the phenylethylamine concentrations in the pork were above the levels currently considered 'safe'.

Depending on the monoamine oxidase status of the individual, a range of 20 – 400 mg of tyramine can cause an adverse reaction (Joosten, 1988). The highest concentrations of tyramine were 58 μg/g in the CO_2-MAP pork (week 6) and 37 μg/g in the vacuum-packaged pork (week 4). In order to ingest 20 mg, one would have to consume 345 g or 540 g of the CO_2-MAP and vacuum-packaged pork, respectively. It is also necessary to

recall that the biogenic amines reported in this study are per gram of pork muscle *surface*; the level of amines in the interior of the pork loin cut would not be expected to be nearly as high. However, as little as 6 mg can cause a hypertensive attack in a susceptible individual (Joosten, 1988). Therefore although the tyramine detected in this study was below the threshold level to be considered as an immediate toxicological concern; the amount of tyramine in the pork study certainly can not be ignored as it is well within the range for a possible toxicological hazard for certain individuals.

C. RECOMMENDATIONS FOR FUTURE RESEARCH

There are several areas of this research for which further immediate investigation is justified. The development of a selective medium for carnobacteria is certainly necessary in order to fully comprehend their role in the microbial ecology of vacuum-packaged and CO_2-MAP pork, and to study their involvement in biogenic amine production. Also, although some of the biogenic amine levels in the pork approach what are considered safe limits in foods, in particular for individuals undergoing MAOI drug therapy, there can be no general recommendations made regarding the suitability of the pork for MAO-compromised individuals. At this point, further research to confirm biogenic amine accumulation in pork, as well as clinical evidence, are needed in order for health care professionals to begin determining the role (if any) of vacuum-packaged or CO_2-MAP pork in MAOI diets. Additionally, a careful examination of the extraction and derivatization method prior to HPLC analysis would be useful. The current HPLC

method has been shown, in this study and others, to be quite suitable for the analysis of amines in fresh meat. However, the method could be applied with even greater success through the improvement of precision. Finally, since some amines were shown to accumulate to possibly toxic amounts, methods for preventing their formation in the pork (and food products in general) should be contemplated. Comprehensive data regarding the occurrence of potentially toxic compounds, such as the biogenic amines, in fresh meat stored in these packaging systems at a low temperature for extended time periods are critical in order to maintain Canada's increasing role in the export of fresh pork to foreign markets.

SECTION VI

REFERENCES

Agriculture Canada, 1993. Enumeration and identification of meat spoilage bacteria. Research Branch Technical Bulletin 1993-8E.

Anonymous, 1968. Headache, tyramine, serotonin, and migraine. Nutr. Rev. 26(2): 40-44.

Ayres, J. C. 1960. The relationship of organisms of the genus *Pseudomonas* to the spoilage of meat, poultry and eggs. J. Appl. Bact. 23(3): 471-486.

Bachrach, U. 1973. *Function of Naturally Occurring Polyamines.* Academic Press, Inc., New York.

Baird, R.M., Cary, J.E.L., Curtis, G.D.W., Mossel, D.A.A., and Skovgaar, N. 1989. Pharmacopeia of culture media for food microbiology – additional monographs. Int. J. Food Microbiol. 9: 85-144.

Bartholomew, B. A., Berry, P.R., Rodhouse, J. C., and Gilbert, R. J. 1987. Scombrotoxic fish poisoning in Britain: features of over 250 suspected incidents from 1976 to 1986. Epidem. Inf. 99: 775-782.

Battaglia, R. 1982. Applications of HPLC. Pp 14-26 in: *Recent Developments in Food Analysis.* Baltes, W., Czedik-Eysenberg, P. B., and Pfannhauser, W. Eds. Verlag Chemie, Weinheim.

Baucom, T. L., Tabacchi, M. H., Cottrell, T. H. E., and Richmond, B. S. 1986. Biogenic amine content of New York State wines. J. Food Sci. 51(5): 1376-1377.

Bjeldanes, L. F., Schutz, D. E., and Morris, M. M. 1978. On the aetiology of scombroid poisoning: cadaverine potentiation of histamine toxicity in the guinea-pig. Food Cosmet. Toxicol. 16: 157-159.

Blackwell, B. 1963. Hypertensive crisis due to monoamine-oxidase inhibitors. Lancet

1963: 849-851.

Blackwell, B. and Mabbitt, L. A. 1965. Tyramine in cheese related to hypertensive crises after monoamine oxidase inhibition. Lancet 1965: 938-940.

Brown, A. L. 1980. The meaning of risk assessment. Oncology 37: 302-305.

Clifford, M. N., Walker, R., Wright, J., Hardy, R., and Murray, C. K. 1989. Studies with volunteers on the role of histamine in suspected scombrotoxicosis. J. Sci. Food Agric. 47: 365-375.

Collins, M.D. Farrow, J.A.E., Phillips, B.A., Ferusu, S., and Jones, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. Int. J. Syst. Bacteriol. 37(4): 310-316.

Dainty, R. H., Edwards, R. A., Hibbard, C. M., and Ramantanis, S. V. 1986. Bacterial sources of putrescine and cadaverine in chill stored vacuum-packaged beef. J. Appl. Bacteriol. 61: 117-123.

Davidson, J. R. T. 1997. Biological therapies for posttraumatic stress disorder: and overview. J. Clin. Psychiatry 58 (suppl. 9): 29-32.

Dixon, N. M. and Kell, D. B. 1989. The inhibition by CO_2 of the growth and metabolism of microorganisms. J. Appl. Bacteriol. 67: 109-36.

Edwards, R. A. and Dainty, R. H. 1987. Volatile compounds associated with the spoilage of normal and high pH vacuum-packaged pork. J. Sci. Food Agric. 38: 57-66.

Edwards, R. A., Dainty, R. H., and Hibbard, C. M. 1983. The relationship of bacterial

numbers and types to diamine concentration in fresh and aerobically stored beef, pork, and lamb. J. Food Technol. 18: 777-788.

Edwards, R. A., Dainty, R. H., Hibbard, C. M., and Ramantanis, S. V. 1987. Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vacuum packs at chill temperatures. J. Appl. Bacteriol. 63: 427-434.

Edwards, S.T. and Sandine, W.E. 1981. Symposium: microbial metabolites of importance in dairy products. J. Dairy Sci. 64: 2431-2438.

Eerola, S., Hinkkanen, R., Lindfors, E., and Hirvi, T. 1993. Liquid chromatographic determination of biogenic amines in dry sausages. J. AOAC Int. 76(3): 575-577.

Eerola, S., Maijala, R., Sagues, A.X.R., Salminen, M., and Hirvi, T. 1996. Biogenic amines in dry sausages as affected by starter culture and contaminant amine-positive *Lactobacillus*. J. Food Sci. 61(6): 1243-1246.

Franzen, F. and Eysell, K. 1969. *Biologically Active Amines Found in Man*. Pergamon Press, London. Pp. 19-20.

Gale, E. F. 1946. The bacterial amino acid decarboxylases. Pp. 1-32 in: *Advances in Enzymology*. Nord, F. F. Ed. Interscience Publishers, New York.

Gardner, D. M., Shulman, K.I., Walker, S. E., and Tailor, S. A. N. 1996. The making of a user friendly MAOI diet. J. Clin. Psychiatry 57(3): 99-104.

Gill, 1983. Meat spoilage and evaluation of the potential storage life of fresh meat. J. Food Prot. 46(5): 444-452.

Gill, C.O. and Newton, K.G. 1977. The development of aerobic spoilage flora on meat stored at chill temperatures. J. Appl. Bacteriol. 43: 189-195.

Greer, G.G., Dilts, B.D., and Jeremiah, L.E. 1993. Bacteriology and retail case life of pork after storage in carbon dioxide. J. Food Prot. 56(8): 689-693.

Greer, G. G. and Murray, A. C. 1991. Freezing effects on quality, bacteriology and retail-case life of pork. J. Food Sci. 56(4): 891-894.

Hall, L. C., Smith, G. C., Dill, C. W., Carpenter, Z. L., and Vanderzant, C. 1980. Physical and sensory characteristics of pork loins stored in vacuum or modified atmosphere packages. J. Food Prot. 43(4): 272-276.

Hathcock, J.N. and Rader, J.I. 1994. Food additives, contaminants, and natural toxins. Pp. 1593-1611 in: *Modern Nutrition in Health and Disease Volume 2.* Shils, M.E., Olson, J.A., and Shike, M. Eds. Williams and Wilkins, Pennsylvania.

Hanington, E. 1967. Preliminary report on tyramine headache. Brit. Med. J. 2: 550-551.

Hernandez-Jover, T., Izquerido-Pulido, M., Veciana-Nogues, M. T., Marine-Font, A., and Vial-Carou, M. C. 1997a. Effect of starter cultures on biogenic amine formation during fermented sausage production. J. Food Prot. 60(7): 825-830.

Hernandez-Jover, T., Izquerido-Pulido, M., Veciana-Nogues, M. T., Marine-Font, A., and Vial-Carou, M. C. 1997b. Biogenic amine and polyamine contents in meat and meat products. J. Agric. Food Chem. 45: 2098-2102.

Hernandez-Jover, T., Izquerido-Pulido, M., Veciana-Nogues, M. T., and Vidal-Carou, M. C. 1996a. Ion-pair high-performance liquid chromatographic determination of biogenic amines in meat and meat products. J. Agric. Food Chem. 44: 2710-2715.

Hernandez-Jover, T., Izquerido-Pulido, M., Veciana-Nogues, M. T., and Vidal-Carou, M.

C. 1996b. Biogenic amine sources in cooked cured shoulder pork. J. Agric. Food Chem. 44: 3097-3101.

Hitchener, B. J., Egan, A.F., and Rogers, P. J. 1982. Characteristics of lactic acid bacteria isolated from vacuum-packaged beef. J. Appl. Bacteriol. 52: 31-37.

Huggett, A. C., Schilter, B., Roberfroid, M., Antignac, E., and Koeman, J. H. 1996. Comparative methods of toxicity testing. Food Chem. Toxic. 34(2): 183-192.

