THE EFFECT OF HIGH-TEMPERATURE SHORT-TIME STERILIZATION ON THE FREE AMINO ACID CONTENT OF MILK

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John G. Parsons, B.Sc.

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A Major Thesis submitted to the

Faculty of Graduate Studies and Research

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in candidacy for the degree of

Master of Science

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ABSTRACT

Twenty samples of raw milk were sterilized at 298-300° F for 4 seconds by direct steam injection using a modified vacreator. Samples of milk were taken before preheating to 175° F and after sterilization. The free amino acids were extracted by dialysis and purified on a cation ion-exchange column. The individual amino acids were separated by one dimensional paper chromatography. The individual amino acids were identified by comparison with known amino acids chromatographed on individual paper strips.

The amino acids on the paper strips were cut out and eluted with distilled water. The optical density of the color was measured with a spectrophotometer at a wavelength of 570 mμ. These units when transformed to mg/100 ml gave a quantitative estimation of the concentration of each amino acid found in each sample.

On the basis of the statistical analysis using a paired "t" test, the increases in concentration between raw and sterilized milk of glutamic and α aminobutyric acids

were found to be significant at the 1% and 5% levels. The increases in the other amino acids were not found to be significant at either level although, in general, there was a higher concentration of amino acids in sterilized than in raw milk.

Consequently, the effect on the free amino acid content of heating milk to 298-300° F for 4 seconds is not significant and, as far as free amino acids are concerned, the biological value of the milk is not greatly changed.

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The Effect of High-Temperature Short-Time Sterilization on the Free Amino Acid Content of Milk

INTRODUCTION

There has been a general trend, over the past ten years, toward high temperatures and shorter heating times for the processing of milk and its products. Pasteurization involves such time-temperature relationships as 143° F for 30 minutes and 161° F for 16 seconds. Sterilization, in which all microorganisms and their spores are destroyed, is accomplished by in-container heating at 241° F for 15 minutes or heating of the product to 300° F for 5 seconds, followed by aseptic packaging.

There are at least two reasons for this trend: firstly, short time operations lend themselves to continuous processing, while at the same time meeting the requirements for the destruction of microorganisms and enzymes; secondly, high temperatures for shorter times minimize the detrimental effects of heat on the flavor and other properties of milk.

As a result of extreme heat treatments, the natural characteristics of milk are changed, e.g. there is increased viscosity, reduced curd tension, increased resistance to oxidation, changed flavor and color and altered protein stability. These changes are usually the result of heat effects, direct or indirect, on the serum proteins of milk.

According to Jenness and Patton (6), pasteurization does not affect the nitrogen distribution in milk. However, increased temperatures produce progressive changes in this distribution. The amounts of proteose-peptone and non-protein nitrogen (free amino acids and ammonia) increase. These increases are the result of fragmentation of the milk proteins. At the same time amino acids are heat labile compounds and temperatures greater than 95° F tend to inactivate and destroy them. Therefore, the action of heat on milk proteins may result in an increase in the content of free amino acids, through fragmentation of serum proteins, or a decrease of existing free amino acids, due to the direct effect of the heat, or there may be a balance between these two actions.

It is possible that a change in the free amino acid content may be responsible for problems in the production of sterile milk. The problem of flavor is of greatest importance in this case. Also a significant change in the content of the essential amino acids may bring about a change in nutritional value.

The purpose of this investigation was to determine the effect on the free amino acid content of heating milk to 298-300° F for 4 seconds.

REVIEW OF LITERATURE

There are three main methods for the determination of amino acids - paper chromatography, ion exchange chromatography and microbiological assay. Block (1) has compared these three methods for determining the amino acids in β-lacto-globulin and found that the results are substantially the same.

Paper chromatography was chosen for this problem because of its simplicity and economy. It is one of the few methods which permit the separation of a large number of substances (up to 20), from each other simultaneously.

Paper chromatography has been used for the separation of amino acids from many biological fluids and food products (10). However, very little work has been done on the determination of the free amino acids in milk.

Lindqvist et al (10) have used buffered paper and buffered solvents with one-dimensional chromatography for the determination of amino acids in cheese. They state that this method uses only 1/16 of the paper required for two-dimension testing. By using buffered paper, they found

that desalting of the sample was not necessary. They adjusted the pH to 6.2 to prevent shifts of the spots of the individual acids, with consequent changes of shifts in the R_f values. The amino acids were extracted by alcohol precipitation. By using a series of standard mixtures of known amino acids (and 5 different pH's) they were able to determine which amino acids were present. They found this method lent itself to a quantitative determination of the amino acids more readily than two-dimensional chromatography.

Kosikowsky (8) studied the free amino acids in American cheddar cheese by two-dimensional descending paper chromatography. With this method he was able to isolate about 20 of the more common amino acids in cheese.

Storgards and Lindqvist (13) investigated the changes in the composition of free amino acids in milk kept in cold storage. To purify the amino acids they used dialysis instead of alcohol precipitation. At equilibrium (after 24 hours) the dialysate was evaporated to approximately 5 ml and analysed by the Spackman, Stein and Moore method in an automatic amino acid analyzer.

