GAS CHROMATOGRAPHIC ANALYSIS OF SOME LOWER MOLECULAR WEIGHT AMINES IN MILK AND THE RELATIONSHIP OF THESE AMINES TO FEED FLAVORS

by

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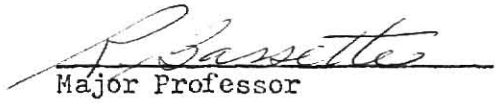

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INTRODUCTION

Definition of the Problem

The modern consumer's acceptance or rejection of milk is based largely upon its flavor. Thus off-flavors in milk are undesirable and even may be unacceptable in commerce. Among the various off-flavors, feedy is the most important from the commercial point of view. However, the term feedy is often misused. Frequently whenever an off-flavor cannot be classified otherwise, it is ascribed to the feed of the cow. In spite of this misrepresentation, off-flavors caused by feeds are very important in the quality of milk.

A fishy flavor in milk is usually classified under the general category of feedy. Some lower aliphatic amines have been associated with this fishiness. However, to date there have been few publications on the accurate quantitation of amine concentrations in milk and their correlation to flavor. Also, no substantial proof has been offered to show a relationship between various feeds, amine concentrations and milk flavor. Thus we hope this study will complement the information available in this area.

Objectives

The general objectives of this investigation are:

1. To develop a suitable quantitative procedure for the isolation, concentration and gas-liquid chromatographic (GLC) analysis of some of the lower aliphatic amines from milk.

2. To determine the amine concentrations of milk from cows fed winter wheat pasture and correlate these with their flavor scores.

3. To compare the flavor quality and amine concentrations in the

mixed herd milk produced on private dairy farms in and around Manhattan, Kansas.

4. To determine the approximate concentrations at which added methylamine (MA) and trimethylamine (TMA) would be organoleptically detectable in milk.

REVIEW OF LITERATURE

Feed flavors

The commercial significance of feed off-flavors in milk is demonstrated by the results of several recent surveys (7, 8, 9, 40, 54) which reveal that between 28 and 93% of all off-flavored milk in the United States is classified as feedy. Many reviews on feed flavors have been published, including ones by Strobel et al. (68) in 1953, by Babcock (6) and others (27, 31).

One of the earliest recorded references to off-flavors in milk is concerned with a feed flavor. In 1757, Bradley (11) reported that milk from cows which were fed turnips was off-flavored.

Kinsella (43) in 1969 compiled a list of the volatile compounds that have been associated with various off-flavors in milk. Sulfides, β-ionone, butanone, hexenol, acetone and octenol have been reported associated with feedy off-flavors. Gordon and Morgan (30) detected 22 chemical compounds in feed flavored milk distillates. By using a wide range of methods, they were able to identify 19 of these compounds. Correlations with flavor scores strongly suggested that the compounds causing the feedy flavor were methylsulfide, butanone, isopropanol, ethanol and propanol.

Although feeds are often responsible for flavor defects, occasionally feeds such as legume and grass silages (29) have been found to enhance the flavor of milk and increase its resistance to oxidative deterioration.

"Zero-milk", milk produced from cows on an odorless, protein-free diet, has been used as a reference material to assist the flavorist in his efforts to study the various off-flavors of milk (25, 27). The taste and smell of this milk is very similar to normal milk and thus will serve

as a good reference material.

Mechanism of Feed Flavor Transmission to Milk

There have been many publications on the transmission time of feed off-flavors to milk (68). In general, the time required to transmit flavor depends upon whether the flavor is transmitted directly from the feed or after the feed has been digested in the rumen.

Work done by Petersen and Brereton (56) and more recently by Shipe, Dougherty and others (3, 22, 62) enables us to understand the mechanism of feed flavor transmission to milk. There are three possible pathways through which flavoring substances may gain entrance to the blood and ultimately the milk:

1. Through the walls of the alimentary tract from ingested material.
2. Through the lungs from inhaled substances.
3. Through the skin from contacted substances.

In the cow, a dynamic equilibrium exists among the blood in the circulatory system, the urine in the bladder, the milk in the udder, the air in the lungs and the feces in the alimentary tract. Volatile materials are easily transmitted from one medium to another depending upon which has a higher concentration. Volatile materials are absorbed from the lungs and the digestive tract by the blood, circulated throughout the body, and are finally transferred to the milk resulting in off-flavors. It has been observed that off-flavors are transmitted much more rapidly by the lung route than by the digestive tract route. When the concentration of volatile materials is higher in the milk than in the blood these volatile materials will pass into the blood and be disposed of in urine, feces and through the lungs.

Fishy Flavors in Milk

Little is known about fishy flavors in milk and there is controversy as to their origin. Some sources have indicated that the fishy taint may be caused by the lower aliphatic amines (16, 49, 52). On the other hand Kinsella (43) has associated n-alkanals (C_5-C_{10}), alk-2-enals (C_5-C_{10}), 2,4-dienal (C_7), 2-alkanones (C_3-C_{14}) and oct-1-ene-3-one with the defect.

As early as 1935, Cranfield and Mackintosh (17) reported that feeding dried molassed beet pulp gave rise to fishy flavored milk and that certain cows were more likely to produce tainted milk than others. They claimed that if pulp was fed midway between milkings, it was more likely to produce a taint than if it were fed just prior to or during milking. These results were verified by Davies (19) in 1936. Trout and Harwood in 1944 (73) reported the pasturing of common rye to produce fishy flavored milk.

In 1955 Corfield (16) indicated that Pseudomonas bacteria could produce fishiness in milk from the breakdown products of lecithin, choline and betaine.

In a 1962 study at Hokkaido University, Japan (2), TMA was found in milk produced on beet-top silage. There were, however, no apparent off-flavors.

Cole et al. (14) in 1961 quantitated the lower aliphatic amines in feed flavored milk. They recovered 68% to 88% of the original concentration of amines. They then added some of these amines to normal milk and evaluated the off-flavors that resulted. Some of their results are shown in Table 1.

Table 1. Effect of added amines on milk flavor.

Compound	Concentration (ppm)	Flavor response
Ammonia	5.1	No effect
	6.8	Slight medicinal
Methylamine	3.1	Metallic
Ethylamine	4.5	Medicinal
	9.0	Slight feed, medicinal
n-Propylamine	10.0	No effect
	13.0	Slight feed
	15.9	Slight feed
	39.5	Unclean, slight cowy
n-Butylamine	7.3	No effect
	11.0	Cowy
	22.0	Feed, malty
n-Hexylamine	13.9	No effect
	18.3	Weed
	22.6	Weed, feed, green grass
	57.4	Chemical, medicinal

Fishy Flavors in other Dairy Products

Lampert (45) claimed that a fishy flavor develops in powdered whole milk if the moisture exceeds 3.5%.

In 1945, Van der Waarden (74) reported TMA to be present in fishy butter, however the addition of TMA to good butter in quantities up to 10 mg/35 gm did not produce fishiness. Thus he refuted the claim that TMA caused fishiness in butter.

Newlander and Atherton (52), while reviewing literature, noted that early workers had found TMA, a breakdown product of choline, to possess a strong fishy odor. Storgards and Hietaranta (67) believed that the tests for TMA were unreliable and that the oily and fishy odor of sour, stored butter resulted from the decomposition products of fat autoxidation.

The oxidation products of linoleic acid appeared to be of prime importance in the development of these flavor defects in butter. Meijboom and Stroink (49) have recently reviewed papers supporting and opposing the theory that TMA is responsible for the fishy off-flavor in autoxidized butter. They concluded from their studies that fishy off-flavors might be caused by the decomposition products of oils containing unsaturated fatty acids. Forss et al. (28) found as a result of GLC analysis, that fishiness in butter was caused by hexanal, heptanal, hex-2-enal and another carbonyl compound. In a 1972 study in the U.S.S.R. (5), a direct relationship was found to exist between the free fatty acid (FFA) content of milk and the incidence of fishy off-flavors. A FFA content of greater than 35 mg% was found to cause this defect. The possibility of using the FFA test to indicate fishiness in butter is being investigated.

In Tilsit cheese, ammonia, MA, dimethylamine (DMA) and traces of TMA have been identified by Schwarz and Thomasow (61). These, among others, have a positive effect on the aroma of the cheese.

Thus all the C_1 through C_6 amines have been detected in milk and dairy products and although these have been associated with feed off-flavors, none except TMA (16, 49, 52) has been specifically associated with fishy off-flavor. To summarize all the above information regarding fishy flavors in milk and its products, it is quite possible that more than one type of defect may occur, i.e. due to the presence of amines or due to the presence of other volatiles such as carbonyl compounds that may be decomposition products.

Fishy Flavors in Other Foods

In some other food products (e.g. fish), fishy odors have been

attributed to amines. Stansby in 1962 (66) reported that only 0.3 mg TMA nitrogen per 100 gm of fish (3 ppm) could cause a fishy odor. In 1936, it was indicated that the fat in fish plays a role in the release of fishiness caused by trimethylamineoxide (47). A 1939 publication reported similar reactions in other non-marine products (47).

Analytical Methods for Amines

In studying specific flavors, the flavor chemist must compare the organoleptic properties of the material and relate them to chemical constituents. Consequently, to contribute appreciably to the solution of flavor problems he must develop both sensory and chemical procedures of analysis.

Sensory evaluation

No single method is suitable for all organoleptic studies. Recent reviews by Pangborn and Dunkley (55) and Lavery (46) outline the various types of sensory tests and how they may be employed. Since the triangular taste test was used in our research, a more thorough review of it was undertaken (33, 58, 59).

The triangular taste test is frequently used to determine whether an experimental product differs from a standard (58). This test has been recommended to detect small flavor differences (59).

Today sensory evaluation methods are often used in conjunction with chemical and instrumental methods of flavor analyses (42) and elaborate statistical procedures have been developed to ensure their relevance (44).

Chemical and Instrumental Methods

The steps in an analytical evaluation of flavor components usually

involve: isolation and concentration of the volatile fraction and separation, identification and characterization of the components.

Isolation and concentration of volatiles. Weurman in 1969 (76) published a review on the isolation and concentration of volatiles in food odor research. He described in detail and gave advantages and disadvantages of various procedures such as distillation, crystallization, solvent extraction, and chromatography. Another review on this subject was presented by Teranishi in 1972 (71). A review dealing specifically with analytical techniques used with dairy products was published by Evans in 1972 (24).

In the experimental procedure described in this thesis, distillation has been used to isolate amines. Honkanen and Karvonen (36) demonstrated that, for high molecular weight compounds, distillation may not be the best method for isolating flavor components. Using GLC examination, they showed that in general, the larger the molecular size of the flavor compound, the lower their percent recovery from milk. On the other hand, they were able to obtain recoveries between 50% and 90% for compounds boiling at 100-250°C at concentrations of 0.005 to 10 ppm. Unfortunately they did not expand their study to include the effect of distillation on amines. Dimick and Walker (21) in 1967 observed that at high distillation temperatures, certain precursor compounds in milk fat are completely converted to their off-flavored derivatives. At low temperatures these reactions can be minimized or avoided, but then the off-flavored compounds in the fat are not completely removed.

Choi et al. in 1946 (13) used a distillation procedure to isolate ammonia from milk. They precipitated the proteins in milk by adding sodium tungstate and sulfuric acid. A 25 ml portion of the filtrate was

steam distilled for 7 min using 10 ml of 8% magnesium hydroxide to liberate the ammonia and 5 ml of 0.01 N sulfuric acid as the absorbing agent to collect amines from the distillate.

Hankinson and Cole (32) in 1958 used solvent extraction and GLC procedures to quantitate ammonia and the lower volatile amines in milk. Extracts were obtained using a modified Roese-Gottlieb procedure, and then were acidified with HCl, evaporated to dryness, and the amine hydrochlorides dissolved in ethyl ether. The free amines were released by alcoholic NaOH just before introducing them to the chromatographic column. This analysis revealed variable concentrations of the lower volatile amines in milk.

It is interesting to note that in 1961, Weurman and Rooij (77) successfully used a trap of 100 ml of 0.2 N HCl to collect the distillate of an amine solution; a similar trap has been used in our research. They also proved experimentally that amine formation did not occur when casein, as well as a number of simple nitrogen-containing non-amine compounds, was steam distilled.

Separation, identification and characterization of flavor components. Among other methods GLC has been commonly used to separate and characterize flavor components. There has been much experimentation with the column packing material and liquid phase to be used to separate amines by GLC. The Varian Aerograph Company (75) recommended Dowfax 9N9 (nonyl phenol polyethylene glycol ether) and Versamid 900 to obtain symmetrical peaks and for the quantitative analysis of free amines and amides. A column (3.05 m X 3.18 mm) packed with 20% Dowfax 9N9 was recommended using a sample size of 0.25 ul. Recently, however, Dowfax 9N9 has been discontinued and Triton X 100 (alkyl phenoxy polyethoxy ethanol) recommended in its place (69).

More recently Chromosorb 103, a porous polymer resin, was developed and was reported to be very good for the separation of amines. However, according to the Applied Science Laboratories, Inc. (4), it could not replace Dowfax 9N9. Thus, Applied Science has developed a stationary solvent, Pennwalt 223 (amine packing) to replace Dowfax 9N9. It has been suggested by Applied Science that, since Chromosorb 103 offers different selectivity from that of Pennwalt 223, these two columns should be used interchangeably to obtain flexibility.

O'Donnell and Mann (53) chromatographically separated mixtures of aliphatic amines, aromatic amines, and aliphatic amides on columns of Dowfax 9N9 with 2.5% NaOH as the stationary solvent. This was found to be superior to Carbowax 400 and 20M columns for the separation of aliphatic amines. The liquid phase constituted 25% of the total support. Keay and Hardy (41) in 1972 also used an alkaline Dowfax 9N9 column to quantitate accurately TMA and DMA at concentrations ranging from 1 to 100 ppm of nitrogen.