Hurst, W. J. 1990. A review of HPLC methods for the determination of selected biogenic amines in foods. J. Liquid Chromat. 13(1): 1-23.

Ingram, M. and Dainty, R.H. Changes caused by microbes in spoilage of meats. J. Appl. Bacteriol. 34(1): 21-39.

Izqueirdo-Pulido, M., Marine-Font, A., and Vidal-Carou, M. C. 1994. Biogenic amines formation during malting and brewing. J. Food Sci. 59(5): 1104-1107.

Jay, J. 1992. *Modern Food Microbiology*. Chapman and Hall, New York. Pp. 371-409.

Jeremiah, L.E., Gibson, L.L., and Argonosa, G. 1995. The influence of inherent muscle quality upon the storage life of chilled pork stored in CO_2 at −1.5°C. Food Res. Int. 28(1): 51-59.

Jeremiah, L. E., Gill, C. O., and Penney, N. 1992a. The effects on pork storage life of oxygen contamination in nominally anoxic packagings. J. Muscle Foods 3: 263-281.

Jeremiah, L. E., Gill, C. O., and Penney, N. 1992b. The effects of prolonged storage under vacuum or CO_2 on the flavour and texture profiles of chilled pork. Food Res. Int. 25: 9-19.

Joosten, H. M. L. J. 1988. The biogenic amine contents of Dutch cheese and their

toxicological significance. Neth. Milk Dairy J. 42: 25-42.

Joosten, H. M. L. J., Gaya, P., and Nunez, M. 1995. Isolation of tyrosine decarboxylaseless mutants of a bacteriocin-producing *Enterococcus faecalis* strain and their application in cheese. J. Food Prot. 58(11): 1222-1226.

Jung, H. Y. P. and Bjeldanes, L. F. 1979. Effects of cadaverine on histamine transport and metabolism in isolated gut sections of the guinea-pig. Food Cosmet. Toxicol. 17: 629-632.

Kennedy, S. H. 1997. Continuation and maintenance treatments in major depression: the neglected role of monoamine oxidase inhibitors. J. Psychiatry Neurosci. 22(2): 127-131.

Kranner, P., Bauer, F., and Hellwig, E. 1991. Investigations on the formation of histamine in raw sausages. Proc. 37th Intl. Congress Meat Sci. Technol., Germany 889-891.

Krikler, D. M. and Lewis, B. 1965. Dangers of natural foodstuffs. Lancet 1965: 1166.

Krizek, A. R., Smith, J. S., and Phebus, R. K. 1995. Biogenic amine formation in fresh vacuum-packaged beef stored at -2°C and 2 °C for 100 days. J. Food Prot. 58(3): 284-288.

Lejonc, J. L., Gusmini, D., and Brochard, P. 1979. Isoniazid and reaction to cheese. Ann. Int. Med. 91: 793.

Lijinsky, W. 1980. Significance of *in vivo* formation of N-nitroso compounds. Oncology 37:223-226.

Lindeberg, J. 1996. Capillary electrophoresis in food analysis. Food Chem. 55(1): 73-94.

Maijala, R., Eerola, S., Lievonen, S., Hill, P., and Hirvi, T. 1995a. Formation of biogenic amines during ripening of dry sausages as affected by starter culture and thawing time of raw materials. J. Food Sci. 60(6): 1187-1190.

Maijala, R., Nurmi, E., and Fischer, A. 1995b. Influence of processing temperature on the formation of biogenic amines in dry sausages. Meat Sci. 39: 9-22.

McCabe, B. J. 1986. Dietary tyramine and other pressor amines in MAOI regimens: a review. J. Am. Diet. Assoc. 86(8): 1059-1064.

McMullen, L.M. and Stiles, M.E. 1991. Changes in microbial parameters and gas composition during modified atmosphere storage of fresh pork loin cuts. J. Food Prot. 54(10): 778-783.

McMullen, L.M. and Stiles, M.E. 1993. Microbial ecology of fresh pork stored under modified atmosphere at −1, 4.4 and 10°C. Int. J. Food Microbiol. 18: 1-14.

Mietz, J. L. and Karmas, E. 1977. Chemical quality index of canned tuna as determined by high-pressure liquid chromatography. J. Food Sci. 42: 155-158.

Montel, M., Talon, R., Fournaud, and Champomier, M. 1991. A simplified key for identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. from meat. J. Appl. Bacteriol. 70: 469-472.

Nout, M. J. 1994. Fermented foods and food safety. Food Res. Int. 27:291-298.

Ordonez, J. A. and Ledward, D. A. 1977. Lipid and myoglobin oxidation in pork stored in oxygen- and carbon dioxide- enriched atmospheres. Meat Sci. 1:41-48.

Ough, C. S. 1971. Measurement of histamine in California wines. J. Agric. Food Chem. 19(2): 241-244.

Paulos, M. A. and Tessel, R. E. 1982. Excretion of β-phenylethylamine is elevated in

humans after profound stress. Science 215: 1127-1129.

Potkin, S. G., Karoum, F., Chuang, L.W., Cannon-Spoor, H. E., Phillips, I., and Wyatt, R. J. 1979. Phenylethylamine in paranoid chronic schizophrenia. Science 206: 470-471.

Recsie, P. A. and Snell, E. E. 1980. Histidine decarboxylase of *Lactobacillus* 30a: Comparative properties of wild type and mutant proenzymes and their derived enzymes. J. Biol. Chem. 257: 7196-7202.

Rice, S. L., Eitenmiller, R. R., and Koehler, P.E. 1975. Histamine and tyramine content of meat products. J. Milk Food Technol. 38(4): 256-258.

Rice, S. L., Eitenmiller, R. R., and Koehler, P.E. 1976. Biologically active amines in food: a review. J. Milk Food Technol. 39(5): 353-358.

Rodriguez-Jerez, J.J., Lopez-Sabater, E.I., Roig-Sagues, A.X., and Mora-Ventura, M.T. 1994. Histamine, cadaverine, and putrescine forming bacteria from ripened Spanish semipreserved anchovies. J. Food Sci. 59(5): 998-1001.

Sandler, M., Youdim, M. B. H., and Hanington, E. 1974. A phenylethylamine oxidising defect in migraine. Nature 250: 335-337.

Schillinger, U. and Holzapfel, W.H. 1990. Antibacterial activity of carnobacteria. Food Microbiol. 7: 305-310.

Schillinger, U. and Lucke, F. 1987. Lactic acid bacteria on vacuum-packaged meat and their influence on shelf life. Fleishwirtschraft 67(10): 1244-1248.

Seideman, S.C., Vanderzant, C., Smith, G.C., Dill, C.W., and Carpenter, Z.I. 1980. Appearance of beef, pork, and lamb stored in vacuum or modified gas atmospheres. J. Food Prot. 43(4): 252-258.

Shalaby, A.R. 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. 29(7): 675-690.

Sharpe, M.E. 1981. The genus *Lactobacillus*. Pp. 1653-1678 in: *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*. Starr, M. P., Stolp, H., Truper, H. G., Balows, A., and Schegel, H. G. Eds. Springer-Verlag, New York.

Shay, B.J. and Egan, A.F. 1986. Studies of possible techniques for extending the storage life of chilled pork. Food Technol. Aust. 38(4): 144-146.

Shelef, L. A. and Jay, J. 1970. Use of a titrimetric method to assess the bacterial spoilage of fresh beef. Appl. Microbiol. 19(6): 902-905.

Sims, G. G., Farn, G., and York, R. K. 1992. Quality indices for canned Skipjack Tuna: correlation of sensory attributes with chemical indices. J. Food Sci. 57(5): 1112-1115.

Smith, T.A. 1981. Amines in food. Food Chem. 6: 169-200.

Smith, J. S., Kenney, P. B., Kastner, C. L., and Moore, M. M. 1993. Biogenic amine formation in fresh vacuum-packaged beef during storage at 1°C for 120 days. J. Food Prot. 56(6): 497-500.

Snell, E.E., Rescei, P.A., and Misono, H. 1975. Histidine decarboxylase from *Lactobacillus* 30a: Nature of conversion of proenzyme to active enzyme. Pp. 213-219 in: *Metabolic Interconversion of Enzymes*. Shaltiel, S. Ed. Springer-Verlag, Berlin.

Soares, V. F. M. and Gloria, M. B. A. 1994. Histamine levels in canned fish available in Belo Horizonte, Minas Gerais, Brazil. J. Food Comp. Anal. 7: 102-109.

Spahl, A., Reineccius, and Tatini, S. 1981. Storage life of pork chops in CO_2-containing atmospheres. J. Food Prot. 44(9): 670-673.

Stratton, J. E., Hutkins, R. W., and Taylor, S. L. 1991. Biogenic amines in cheese and other fermented foods: a review. J. Food Prot. 54(6): 460-470.

Sullivan, E. A. and Shulman, K. I. 1984. Diet and monoamine oxidase inhibitors: a re-examination. Can. J. Psych. 29(8): 707-711.

Tannenbaum, S. R. 1980. A model for estimation of human exposure to endogenous N-nitrosodimethylamine. Oncology 37: 232-235.

Taylor, S.L. 1986. Histamine food poisoning: toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17(2): 91-128.

Voigt, M. N. and Eitenmiller, R. .R. 1978. Role of histidine and tyrosine decarboxylases and mono- and diamine oxidases in amine build-up in cheese. J. Food Prot. 41(3): 182-186.