Van Der Zant and Nelson (14) reported the presence of alanine, glutamic acid, glycine, leucines and valine in fresh skimmilk heated for 20 mins. at 85° C. Block (2) reported the presence of free amino acids in protein free extract of milk with glutamic acid being present in the largest quantity of any of the free amino acids. Deutsch and Samuelsson (3) confirmed the presence of free amino acids in milk produced under practically sterile conditions.

Hetzel (5) states that both fresh and pasteurized skimmilk contain distinct amounts of the common amino acids, with a preponderance of glutamic acid. He reported some unidentified material, probably peptide, was also present. Sterilized milk was found to contain more free amino acids, especially proline, than raw milk. Kon (7) suggested that some of the more drastic heat treatments of milk, such as in-bottle sterilization and evaporation reduced the protein value of milk, particularly through their effect on lysine.

METHOD

The free amino acids were determined according to the method of Hetzel (5), except that dialysis was used to obtain the crude amino acid extract instead of acid and alcohol precipitation, and the project was designed to give quantitative rather than qualitative results.

Twenty samples of raw milk were used in the project. Sixteen samples were obtained from individual can milk shippers in the Winnipeg milkshed and four samples from the University of Manitoba Dairy. The samples were taken one day and stored at 40° F until the following day.

The individual milk samples (80 lbs/8 gal. can) were preheated to a temperature of 175° F by immersing the cans in hot water. The samples were held about 15 minutes at this temperature and then were sterilized by direct steam injection using a modified vacreator.

The milk was pumped under pressure by an homogenizer into the bottom of a stainless steel tube 2 feet long and 3 inches in diameter. High pressure steam (75 lbs/sq. in.) entered the tube at right angles, directly into the milk and

heated it to 300° F. A small orifice (1/4" dia.) at the top of the tube created the high pressure required to reach this temperature. The length of the tube was such that a holding time of 4 seconds was maintained. The milk was discharged from the holding tube into the vacreator where it was flash cooled under vacuum to 90° F.

Five hundred ml aliquots were drawn off before preheating and after sterilization. A total of twenty pairs of samples, one raw and one sterilized, were obtained. Toluene was added at the rate of 1% to each sample as a preservative. The samples were then cooled to 40° F and a 100 ml aliquot was poured into a dialysis cylinder. A dialysis tube containing 40 ml of distilled water was placed in this cylinder. The cylinder was then stored in a refrigerator at 35.6° F for 16 hours. A narrow cylinder was used as the dialysis vessel, so that the dialysis tube would be surrounded by a relatively thin layer of milk, thus eliminating the need of shaking for the system to reach equilibrium. The volume of the dialysate was then measured and the pH adjusted to 6.2 with 0.5 N HCl.

The amino acid extracts were purified on an ion

exchange column (12 cm x 0.9 cm). The cation exchange resin, Dowex 50WX4 (200-400 mesh) was used. The dialysate, pH 6.2, was passed through the column at the rate of 6-8 drops per minute. The column was then washed with 200 ml of distilled water or until an aliquot of the washings showed a negative Molisch test.

The adsorbed amino acids were eluted from the ion exchange resin with 50 ml of 4% NH_4OH, followed by 100 ml of distilled water. The column was regenerated by the addition of 50 ml of 2 N HCl, followed by water until the washings were neutral.

The purified amino acid extracts were evaporated to dryness in vacuum at a temperature not over 95° F. The amino acids were taken up in 0.9 ml distilled water and 0.1 ml isopropyl alcohol. Isopropyl alcohol was added as a preservative and as a solvent. Most amino acids are more soluble in a 10% isopropyl alcohol solution than in distilled water.

The one dimensional method of paper chromatography was used with buffered filter paper and buffered solvents as suggested by Lindqvist et al (10).

Whatman No. 1 chromatography paper was used throughout this investigation. The dimensions of the paper strips were 22" x 1.5". A spot was marked 6.5 cm from one end of the strip to denote the position of the amino acid mixture. The strip was folded at a distance 4.7 cm from the same end so that the required end would fit in the trough of the chromatographic apparatus and the rest of the strip would hang over the support rod, as in descending paper chromatography.

A phosphate buffer was used to buffer the paper strips and to make up the solvents. The buffer consisted of 8 ml of 0.067 M KH_2PO_4 and 2 ml of 0.067 M Na_2HPO_4 which gave a pH of 6.2. The paper strips were dipped in this phosphate buffer and dried in a fume chamber. These could be prepared ahead of time and stored in a clean, dark place.

Several solvents were used in the separation and identification of the individual amino acids on the strips. Water saturated phenol failed to give discrete spots and few amino acids were completely separated. The solvent suggested by Hardy *et al* (4), ethanol-butanol-H_2O-dicyclohexylamine (10:10:5:2), separated several amino acids

because of the fact that they showed up as differently colored spots after development with ninhydrin. Lysine and arginine were identified in this way. The 4-1-5 solvent of Partridge (Butanol-Acetic Acid-H_2O) gave varying results and also very low R_f values.