In 1972, MA, DMA and TMA were separated by Miller et al. (51) using columns containing Graphon and tetraethylenepentamine. They used a GLC procedure similar to the one we used, to analyze fish for these amines. The standard curves obtained for DMA and TMA were linear.

Sze et al. (70) reported that, of the lower aliphatic amines, the methylamines and particularly MA, were the most tenaciously held by the packing. They concluded that two kinds of packing were effective in reducing the adsorption effects. The first was a packing of 2% potassium hydroxide used with 15% Carbowax 400 or 1540, and the second was 5% tetraethylenepentamine with 15% diglycerol and Carbowax 400. They also reviewed the results of previous work (12, 37, 38) concerning the use of

different kinds of packing material and problems encountered while attempting deactivation of the support.

To separate the methylamines from ammonia, James et al. (39) employed a mixture of 5-ethyl-nonen-2-ol and liquid paraffin with Celite 545 support. Celite 545 was washed with methanolic sodium hydroxide to reduce support adsorptive effects. Other workers (20, 57, 64) have reported a more effective deactivation with other amine samples by depositing an alkali hydroxide on the support. In other cases the deactivation problem was avoided by using Teflon (26) instead of the usual diatomite support.

In addition to the GLC method, other techniques also have been employed to determine amines in milk. Choi et al. (13) employed a colorimetric method to quantitate ammonia in milk. Cole et al. (14) used Infrared Spectroscopy to identify the amines they found in milk. Several others (18, 23, 60, 63, 78) have used methods such as spectrophotometry, colorimetry, and photometry by which amines could be quantitated from systems other than milk.

Toan et al. (72) have described a procedure by which the volatiles from milk can be quantitated by GLC examination of head-space gas (HSG). Loney et al. (48) speculated that it was unlikely that the HSG method would be effective in the quantitation of amines due to their high degree of polarity. As a result of testing Loney's hypothesis, we developed the analytical method employed in this research.

EXPERIMENTAL

Chapter I. Analytical Methods

Apparatus and Materials.

An improved Kemmerer-Hallett type micro-Kjeldahl nitrogen distillation apparatus along with digestion-distillation flasks (all made of borosilicate glass) was used. Other apparatus included a waterbath, plastic 50 ml centrifuge tubes, 5 ml serum bottles with self sealing rubber stoppers, and syringes of 1 ml (Hamilton #1001, gas-tight with a 25 gauge needle), 2 ml (glass), and 10 ml (glass) capacities, respectively. The reagents were ACS grade KOH, NaOH, and HCl. Aliphatic amine salts were from Eastman Kodak.

For gas chromatography, an Aerograph model 600-B gas chromatograph with a flame ionization detector was used with a 1.05 mv Brown-Honeywell recorder. The column was stainless steel (3.05 m X 3.18 mm) packed with 80/100 mesh HMDS Chromosorb W coated with 25% Triton X 100 and 5.6% KOH as the stationary solvents. It was conditioned at 200°C for 60 hrs prior to its use in analytical work.

Procedure.

The method developed for the isolation and quantitation of some of the lower molecular weight aliphatic amines in milk can be divided into the following three parts:

1. Acid precipitation and distillation of milk. The micro-Kjeldahl apparatus was first pre-cleaned by refluxing distilled water for 5 min. Fifty milliliters of milk, at or below 15°C, was then poured into the dry distillation flask and acidified with 2 ml of 1N HCl. The curd was precipitated and the amines were converted to their hydrochloride salts.

A 50 ml plastic centrifuge tube containing 2 ml of 1N HCl was set under the outlet of the condenser to trap all the basic volatiles distilled from the milk sample. After making sure that the distillation system was air-tight and the condenser outlet was in the HCl trap, 5 ml of 1N NaOH was added to the curd-whey mixture to liberate the amines from their hydrochloride salt forms and heating of the steam-generating apparatus was started. The milk was distilled for about 30 min to obtain 20 to 50 ml of distillate.

2. Concentration of the distillate. The distillate was dried in the centrifuge tubes in a 60°C water bath using a stream of air and was then reconstituted to 5 ml by adding boiled, distilled water.

3. Gas-liquid chromatographic analysis of the amines. GLC conditions were: column temperature, 143°C; injector temperature, 193°C; detector temperature, 188°C; hydrogen flow, 8.4 ml/min; nitrogen carrier gas flow, 6.6 ml/min.

The GLC procedure was a modification of that employed by Toan et al. (72). A 2 ml aliquot of saturated KOH which had been boiled for 5 min to eliminate volatile contaminants and cooled to room temperature, was added to a 5 ml serum bottle. The bottles or vials were sealed with new rubber stoppers. A 2-day supply of vials was prepared in this manner. A 2 ml sample to be analyzed was injected through the cap into the vial, using a 2 ml syringe. The amines are liberated rapidly from the salt forms by the action of KOH. The vial was maintained at room temperature for 1 min ± 5 sec. One milliliter of the head-space gas (HSG) was removed from the vial with a 1 ml gas-tight syringe and injected into the gas chromatograph.

Results and Discussion.

Acid precipitation and distillation of milk. In an attempt to determine the optimum conditions necessary to obtain highest recoveries of amines from milk, the following variables were tested in the micro-Kjeldahl distillation procedure:

1. Determining the quantity of distillate necessary for complete recovery of the amines.
2. Acidifying cold milk vs warm milk prior to distillation.
3. Distilling a curd-whey mixture vs only whey.
4. Testing the effect of the variables employed on different amines and checking to determine if all of the amines respond to the same degree.
5. Testing the effect of adding NaOH before or after the start of steam generation.

To establish the amount of distillate to be collected, an experiment was conducted using a model system of butylamine hydrochloride salt solution in water. Recovery of free butylamine after distillation and subsequent GLC analysis by the above procedure yielded the following results:

	GLC peak height (average of two injections
1. Control (100 ppm solution - with no distillation)	142,000
2. 10 ml of 100 ppm solution distilled, dried and reconstituted to 10 ml with only 8 ml distillate collected.	110,000
3. Same as #2 except that 28 ml distillate was collected.	146,000
4. Same as #2 except that condenser water in the distillation apparatus was not turned on and 20 ml distillate was collected.	60,500

From the above experiment with the aqueous model system, it was

concluded that the quantity of distillate is a critical factor. Complete recoveries are obtained by distilling as outlined in the experimental procedure. It is also essential to utilize the condenser.

A series of experiments then was designed to establish a procedure for recovering the amines from milk (detailed procedure in Appendix A). The 1-, 2-, 3-, 4- and 6-carbon amine hydrochloride salts were added in known quantities to milk samples. Some of the observations recorded were as follows:

1. Acidifying a cold milk (9°C) sample resulted in better recovery than acidifying a warm (30°C) sample. Compare peak heights for treatment 1 and treatment 2 in Table 2.

2. Distillation of acid whey-curd mixture resulted in better recoveries than the distillation of only acid whey. Compare treatments 1 and 2 with 4 and 5 in Table 2.

3. Treatment variation resulted in different relative responses for the several amines analysed. This can be seen by comparing peak response ratios relative to C-4 at 100 (Table 2).

4. In the micro-Kjeldahl system, adding NaOH before starting to heat the distillation flask was superior to heating the distillation flask a few minutes prior to adding NaOH. Compare treatments 4 and 5 in Table 2.

Concentration of the distillate. In an attempt to determine the optimum conditions necessary to obtain highest recoveries of amines from milk distillates, the following variables were tested:

1. Exposing the concentrated salts to room air after concentration for varying periods.

2. Comparison of 60°C and 83°C water baths for evaporating the amine salt distillates.

Table 2. Effect of distillation procedures on recovery of amines as measured by GLC analysis.

Treatment[1]	Carbon chain length of amines					Volume whey	Volume of distillate
	C_1	C_2	C_3	C_4	C_6		
	(Peak heights)					(ml)	(ml)
1 Acidified 9°C milk plus 100 ppm added amines	1,200[2] 1.9[3]	23,400 37.5	57,600 92.3	62,400 100	11,100 17.8		35
2 Acidified 30°C milk plus 100 ppm added amines	1,200 3.13	13,900 36.2	37,000 96.4	38,400 100	5,800 15.1		40
3 Filtered whey from milk acidified at 30°C[4]		10 6.0	90 53.3	168 100	30 17.8	29	34
4 Filtered whey from milk acidified at 30°C and containing added amines	400 2.9	5,100 36.4	14,500 103.6	14,000 100	1,900 13.6	34	19
5 Filtered whey plus added amines, steam distillation delayed 2 min.	600 3.1	7,750 40.6	19,200 100.5	19,100 100	2,450 12.8	34.5	44
6 Control, response from 100 ppm amines, no distillation.	4,400 2.9	60,500 40.4	141,200 94.3	149,760 100	16,100 10.7		

[1] Detailed treatment procedure in Appendix.

[2] Average of two injections; % of full scale deflection x attenuation factor.

[3] Ratios are based upon the C_4 amine as 100.

[4] No amines were added to this sample.

3. Concentrating distillates to different volumes.

Experiments were initiated in which 10 ml of a 100 ppm butylamine hydrochloride solution was subjected to different treatments to test the above variables. Butylamine was used as reference amine in preliminaries since in terms of the number of carbon atoms, it was located midway in the C_1 through C_6 series of amines. The treatments and results of this study are summarized in Table 3.

From this experiment, it was concluded that there was no appreciable difference between evaporated samples of amine hydrochloride salt solutions kept at room temperature and those exposed to room atmosphere for 0, 1 and 8 days. Increasing water bath temperature from 60° to 83°C during evaporation resulted in an increase in losses of amines by approximately 8%. Concentration of amine salt solution from 10 to 3 ml resulted in loss of about 3% amines; the losses were as much as 10% when volume decreased to 1 ml and when reduced to dryness, losses were as high as 20-25%.

Gas-liquid chromatographic analysis of the amines. The GLC procedure that was first used was that of Toan et al. (72) with the following modifications:

1. Prior to the 8 min incubation period in the water bath, approximately 0.2 ml of saturated KOH was added to the sample in the vial.

2. The gas-tight syringe was heated to approximately 65°C before using it to inject HSG into the gas chromatograph.

During the course of the GLC analyses, however, many problems were encountered, some of which are listed below along with some suggestions for their solution:

Impurities: Many impurity peaks were observed on chromatograms when analysed by this procedure.

Table 3. Effect of concentration variables on recovery of butylamine as measured by GLC analysis.

Date of GLC anal.	Water bath temperature (°C)	Original volume (ml)	Final volume (ml)	Exposure to air (days)	Peak height[1]
9-8-72	Control[2]				168,000
	60	10	dry	8	132,000
	63	10	dry	1	132,000
	83	10	dry	1	120,000
9-9-72	Control				152,000
	Control				152,000
	63	10	dry	1	140,000
9-16-72	60	10	dry	0	136,000
	60	10	dry	0	128,000
	60	10	0.25	0	140,000
	60	10	1	0	144,000
	60	10	3	0	154,000
	Control[3]				160,000
	Control				160,000
	Control				157,000
	Control[4]				168,000

[1]Percent of full scale recorder deflection x attenuation factor.

[2]Control sample is not subjected to evaporation.

[3]Due to lower room temperature, saturation point decreased on 9-16-72 resulting in a lower concentration of alkali.

[4]Alkali was from 9-9-73.

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1. The major cause for these peaks was impurities in the saturated alkali used in vial.

These impurities could be eliminated by:

a. Boiling alkali for 5 minutes.

b. Thoroughly washing the pipette or syringe used to transfer alkali from stock solution flask to sample vials between consecutive usage.

c. Keeping the stock-solution flask sealed when not in use.

d. Reboiling the stock-solution every two or three days.

e. Washing the syringe used to inject amine salt solution into the vial containing alkali after every injection to wash off alkali spray sticking to tip of needle.

2. Some spurious peaks occurred from the diluent water. Distilled water may be kept relatively pure for about 20 days by boiling it and keeping it sealed.

3. Air peaks at 0 and 1 min could not be eliminated. No impurity peaks were observed due to KOH or water per se.

Response increase: Another major problem encountered was that of increasing the instrument response and improving reproducibility so that adequate sensitivity could be obtained.

1. No difference was found among the various alkalis tested.

2. Only slight differences were observed between syringes with Teflon or glass plungers with the former having a slight advantage over the latter. Consequently the Teflon plunger was selected. The slight advantage obtained by heating the Teflon plunger was eliminated by manipulating the Teflon tip of the plunger to obtain a "gas-tight fitting" in the syringe. Thus it was decided not to heat the plunger and syringe.

3. It was observed that there was a large loss of volatile vapors

during the changing of stoppers prior to the 8 min water bath incubation period. Thus it was decided to modify the previously described Toan procedure entirely.

4. Saturated alkalis to which the samples were added, yielded significantly higher responses over lower alkali concentration.

5. Different volume combinations of alkali and amine salt solution were tried. Responses increased proportionately until a ratio of 1.5 ml alkali to 2 ml sample was attained, after which there was no increase in response by increasing alkali. Thus a 2 ml alkali - 2 ml sample combination was adopted.

6. Combinations of 5, 8, 10 and 15 min incubations in a water bath at 60, 70, 80 and 90°C were tried. No variation was observed after sample introduction due to change in water bath temperature; however, responses decreased considerably with an increase in time.

When the vial was maintained at room temperature, a maximum and relatively constant response was obtained within an interval of 0.5 min to 1.5 min between introduction of the sample into vial and injection into the gas chromatograph. To minimize the chance that sensitivity may be influenced by time, an interval of 1 min $\pm$ 5 sec was adopted for our procedure.

It was on the basis of all of these findings that the Toan method was modified as presented in the procedure.