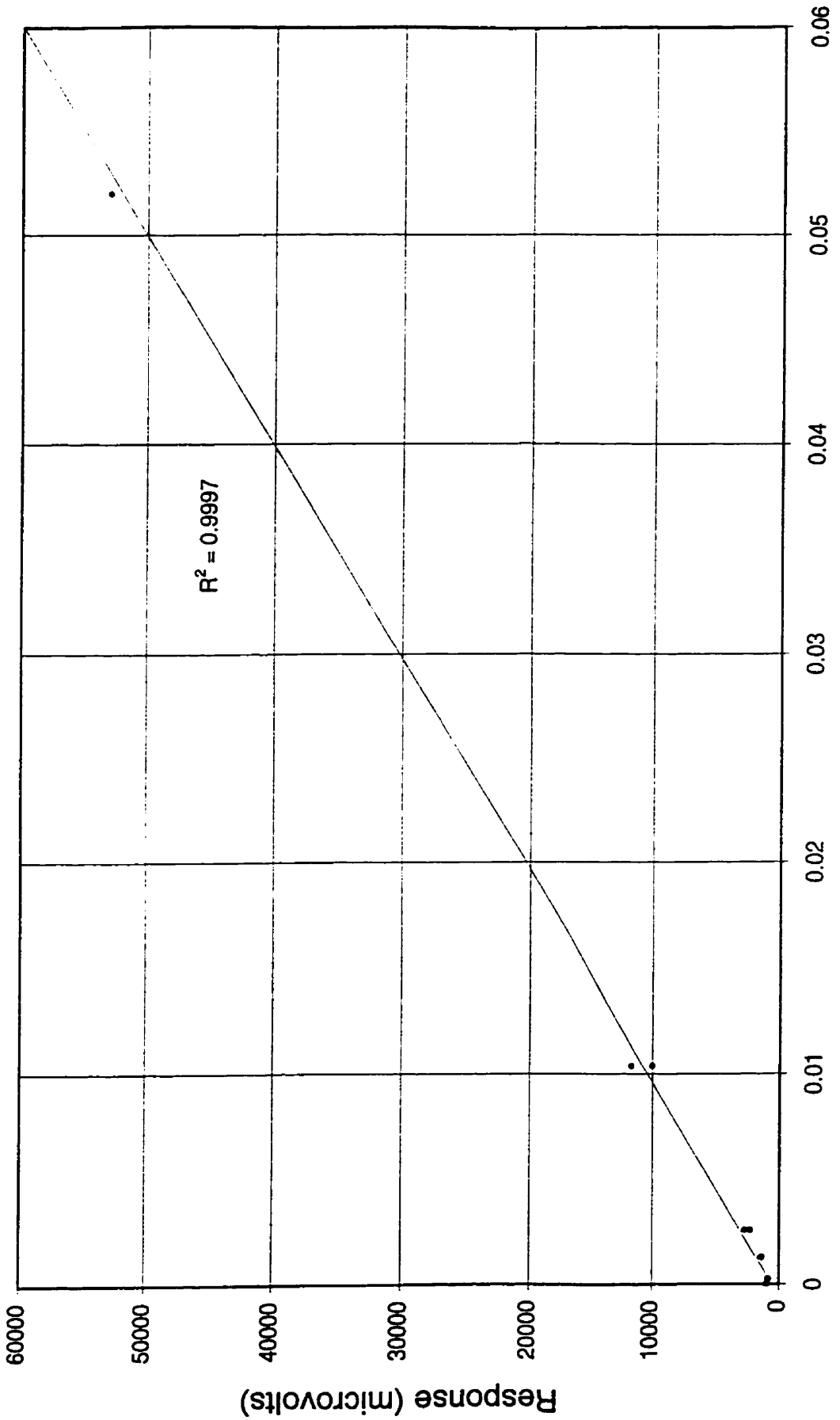
Warthesen, J. J., Scanlan, R. A., Bills, D. D., and Libbey, L. M. 1975. Formation of heterocyclic N-nitrosamines from the reaction of nitrite and selected primary diamines and amino acids. J. Agric. Food Chem. 23(5): 898-902.

Wei, C.I., Chen, C.M., Koburger, J.A., Otwell, W.S., and Marshall, M.R. 1990. Bacterial growth and histamine production on vacuum packaged tuna. J. Food Sci. 55(1): 59-63.

Zee, J. A., Simard, R. E., and Desmarais, M. 1981. Biogenic amines in Canadian, American, and European beers. Can. Inst. Food Sci. Technol. J. 14(2): 119-122.