For the quantitative part of the project a butanol-acetic acid solvent (Butanol-Acetic Acid-water (48-12-20)) (12) was used, modified by the use of phosphate buffer instead of distilled water. This solvent mixture formed a monophasic solvent stable down to 32° F, and also gave the most discrete spots for quantitative work.

Standard solutions of known amino acids were made up as suggested by Lindqvist et al (10). A total of 19 amino acids was used to make up eleven standards. For the qualitative determination of the amino acids in the samples, a 5 aliquot of each standard was placed on a separate strip of filter paper and these were chromatographed together with those containing the amino acid extracts.

Twenty of the amino acid extracts were spotted onto paper strips in duplicate. These strips were placed in the chromatographic tank with the standards, a short time

before the solvent was added, to allow for equilibration of the system. With a 15-hour (overnight) run, the solvent travelled nearly the length of the strip, 18-19 inches.

The strips were next dried for 1/2 hour in a fume chamber. After drying, they were developed by a dipping procedure in an acetone solution containing 0.4% ninhydrin and 2% acetic acid. The strips were dried again in a fume chamber for 10 minutes and finally placed in a hot air oven at 158° F for 10 minutes, to develop the colored spots. R.F. values were calculated and the amino acids identified by comparison with corresponding results obtained for each standard strip.

The individual spots were cut out and the color was eluted with 6 ml of distilled water in three successive washings, 2 ml at a time. The optical density of the color was measured with a Beckman DU Spectrophotometer at a wavelength of 570 mμ (Naftalin, 11).

To prepare standard curves for each amino acid, 20λ of the standards were spotted onto paper strips and chromatographed. The individual spots corresponding to the various amino acids were cut out and eluted with distilled

water as were the samples. The 6 ml quantity was measured in the spectrophotometer and then the sample was diluted to 10, 15, 20 ..... 50 ml and the corresponding optical density recorded. These values were spotted against the known concentration of the amino acids contained in the standard in mg/100 ml.

From the optical density measurements the individual amino acid contents of the milk samples were determined as follows: Dialysis of 100 ml of milk with 40 ml of distilled water gave a quantity of dialysate (which was measured) corresponding to a number of ml of normal milk. For example: 30 ml of dialysate correspond to $30 \times \frac{100}{140}$ or 21.49 ml normal milk. We used a 20λ sample equivalent to $\frac{1000}{20} \times 21.49$ or 0.43 ml normal milk. This sample was taken into 6 ml distilled H_2O for measurement and became equivalent to $\frac{0.43}{6} \times 100$ or 7.17 ml normal milk, since the standards were in 100 ml. Thus the O.D. reading corresponds to that for a sample of 7.17 ml normal milk. From the standard curves, the O.D. reading is converted to mg/100 ml. By applying a correction factor of, in this case, 13.947, we can quote the true amino acid content of normal milk as

mg/100 ml.

The original data on the free amino acid content of raw and sterilized milk is given in Appendix I.

RESULTS

The amounts of the various free amino acids found in the raw and sterilized milk are presented in Tables 1.1 to 1.9. There is a considerable variation in the individual amino acid content between samples. We were not concerned in this investigation with the amino acid content of the original milk, but rather with the changes which occurred due to sterilization. The variation between samples may possibly be influenced by the amino acids liberated through the growth of microorganisms. The amount of amino acid present would then depend to some extent on the number of microorganisms present in the raw milk, i.e. the quality of the raw milk. The change in the amino acid content due to sterilization is in most cases slight. The increase in concentration of the two amino acids (glutamic and α aminobutyric) between raw and sterilized milk is statistically significant.

The measurement of several amino acids present in trace amounts in some samples was difficult. These could not be measured with the spectrophotometer, so they were

Table 1.1

Lysine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	0.13	--	-0.13
2	+	0.04	+0.04
3	0.33	+	-0.33
4	+	+	--
5	+	+	--
6	+	+	--
7	0.12	0.04	-0.08
8	+	+	--
9	0.05	0.03	-0.02
10	0.06	0.06	--
11	+	+	--
12	--	--	--
13	+	+	--
14	+	0.08	+0.08
15	+	--	--
16	0.04	0.05	+0.01
17	+	0.06	+0.06
18	+	0.04	+0.04
19	--	+	--
$\sum(x)$	0.73	0.40	-0.33
			$\sum(d^2) =$ 0.146
$\bar{x}$	0.04	0.02	-0.017

$$t = \frac{-0.017}{0.0202} = -0.84$$

Table 1.2

Arginine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	0.45	0.31	-0.14
2	0.06	0.39	+0.33
3	0.55	0.61	+0.06
4	0.61	0.54	-0.07
5	0.62	0.37	-0.25
6	0.04	0.41	+0.37
7	0.37	0.55	+0.18
8	0.28	0.46	+0.18
9	0.66	0.62	-0.04
10	0.36	0.20	-0.16
11	0.47	0.39	-0.08
12	0.29	0.35	+0.06
13	0.75	0.77	+0.02
14	0.66	0.81	+0.15
15	0.12	0.76	+0.64
16	0.89	1.02	+0.13
17	0.65	0.77	+0.12
18	1.18	0.73	-0.45
19	0.58	0.76	+0.18
$\sum(x)$	9.59	10.82	+1.23
			$\sum(d^2) =$ 1.137
$\bar{x}$	0.50	0.57	0.065