Chapter II. Analysis of Feed Flavored Milk

Objectives.

1. To study the flavor and amine concentrations of milk porduced by cows on winter wheat pasture.

2. To correlate the amine concentrations in the milk with the flavor scores.

Procedure.

Two cows (A and B) were put on winter wheat pasture on October 18, 1972. The cows were milked the day before being put on pasture and subsequently hand milked until October 25, 1972. Evening milk from these cows was evaluated organoleptically the following day. For organoleptic evaluation, milk was judged according to the procedure followed in the National Collegiate Student Judging Contest. A score of 40 points indicated excellent quality milk with no flavor defect, whereas a score of 30 points indicated unacceptable milk. The taste panel consisted of 4 judges experienced in milk flavor analysis.

Besides flavor analysis by the above method, four of the milk samples were evaluated by the KOH pellet test of Bassette (10). This test consists of adding a pellet of KOH to 20 ml of milk in a stoppered flask at room temperature. The aromas of the KOH-treated milk were examined and scored on a 1 to 5 scale according to the intensity of fishiness. The milk was distilled the next day and after concentration and reconstitution to 5 ml, was refrigerated until all samples were ready for GLC analysis. The analytical procedure for GLC outlined in Chapter I was used.

To ascertain the relative response to the C_1 through C_6 aliphatic amines by the above GLC procedure, 100 ppm solutions of the amines in the

salt form were analyzed. The peak heights were compared to the physical properties of the amines.

Results and Discussion.

Table 4 summarizes the observations on the physical characteristics and GLC analyses of the standard amines. As can be seen from these results, retention times (RT) of the primary amines increased with increases in boiling points (BP) and the number of carbon atoms in the chain. The RT of TMA, on the other hand, seemed to be influenced by its pKa. This perhaps was due to the polar nature of the GLC column causing less interaction with TMA. No relationships were observed between the peak heights and BP, pKa's or the number of carbon atoms in the amine molecules.

Table 4. GLC analyses of some aliphatic amines.

Name	Boiling points[1] (°C)	pKa[2]	Retention time (min)	Peak height[3]	Peak heights relative to butylamine
Trimethylamine	3.5	9.80	1.5	210,000	142
Methylamine	-6.3	10.62	1.7	4,400	2.9
Ethylamine	16.6	10.63	2.0	60,500	40.4
Propylamine	48		2.8	141,200	94.3
Butylamine	78		4.2	149,760	100
Pentylamine	104		7.0		
Hexylamine			11.3	16,100	10.7

[1]Merck Index (50)

[2]Albert and Serjeant (1)

[3]100 ppm amine solutions used.

For quantitative analysis of amines, standard curves for TMA and MA were plotted (Figs. 1 and 2). Each point on these standard curves represented the average of duplicate injections of the standard. The probable reason for the curvilinear nature of the standard curve for MA is that it

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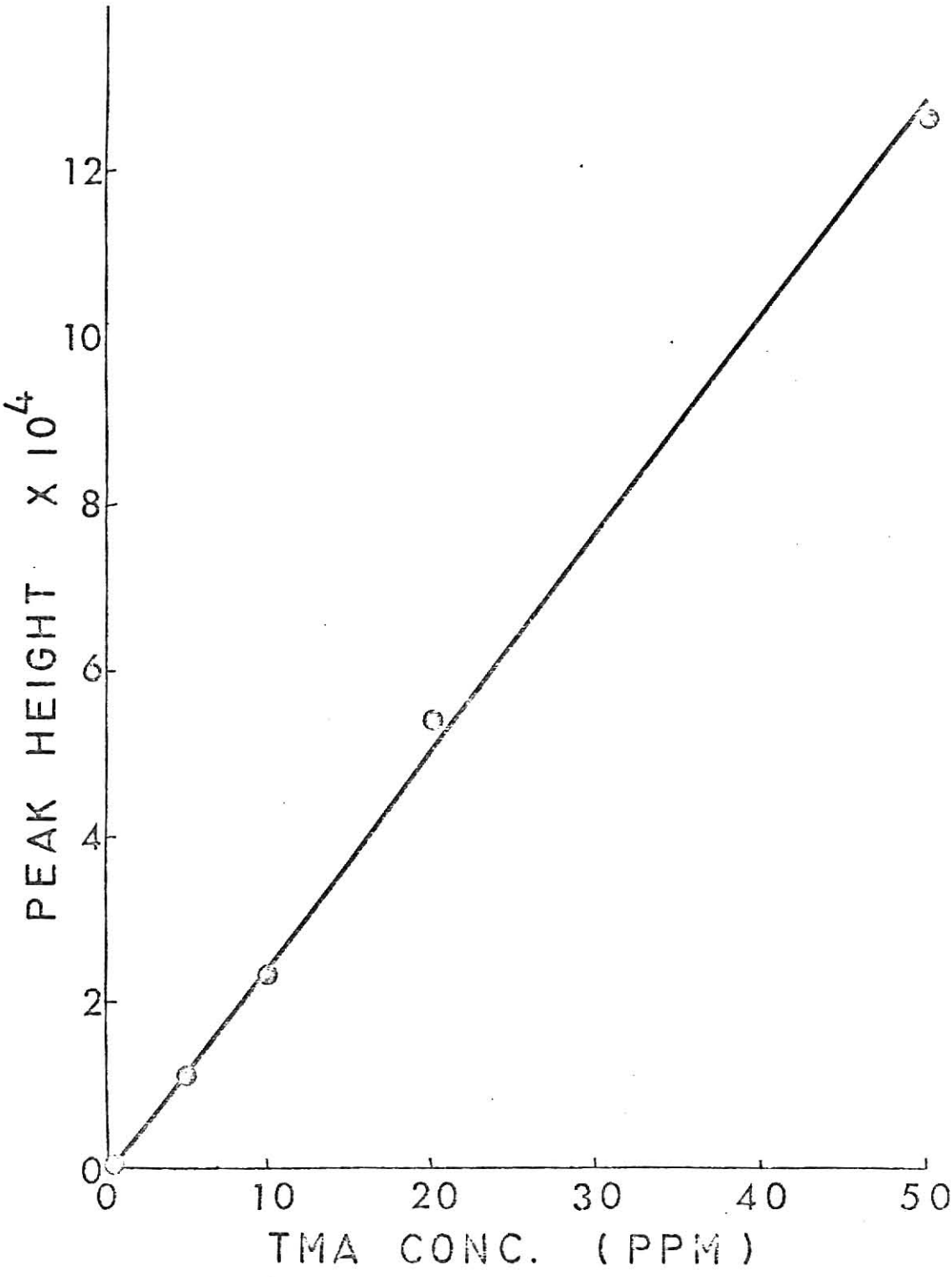

Fig. 1. Comparison of GLC peak heights with trimethylamine (TMA) concentrations.

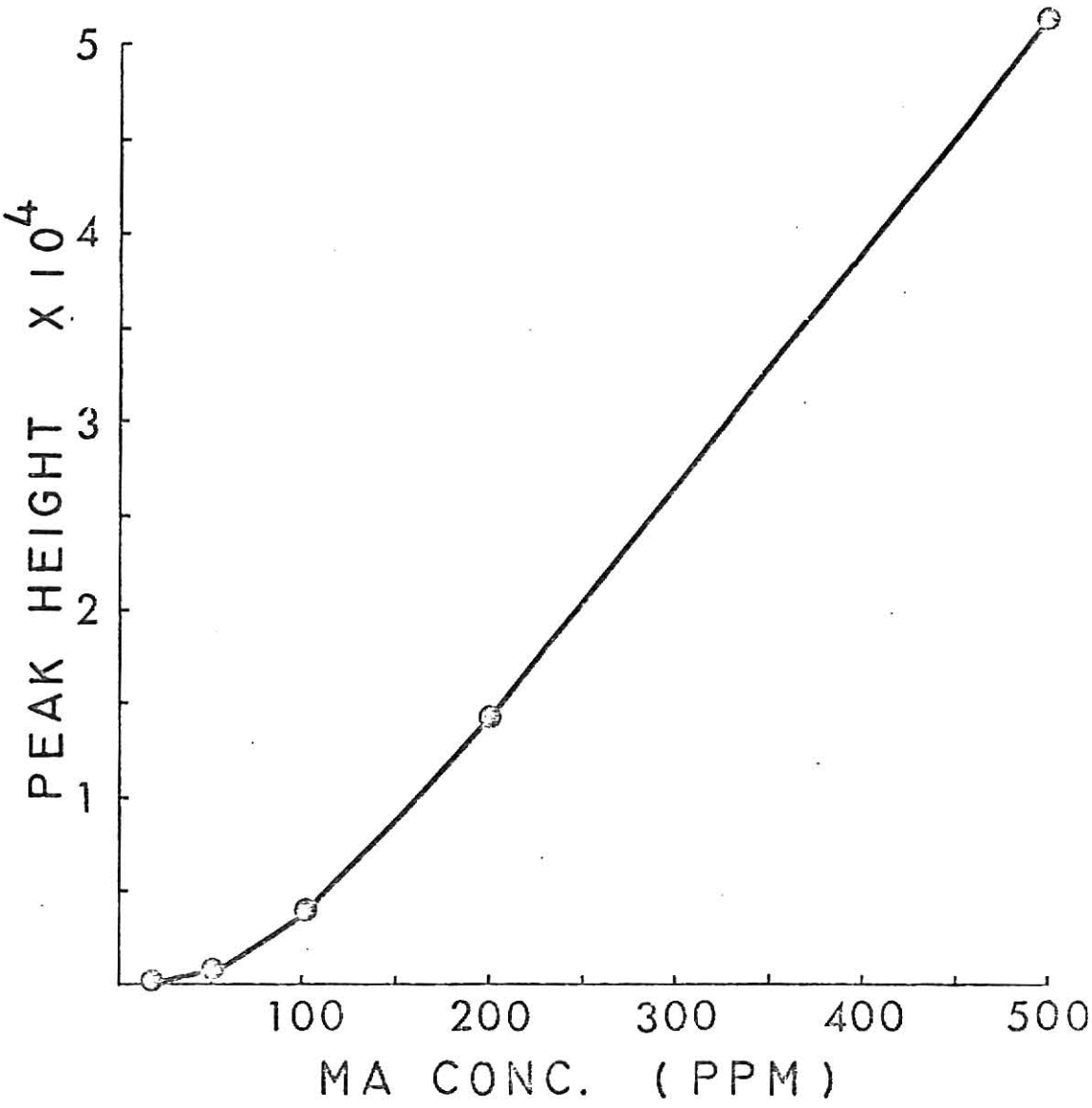

Fig. 2. Comparison of GLC peak heights with methylamine (MA) concentrations.

was adsorbed to a larger degree on the GLC column packing than TMA (70).

GLC analyses of the milk from the 2 cows on winter wheat pasture are summarized in Tables 5 through 8. For each date and each cow, 2 samples of milk were distilled and after concentration and reconstitution, 5 ml of sample was obtained from each distillate. Thus 2 injections were made from each distillate and the value reported in the tables for each distillation represents the average of 2 injections.

To determine the TMA and MA concentrations, their respective standard curves were used. The propylamine and butylamine peaks (RT 2.8 and 4.2 min, respectively) were also identified and recorded but, since they did not seem to be consistent, it was decided that no useful information could be gleaned from them in this study. Figures 3 through 6 show the daily changes in amine concentrations as well as the flavor scores over the 8 day trial period. Similarly Figs. 7 and 8 show the daily changes in peak heights and flavor scores for two unidentified basic compounds with a RT of 3.5 and 5.2 min. Figures 9 and 10 show an inverse relationship between the flavor scores and TMA and MA concentrations, respectively. The correlation coefficients (4) calculated by Snedecor and Cochran's method (64) were as follows:

Cow	Amine	r	P <
A	TMA	-0.76	.02
A	MA	-0.79	.02
B	TMA	-0.66	.06
B	MA	-0.84	.01

When calculating the correlation coefficients for the TMA of cow B, if the observations on 10-19-72 (day 2) were not included, the correlation was 0.97. Thus it is probable that an error occurred on that particular

Table 5. GLC analyses of milk produced on wheat pasture (Cow A).

Days on Pasture	Distillation	GLC peak retention time (min)[1]						
		1.5 (TMA	1.7 (MA)	2.8	3.5	4.2	5.2	13.1
0	a[2]	8,480	592	800	-	120	-	-
	b[3]	8,800	300	16	14	97	10	-
	avg[4]	8,640	446	408	7	108	5	-
1	a	36,160	3,400	27	33	10	40	-
	b	24,080	1,580	27	-	-	24	-
	avg	30,120	2,490	27	17	5	32	-
2	a	29,520	2,720	40	80	40	85	12
	b	20,320	550	19	12	17	38	-
	avg	24,920	1,635	30	46	28	62	6
3	a	38,400	12,000	-	46	8	118	20
	b	18,320	2,000	13	13	4	74	-
	avg	28,360	7,000	7	30	6	96	10
4	a	27,360	2,400	8	18	-	74	-
	b	25,600	1,360	9	10	-	55	-
	avg	26,480	1,880	9	14	-	65	-
5	a	29,920	1,540	16	8	7	56	12
	b	27,600	1,600	7	8	-	49	12
	avg	28,760	1,570	12	8	4	53	12
6	a	55,040	10,640	-	-	-	69	17
	b	59,840	8,840	-	-	-	68	-
	avg	57,440	9,740	-	-	-	69	9
7	a	46,240	7,520	-	7	4	90	16
	b	43,360	7,000	-	4	-	72	8
	avg	44,800	7,260	-	6	2	81	12
8	a	55,840	14,240	8	10	-	129	10
	b	54,560	11,440	-	11	-	125	10
	avg	55,200	12,840	4	11	-	127	10

[1]RT 1.5 min corresponds to trimethylamine; 1.7 min, methylamine; 2.8 min, n-propylamine and 4.2 min, n-butylamine.