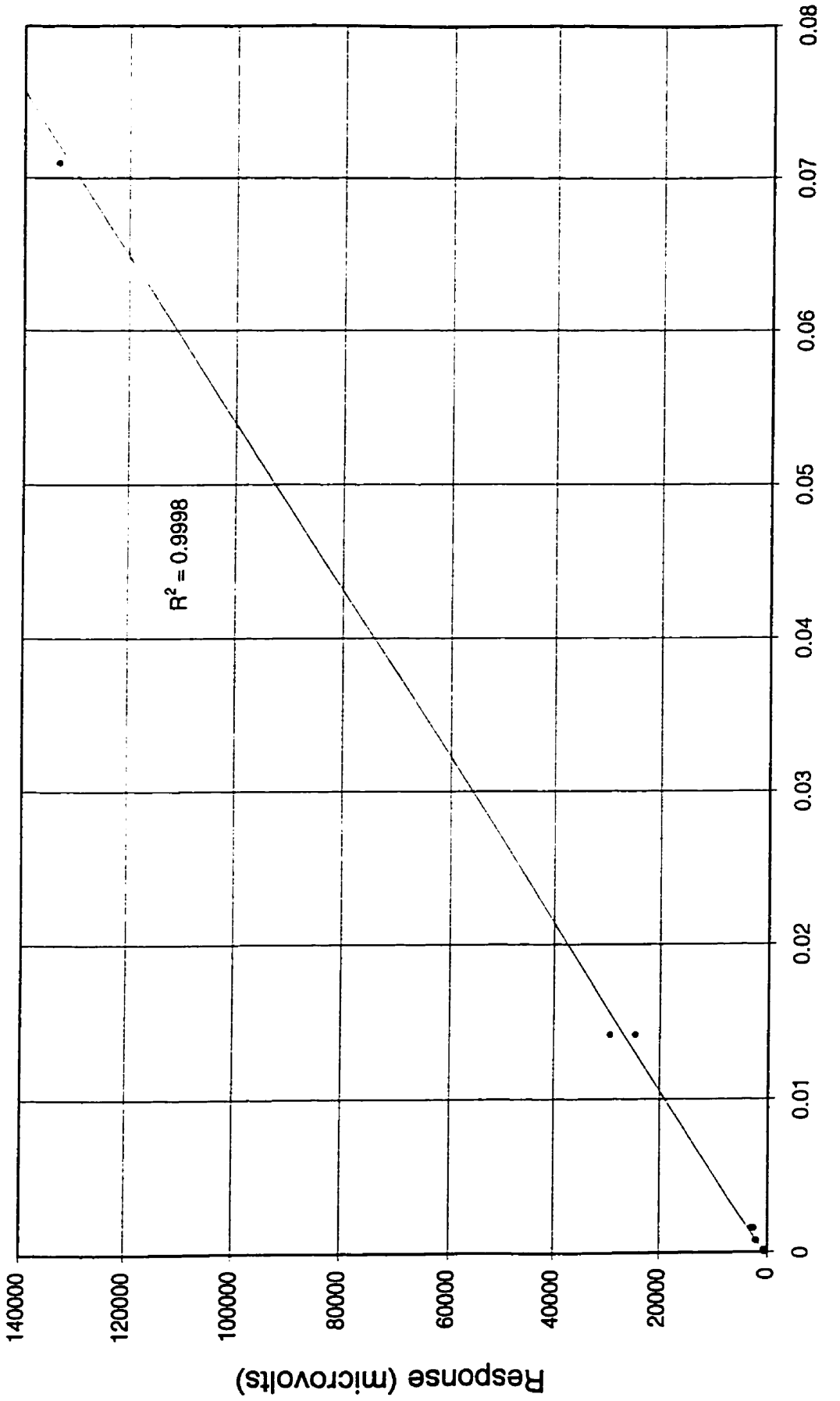
SECTION VII

APPENDICES

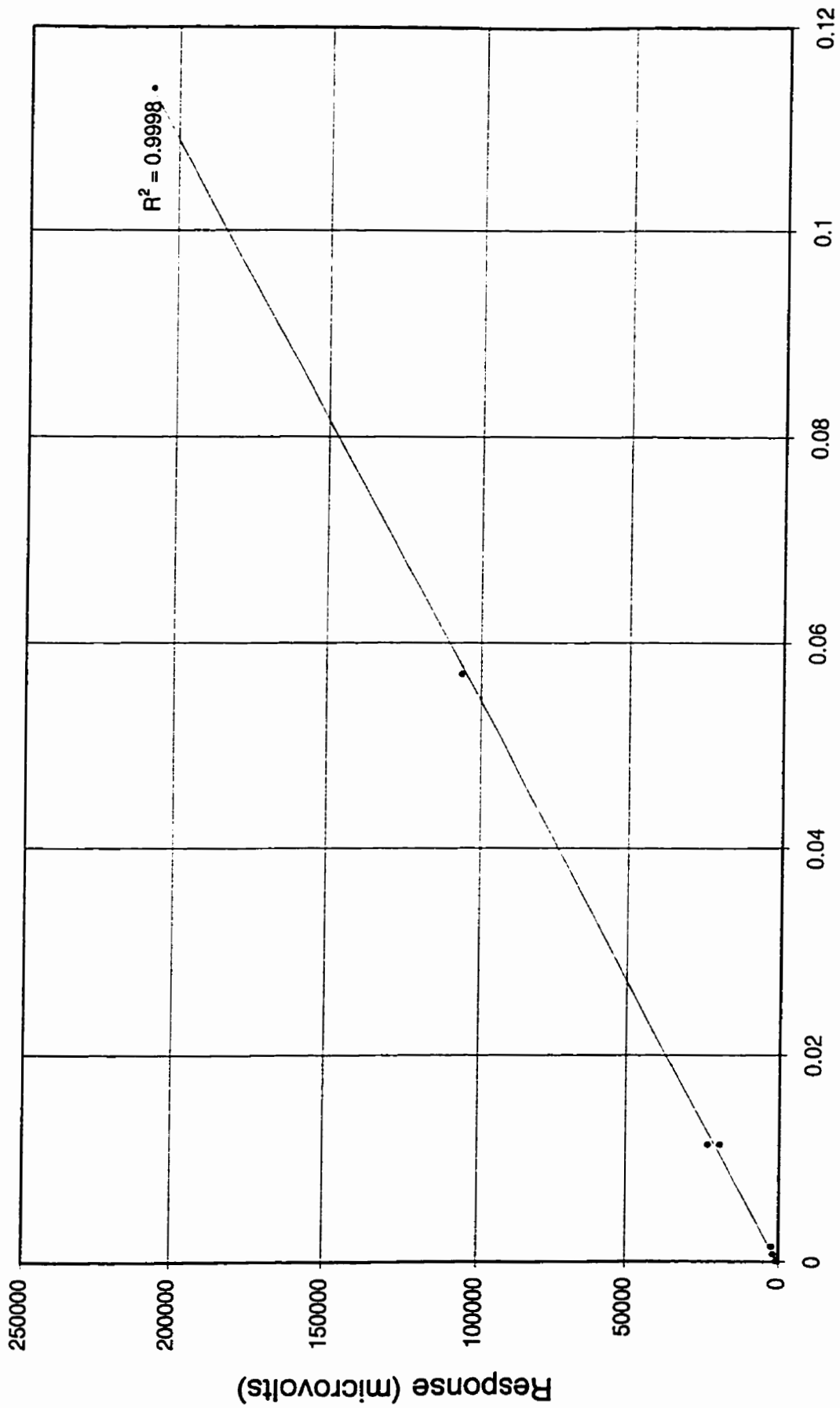


Appendix 1. Calibration curve for HPLC determination of phenylethylamine

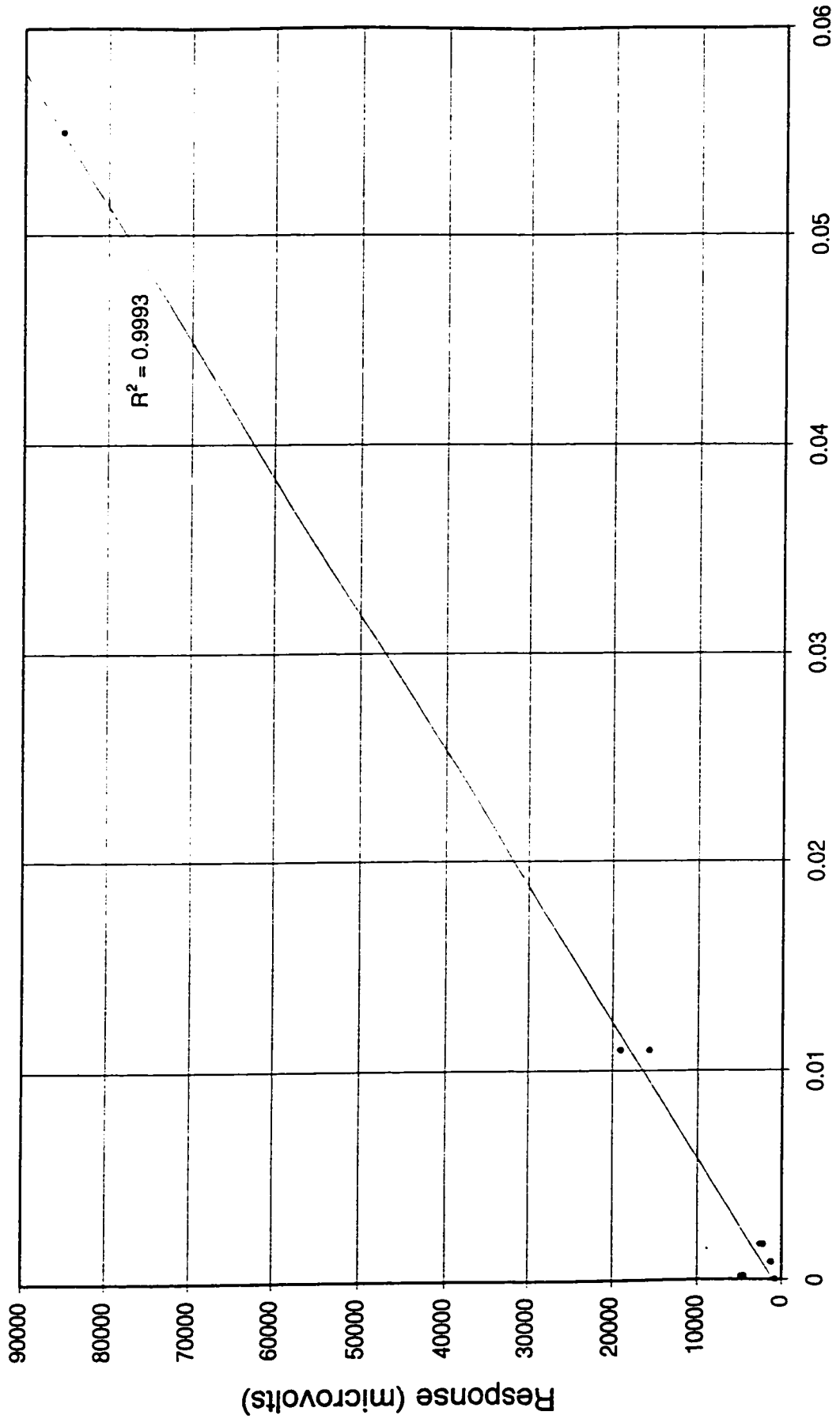
Appendix 2. Calibration curve for HPLC determination of putrescine



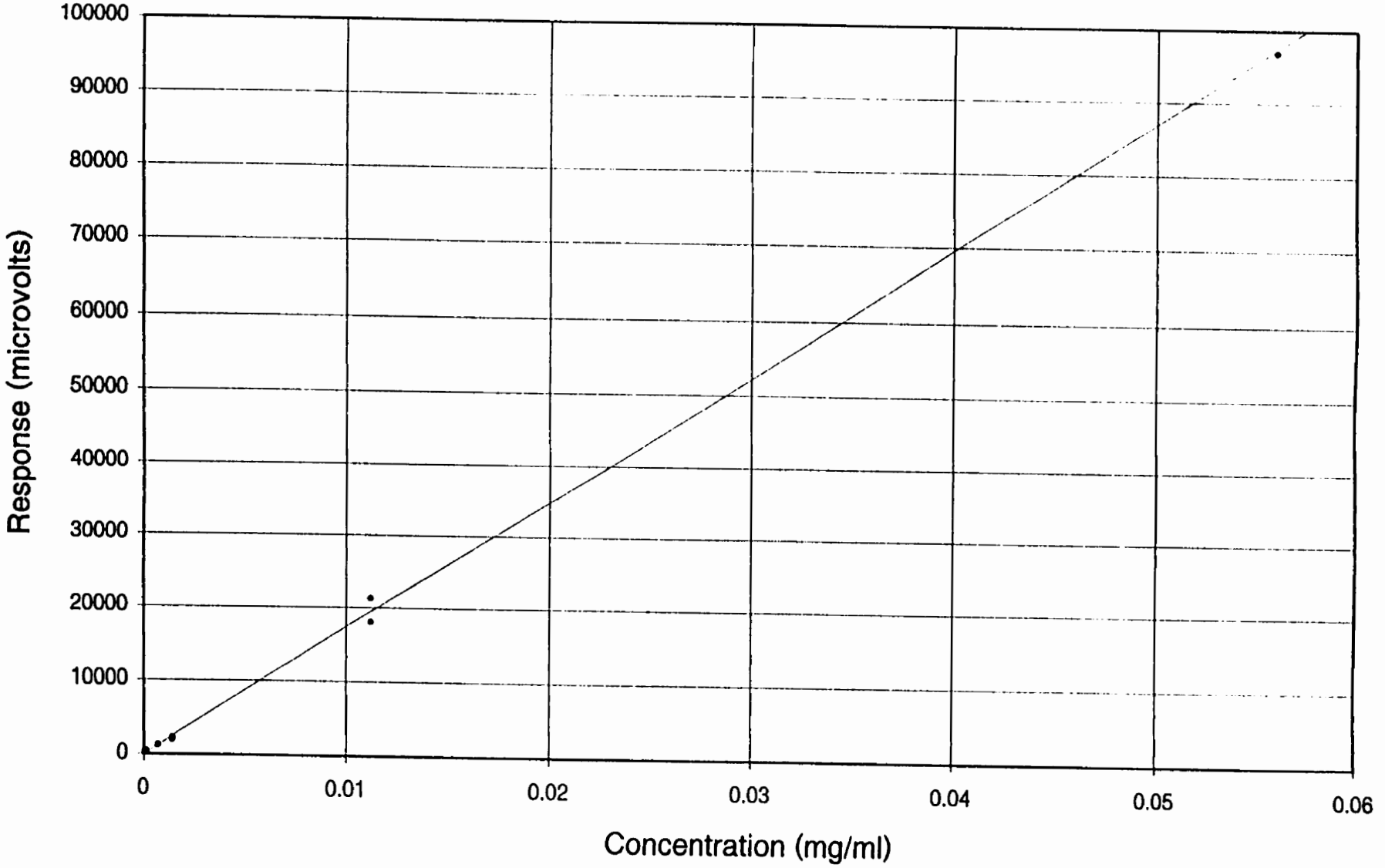
Appendix 3. Calibration curve for HPLC determination of cadaverine