$$t = \frac{0.065}{0.0556} = 1.17$$

Table 1.3

Serine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	0.24	0.11	-0.13
2	+	0.10	+0.10
3	0.15	0.15	--
4	0.12	0.19	+0.07
5	0.26	0.14	-0.12
6	+	+	--
7	0.12	0.28	+0.16
8	0.01	0.14	+0.13
9	0.20	0.13	-0.07
10	0.10	0.09	-0.01
11	0.15	0.09	-0.06
12	0.08	0.11	+0.03
13	0.18	0.14	-0.04
14	0.13	0.26	+0.13
15	+	0.05	+0.05
16	0.21	0.21	--
17	0.09	0.12	+0.04
18	0.20	0.15	-0.05
19	0.09	0.20	+0.11
$\sum(x)$	2.33	2.67	+0.34
			$\sum(d^2) =$ 0.135
$\bar{x}$	0.12	0.14	+0.018

$$t = \frac{0.018}{0.0194} = 0.93$$

Table 1.4

Threonine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	1.57	1.08	-0.49
2	0.52	1.59	+1.07
3	2.62	2.17	-0.45
4	1.49	2.02	+0.53
5	1.64	2.64	+1.00
6	4.07	2.93	-1.14
7	1.63	2.10	+0.47
8	1.38	2.38	+1.00
9	2.47	2.68	+0.21
10	2.72	2.68	-0.04
11	1.42	1.96	+0.54
12	0.69	1.08	+0.39
13	1.29	1.47	+0.18
14	1.26	1.96	+0.70
15	2.96	3.39	+0.43
16	1.84	1.90	+0.06
17	2.17	2.56	+0.39
18	3.12	2.30	-0.82
19	0.80	1.12	+0.32
$\sum(x)$	35.66	40.01	+4.35
			$\sum(d^2) =$ 7.516
$\bar{x}$	1.88	2.11	0.229

$$t = \frac{0.229}{0.1381} = 1.66$$

Table 1.5

Glutamic Acid Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	6.90	6.23	-0.67
2	3.08	4.34	+1.26
3	5.42	5.98	+0.56
4	8.67	9.45	+0.78
5	7.48	6.31	-1.17
6	9.21	8.33	-0.88
7	7.38	8.61	+1.23
8	6.36	8.19	+1.83
9	8.60	10.64	+2.04
10	8.40	8.90	+0.50
11	7.66	8.89	+1.23
12	7.12	9.21	+2.09
13	6.50	7.75	+1.25
14	7.91	9.55	+1.64
15	8.27	7.99	-0.28
16	4.59	6.76	+2.17
17	10.09	12.82	+2.73
18	9.58	8.67	-0.91
19	6.44	8.07	+1.63
$\sum(x)$	139.66	156.69	+17.03
			$\sum(d^2) =$ 40.234
$\bar{x}$	7.35	8.25	0.896

$$t = \frac{0.896}{0.2702} = 3.32^*$$

Table 1.6

Alanine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	1.05	0.64	-0.41
2	0.50	1.14	+0.64
3	1.18	1.35	+0.17
4	0.99	1.47	+0.48
5	0.98	1.06	+0.08
6	1.08	0.95	-0.13
7	0.88	1.54	+0.66
8	0.88	1.12	+0.24
9	1.12	1.37	+0.25
10	1.16	1.16	--
11	1.22	1.32	+0.10
12	0.95	1.15	+0.20
13	1.08	1.02	-0.06
14	1.04	1.19	+0.15
15	1.21	1.02	-0.19
16	0.80	1.15	+0.35
17	1.12	1.40	+0.28
18	1.64	1.22	-0.42
19	0.85	1.02	+0.17
$\sum(x)$	19.73	22.29	$\sum(d^2)=$ +2.56 1.934
$\bar{x}$	1.04	1.17	0.135

$t = \frac{0.135}{0.0681} = 1.98$

Table 1.7

α Aminobutyric Acid Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	--	0.24	+0.24
2	--	0.40	+0.40
3	--	0.18	+0.18
4	--	0.60	+0.60
5	0.63	0.80	+0.17
6	0.45	0.80	+0.35
7	0.30	0.18	-0.12
8	+	0.38	+0.38
9	0.81	0.67	-0.14
10	0.72	0.25	-0.47
11	+	0.37	+0.37
12	+	0.10	+0.10
13	0.20	0.33	+0.13
14	0.35	0.57	+0.22
15	0.31	0.68	+0.37
16	0.35	0.63	+0.28
17	0.49	0.60	+0.11
18	0.73	0.91	+0.18
19	0.56	0.77	+0.21
$\sum(x)$	5.90	9.46	+3.56
			$\sum(d^2) = 1.677$
$\bar{x}$	0.31	0.50	0.187

