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TECHNICAL REPORT

75-102 FEL

OBJECTIVE METHODOLOGY TO DIFFERENTIATE BETWEEN FRESH AND FROZEN-AND-THAWED MEATS

Western Regional Research Center

Berkeley, California 94710

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FEL-24

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The basic knowledge that mitochondrial enzymes are released by freezing-and-thawing muscle tissue was used to develop a fairly simple objective test to distinguish between meat that has been frozen-and-thawed and that which has never been frozen. Previous studies indicate that glutamic-oxalacetic transaminase (GOT) of muscle press juice (MPJ) can be used for this. Our tests showed that the increase in total GOT activity in MPJ

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ranged from 25-60%. The low increase of 25% was not considered to be sufficiently reliable as an indicator. Therefore, a more reliable and sensitive method was developed and tested on meat from five species.

The test requires the determination of the relative amounts of mitochondrial isozyme (GOT_m) and sarcoplasmic isozyme (GOT_s) that is present in MPJ from the original meat sample of unknown history as received, and in MPJ from a portion of the original sample that has been intentionally frozen-and-thawed in the laboratory. Freezing-and-thawing does not alter the amount of GOT_s in MPJ but increases the amount GOT_m in the juice from 50-150% or more. The two isozymes are separated by electrophoresis and the relative amount of each isozyme present in the MPJ is determined by measuring its enzymatic activity. The values for percent GOT_m in the MPJ's will be similar or show only a small increase if the unknown had been frozen. Over 140 muscle samples including samples of beef, pork, lamb chicken and turkey gave this result consistently. Variations in freezing temperature and holding time in either the frozen or refrigerated condition were not critical. A series of samples could be analyzed conveniently in one day.

PREFACE

The freezing or thawing of muscle is known to result in a number of subtle changes. In commercial operations a number of adverse changes affecting the functional and sensory properties of mammalian and poultry meats are attributed to freezing and thawing. As a consequence, specifications for several processed items prohibit the use of meat which has previously been frozen. Aside from the issue of validity, this restriction brings into focus the collateral problem that a test, preferbly an objective test suitable for citation in a specification, is not currently available to identify meat which has been frozen and thawed. The development of such a test is the objective of this investigation.

The investigation here reported was performed at the Western Regional Center of the US Department of Agriculture at Berkeley, CA 94710. Funds were provided by Project 1T762724AH99, titled: Food Technology. Dr. Kenneth E. Berry served as Principal Investigator under the general direction of Dr. Hans Lineweaver. Drs. Maxwell C. Brockmann and Larry Hinnergardt of the US Army Natick Research and Development Command, formerly the US Army Natick Development Center, served respectively, as Project Officer and Alternate Project Officer.

Appreciation is given to Dr. Max Brockmann of the US Army Natick Research and Development Command for his support of this research. Grateful acknowledgement is given to Drs. Jim Zahnley and Ladell Crawford for their technical assistance and to Mr. Bill Nelson, Solano Laboratories, Berkeley, for his assistance and the use of his densitometer.

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INTRODUCTION

Meat purchases by the Department of Defense, through "purchase specifications", represent the most significant food expenditure. A requirement of several of these military or federal specifications is that the meat or poultry used in the manufacture of processed products must not have been frozen prior to manufacture. Presently, an acceptable objective method to determine if the meat is fresh or frozen-and-thawed is not available. Nor is there a reliable subjective method, since the physical appearance of frozen-and-thawed meat can quite closely resemble fresh meat. This investigation was undertaken to develop a rapid and objective procedure capable of determining if meat has ever been frozen-and-thawed.