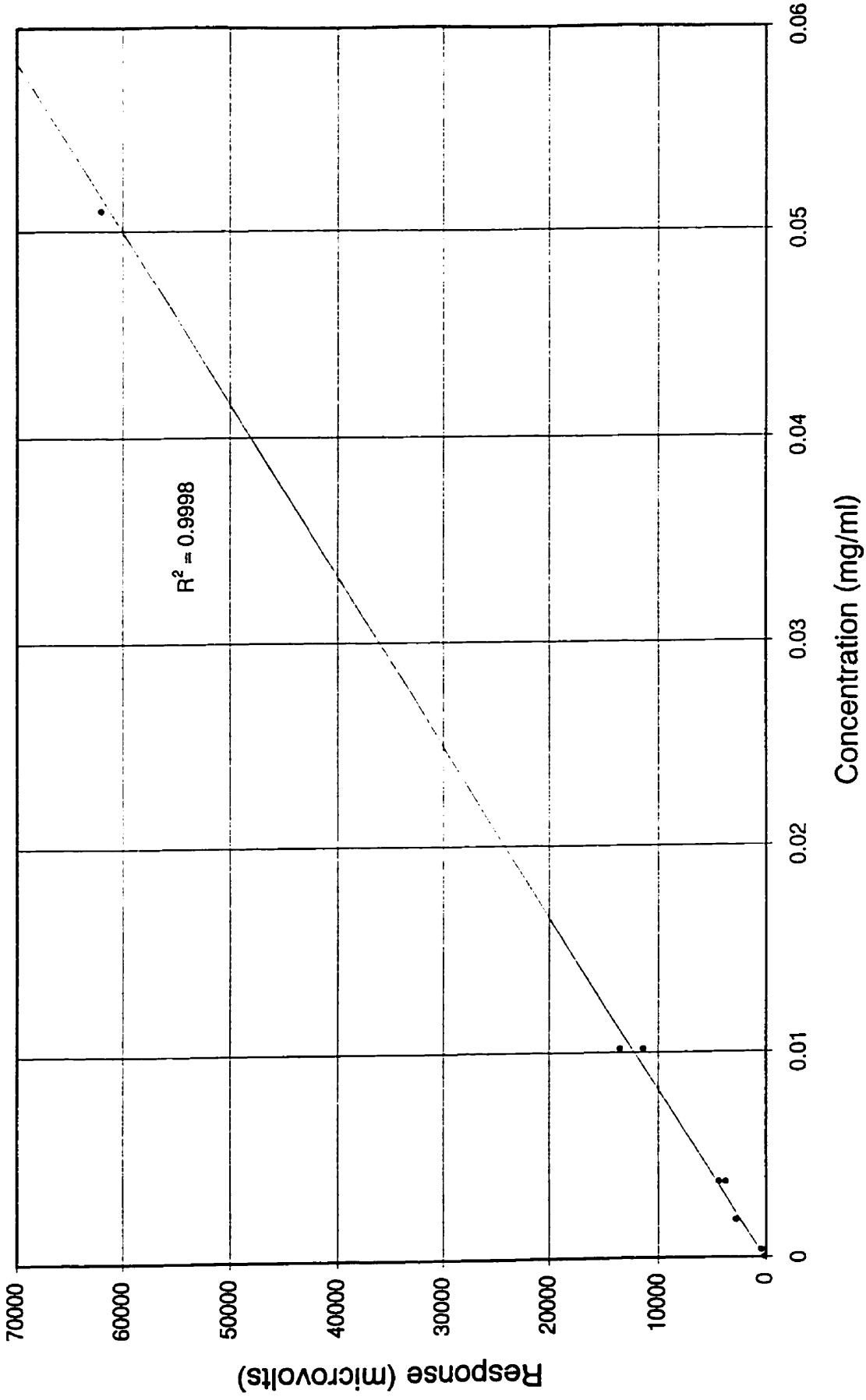
Appendix 4. Calibration curve for HPLC determination of histamine

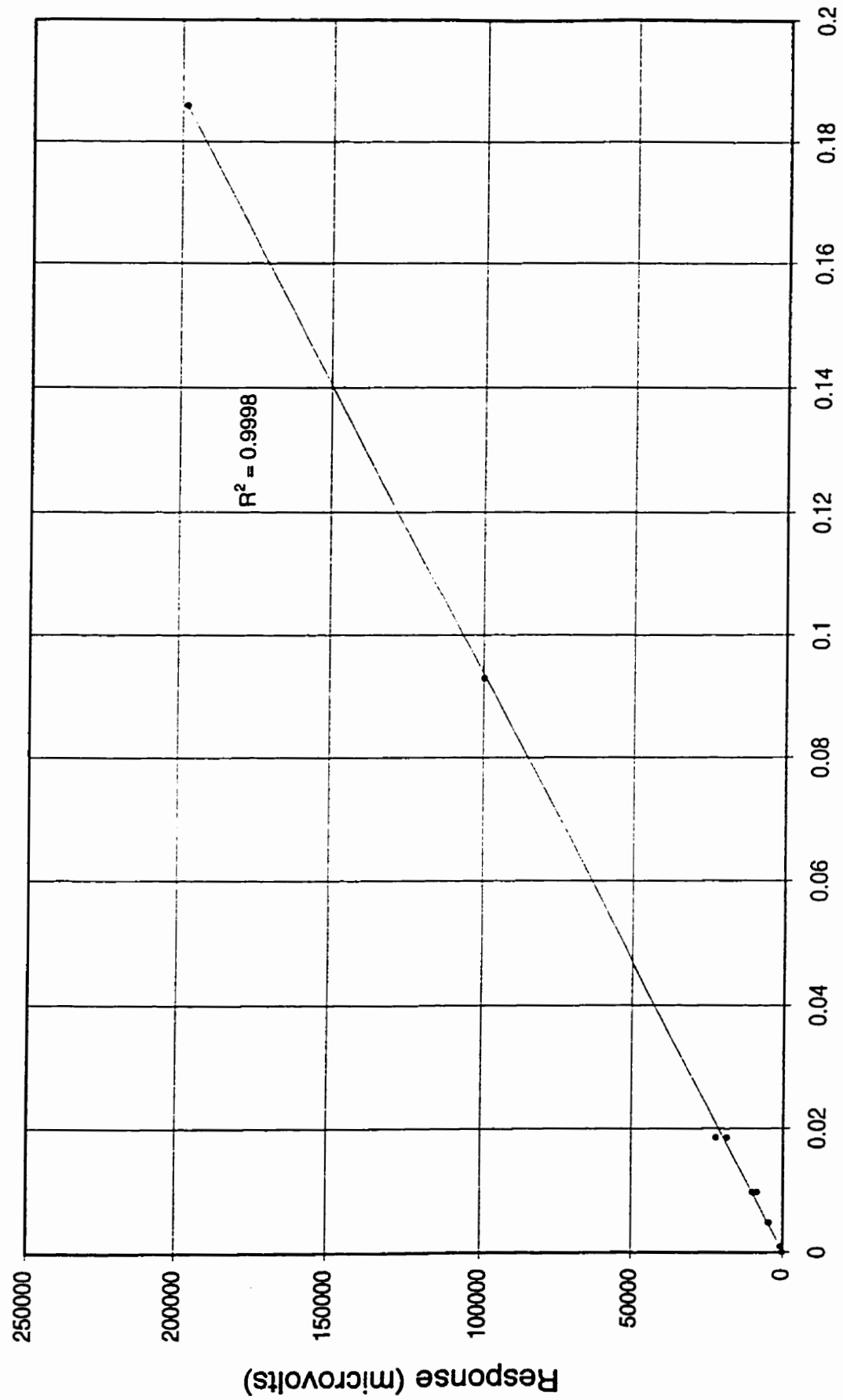


Appendix 5. Calibration curve for HPLC determination of tyramine



Appendix 6. Calibration curve for HPLC determination of spermidine





Appendix 7. Calibration curve for HPLC determination of spermine

Appendix 8. Information sheet for research participants

Fresh Chilled Pork in Vacuum Packaging and Carbon Dioxide Modified-Atmosphere Packaging

STORAGE LIFE STUDY

Information for Research Participants

This sheet contains the general information recommended by the Ethics Review Committee for participants of research involving human subjects. Please read the information thoroughly before signing consent.

Research Intent

The objective of the study is to determine the storage life of packaged, fresh pork loin cuts using sensory evaluation. The pork to be evaluated was stored at -1.5 ± 0.5°C in two types of packaging systems: vacuum and carbon dioxide modified atmosphere for 9 and 13 weeks, respectively. Each week 4 samples of pork in each type of package were frozen. The anticipated results will be the storage life, in weeks, of the pork for each type of packaging. These results will be used to conclude the relative effectiveness of the packaging systems and will appear in the researcher's thesis and a journal publication.

Participant Risks and Benefits

The risks associated with participating in this study are extremely minimal. There is a risk that you may become nauseous or ill as a result of the sample odours. There is a risk of personal distress as a result of potential differences of opinions or debates during the discussion period. Finally, there is a risk that your personal tastes regarding pork may be altered indefinitely.

The benefits associated with participation include: contributing to the general advancement of scientific knowledge; contributing to specific research regarding meat science; and expanding your personal knowledge and experience regarding sensory evaluation. There will also be remuneration in the form of a gift certificate for each person when your participation in the study is finished.

Procedures and Requirements of Participation

As a participant in this study, you will be required to attend the panelist training session and contribute to the group discussion (approximately 1 hour). The training will also involve viewing and smelling various samples of raw pork. You will learn the specific instructions for completing the procedures during the actual test. Generally, the test will involve assessing samples of raw pork loin chops for odour and appearance and then filling out a ballot with your responses. The day immediately following the training you will evaluate the experimental samples using the acquired sensory techniques (2 days, approximately two 30-minute sessions per day).

Participant Rights

As a participant of this study, you have the following rights:

1. The right to refuse participation at any stage during the study without explanation.
2. The right to have all information remain confidential at all times, including the dissemination of research results.

Additionally, any rights the subject may have access to now or in the future will not be affected by participation in this study.

Appendix 9. Consent form for research participants

CONSENT FORM

Project Title: Pork storage life study

Principal Investigator: Céline Nadon (candidate, M.Sc.)