[2]Distillation unit a, avg of duplicate GLC analyses.

[3]Distillation unit b, avg of duplicate GLC analyses.

[4]Average of distillations a and b.

Table 6. Trimethylamine and methylamine concentrations, and organoleptic tests of milk from cow A on wheat pasture.

Days on Pasture	TMA (ppm)[3]	MA	Flavor Score[1]	Pellet Test[2]
0	0.38	3.5	36.88	----
1	1.22	8.0	37.63	----
2	1.01	6.5	35.25	----
3	1.14	13.1	35.50	2.2
4	1.07	6.8	35.63	1.2
5	1.16	6.3	37.00	1.2
6	2.27	15.8	34.00	2.6
7	1.78	13.3	34.25	----
8	2.19	18.8	34.00	----

[1]Average scores of four experienced judges

[2]Scale 1 (no odor) to 5 (strong fishy), Bassette (10)

[3]Concentration of amines in milk (ppm)

Table 7. GLC analyses of milk produced on wheat pasture (cow B)

Days on Pasture	Distillation	Retention time (min)						
		TMA 1.5	MA 1.7	2.8	3.5	4.2	5.2	13.1
17	a[2]	15,200	1,030	29	45	112	23	-
	b[3]	4,760	460	36	24	40	8	-
	avg[4]	9,980	745	33	35	76	16	-
18	a	10,880	110	64	31	51	161	-
	b	11,000	425	18	17	88	19	-
	avg	10,940	268	41	24	70	90	-
19	a	88,320	2,240	-	92	4	218	22
	b	27,680	3,600	-	11	58	43	-
	avg	58,000	2,920	-	53	31	131	11
20	a	41,120	5,880	21	9	74	49	16
	b	55,360	7,600	-	56	4	160	8
	avg	48,240	6,740	11	33	39	105	12
21	a	60,160	27,200	-	30	-	194	8
	b	46,560	12,560	-	42	-	194	9
	avg	53,360	19,880	-	36	-	194	9
22	a	59,520	37,440	-	10	12	126	10
	b	62,720	23,360	-	18	-	172	12
	avg	61,120	30,400	-	14	6	149	11
23	a	48,640	61,120	-	60	-	140	-
	b	100,800	50,240	-	20	-	214	18
	avg	74,720	55,680	-	40	-	177	9
24	a	41,440	23,040	-	8	-	143	6
	b	64,960	24,480	-	-	-	166	9
	avg	53,200	23,760	-	4	-	155	8
25	a	104,320	45,440	-	16	-	153	7
	b	87,680	35,200	-	40	-	254	12
	avg	96,000	40,320	-	28	-	204	10

[1]R.T. 1.5 min corresponds to trimethylamine; 1.7 min, methylamine; 2.8 min n-propylamine and 4.2 min, n-butylamine.

[2]Distillation unit a, avg of duplicate GLC analyses.

[3]Distillation unit b, avg of duplicate GLC analyses.

[4]Average of distillations a and b.

Table 8. Trimethylamine and methylamine concentrations, and organoleptic tests of milk from cow B on wheat pasture.

Days on Pasture	TMA (ppm)[3]	MA	Flavor Score[1]	Pellet Test[2]
0	0.44	4.7	36.38	----
1	0.47	3.0	36.38	----
2	2.30	8.5	37.75	----
3	1.91	12.8	34.75	2.0
4	2.05	24.5	35.38	3.8
5	2.41	33.0	34.88	2.4
6	2.94	50.2	33.75	3.0
7	2.11	27.6	35.00	----
8	3.76	40.9	33.25	----

[1]Average scores of four experienced judges

[2]Scale 1 (no odor) to 5 (strong fishy), Bassette (10)

[3]Concentration of amines in milk (ppm).

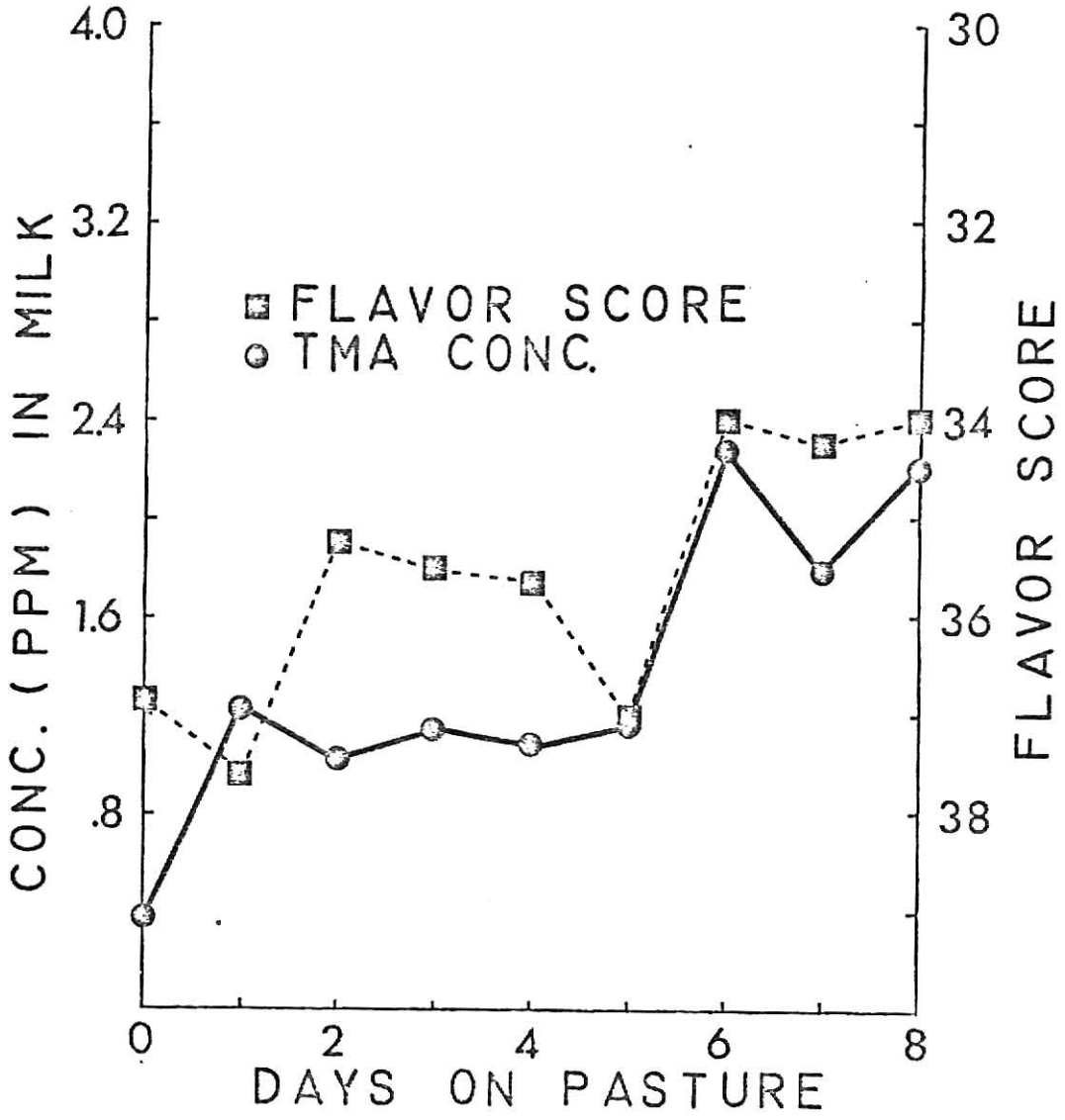

Fig. 3. Changes in flavor scores and trimethylamine concentrations in milk from cow A while on wheat pasture.

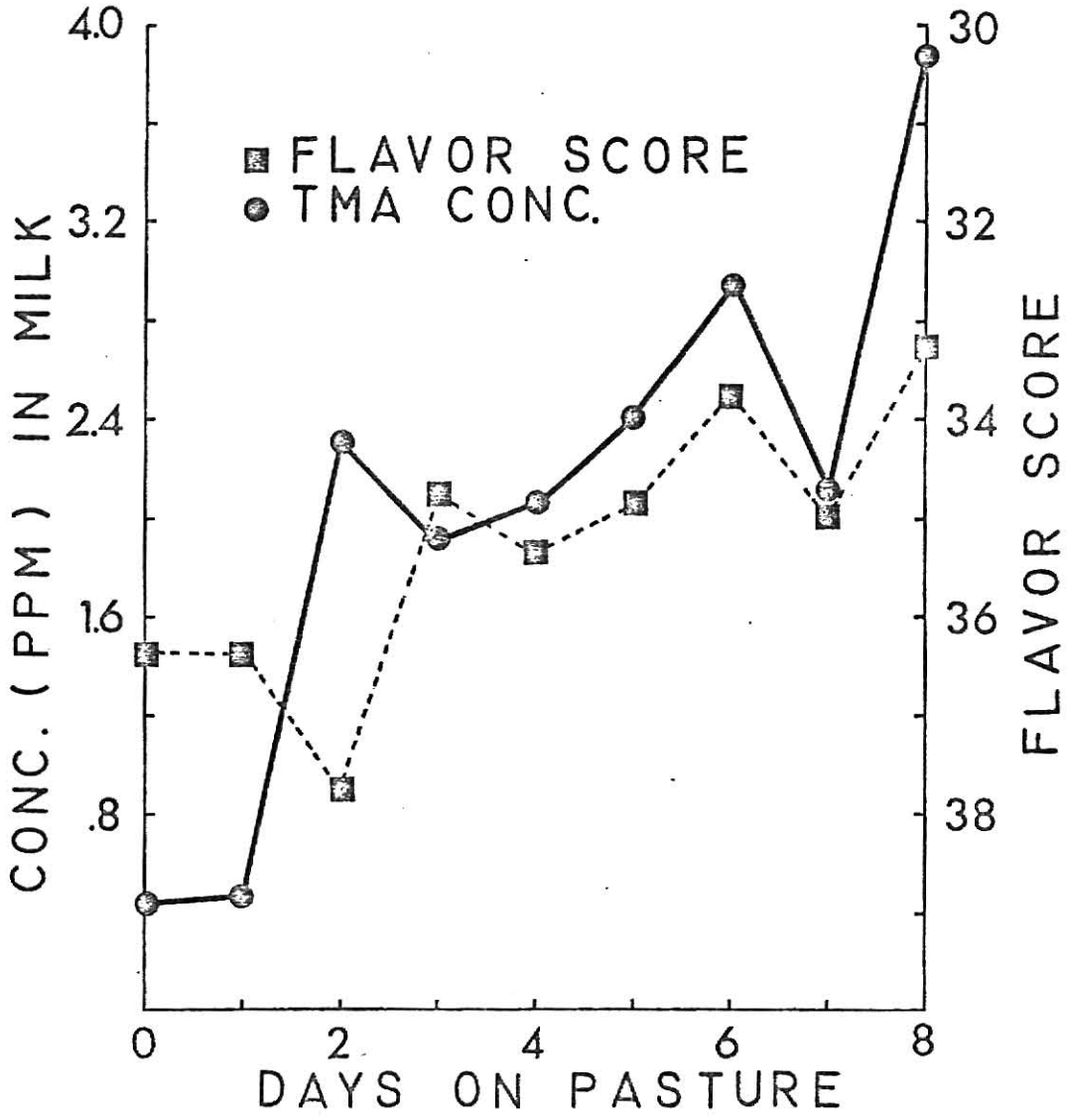

Fig. 4. Changes in flavor scores and trimethylamine concentration in milk from cow B while on wheat pasture.

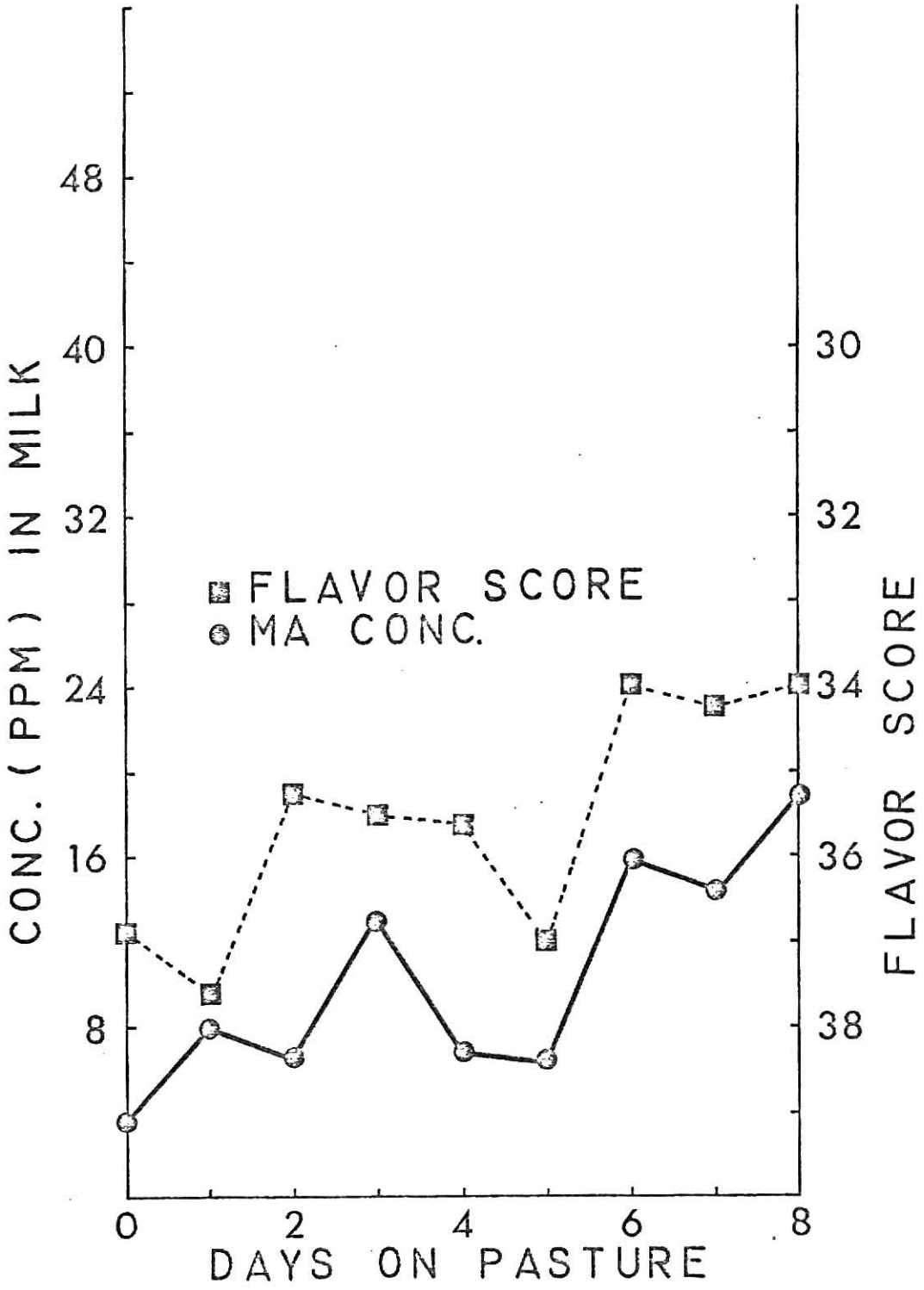

Fig. 5. Changes in flavor scores and methylamine concentration in milk from cow A while on wheat pasture.