$$t = \frac{0.187}{0.0543} = 3.44^*$$

Table 1.8

Valine Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	0.72	0.61	-0.11
2	+	+	--
3	0.70	0.65	-0.05
4	+	0.90	+0.90
5	0.92	0.54	-0.38
6	0.73	0.38	-0.35
7	0.41	0.41	--
8	0.55	0.49	-0.06
9	1.15	0.85	-0.30
10	0.70	0.54	-0.16
11	0.76	0.34	-0.42
12	0.42	0.54	+0.12
13	0.57	0.46	-0.11
14	0.63	0.73	+0.10
15	0.53	0.54	+0.01
16	0.66	0.58	-0.08
17	0.41	0.56	+0.15
18	0.80	0.41	-0.39
19	0.76	0.73	-0.03
$\sum(x)$	11.42	10.26	$\sum(d^2)=$ -1.16 1.606 -0.061
$\bar{x}$	0.60	0.54	

$$t = \frac{-0.061}{0.067} = -0.91$$

Table 1.9

Leucine* Content of Raw and Sterilized Milk (mg/100 ml)

Sample no.	Raw x_1	Sterilized x_2	Difference $x_1 - x_2 = d$
1	0.12	0.11	-0.01
2	--	+	--
3	0.13	0.09	-0.04
4	0.26	0.28	+0.02
5	0.24	0.31	+0.07
6	0.18	0.10	-0.08
7	0.05	0.04	-0.01
8	0.09	0.13	+0.04
9	0.21	0.27	+0.06
10	0.14	0.18	+0.04
11	0.19	0.11	-0.08
12	0.05	0.18	+0.13
13	0.09	0.11	+0.02
14	0.17	0.18	+0.01
15	0.13	0.18	+0.05
16	0.08	0.08	--
17	0.03	0.10	+0.07
18	0.08	0.09	+0.01
19	0.08	0.09	+0.01
$\sum(x)$	2.32	2.62	+0.31
			$\sum(d^2) =$ 0.0517
$\bar{x}$	0.12	0.14	+0.016

$t = \frac{0.016}{0.0117} = 1.37$

*Leucine and Isoleucine

recorded merely as a plus (+). In some samples various amino acids, e.g. lysine, were not detected at all. In this case they were recorded as a minus (-).

Values for the amino acid content of total milk proteins are presented in Table 2. These show the relative proportion of the different amino acids in the total milk proteins. The free amino acids in raw and sterilized milk might be expected to be found in the same relative proportion as they occur in the total milk proteins.

A paired "t" test was used for the statistical analysis of the individual amino acids found free in raw and sterilized milk. The null hypothesis tested was that the mean of a population of differences was zero; the alternative was that the mean was not zero. The test criterion was distributed as "t" when the assumption that differences were normally distributed was correct and the null hypothesis was true. $S_{\bar{d}}$ in Tables 1.1 to 1.9 was computed as follows:

Table 2

The amino acid composition (g/16 g nitrogen) of total milk proteins*

Amino acid	Block & Weiss	Orr & Watt
Alanine	3.6	3.5
Arginine	3.5	3.7
Aspartic acid	7.5	7.4
Cystine	0.9	0.9
Methionine	2.4	2.5
Total sulphur amino acids	3.3	3.4
Glutamic acid	21.7	23.9
Glycine	2.1	2.0
Histidine	2.7	2.7
Isoleucine	6.5	6.5
Leucine	9.9	10.0
Lysine	8.0	7.9
Phenylalanine	5.1	4.9
Proline	9.2	11.3
Serine	5.2	6.0
Threonine	4.7	4.7
Tryptophane	1.3	1.4
Tyrosine	4.9	5.2
Valine	6.7	7.0

*Kon, S. K. (7)

$$s_{\bar{d}} = \sqrt{\frac{\sum d^2 - \frac{(\sum d)^2}{n}}{n\,(n-1)}}$$

e.g. Calculations for Table 1.1 are

$$s_{\bar{d}} = \sqrt{\frac{0.146 - \frac{(-0.33)^2}{19}}{19 \times 18}} = 0.0202$$

In the divisor, n-1 is the degrees of freedom and n is the number of sample differences or pairs.

"t" was calculated as follows:

$$t = \frac{\bar{d}}{s_{\bar{d}}} = \frac{-0.017}{0.0202} = -0.84, \text{ for 18 df.}$$

The tabulated $t_{.01}$ and $t_{.05}$ for 18 degrees of freedom and a two-tailed test are 2.878 and 2.101.

A summary of the paired "t" tests for each amino acid is presented in Table 3. The differences for glutamic acid and α aminobutyric acid were both found to be significant at the 1% and 5% levels and therefore the null hypothesis was rejected on the basis of the evidence presented for these two amino acids. The differences for

Table 3

Summary of the Paired "t" Tests

Amino acid	"t"	Critical Value	
		5%	1%
Lysine	-0.84	2.101	2.878
Arginine	1.17	2.101	2.878
Serine	0.93	2.101	2.878
Threonine	1.66	2.101	2.878
Glutamic Acid	3.32*	2.101	2.878
Alanine	1.98	2.101	2.878
α Aminobutyric acid	3.15*	2.101	2.878
Valine	-0.91	2.101	2.878
Leucine and Isoleucine	1.37	2.101	2.878

the other amino acids were not found to be significant at either level and therefore the null hypothesis was accepted.