Telephone: 694-3832 (home)

Address: 213 Ellis Building

E-mail: umnadon1@cc.umanitoba.ca

Co-investigator: Dr. M.A.H. Ismond (thesis advisor)

PLEASE CHOOSE EITHER YES OR NO IN RESPONSE TO EACH OF THE FOLLOWING QUESTIONS:

1. Do you understand that you have been asked to be in a research study?
   YES ☐ NO ☐

2. Have you read the information sheet or heard the verbal explanation of the investigator?
   YES ☐ NO ☐

3. Do you understand the benefits and risks involved in taking part in the research study?
   YES ☐ NO ☐

4. Have you had an opportunity to ask questions and discuss the study?
   YES ☐ NO ☐

5. Do you understand that you are free to withdraw from the study at any time without having to give a reason and without any detriment to your ongoing association with the University of Manitoba?
   YES ☐ NO ☐

6. Do you understand that you can refuse to answer any questions or provide information or samples during you participation in the study?
   YES ☐ NO ☐

7. Has the issue of confidentiality been described to you and do you understand: a) who will have access to the information you provide, b) that no reports will identify you as an individual?
   YES ☐ NO ☐

I agree to take part in this study
YES ☐ NO ☐

Signature of participant _____ Printed name of participant _____ Date _____

Appendix 10. Ballot form for sensory evaluation of sample odour

Panelist Code #: ☐☐

Date: February xx, 1998

Type of Sample: pork loin, raw

Attribute Studied: **ODOUR**

INSTRUCTIONS

1. RECEIVE THE SAMPLE TRAY AND NOTE EACH SAMPLE CODE BELOW ACCORDING TO ITS POSITION ON THE TRAY.

2. SMELL THE SAMPLE BY OPENING THE BAG SLIGHTLY AND TAKING SEVERAL SHORT SNIFFS. IMMEDIATELY RE-SEAL THE BAG.

3. SMELL THE SAMPLES FROM LEFT TO RIGHT AND INDICATE YOUR RESPONSE FOR EACH SAMPLE'S **ODOUR** USING THE SCALE BELOW.

4. PLEASE WAIT 10-20 SECONDS BETWEEN SAMPLES.

	SAMPLE XXX	SAMPLE YYY	SAMPLE ZZZ
5 Acceptable	5 ☐	5 ☐	5 ☐
4	4 ☐	4 ☐	4 ☐
3 Neither acceptable or unacceptable	3 ☐	3 ☐	3 ☐
2	2 ☐	2 ☐	2 ☐
1 Unacceptable	1 ☐	1 ☐	1 ☐

COMMENTS_____

Appendix 11. Ballot form for sensory evaluation of sample appearance

Panelist Code #: ☐☐

Date: February xx, 1998

Type of Sample: pork loin, raw

Attribute Studied: **APPEARANCE**

INSTRUCTIONS

1. RECEIVE THE SAMPLE TRAY AND NOTE EACH SAMPLE CODE BELOW ACCORDING TO ITS POSITION ON THE TRAY.

2. VIEW THE SAMPLE THROUGH THE PLASTIC BAG. DO NOT OPEN THE BAG. YOU MAY PICK UP THE SAMPLE AND VIEW IT FROM ANOTHER ANGLE.

3. VIEW THE SAMPLES FROM LEFT TO RIGHT AND INDICATE YOUR RESPONSE FOR EACH SAMPLE'S **APPEARANCE** USING THE SCALE BELOW.

	SAMPLE 701	SAMPLE 638	SAMPLE 125
7-Extremely desirable	7 ☐	7☐	7 ☐
6-Desirable	6 ☐	6 ☐	6 ☐
5-Slightly desirable	5 ☐	5 ☐	5 ☐
4-Neither desirable or undesirable	4 ☐	4 ☐	4 ☐
3-Slightly undesirable	3 ☐	3 ☐	3 ☐
2-Undesirable	2 ☐	2 ☐	2 ☐
1-Extremely undesirable	1 ☐	1 ☐	1 ☐

COMMENTS_____

Appendix 12. The growth of lactic acid bacteria in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C[1].

Week	Lactic Acid Bacteria CFU*/cm^2
1	$2.32 \times 10^{2\,b}$
2	$1.08 \times 10^{3\,b}$
3	$4.86 \times 10^{4\,b}$
4	$5.44 \times 10^{5\,b}$
5	$1.49 \times 10^{6\,b}$
6	$4.77 \times 10^{5\,b}$
7	$4.13 \times 10^{6\,a}$
8	$4.35 \times 10^{6\,a}$
9	$5.24 \times 10^{6\,a}$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* CFU; colony forming units
[1]Results are an average of 6-8 replications per week

Appendix 13. The growth of lactic acid bacteria in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C[1].

Week	Lactic Acid Bacteria CFU*/cm^2
1	$2.07 \times 10^{2\,c}$
2	$2.16 \times 10^{2\,c}$
3	$2.23 \times 10^{2\,c}$
4	$7.98 \times 10^{2\,c}$
5	$1.40 \times 10^{3\,c}$
6	$3.52 \times 10^{4\,bc}$
7	$2.46 \times 10^{4\,c}$
8	$5.22 \times 10^{4\,bc}$
9	$8.43 \times 10^{3\,c}$
10	$5.94 \times 10^{4\,bc}$
11	$3.19 \times 10^{5\,a}$
12	$3.45 \times 10^{5\,a}$
13	$1.79 \times 10^{5\,a}$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* CFU; colony forming units
[1]Results are an average of 6-8 replications per week

Appendix 14. Comparison of lactic acid bacteria in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C.

Week	CO_2-MAP Lactic Acid Bacteria CFU [†]/cm^2		Vacuum-Packaging Lactic Acid Bacteria CFU/cm^2
1	2.07×10^2		2.32×10^2
2	2.16×10^2	***	1.08×10^3
3	2.23×10^2	***	4.86×10^4
4	7.98×10^2	***	5.44×10^5
5	1.40×10^3	***	1.49×10^6
6	3.52×10^4	***	4.77×10^5
7	2.46×10^4	***	4.13×10^6
8	5.22×10^4	***	4.35×10^6
9	8.43×10^3	***	5.24×10^6
10	5.94×10^4		
11	3.19×10^5		
12	3.45×10^5		
13	1.79×10^5		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[†] CFU; colony forming units

Appendix 15. Phenylethylamine in vacuum-packaged fresh pork stored for 9 weeks at –1.5°C determined by reversed-phase HPLC[1]

Week in Storage	Phenylethylamine (μg/g) ± SD*
1	21.561[b] ± 17.828
2	4.248[b] ± 3.123
3	9.657[b] ± 3.762
4	39.985[a] ± 48.217
5	11.388[b] ± 3.547
6	12.789[b] ± 5.111
7	12.854[b] ± 3.847
8	16.258[b] ± 3.712
9	18.168[b] ± 2.557

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 16. Phenylethylamine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Phenylethylamine (μg/g) ± SD*
1	$44.108^{a} \pm 40.861$
2	$7.663^{cd} \pm 4.667$
3	$8.319^{cd} \pm 4.408$
4	$9.598^{bcd} \pm 4.984$
5	$17.051^{bcd} \pm 9.925$
6	$26.936^{abc} \pm 17.433$
7	$16.181^{bcd} \pm 7.237$
8	$20.432^{bcd} \pm 5.406$
9	$31.335^{ab} \pm 18.211$
10	$27.617^{abc} \pm 8.745$
11	$23.570^{abcd} \pm 18.380$
12	$2.619^{d} \pm 1.558$
13	$5.461^{cd} \pm 3.654$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 17. Comparison of phenylethylamine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	CO_2-MAP Phenylethylamine (μg/g) ± SD[1]		Vacuum-Packaging Phenylethylamine (μg/g) ± SD
1	44.108 ± 40.861	†	21.561 ± 17.828
2	7.663 ± 4.667		4.248 ± 3.123
3	8.319 ± 4.408		9.657 ± 3.762
4	9.598 ± 4.984		39.985 ± 48.217
5	17.051 ± 9.925		11.388 ± 3.547
6	26.936 ± 17.433		12.789 ± 5.111
7	16.181 ± 7.237		12.854 ± 3.847
8	20.432 ± 5.406		16.258 ± 3.712
9	31.335 ± 18.211		18.168 ± 2.557
10	27.617 ± 8.745		
11	23.570 ± 18.380		
12	2.619 ± 1.558		
13	5.461 ± 3.654		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)

[1] SD; standard deviation

† no comparisons for entire study were significantly different in phenylethylamine

Appendix 18. Putrescine in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Putrescine (μg/g) ± SD*
1	$8.594^{bc} \pm 8.783$
2	$1.618^{c} \pm 0.530$
3	$6.120^{bc} \pm 0.936$
4	$21.700^{a} \pm 23.792$
5	$3.993^{bc} \pm 3.765$
6	$3.067^{bc} \pm 1.736$
7	$8.182^{bc} \pm 2.734$
8	$14.358^{ab} \pm 6.994$
9	$20.308^{a} \pm 6.911$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 19. Putrescine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Putrescine (μg/g) ± SD*
1	$13.488^{ab} \pm 14.968$
2	$1.999^{c} \pm 1.360$
3	$4.608^{bc} \pm 5.809$
4	$11.957^{bc} \pm 15.394$
5	$9.996^{bc} \pm 5.274$
6	$21.779^{a} \pm 2.454$
7	$6.475^{bc} \pm 1.364$
8	$7.849^{bc} \pm 2.750$
9	$13.003^{ab} \pm 12.316$
10	$10.074^{bc} \pm 4.608$
11	$5.435^{bc} \pm 1.344$
12	$8.805^{bc} \pm 2.940$
13	$6.545^{bc} \pm 4.450$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 20. Comparison of putrescine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	CO_2-MAP Putrescine (μg/g) ± SD[1]		Vacuum-Packaging Putrescine (μg/g) ± SD
1	13.488 ± 14.968		8.594 ± 8.783
2	1.999 ± 1.360		1.618 ± 0.530
3	4.608 ± 5.809		6.120 ± 0.936
4	11.957 ± 15.394		21.700 ± 23.792
5	9.996 ± 5.274	***	3.993 ± 3.765
6	21.779 ± 2.454	***	3.067 ± 1.736
7	6.475 ± 1.364		8.182 ± 2.734
8	7.849 ± 2.750	***	14.358 ± 6.994
9	13.003 ± 12.316		20.308 ± 6.911
10	10.074 ± 4.608		
11	5.435 ± 1.344		
12	8.805 ± 2.940		
13	6.545 ± 4.450		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 21. Cadaverine in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Cadaverine (μg/g) ● SD*
1	$10.586^{cd} \pm 11.684$
2	$9.759^{cd} \pm 8.130$
3	$5.524^{cd} \pm 2.515$
4	$23.643^{c} \pm 23.535$
5	$8.636^{cd} \pm 7.176$
6	$4.551^{d} \pm 2.145$
7	$7.666^{cd} \pm 2.636$
8	$43.683^{b} \pm 25.814$
9	$68.335^{a} \pm 15.782$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 22. Cadaverine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Cadaverine (μg/g) ± SD*
1	$17.613^{ab} \pm 17.349$
2	$2.299^{d} \pm 1.086$
3	$6.155^{cd} \pm 7.037$
4	$0.728^{d} \pm 0.824$
5	$8.565^{bcd} \pm 8.276$
6	$20.821^{a} \pm 14.005$
7	$5.808^{cd} \pm 1.618$
8	$6.648^{cd} \pm 3.449$
9	$14.852^{abc} \pm 16.263$
10	$6.329^{cd} \pm 4.494$
11	$3.357^{cd} \pm 1.788$
12	$4.271^{cd} \pm 1.997$
13	$4.971^{cd} \pm 3.331$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 23. Comparison of cadaverine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	**CO_2-MAP** Cadaverine (μg/g) ± SD[1]		**Vacuum-Packaging** Cadaverine (μg/g) ± SD
1	17.613 ± 17.349		10.586 ± 11.684
2	2.299 ± 1.086		9.759 ± 8.130
3	6.155 ± 7.037		5.524 ± 2.515
4	0.728 ± 0.824		23.643 ± 23.535
5	8.565 ± 8.276		8.636 ± 7.176
6	20.821 ± 14.005	***	4.551 ± 2.145
7	5.808 ± 1.618		7.666 ± 2.636
8	6.648 ± 3.449	***	43.683 ± 25.814
9	14.852 ± 16.263	***	68.335 ± 15.782
10	6.329 ± 4.494		
11	3.357 ± 1.788		
12	4.271 ± 1.997		
13	4.971 ± 3.331		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 24. Histamine in vacuum-packaged fresh pork stored for 9 weeks at –1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Histamine (μg/g) ± SD*
1	5.168[b] ± 3.426
2	13.886[a] ± 3.269
3	4.495[b] ± 3.406
4	15.573[a] ± 12.018
5	3.240[b] ± 2.345
6	1.354[b] ± 1.194
7	1.908[b] ± 1.406
8	1.157[b] ± 0.665
9	0.821[b] ± 0.664