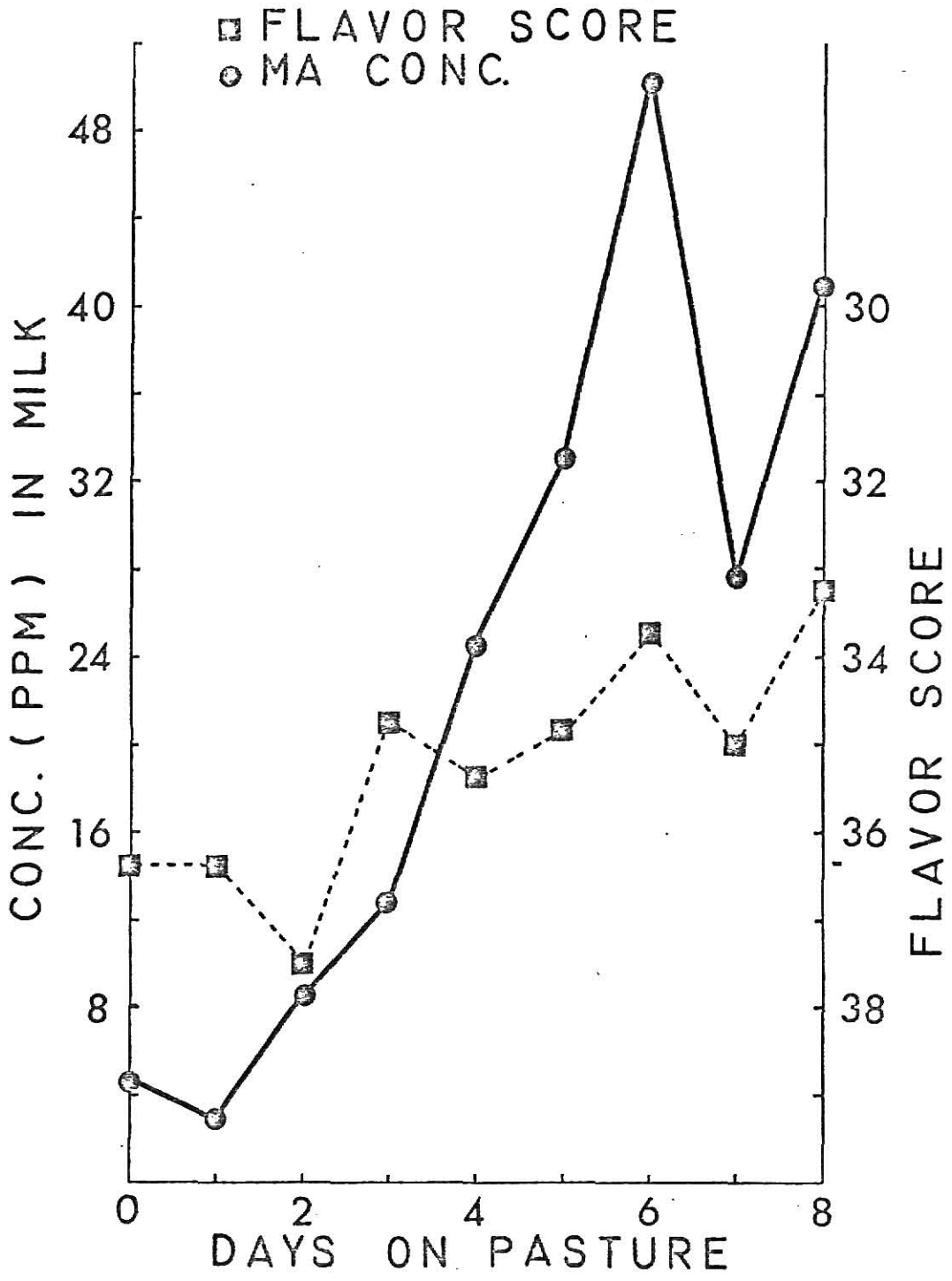

Fig. 6. Changes in flavor scores and methylamine concentration in milk from cow B while on wheat pasture.

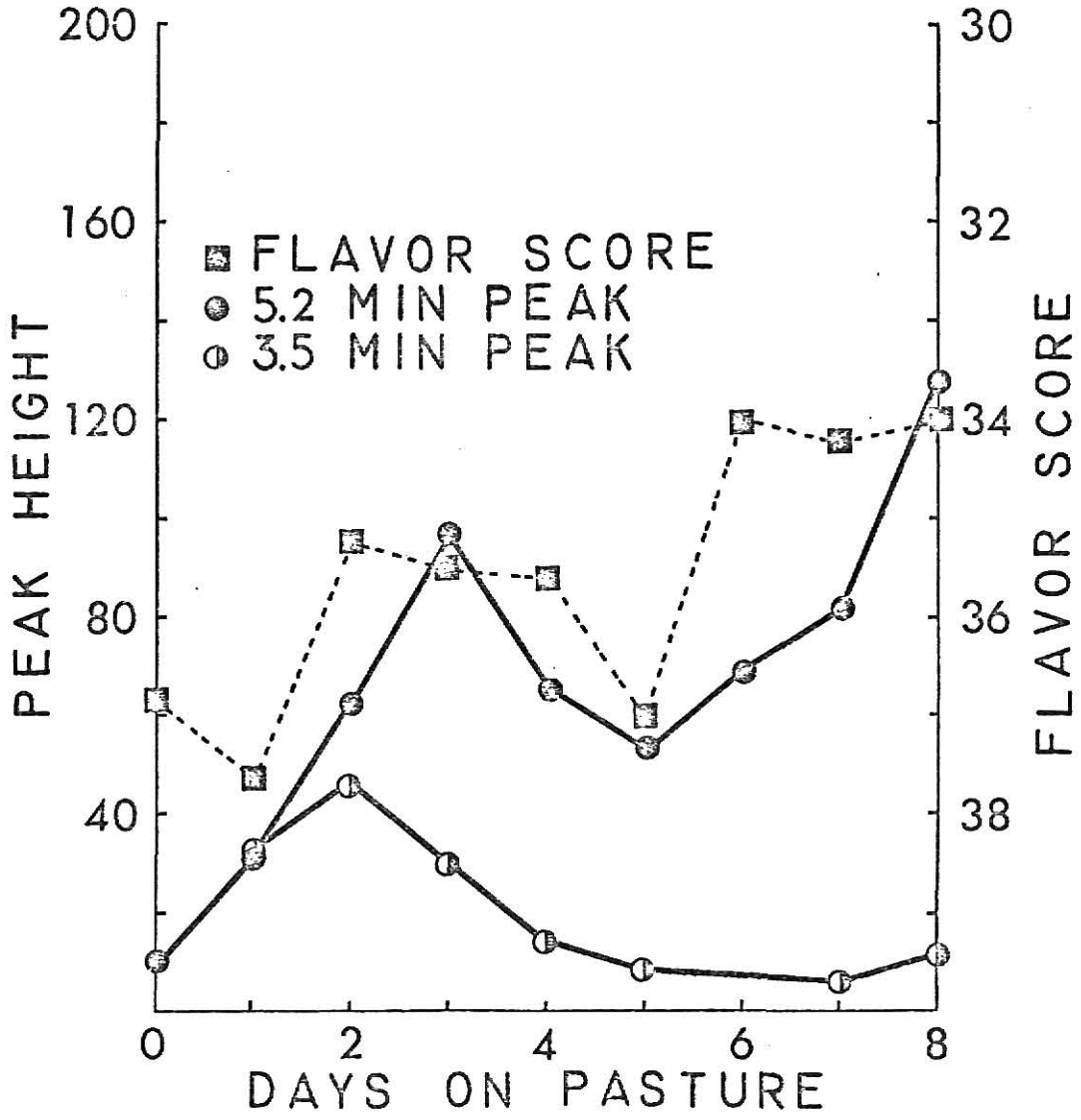

Fig. 7. Changes in flavor scores and peak heights of two unidentified peaks from milk of cow A on wheat pasture.

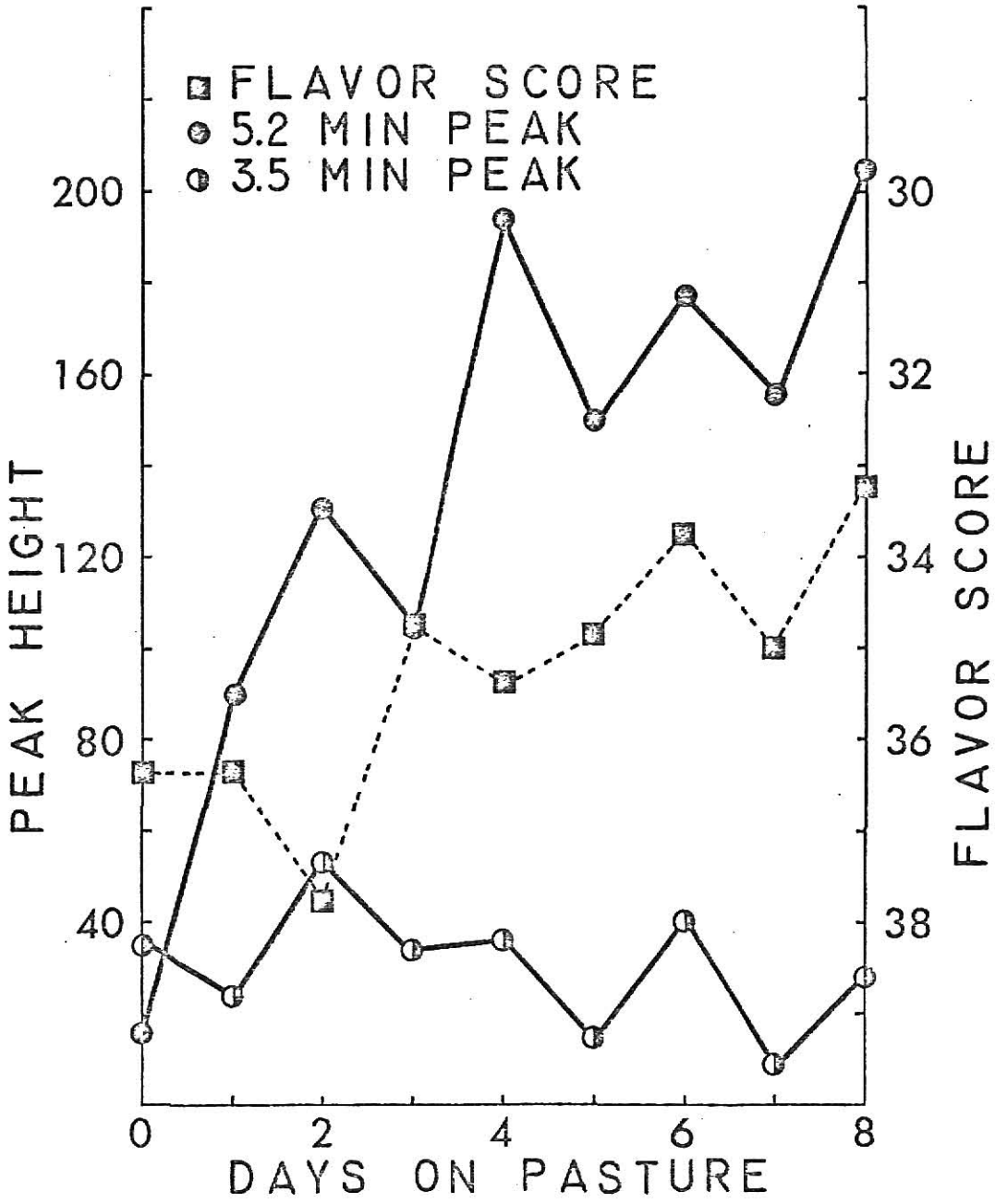

Fig. 8. Changes in flavor scores and peak heights of two unidentified peaks from milk of cow B on wheat pasture.