DISCUSSION

Data for nineteen samples (Table 1.1 to 1.9) instead of twenty are presented because the free amino acid content of sample number 20 was so low that results could not be recorded.

Some difficulty was experienced in identifying certain spots on the paper strips. A total of 19 amino acids in a combination of 11 standards was used to identify the individual amino acids. Trace amounts of ninhydrin positive compounds were detected usually at a position lying between alanine and α aminobutyric acid. Also two spots were present in the region between α aminobutyric acid and valine. These spots did not correspond to any of our standards. Further work would be necessary to identify these compounds but, since they are present only in trace amounts and varied little from raw to sterilized samples, they were not explored further in this investigation. Leucine and isoleucine were measured together since they could not be separated by our methods.

In general the methods used for the quantitative

determination of amino acids are not too accurate. The greatest source of error is in the method of eluting the color from the spots for measurement. The large error in the blank determination may be minimized by a careful extraction procedure.

This method of determining the free amino acid content has several advantages. One is that the colored compounds on the strips are easily eluted for measurement in a spectrophotometer or similar instrument. In addition it is relatively rapid and inexpensive as compared with column chromatography. The latter is more accurate but, if several analyses are to be carried out, the time and labor required increase greatly.

The general patterns showing the distribution of the amino acids on the developed strips for each sample were remarkably similar, however the concentrations of the individual amino acids varied considerably.

The temperatures used in sterilization would be expected both to produce and to destroy amino acids. Sterilization temperatures used can cause fragmentation of the milk proteins thus producing more amino acids. At the

same time amino acids are heat labile compounds and as such are destroyed or inactivated by heat.

There are, therefore, four possible effects on the concentrations of the amino acids in sterilized milk. First, more are produced than are destroyed and we would find a greater concentration of amino acid in the sterilized milk, as happened with glutamic acid. On the other hand, the situation could be reversed. The original amino acid content may be changed in that more amino acids are destroyed than are produced. We would then find a lower amino acid content in the sterilized milk. This is probably the case with lysine and valine. Most of the amino acids give evidence of little change. This could be due to a third possibility that the temperature and time used in sterilization were not sufficient to destroy the amino acids and to produce fragmentation. Or it might be due to a fourth possibility that a combination of fragmentation and destruction took place to the same extent.

There was a statistically significant increase in the glutamic acid and α-aminobutyric acid content of milk (Table 3) due to the effect of sterilization. The

concentration of lysine and valine were found to have decreased after sterilization of milk. The other amino acids were present in nearly the same concentrations before and after treatment. The amounts of amino acids detected in this investigation agree closely with the findings of other authors (2, 3, 14). In general there was a higher concentration of amino acids in sterilized than in raw milk.

A significant increase in the content of glutamic acid was found between raw and sterilized milk. Table 2 shows that glutamic acid is present in a far greater concentration in the total milk proteins than is any other amino acid. This may account for the increase in concentration of glutamic acid due to sterilization because of the fact that glutamic acid has a larger chance of being produced by fragmentation of the milk proteins.

A large change in the free amino acid content might affect the flavor of the sterilized product. In this investigation very little change was found in the amino acid pattern and also a relatively small change in the concentration of the individual amino acids. From these results, it would appear that insofar as the free amino

acids are concerned, the biological value of the milk has not been greatly changed and the effect of heat on the free amino acid content of milk has not been significant.

SUMMARY AND CONCLUSIONS

1. Sterilizing of raw milk at 298-300° F for 4 seconds by direct steam injection using a modified vacreator produced increases in the concentrations of glutamic and α aminobutyric acids which were found to be significant at the 1% and 5% levels.

2. Seven other amino acids or groups of amino acids--alanine, arginine, leucine and isoleucine, lysine, serine, threonine, valine--did not show significant increases although, in general, there was a higher concentration of amino acids in sterilized than in raw milk.

3. The effect on the free amino acid content of milk produced by heating at 298-300° F for 4 seconds is not significant and, as far as free amino acids are concerned, the biological value of the milk is not changed significantly.

BIBLIOGRAPHY

1. Block, R. J., Durrum, E. L. and Zweig, G. Paper Chromatography and Paper Electrophoresis, 2nd ed. Academic Press, New York. 1958.

2. Block, Richard J. Some Amino Acids, Peptides and Amines in Milk, Concentrated Milks and Cheese. J. Dairy Sci., 34: 1-10. 1951.

3. Deutsch, A. and Samuelsson, E. G. Amino Acids and Low-Molecular Amino Acid Derivatives in Cows' Milk. Proc. XV Intern. Dairy Congr. (London) Vol. 3, Sec. 5: 1650-1652. 1959.

4. Hardy, T. L., Holland, D. O., and Nayler, J. H. C. One-Phase Solvent Mixtures for the Separation of Amino Acids. Anal. Chem. 27: 971-974. 1955.