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 25. Histamine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Histamine (μg/g) ± SD*
1	$7.692^{abcd} \pm 6.088$
2	$0.158^{f} \pm 0.316$
3	$10.029^{abc} \pm 6.149$
4	$6.536^{bcde} \pm 4.077$
5	$4.491^{cdef} \pm 2.916$
6	$12.095^{a} \pm 8.563$
7	$1.609^{ef} \pm 1.067$
8	$2.434^{def} \pm 2.134$
9	$9.270^{abc} \pm 4.856$
10	$3.796^{cdef} \pm 3.682$
11	$2.259^{def} \pm 1.551$
12	$0.671^{f} \pm 0.256$
13	$0.593^{f} \pm 1.028$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 26. Comparison of histamine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	**CO_2-MAP** Histamine (μg/g) ± SD[1]		**Vacuum-Packaging** Histamine (μg/g) ± SD
1	7.692 ± 6.088		5.168 ± 3.426
2	0.158 ± 0.316	***	13.886 ± 3.269
3	10.029 ± 6.149		4.495 ± 3.406
4	6.536 ± 4.077		15.573 ± 12.018
5	4.491 ± 2.916		3.240 ± 2.345
6	12.095 ± 8.563	***	1.354 ± 1.194
7	1.609 ± 1.067		1.908 ± 1.406
8	2.434 ± 2.134		1.157 ± 0.665
9	9.270 ± 4.856	***	0.821 ± 0.664
10	3.796 ± 3.682		
11	2.259 ± 1.551		
12	0.671 ± 0.256		
13	0.593 ± 1.028		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 27. Tyramine in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Tyramine (μg/g) ± SD*
1	$3.835^{c} \pm 3.626$
2	$0.808^{c} \pm 0.432$
3	$6.603^{c} \pm 3.480$
4	$37.350^{a} \pm 36.819$
5	$34.904^{ab} \pm 14.004$
6	$17.516^{bc} \pm 7.488$
7	$17.982^{bc} \pm 3.796$
8	$37.921^{a} \pm 20.029$
9	$26.373^{ab} \pm 14.239$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 28. Tyramine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Tyramine (μg/g) ± SD*
1	$10.791^{bc} \pm 3.920$
2	$1.029^{c} \pm 0.751$
3	$2.307^{c} \pm 0.923$
4	$1.484^{c} \pm 0.986$
5	$2.156^{c} \pm 2.325$
6	$57.994^{a} \pm 40.232$
7	$2.836^{c} \pm 1.067$
8	$4.188^{c} \pm 2.186$
9	$15.157^{bc} \pm 18.800$
10	$17.703^{bc} \pm 6.985$
11	$16.388^{bc} \pm 3.982$
12	$21.415^{b} \pm 14.836$
13	$14.453^{bc} \pm 14.826$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 29. Comparison of tyramine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	**CO_2-MAP** Tyramine (μg/g) ± SD[1]		**Vacuum-Packaging** Tyramine (μg/g) ± SD
1	10.791 ± 3.920	***	3.835 ± 3.626
2	1.029 ± 0.751		0.808 ± 0.432
3	2.307 ± 0.923	***	6.603 ± 3.480
4	1.484 ± 0.986		37.350 ± 36.819
5	2.156 ± 2.325	***	34.904 ± 14.004
6	57.994 ± 40.232	***	17.516 ± 7.488
7	2.836 ± 1.067	***	17.982 ± 3.796
8	4.188 ± 2.186	***	37.921 ± 20.029
9	15.157 ± 18.800		26.373 ± 14.239
10	17.703 ± 6.985		
11	16.388 ± 3.982		
12	21.415 ± 14.836		
13	14.453 ± 14.826		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 30. Spermidine in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Spermidine (μg/g) ± SD*
1	$7.638^{ab} \pm 6.482$
2	$5.840^{abc} \pm 3.247$
3	$1.541^{c} \pm 1.139$
4	$9.116^{a} \pm 9.893$
5	$2.723^{bc} \pm 2.877$
6	$3.926^{bc} \pm 2.350$
7	$3.098^{bc} \pm 1.440$
8	$3.431^{bc} \pm 1.371$
9	$2.268^{c} \pm 0.865$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 31. Spermidine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Spermidine (μg/g) ± SD*
1	9.799[b] ± 5.212
2	5.962[bc] ± 2.124
3	5.398[c] ± 2.507
4	2.652[c] ± 1.651
5	4.687[c] ± 2.676
6	14.656[a] ± 10.113
7	2.169[c] ± 0.710
8	3.801[c] ± 1.080
9	3.083[c] ± 1.089
10	5.066[c] ± 2.186
11	4.263[c] ± 1.871
12	2.384[c] ± 0.881
13	4.255[c] ± 2.561

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 32. Comparison of spermidine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	**CO_2-MAP** Spermidine (μg/g) ± SD[1]		**Vacuum-Packaging** Spermidine (μg/g) ± SD
1	9.799 ± 5.212		7.638 ± 6.482
2	5.962 ± 2.124		5.840 ± 3.247
3	5.398 ± 2.507	***	1.541 ± 1.139
4	2.652 ± 1.651		9.116 ± 9.893
5	4.687 ± 2.676		2.723 ± 2.877
6	14.656 ± 10.113	***	3.926 ± 2.350
7	2.169 ± 0.710		3.098 ± 1.440
8	3.801 ± 1.080		3.431 ± 1.371
9	3.083 ± 1.089		2.268 ± 0.865
10	5.066 ± 2.186		
11	4.263 ± 1.871		
12	2.384 ± 0.881		
13	4.255 ± 2.561		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 33. Spermine in vacuum-packaged fresh pork stored for 9 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Spermine (μg/g) ± SD*
1	636.21[a] ± 116.383
2	455.82[b] ± 92.377
3	169.45[c] ± 28.943
4	62.79[d] ± 17.620
5	35.19[d] ± 8.313
6	43.97[d] ± 17.401
7	22.61[d] ± 8.187
8	21.76[d] ± 5.038
9	25.65[d] ± 6.935

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 34. Spermine in carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C determined by reversed-phase HPLC[1].

Week in Storage	Spermine (μg/g) ± SD*
1	646.43[a] ± 250.940
2	652.11[a] ± 105.145
3	374.68[b] ± 114.209
4	108.39[c] ± 54.737
5	39.78[c] ± 11.939
6	29.60[c] ± 4.000
7	20.81[c] ± 4.846
8	22.84[c] ± 5.665
9	30.22[c] ± 6.684
10	22.79[c] ± 3.466
11	50.44[c] ± 33.239
12	14.53[c] ± 6.102
13	22.38[c] ± 6.666

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
* SD; standard deviation
[1]Results are an average of 6-8 replications per week

Appendix 35. Comparison of spermine in carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C determined by reversed-phase HPLC.

Week	**CO_2-MAP** Spermine (μg/g) ± SD[1]		**Vacuum-Packaging** Spermine (μg/g) ± SD
1	646.43 ± 250.940		636.21 ± 116.383
2	652.11 ± 105.145	***	455.82 ± 92.377
3	374.68 ± 114.209	***	169.45 ± 28.943
4	108.39 ± 54.737		62.79 ± 17.620
5	39.78 ± 11.939		35.19 ± 8.313
6	29.60 ± 4.000		43.97 ± 17.401
7	20.81 ± 4.846		22.61 ± 8.187
8	22.84 ± 5.665		21.76 ± 5.038
9	30.22 ± 6.684		25.65 ± 6.935
10	22.79 ± 3.466		
11	50.44 ± 33.239		
12	14.53 ± 6.102		
13	22.38 ± 6.666		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[1] SD; standard deviation

Appendix 36. The odour scores for vacuum-packaged fresh pork stored for 9 weeks at –1.5°C[1].

Week in Storage	Odour Score † ± SD*
0	$4.06^{a} \pm 0.95$
1	$3.78^{ab} \pm 1.04$
2	$3.66^{ab} \pm 1.18$
3	$3.50^{bc} \pm 1.14$
4	$2.84^{d} \pm 1.39$
5	$3.06^{cd} \pm 1.22$
6	$2.16^{e} \pm 1.08$
7	$1.38^{f} \pm 0.87$
8	$1.28^{f} \pm 0.52$
9	$1.25^{f} \pm 0.62$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)

† Odour scores on a scale where 1 = unacceptable, 3 = neither acceptable nor unacceptable, 5 = acceptable

* SD; standard deviation

[1]Results are an average of 32 replications per week

Appendix 37. The odour scores for carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at −1.5°C[1].