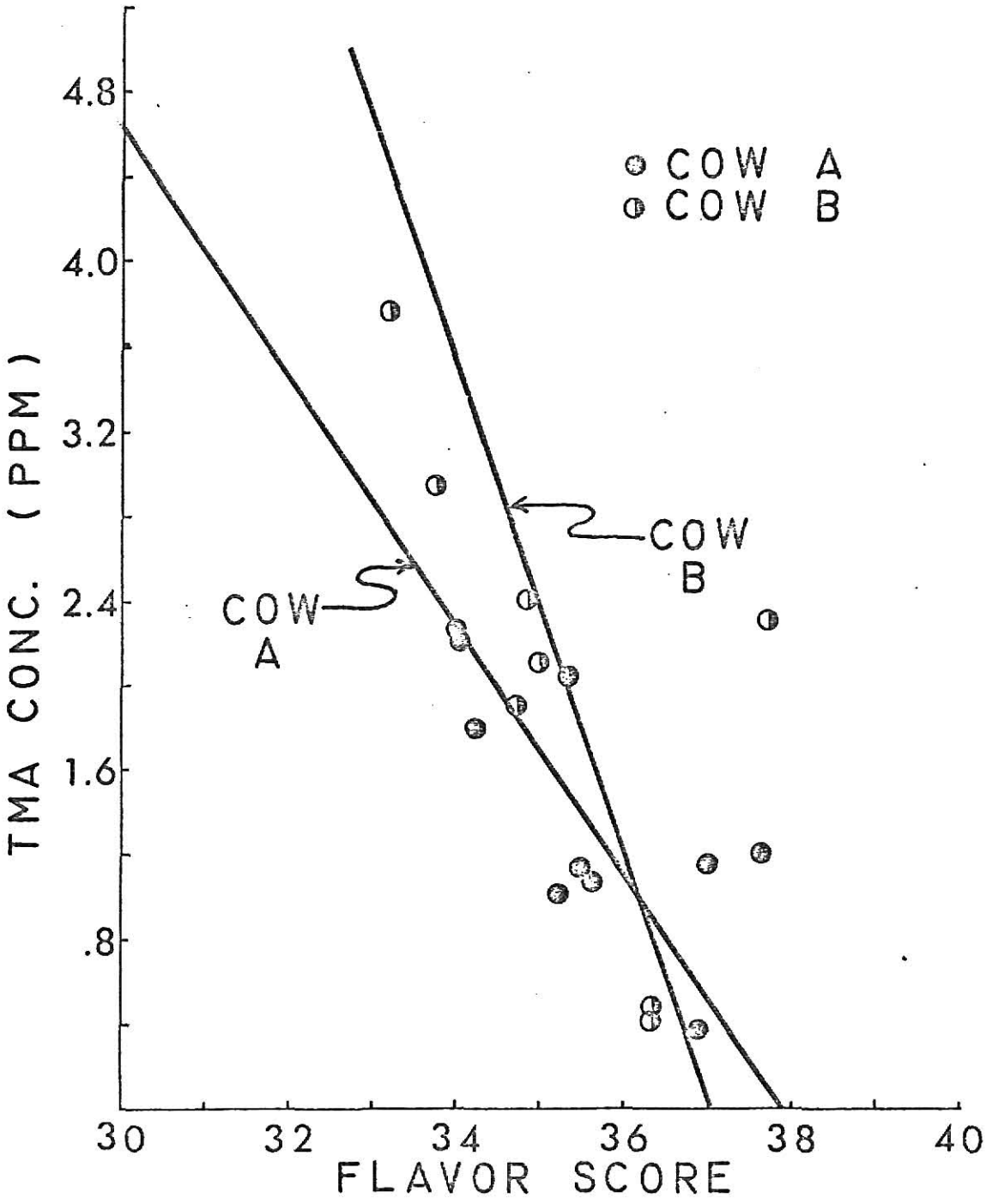

Fig. 9. Comparison of trimethylamine concentrations with respective flavor scores for milk from the two cows on wheat pasture.

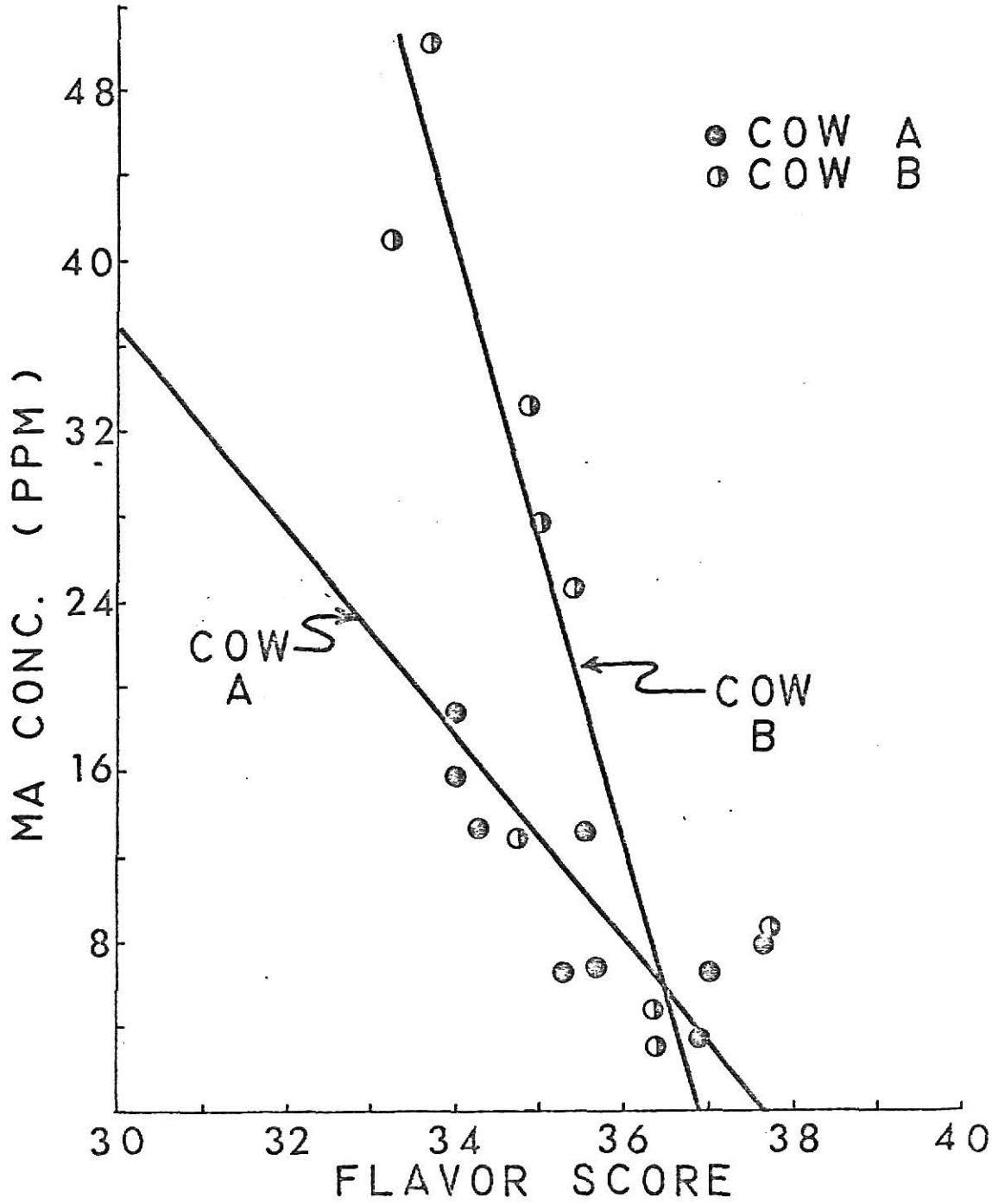

Fig. 10. Comparison of methylamine concentrations with respective flavor scores for milk from the two cows on wheat pasture.

day, either in the sensory evaluation or determination of amine concentration.

Observations from Figs. 3 through 10 and Tables 5 through 8 may be summarized as follows:

1. The milk from cows on wheat pasture seemed to deteriorate in flavor quality during the pasture period (Tables 6 and 8 and Figs. 3 through 8).

2. In general, concentrations of TMA, MA and the unidentified compound with a RT of 5.2 min increased in the milk of cows on wheat pasture.

3. There was an inverse correlation between flavor score and concentrations of TMA and MA. It also appeared that there was a direct relationship between peak heights of 5.2 min component and flavor scores.

4. The compound which had a RT of 3.5 min decreased in concentration during the 8 day trial period. It is possible that this compound might have been a precursor for an off-flavor component.

5. TMA and MA could be responsible for the fishy flavor in milk. This is a strong possibility because, when milk was made alkaline using the KOH pellet test of Bassette (10) (see Tables 6 and 8), the intensity of fishiness (odor) seemed to be related to the flavor scores and thus indirectly to the concentration of amines.

Chapter III. Development of a Gas-liquid Chromatographic Method for Analysis of Trimethylamine and Methylamine

Objective.

As discussed in Chapter II, it was observed that TMA and MA were suspect as the principal basic components related to fishy flavor. Thus, it was decided to modify the GLC instrumental procedure so that it would yield greater resoltuion, and to change the GLC sampling method to give better response for these two amines.

Procedure.

In attempting to give greater resolution of TMA and MA, the GLC column temperature was decreased and the nitrogen gas flow increased. Various combinations of gas-flow and column temperature were tested. Finally it was found that best resolution was obtained at a column temperature of 93°C and a nitrogen gas flow of 9 ml/min and these conditions were incorporated into the procedure.

To increase the response, a study was made of the GLC sampling procedures. It was decided to pre-heat the alkali before adding the amine salts sample to it, so that more volatile material would be obtained in the vapor phase.

Results and Discussion.

Studies of sample preparation revealed a number of factors that had to be considered in developing this portion of the analysis. The following are the major problems that had to be dealt with, particularly upon heating the alkali and sample prior to GLC analysis.

1. It was found advantageous to leave the rubber stopper off the alkali vial while it was kept in the water bath prior to the introduction

of the sample. When the vial remained sealed there was an erratic GLC response perhaps due to large fluctuations in the amount of water-vapor formed in the HSG. Also, the HSG was under positive pressure after the sample was introduced into the vial of alkali. Consequently, between the time that the HSG sample was withdrawn and transferred from vial to the injection port, an appreciable proportion of the vapors may have been lost, thereby causing a fluctuation in response. One of the principal advantages in leaving the vial unstoppered is that the small proportion of volatile impurities absorbed by the alkali may be removed during this tempering period, thus resulting in smaller impurity peaks.

2. It was found that with the preheating step, higher water bath temperature for the alkali resulted in higher total response. However, above 70°C, there was too much fluctuation and, it was decided to heat the vial of saturated KOH for 5 min at 70°C.

3. If the amine salts solution was preheated to 70°C, the response was increased considerably. The time necessary to bring the sample and the alkali to 70°C was 3 min. To ensure that these solutions were 70°C, a water bath residence time of 5 min was established.

From these findings the following GLC procedure for analysis of TMA and MA was developed.

Sample preparation. 1. Prior to injecting the 2 ml solution of amine hydrochloride into the vial, the sample solutions were placed in test tubes and tempered in a water bath to 70°C.

2. The vials of alkali also were equilibrated to 70°C in the water bath for 5 min, unstoppered, so that the warmed alkali was exposed to air.

3. After the 5 min pre-heating period, the time necessary to achieve 70°C, the vials were stoppered and a 2 ml preheated sample of amine

hydrochlorides was injected through the cap into the vial.

4. The vials were held for 1 min in a beaker containing water at 70°C and then 1 ml of the head space gas was removed and injected into the gas chromatograph.

Gas chromatography. A column temperature of 93°C was selected, with injector and detector temperatures of 176°C and 198°C, respectively. The hydrogen flow rate was 15 ml/min and the nitrogen flow rate was 9 ml/min. The GLC column was the same as that described in Chapter I.

Chapter IV. Flavor of Milk with Added Amines

Objectives.

1. To study the effect of added TMA and MA, at concentrations found in feed flavored milk (Chapter II), on the flavor score of normal milk.

2. To determine approximate flavor threshold levels for TMA and MA in milk.

Procedure.

Effect of added amines on flavor scores. For the first part of the experiment, TMA and MA, at concentrations found in feed flavored milk in Chapter II, were added to four of the mixed herd milks analyzed in Chapter V and the effect on flavor score determined. The raw milk samples were refrigerated and were evaluated organoleptically within a day or two after collection.

The same panel used for flavor analysis in Chapters II and V scored the milk samples with the added amines. For the panel test, 2 series of samples were examined each time the panel met. Thus the panel had to score a total of 6 samples (2 controls, 2 with added TMA and 2 with added MA). The amine levels added were based on maximum amine concentrations recorded in the wheat pasture trial (Chapter II) and also on the presumption that the control milk in this experiment had 0.5 ppm TMA and 5 ppm MA.

Threshold studies on trimethylamine and methylamine. In the second part of the experiment, 5 samples of KSU raw mixed herd milk were obtained over a period of 3 weeks (from 3-13-73 to 4-5-73), and a series of triangular taste tests were conducted using a panel of 5 experienced milk flavor judges. The milk was analyzed by GLC as outlined in Chapter III and the triangular taste tests were conducted in the following manner:

1. When the panel met, 4 sets of triangular taste tests were organized using fresh milk obtained on the same day.

2. To identify the 4 triangular taste tests, 4 different colored tapes were used and 3 consecutive alphabet letters were assigned to distinguish among the 3 samples of each test. The same letters were used to identify the other 3 milk samples on the particular day. The colors and letters were changed for every experiment.

3. Out of a series of 4 sets, 2 sets were assigned to TMA and MA, respectively (except for one day when all 4 sets were assigned to TMA). For each amine, one set had 2 controls and 1 sample with the added amine whereas the other set had 1 control and 2 samples with added amines.