5. Hetzel, H. F. Papierchromatographische Nachweis der Freien Aminosauren des Milchserums nach Vorreinigung uber Ionenaustauscher. Milchwissenschaft, 13: 268. 1958.

6. Jenness, R. and Patton, S. Principles of Dairy Chemistry. John Wiley & Sons, Inc., New York. 1959.

7. Kon, S. K. Amino Acid Make-up of Milk Proteins in Relation to their Nutritive Values. Intern. Dairy Federation, Ann. Bull. 131-138. 1962.

8. Kosikowsky, F. V. Paper Partition Chromatography of the Free Amino Acids in American Cheddar Cheese. J. Dairy Sci., 34: 228-234. 1951.

9. Lederer, E. and Lederer, M. Chromatography. Elsevier Pub. Co., New York. 1957.

10. Lindqvist, B., Storgards, T. and Goransson, Maj-Britt. Paper Chromatographic Determination of the Amino Acids in Cheese. Proc. XII Intern. Dairy Congr. (The Hague) Vol. 3, Sec. 4: 1254-1260. 1953.

11. Naftalin, L. Quantitative Chromatographic Estimation of Amino Acids. Nature, 161: 763. 1948.

12. Smith, I. Chromatography and Electrophoretic Techniques. Interscience Publ., New York. 1960.

13. Storgards, T. and Lindqvist, B. Changes in Composition of Free Amino Acids in Milk during Cold Storage. Proc. XVI Intern. Dairy Congr. (Copenhagen) Vol. A, Sec. 2: 793-798. 1962.

14. Van der Zant, W. C. and Nelson, F. E. Amino Acids and Peptides in the Protein Free Fraction of Milk Before and After Incubation with Streptococcus lactis. J. Dairy Sci., 37: 790-794. 1954.

APPENDIX I

Lysine and Arginine Content of Raw and Sterilized Milk

	Lysine				Arginine			
	Raw		Sterilized		Raw		Sterilized	
Sample no.	O.D.*	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml
1	0.008	0.13	-	-	0.033	0.45	0.022	0.31
2	+	+	0.002	0.04	0.005	0.06	0.027	0.39
3	0.024	0.33	+	+	0.040	0.55	0.039	0.61
4	+	+	+	+	0.042	0.61	0.035	0.54
5	+	+	+	+	0.044	0.62	0.027	0.37
6	+	+	+	+	0.003	0.04	0.031	0.41
7	0.007	0.12	0.002	0.04	0.027	0.37	0.037	0.55
8	+	+	+	+	0.020	0.28	0.032	0.46
9	0.003	0.05	0.001	0.03	0.046	0.66	0.040	0.62
10	0.003	0.06	0.003	0.06	0.026	0.36	0.015	0.20
11	+	+	+	+	0.034	0.47	0.028	0.39
12	-	-	-	-	0.022	0.29	0.026	0.35
13	+	+	+	+	0.051	0.75	0.050	0.77
14	+	+	0.005	0.08	0.048	0.66	0.056	0.81
15	+	+	-	-	0.009	0.12	0.052	0.76
16	0.002	0.04	0.003	0.05	0.062	0.89	0.071	1.02
17	+	+	0.003	0.06	0.045	0.65	0.051	0.77
18	+	+	0.002	0.04	0.083	1.18	0.050	0.73
19	-	-	+	+	0.040	0.58	0.054	0.76

Legend- (+) trace amounts
(-) not detected
*O.D.optical density

APPENDIX I cont'd

Serine and Threonine Content of Raw and Sterilized Milk

	Serine				Threonine			
	Raw		Sterilized		Raw		Sterilized	
Sample no.	O.D.*	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml
1	0.023	0.24	0.010	0.11	0.062	1.57	0.041	1.08
2	0.001	+	0.007	0.10	0.020	0.52	0.056	1.59
3	0.015	0.15	0.013	0.15	0.111	2.62	0.076	2.17
4	0.011	0.12	0.016	0.19	0.055	1.49	0.069	2.02
5	0.027	0.26	0.013	0.14	0.063	1.64	0.103	2.64
6	+	+	+	+	0.180	4.07	0.129	2.93
7	0.011	0.12	0.027	0.28	0.062	1.63	0.076	2.10
8	0.002	0.01	0.012	0.14	0.052	1.38	0.085	2.38
9	0.018	0.20	0.011	0.13	0.092	2.47	0.096	2.68
10	0.009	0.10	0.006	0.09	0.101	2.72	0.095	2.68
11	0.014	0.15	0.007	0.09	0.052	1.42	0.074	1.96
12	0.006	0.08	0.010	0.11	0.028	0.69	0.040	1.08
13	0.016	0.18	0.013	0.14	0.048	1.29	0.052	1.47
14	0.012	0.13	0.026	0.26	0.049	1.26	0.074	1.96
15	+	+	0.004	0.05	0.130	2.96	0.143	3.39
16	0.020	0.21	0.021	0.21	0.071	1.84	0.073	1.90
17	0.007	0.09	0.011	0.13	0.081	2.17	0.093	2.56
18	0.018	0.20	0.014	0.15	0.135	3.12	0.085	2.30
19	0.008	0.09	0.018	0.20	0.031	0.80	0.044	1.12