Week in Storage	Odour Score † ± SD*
0	$4.00^{abcd} \pm 1.16$
1	$4.22^{ab} \pm 0.91$
2	$3.75^{bcd} \pm 1.02$
3	$4.34^{a} \pm 0.65$
4	$4.28^{ab} \pm 0.68$
5	$3.16^{e} \pm 1.14$
6	$4.06^{abc} \pm 0.76$
7	$4.00^{abcd} \pm 0.80$
8	$4.16^{ab} \pm 0.88$
9	$4.09^{abc} \pm 1.00$
10	$3.97^{abcd} \pm 1.06$
11	$3.53^{cde} \pm 1.22$
12	$3.44^{de} \pm 1.13$
13	$3.56^{cde} \pm 1.39$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)

† Odour scores on a scale where 1 = unacceptable, 3 = neither acceptable nor unacceptable, 5 = acceptable

* SD; standard deviation

[1]Results are an average of 32 replications per week

Appendix 38. Comparison of odour scores for carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C.

Week	CO_2-MAP Odour Score [†] ± SD [1]		Vacuum-Packaging Odour Score [†] ± SD
0	4.00 ± 1.16		4.06 ± 0.95
1	4.22 ± 0.91		3.78 ± 1.04
2	3.75 ± 1.02		3.66 ± 1.18
3	4.34 ± 0.65	***	3.50 ± 1.14
4	4.28 ± 0.68	***	2.84 ± 1.39
5	3.16 ± 1.14		3.06 ± 1.22
6	4.06 ± 0.76	***	2.16 ± 1.08
7	4.00 ± 0.80	***	1.38 ± 0.87
8	4.16 ± 0.88	***	1.28 ± 0.52
9	4.09 ± 1.00	***	1.25 ± 0.62
10	3.97 ± 1.06		
11	3.53 ± 1.22		
12	3.44 ± 1.13		
13	3.56 ± 1.39		

*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[†] Odour scores on a scale where 1 = unacceptable, 3 = neither acceptable nor unacceptable, 5 = acceptable
[1] SD; standard deviation

Appendix 39. The appearance scores for vacuum-packaged fresh pork stored for 9 weeks at −1.5°C[1].

Week in Storage	Appearance Score [†] ± SD*
0	$5.22^{abc} \pm 1.29$
1	$4.56^{bcd} \pm 1.78$
2	$5.38^{ab} \pm 1.26$
3	$5.44^{a} \pm 0.91$
4	$4.81^{abcd} \pm 1.66$
5	$5.39^{ab} \pm 1.12$
6	$4.03^{d} \pm 1.66$
7	$4.75^{abcd} \pm 1.30$
8	$4.44^{cd} \pm 1.85$
9	$4.35^{d} \pm 1.78$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)

[†] Appearance scores on a scale where 1 = extremely undesirable, 4 = neither desirable nor undesirable, 7 = extremely desirable

* SD; standard deviation

[1]Results are an average of 32 replications per week

Appendix 40. The appearance scores for carbon dioxide modified atmosphere-packaged fresh pork stored for 13 weeks at –1.5°C.

Week in Storage	Appearance Score [†] ● SD*
0	$5.06^{abc} \pm 1.74$
1	$4.66^{bc} \pm 1.64$
2	$5.56^{a} \pm 1.13$
3	$5.66^{a} \pm 1.26$
4	$5.66^{a} \pm 1.07$
5	$4.38^{c} \pm 1.39$
6	$5.38^{ab} \pm 0.79$
7	$5.38^{ab} \pm 1.19$
8	$5.78^{a} \pm 0.98$
9	$5.50^{a} \pm 1.16$
10	$5.56^{a} \pm 1.27$
11	$5.25^{ab} \pm 1.65$
12	$5.06^{abc} \pm 1.74$
13	$5.38^{ab} \pm 1.10$

Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)

[†] Appearance scores on a scale where 1 = extremely undesirable, 4 = neither desirable nor undesirable, 7 = extremely desirable

* SD; standard deviation

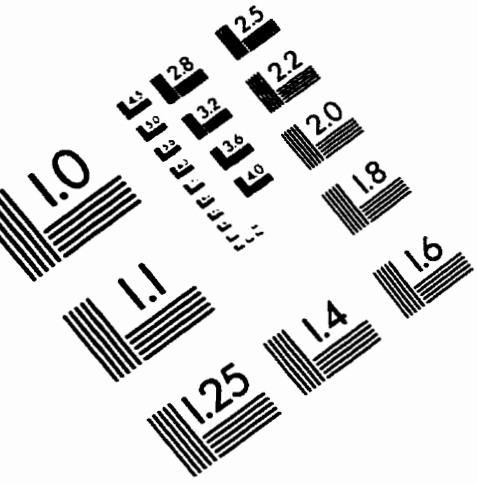
[1]Results are an average of 32 replications per week

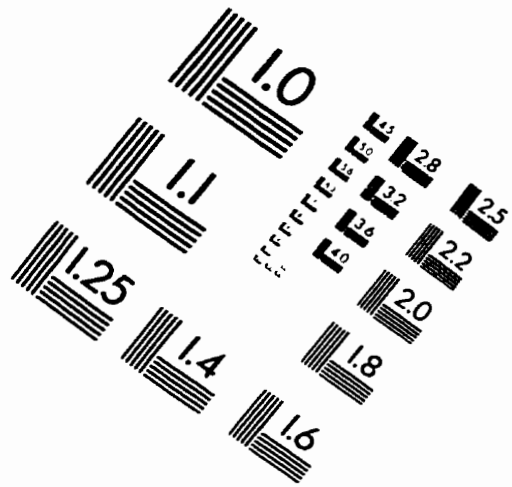
Appendix 41. Comparison of appearance scores for carbon dioxide modified atmosphere-packaged and vacuum-packaged fresh pork stored at −1.5°C.

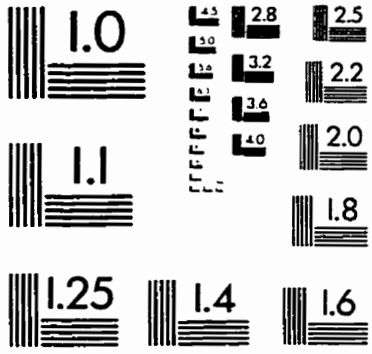
Week	CO_2-MAP Appearance Score [†] ± SD [‡]		Vacuum-Packaging Appearance Score ± SD
0	5.07 ± 1.74		5.22 ± 1.29
1	4.66 ± 1.64		4.56 ± 1.78
2	5.56 ± 1.13		5.38 ± 1.26
3	5.66 ± 1.26		5.44 ± 0.91
4	5.66 ± 1.07	***	4.81 ± 1.66
5	4.38 ± 1.39	***	5.39 ± 1.12
6	5.38 ± 0.79	***	4.03 ± 1.66
7	5.38 ± 1.19	***	4.75 ± 1.30
8	5.78 ± 0.98	***	4.44 ± 1.85
9	5.50 ± 1.16	***	4.35 ± 1.78
10	5.56 ± 1.27		
11	5.25 ± 1.65		
12	5.06 ± 1.74		
13	5.38 ± 1.10		

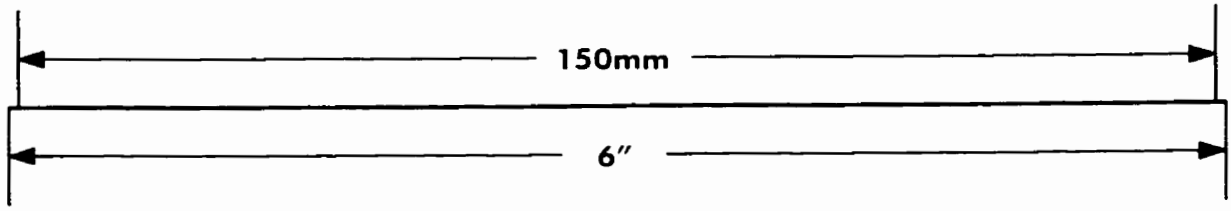
*** comparison is significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$)
[†] Appearance scores on a scale where 1 = extremely undesirable, 4 = neither desirable nor undesirable, 7 = extremely desirable
[‡] SD; standard deviation

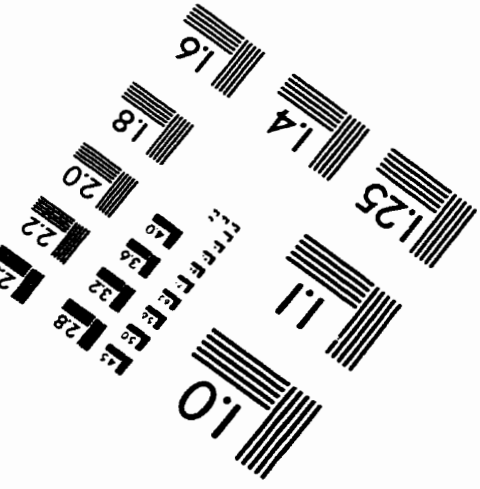
IMAGE EVALUATION
TEST TARGET (QA-3)

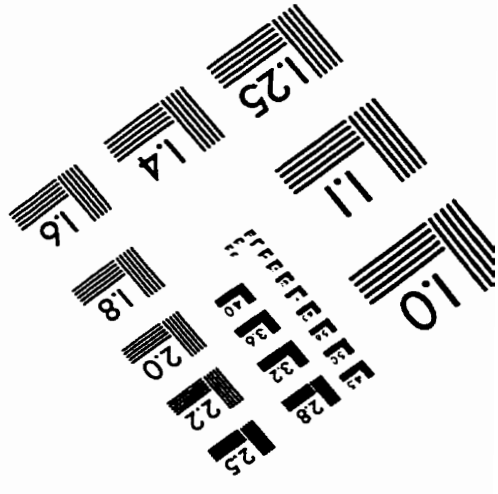












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989