To determine the effectiveness, if any, of pasteurization in reducing off-flavors detected by the panel, the milk samples of 3-15-73 (control, control + TMA and control + MA) were pasteurized in the laboratory at 61.7°C for 30 min. After cooling, 4 sets of triangular taste tests were organized on 3-20-73 following the same procedure used for the raw milk samples.

Results and Discussion.

Effect of added amines on flavor scores. Some of the effects of added amines on flavor score are tabulated in Table 9. The results indicated that TMA affected the flavor score considerably, whereas MA did not. Frequently, the comments made by the panel for added TMA were fishy, indicating that TMA does cause a fishy off-flavor.

Table 9. Effect of TMA and MA on flavor scores

Sample	Flavor Scores					
	Control (C)		C + 3.5 ppm TMA		C + 35 ppm MA	
III	36.38	sl.[1] feed, medicinal	32.75	str.[2] fishy, str. feed	37.00	sl. feed, medicinal
IV	37.00	sl. feed	32.25	fishy, feed	37.00	feed
V	36.63	sl. feed	31.00	fishy	37.75	sl. cooked, weak oxidized, feed
VI	36.25	feed, cooked	31.75	sl. fishy, unclean	36.50	feed, sl. cooked

[1] slight

[2] strong

Threshold studies on trimethylamine and methylamine. The triangular taste test was set up to determine approximate flavor threshold levels at which TMA and MA could be detected in milk. The levels of amines tested were within the normal ranges expected to be found in off-flavored milk. These results are summarized in Table 10.

In Table 10, one can see that at a total level of 4 ppm, TMA was detected by all panel members. Laboratory pasteurization did not alter the detectability of TMA at this level (4 ppm). However, at the 1.5 ppm added level (i.e., a total conc. of ca 2 ppm), the amine was not consistently detected. Thus it seems probable that the threshold level for TMA is near 2 ppm.

For MA, panel members could not detect the amine even at 80 ppm. The maximum concentration detected in the feed flavored milk in Chapter II was 40 ppm. Thus this did not seem to be the cause of the so-called feed flavor. In this respect the threshold studies of Cole et al. (14) were

Table 10. Results of triangular taste test for determining significance of trimethylamine and methylamine in raw milk.

Date	Conc. in control[1] (ppm)	Conc. added (ppm)	Total amine conc. in milk (ppm)	Correct responses in triangle: 2 control 1 amine	Correct responses in triangle: 2 amines 1 control	Total correct
	TMA					
3-13-73	0.46	3.5	3.96	5	5	10***
3-15-73	0.42	3.5	3.92	5	5	10***
3-22-73	0.38	1.5	1.88	1	2	3
4-2-73[2]	0.50	1.5	2.00	4	4	8***
4-5-73	0.56	1.5	2.06	3	3	6**
4-5-73	0.56	0.75	1.31	1	1	2
3-20-73[3]	0.42	3.5	3.92	5	5	10***
	MA					
3-13-73	6.4	35	41.4	2	2	4
3-15-73	5.3	35	40.3	1	2	3
3-22-73	4.7	75	79.7	1	1	2
4-2-73[2]	7.7	75	82.7	1	0	1
3-20-73[3]	5.3	35	40.3	0	1	1

[1]Values obtained from GLC analysis of raw milk sample, see Tables 14 and 15.

[2]One panel member was sick thus his results were negative.

[3]Samples pasteurized in laboratory

*P < .05

**P < .01

***P < .001

puzzling. It was surprising that the experienced panel used could not detect MA even at 80 ppm, whereas the panel used by Cole et al. ascribed a metallic taste when MA was added at just 3.1 ppm (1 uM/10 ml).

The results of the GLC analyses of the control milk samples of the above are included in Tables 13 and 14 (Appendix B).

Chapter V. Analysis of Mixed Herd Milk for Trimethylamine and Methylamine

Objective.

To study and compare the flavor quality and amine concentrations in mixed herd milk produced by private dairy farms near Manhattan, Kansas.

Procedure.

Ten mixed herd milk samples were obtained from dairy farms between February 28 and March 29, 1973. The samples were refrigerated and within a day or two after collection the raw milk was evaluated organoleptically and distilled for further GLC studies.

For the organoleptic evaluation of the milk, a panel consisting of 4 experienced flavor judges was used. The same panel had been used in organoleptic studies in Chapter II. The milk was scored according to the National Collegiate Student Dairy Judging Contest procedures.

Duplicate distillations were made for each milk sample. After distillation, the samples were concentrated, reconstituted to 5 ml and refrigerated until all the samples were ready for analysis. For GLC analysis, the procedure outlined in Chapter III was used.

Results and Discussion.

Table 11 presents the rations that were fed milking herds I through X as well as the results of the flavor analyses of corresponding milk samples. Each milk sample was obtained from a different herd and thus milk produced from herds on a wide variety of feeds was analyzed. The last two columns in the table list the average flavor scores and some comments made by the panel members. Attempts were made to list the comments generally according to the degree of importance attached by the

Table 11. Rations fed to milking herds and flavor analyses of their milk.

Sample number	Ration	Flavor[1]	
		Score[2]	Comments
I	Unknown	--	--
II	Milo, corn, oats, bran, soybean meal, urea, molasses, alfalfa and sorghum silage	--	--
III	Alfalfa, prairie hay, Cargill 13% dairy ration	36.4	Slight feed, medicinal
IV	Alfalfa, milo 16% Master Mix dairy ration supplement	37.0	Slight feed
V	Alfalfa haylage hay, free choice of grain mix[4]	36.6[3]	--
VI	Day: hay 3rd cutting. Night: choice of corn silage and haylage (4th cutting)	36.3	Feed
VII	Alfalfa and sorghum silage	34.0	Oxidised, feed, soapy, chemical
VIII	Same as VII	35.5	Oxidised, feed
IX	Free choice corn silage, 10 lbs alfalfa hay 17 lbs grain mix[5]	38.3	No criticism, feed, sweet
X	Free choice corn silage, 5 lbs crimped milo, 10 lbs alfalfa hay, 20 lbs grain mix[6]	36.7	Feed

[1]Milks were judged on same day collected except samples VII which were examined after 3 days and VIII after four days.
[2]Average of four flavor scores
[3]Two panel members were substituted by others on this day.
[4]Cracked milo, cracked corn, soy-meal, urea, calcium, salt, ruminant concentrate, black strap molasses, monosodium phosphate salt, trace mineral phosphate
[5]Corn, milo, bone meal, Starea, soybean oil meal, vitamins A, D and E, salt, mineral mixture
[6]Corn, milo, soybean oil meal, Starea, molasses, mineral mixture, vitamins A, D and E.

panel members and to give consideration to the number of panel members making the same comment.

The possible reason for the low flavor scores for samples VII and VIII could be that these two samples were stored for 3 and 4 days, respectively, prior to being tasted. Thus the milk could have been oxidized resulting in poor flavor.

Table 12 shows the peak heights and concentrations of MA and TMA in the distillates, calculated from the standard curves (Figs. 11 and 12) and the concentration of TMA and MA in milks. The standard curves (Figs. 11 and 12) are for the amine samples (i.e., the 5 ml distillate sample) actually injected into the gas chromatograph. Since this 5 ml of distillate contains amines from 50 ml of milk, the amines in the milk are 0.1 of those in the distillate.

The results in Table 12 indicate that the normal levels of TMA and MA in these milks were well below their flavor thresholds (see Chapter IV) and hence would not effect a fishy flavor in milk. Thus the milk produced in March around Manhattan seemed generally to be of good flavor quality, with respect to amines. It would have been interesting to check amine concentrations over a 12 month period to observe the seasonal variation in milk flavor.

There were 11 other peaks observed on the gas chromatograms and the retention times and peak heights for these were recorded in Table 15 (Appendix B). These do not appear to be significant so far as flavor is concerned (see results of Chapter III) and thus there was no attempt to analyze these data further.

Table 12. Trimethylamine and methylamine peak heights and concentration from GLC analyses of mixed herd milk samples.

Sample #	Distillation	Trimethylamine[1] Peak height[2]	Trimethylamine[1] Conc. in distillate (ppm)	Trimethylamine[1] Conc. in milk	Methylamine[1] Peak height[2]	Methylamine[1] Conc. in distillate (ppm)	Methylamine[1] Conc. in milk
I	1	15,440	2.6		5,200	52.5	
	2	15,200	2.55		6,880	61	
	avg	15,320	2.58	0.26	6,040	56.8	5.68
II	1	18,080	2.9		4,140	47	
	2	20,240	3.2		4,780	50	
	avg	19,160	3.05	0.31	4,460	48.5	4.85
III	1	110,400	13.6		23,040	131	
	2	96,000	12.0		31,520	161.5	
	avg	103,200	12.8	1.28	27,280	146.3	14.63
IV	1	44,800	6.1		13,400	96.5	
	2	22,560	3.4		17,040	109.5	
	avg	33,680	4.8	0.48	15,220	103	10.30
V	1	24,000	3.6		3,560	49	
	2	18,800	3.05		4,240	53.5	
	avg	21,440	3.25	0.33	3,900	51.5	5.15
VI	1	49,600	6.65		5,840	63	
	2	47,040	6.35		4,400	54.5	
	avg	48,320	6.6	0.66	5,120	58.5	5.85
VII	1	21,600	3.3		3,620	50	
	2	11,760	2.1		3,700	50.5	
	avg	16,680	2.7	0.27	3,660	50.3	5.03
VIII	1	13,280	2.3		3,920	51.5	
	2	18,880	2.95		4,220	53.5	
	avg	16,080	2.65	0.27	4,070	52.5	5.25
IX	1	52,640	7		8,120	74.5	
	2	46,240	6.25		9,160	80	
	avg	49,440	6.6	0.66	8,540	77.3	7.73
X	1	40,640	5.6		5,680	62	
	2	29,600	4.3		5,360	60	
	avg	35,120	5		5,520	61	6.10

[1]Retention times; TMA, 1.7 min; MA, 2.2 min.

[2]Peak height values represent average of duplicate GLC analyses for each distillation.

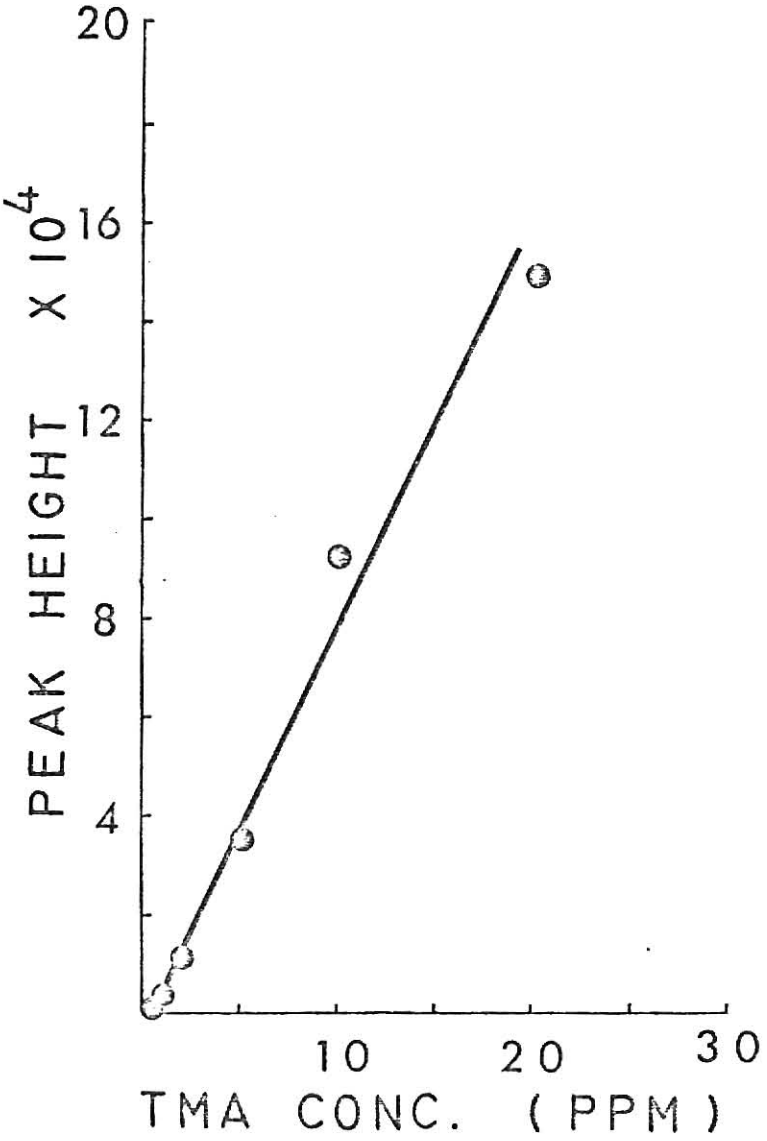

Fig. 11. Comparison of GLC peak heights with concentrations of trimethylamine in the modified GLC procedure.