Legend- (+) trace amounts
(-) not detected
*O.D. optical density

APPENDIX I cont'd

Glutamic Acid and Alanine Content of Raw and Sterilized Milk

	Glutamic Acid				Alanine			
	Raw		Sterilized		Raw		Sterilized	
Sample no.	O.D.*	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml
1	0.272	6.90	0.233	6.23	0.053	1.05	0.048	0.64
2	0.099	3.08	0.138	4.34	0.018	0.50	0.051	1.14
3	0.212	5.42	0.203	5.98	0.067	1.18	0.067	1.35
4	0.341	8.67	0.334	9.45	0.045	0.99	0.070	1.47
5	0.298	7.48	0.236	6.31	0.047	0.98	0.050	1.06
6	0.365	9.21	0.349	8.33	0.056	1.08	0.047	0.95
7	0.284	7.38	0.325	8.61	0.038	0.88	0.084	1.54
8	0.248	6.36	0.308	8.19	0.039	0.88	0.055	1.12
9	0.339	8.60	0.398	10.64	0.054	1.12	0.068	1.37
10	0.317	8.40	0.326	8.90	0.056	1.16	0.055	1.16
11	0.296	7.66	0.351	8.89	0.065	1.22	0.071	1.32
12	0.292	7.12	0.365	9.21	0.047	0.95	0.058	1.15
13	0.244	6.50	0.288	7.75	0.052	1.08	0.045	1.02
14	0.317	7.91	0.381	9.55	0.051	1.04	0.061	1.19
15	0.348	8.27	0.309	7.99	0.068	1.21	0.047	1.02
16	0.166	4.59	0.266	6.76	0.035	0.80	0.061	1.15
17	0.404	10.09	0.508	12.82	0.054	1.12	0.073	1.40
18	0.396	9.58	0.342	8.67	0.100	1.64	0.067	1.22
19	0.239	6.44	0.324	8.07	0.036	0.85	0.050	1.02

Legend- (+) trace amounts
(-) not detected
*O.D. optical density

APPENDIX I cont'd

α Aminobutyric Acid and Valine Content of Raw and Sterilized Milk

	α Aminobutyric Acid				Valine			
	Raw		Sterilized		Raw		Sterilized	
Sample no.	O.D.*	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml	O.D.	mg/ 100 ml
1	-	-	0.025	0.24	0.027	0.72	0.023	0.61
2	-	-	0.038	0.398	0.005	+	0.007	+
3	-	-	0.018	0.18	0.027	0.70	0.022	0.65
4	-	-	0.053	0.599	0.008	+	0.030	0.90
5	0.064	0.63	0.079	0.799	0.037	0.92	0.020	0.54
6	0.044	0.45	0.083	0.795	0.026	0.73	0.015	0.38
7	0.030	0.30	0.018	0.18	0.015	0.41	0.016	0.41
8	+	+	0.037	0.38	0.021	0.55	0.018	0.49
9	0.085	0.81	0.062	0.67	0.044	1.15	0.031	0.85
10	0.069	0.72	0.024	0.25	0.025	0.70	0.019	0.54
11	+	+	0.037	0.37	0.028	0.76	0.012	0.34
12	+	+	0.010	0.10	0.017	0.42	0.020	0.54
13	0.020	0.20	0.030	0.33	0.021	0.57	0.017	0.46
14	0.037	0.35	0.057	0.57	0.024	0.63	0.026	0.73
15	0.031	0.31	0.067	0.68	0.021	0.53	0.020	0.54
16	0.036	0.35	0.064	0.63	0.025	0.66	0.022	0.58
17	0.049	0.49	0.058	0.595	0.015	0.41	0.020	0.56
18	0.073	0.73	0.089	0.91	0.032	0.80	0.015	0.41
19	0.053	0.56	0.079	0.77	0.028	0.76	0.028	0.73

Legend- (+) trace amounts
(-) not detected
*O.D. optical density

APPENDIX I cont'd

Leucine and Isoleucine Content of Raw and Sterilized Milk

	Raw		Sterilized	
Sample no.	O.D.*	mg/ 100 ml	O.D.	mg/ 100 ml
1	0.014	0.12	0.013	0.11
2	-	-	+	+
3	0.015	0.13	0.008	0.09
4	0.027	0.26	0.028	0.28
5	0.028	0.24	0.035	0.31
6	0.009	0.18	0.013	0.10
7	0.005	0.05	0.004	0.04
8	0.010	0.09	0.014	0.13
9	0.023	0.21	0.028	0.27
10	0.015	0.14	0.017	0.18
11	0.020	0.19	0.013	0.11
12	0.005	0.05	0.018	0.18
13	0.010	0.09	0.011	0.11
14	0.018	0.17	0.018	0.18
15	0.015	0.13	0.018	0.18
16	0.008	0.08	0.008	0.08
17	0.003	0.03	0.010	0.10
18	0.008	0.08	0.009	0.09
19	0.008	0.08	0.010	0.09

Legend - (+) trace amounts
(-) not detected
*O.D. optical density