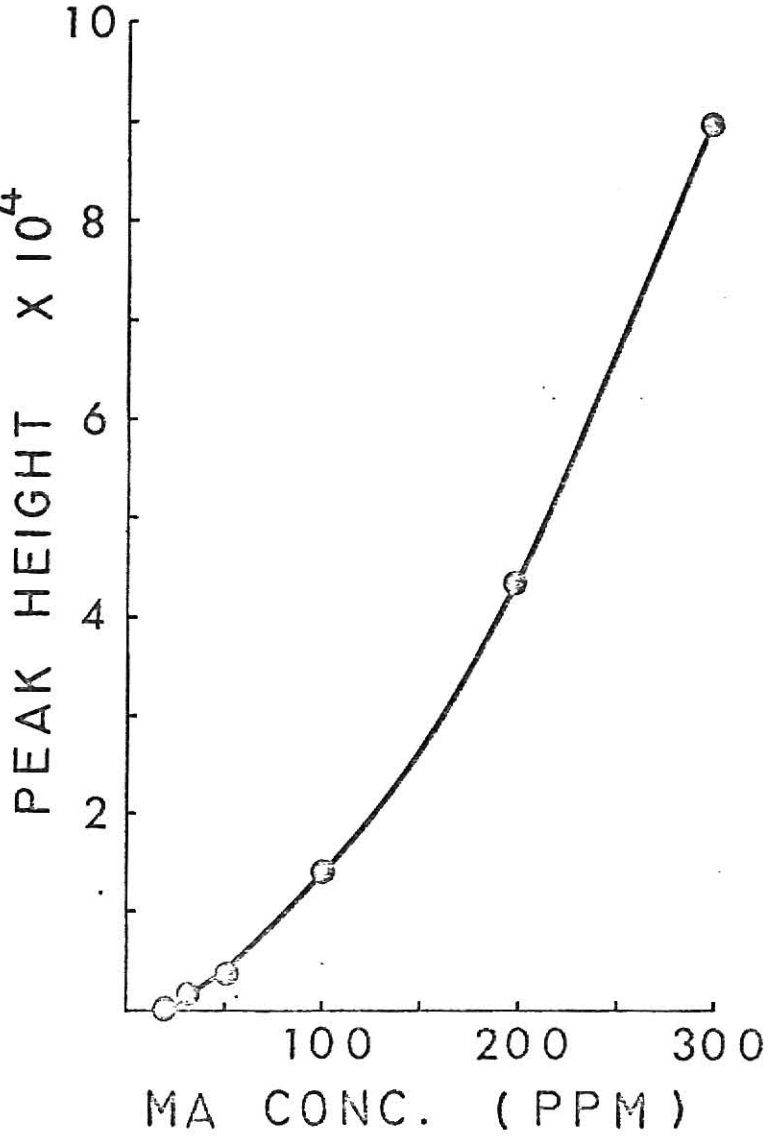

Fig. 12. Comparison of GLC peak heights with concentrations of methylamine in the modified GLC procedure.

CONCLUSIONS

Based on the data presented in this thesis, the following conclusions may be drawn:

1. A satisfactory analytical procedure was developed for quantitatively measuring the lower molecular weight aliphatic amines in milk.

2. Feeding cows on wheat pasture produced off-flavored milk with a simultaneous increase in the concentrations of TMA, MA and an unidentified basic compound. Another unidentified basic compound decreased in concentration with deterioration in flavor quality of the milk.

3. The flavor threshold for TMA was approximately 2 ppm; MA could not be detected up to 80 ppm.

4. Amounts of TMA and MA found in commercial milk produced during March around Manhattan, Kansas were found to be below flavor threshold levels.

5. TMA was found to be a cause of fishy flavor characterized in milk from cows fed wheat pasture. This result was confirmed when "fishy" flavor was introduced by the addition of TMA to normal flavored milk.

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APPENDIX A

APPENDIX A

Treatment 1

1. a. Forty-five milliliters of pasteurized, homogenized whole milk (at 9°C) was transferred into a micro-Kjeldahl distillation flask with 5 ml of a 100 ppm solution of the amine salts.

b. Two milliliters of 1N HCl was pipetted into the cold milk-amines salts mixture.

2. The reaction products so obtained were not filtered.

3. a. The steam distillation apparatus (micro-Kjeldahl) was connected and the steam generator flask heated until the contents of the flask boiled and steam was passed through the sample for 2 min.

b. At this stage, 5 ml of 1N NaOH was introduced into the flask through a thistle funnel.

c. The distillate was directed into a trap of 2 ml 1N HCl.

d. The condenser water was kept flowing.

e. Distillate was collected for 30 min after the addition of NaOH. A distillate of approximately 35 ml was obtained.

4. The distillate was evaporated to dryness in a water bath and reconstituted to 5 ml by addition of distilled water. The solution was analysed by the modified GLC procedure of Toan et al. outlined in the procedure of Chapter I.

Treatment 2

The procedure for treatment 2 was identical to treatment 1 with the following exceptions:

1. b. The amine-milk sample was tempered to 30°C in the distillation flask before acid was added.

3. e. Volume of distillate collected was 40 ml.

Treatment 3

The procedure for treatment 3 was identical to treatment 1 with the following exceptions:

1. a. Forty-five milliliters of milk was poured into a beaker with 5 ml of distilled water.

b. The diluted milk sample was tempered to 30°C in the beaker before acid was added.

2. The reaction products so obtained were filtered through Whatman #42 filter paper, thus separating the curd and the acid whey. The curd was discarded.

3. a. The acid whey (29 ml) was transferred to the distillation flask and distilled by the usual procedure.

e. Volume of distillate collected was 34 ml.

Treatment 4

The procedure for treatment 4 was identical to treatment 3 with the following exceptions:

1. a. Five milliliters of the 100 ppm amine salts solution was added to the milk instead of distilled water.

3. a. Thirty-four milliliters of acid whey was transferred to the distillation flask.

e. Volume of distillate collected was 27.5 ml.

Treatment 5

The procedure for treatment 5 was identical to treatment 3 with the following exceptions:

1. a. Five milliliters of the 100 ppm amine salts solution was added to the milk instead of distilled water.

3. a. The acid whey (34.5 ml) was transferred to the distillation flask. The generator flask was not heated at this stage and the steam was not passed through the sample for 2 min.

b. After 5 ml of 1N NaOH was introduced into the flask through a thistle funnel, the steam generator was heated until the contents of the flask boiled and steam was passed through the sample for 30 min. After this the usual procedure was followed.

e. Volume of distillate collected was 35 ml.

APPENDIX B

Table 13. Trimethylamine and methylamine peak heights and concentration of raw mixed herd milk used for triangular taste tests.

Date of sample	Distillation	Trimethylamine			Methylamine		
		Peak[1] height	Conc. in distillate (ppm)	Conc. in milk (ppm)	Peak[1] height	Conc. in distillate (ppm)	Conc. in milk (ppm)
3-13-73	1	30,720	4.45		5,360	60	
	2	32,800	4.70		6,720	67.5	
	avg	31,760	4.55	0.46	6,040	64.0	6.4
3-15-73	1	28,000	4.1		5,100	58.5	
	2	29,760	4.3		3,140	46.5	
	avg	28,880	4.2	0.42	4,120	53	5.3
3-22-73	1	31,040	4.5		2,760	43.5	
	2	20,000	3.1		3,700	50.5	
	avg	25,520	3.8	0.38	3,230	47	4.7
4-2-73	1	27,520	4.05		7,880	73.5	
	2	41,920	5.75		9,480	81	
	avg	34,720	4.95	0.50	8,680	77	7.7
4-5-73	1	40,960	5.65		6,540	66.5	
	2	39,520	5.5		7,200	70	
	avg	40,240	5.6	0.56	6,870	68.5	6.85

[1]Peak height values represent average of duplicate GLC analyses for each distillation.

Table 14. Peak heights[1] for GLC peaks other than trimethylamine and methylamine from raw mixed herd milk used for the triangular taste test.

Date of sample	Distillation	Retention time (min) 2.8	4.5	5.4	7.0	7.8	9.8	10.3	24.5
		(peak heights)[1]							
3-13-73	1	3,620	110	35	265	108	60	-	38
	2	4,920	135	33	250	132	55	-	65
	avg	4,270	123	34	258	120	58	-	102
3-15-73	1	3,940	123	35	283	120	43	10	78
	2	2,480	85	20	250	100	10	-	42
	avg	3,210	104	28	267	110	27	5	60
3-22-73	1	2,220	25	25	203	150	20	-	43
	2	3,080	28	15	170	88	20	15	90
	avg	2,650	27	20	187	119	20	8	67
4-2-73	1	3,280	18	33	193	175	38	-	75
	2	4,160	60	30	240	250	37	-	123
	avg	3,720	39	32	217	213	38	-	99
4-5-73	1	2,060	45	33	145	235	35	-	70
	2	2,860	30	15	180	228	20	135	40
	avg	2,460	38	24	163	232	28	68	55

[1]Peak height values represent averages of duplicate GLC analyses for each distillation.

Table 15. Peak heights[1] of GLC peaks other than trimethylamine and methylamine from analysis of mixed herd milk.

Sample	Distillation	Retention time (min)								
		2.8	4.5	5.1	6.1	7.0	7.8	9.8	10.3	24.5
		(peak heights[1])								
I	1	3,960	35	-	40	250	190	70	-	235
	2	5,360	20	-	35	270	220	75	-	430
	avg	4,660	28	-	38	260	205	73	-	333
II	1	3,960	20	-	40	335	-	125	-	160
	2	4,480	80	-	45	325	-	90	-	255
	avg	4,220	50	-	43	330	-	107	-	208
III	1	8,400	70	10	5	610	-	50	10	75
	2	10,080	10	10	10	610	-	145	15	200
	avg	9,240	40	10	8	610	-	98	13	138
IV	1	6,520	110	20	15	375	-	30	20	110
	2	6,000	95	20	10	345	-	35	-	115
	avg	6,260	103	20	13	360	-	33	10	113
V	1	2,360	115	20	-	215	150	35	10	45
	2	2,900	165	-	-	255	150	30	15	75
	avg	2,630	140	10	-	235	150	33	13	60
VI	1	4,020	80	20	10	250	160	65	70	275
	2	3,080	125	70	-	250	95	110	-	170
	avg	3,550	103	45	5	250	128	88	35	223
VII	1	2,260	110	20	10	290	75	25	-	95
	2	2,480	70	10	-	270	75	20	-	140
	avg	2,370	90	15	5	280	75	23	-	118
VIII	1	4,240	75	10	-	335	300	15	20	55
	2	4,720	80	15	-	360	335	10	10	75
	avg	4,480	78	13	-	348	318	13	15	65
IX	1	4,120	40	20	-	320	55	30	-	70
	2	5,200	30	20	-	355	45	-	105	50
	avg	4,660	35	20	-	338	50	15	53	60
X	1	5,200	115	40	10	345	75	65	-	90
	2	5,120	105	25	5	350	60	30	15	65
	avg	5,160	110	33	8	348	68	48	8	78

[1]Peak height values represent averages of duplicate GLC analyses for each distillation.

GAS CHROMATOGRAPHIC ANALYSIS OF SOME LOWER MOLECULAR WEIGHT AMINES IN MILK AND THE RELATIONSHIP OF THESE AMINES TO FEED FLAVORS

by

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This study was designed to develop a suitable procedure for the isolation, concentration and gas-liquid chromatographic (GLC) analysis of some of the lower aliphatic amines of milk. The effect of winter wheat pasture on the flavor and amounts of some individual amines of the milk was determined. Subsequently an amine causing a fishy off-flavor was identified as trimethylamine (TMA) and its approximate threshold level in normal milk determined. Finally, the levels of TMA and methylamine (MA) present in milks from commercial milking herds were determined and related to milk flavor quality.

The initial analytical procedure developed was as follows. Fifty milliliters of milk at 9°C was acidified with 2 ml 1N HCl and the amines subsequently liberated from their hydrochloride salts by addition of 5 ml 1N NaOH in a closed micro-Kjeldahl distillation system. The amines were distilled into a trap of 2 ml of 1N HCl, the distillate reduced to dryness and then reconstituted to 5 ml with distilled water. The sample was then prepared for chromatography, and the amines separated and quantitatively analysed by GLC on a stainless steel column (3.05 m x 3.18 mm) packed with 25% Triton X 100 and 5.6% KOH on HMDS Chromosorb W (80/100 mesh). The GLC procedure was developed to analyze milk for the C_1 thru C_6 amines. For the GLC procedure a column temperature of 143°C was used. The sample for chromatography was prepared as follows: 2 ml of recently boiled saturated KOH solution at room temperature was added to a 5 ml serum vial. After the vial was capped, 2 ml of the sample to be analysed was injected through the serum cap into the vial. After the mixture had been held at room temperature for 1 min, 1 ml of the

head-space gas was withdrawn and injected into the chromatograph.

This procedure was employed to measure amines in milk from two cows that were on winter wheat pasture for eight days. A relationship was found between TMA, MA and the milk flavor scores. During the 8-day period when cows were on wheat pasture, there was a decrease in milk flavor scores and an increase in the concentrations of the two amines. There was also an increase in concentration of an unidentified compound (5.2 min retention time) and a decrease in another component (3.5 min retention time). The significance of these two changes was not determined.

As a result of these observations, it was decided to modify the initial GLC procedure to determine principally TMA and MA. The modifications included reducing the column temperature and increasing the carrier gas flow rate. A modification in the sample preparation method involved warming the alkali and sample separately for 5 min in a 70°C bath before mixing. After the sample was injected into the alkali vial, the mixture was held at 70°C for 1 min prior to drawing the headspace gas sample for the GLC analysis.

Using the modified procedure, 10 commercial milk samples were analysed. Flavor scores ranged between 34 and 38.33, maximum possible score being 40. TMA and MA concentrations ranged from 0.26 to 1.28 and 4.85 to 14.63 ppm, respectively. These concentrations were well below the approximate flavor threshold levels in milk, as determined by triangular taste tests. The flavor threshold for TMA was found to be approximately 2 ppm, whereas MA could not be detected at 80 ppm. In another experiment, the effect of adding TMA and MA to normal milk at levels found in milk produced on wheat pasture was determined. A 4.0 ppm

TMA concentration level resulted in a decrease in flavor score from 36.55 (in normal milk) to 31.95, while a 40 ppm MA level resulted in a flavor score of 37.05. The addition of TMA elicited remarks such as fishy and unclean. Thus it is concluded that TMA is a cause of the fishy flavor characterized in milk.