The freezing of living plant and animal tissues can have a profound effect on their biological survival as evidenced by frost damage and frost bite, respectively. Yet, the freezing of muscle for food preservation yields products that are widely accepted as being of high quality. Visual and organoleptic examinations are not guides capable of classifying meat as having been frozen-and-thawed or never frozen. Such measures as volume of exudate, total proteins or solids in the weep or exudate are too variable to be reliable indices. But cells are ruptured by freezing-and-thawing, and various proteins and perhaps other substances are

released or solubilized on freezing-and-thawing. Some enzymes are among the proteins released and their catalytic activity makes them readily detected and measured. Various workers (Gantner, et al., 1964;[1] Hamm, 1969;[2] Hamm and Kormendy, 1969;[3] Hamm et al., 1969[4]) have reported the release of mitochondrial enzymes by freezing. The findings by these workers on the release of glutamic-oxalacetic transaminase (GOT) (L-Aspartate: 2-oxoglutarate aminotransferase EC 2.6.1.1) led us to study the differences in the amounts of total GOT, including both the sarcoplasmic (GOT_s) and mitochondrial (GOT_m) isozyme forms, in the muscle press juice (MPJ) of fresh and frozen-and-thawed meat. This has led to a recommended objective method that can be used to distinguish between fresh and frozen-and-thawed meat.

This report will include results on variations in total GOT activity in MPJ as well as data on GOT_m activity in MPJ and muscle exudate. Results are reported on tissue from 5 species.

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1 Gantner, G. and Hamm, R. 1964. Transaminasen des Fleisches. Extrahierbarkeit und Eigenschaften von Transaminasen (GOT und GPT) aus Schweinemuskel. Zeitschr. Lebensmittel-Untersuch. u. Forsch. 126:1.

2 Hamm, R. 1969. Transaminases of Skeletal Muscle. 2. Transaminase Activities in White and Red Muscles of Pigs and Cows. J. Food Sci. 34:449.

3 Hamm, R. and Kormendy, L. 1969. Transaminases of Skeletal Muscle. 3. Influence of Freezing and Thawing on the Subcellular Distribution of Glutamic-Oxaloacetic Transaminase in Bovine and Porcine Muscle. J. Food Sci. 34:452.

4 Hamm, R., Kormendy, L. and Gantner, G. 1969. Transaminases of Skeletal Muscle. 1. The Activity of Transaminases in Post-Mortem Bovine and Porcine Muscles. J. Food Sci. 34:446.

The recommended test was reliable even when samples were subjected to rather wide variations in freezing temperatures and holding times both in frozen and refrigerated condition. Chicken and turkey meat were studied most extensively because samples of known thermal history were readily available.

Considerable time and effort was spent in attempting to develop a method based on the total GOT in the MPJ before it was decided to develop a test based on the relative amount of GOT_m in the MPJ. The results on the mitochondrial isozyme are limited but we believe they are sufficient to establish the soundness of the method recommended.

MATERIALS

Meat of known thermal history was procured through local packing plants either immediately after slaughter or after routine plant carcass cooling. Muscle samples for analysis were obtained from the beef Semimembranosus, pork and lamb Longissimus dorsi, and chicken and turkey Pectoralis major and turkey leg muscles. The samples were taken to the laboratory, placed in a 2.2°C (36°F) coldroom, and cut so that serial samples approximately 2.5 cm^3 (ca. 15 gms), could be made from the same muscle (or cut). However, the chicken breast was cut perpendicular to the fiber direction into four equal size pieces.

The turkey legs were sampled by making cross section cuts around the bone, approximately 1.27 cm thick. Normally four samples were taken: one control (non-frozen, original); two for conventional freezing [-17.8°C (0°F) and -34.4°C (-30°F)]; and the fourth to be frozen in liquid nitrogen (LN_2). The samples destined for freezing were then placed in small plastic bags, the bags evacuated and placed in a -17.8°C (0°F) or -34.4°C (-30°F) freezer. The control and LN_2 frozen samples were left uncut in the 2.2°C coldroom until the frozen samples were removed from the freezer. At that time they were prepared by trimming the surface and then cut, as above, to provide the control and LN_2 samples. The LN_2 sample was frozen and then immediately allowed to thaw in the coldroom or under observation at room temperature. The other samples were thawed in the same manner.

1. Previously Frozen-and-Thawed Meats

Frozen samples of known history were thawed and sampled in the same manner as original unknown samples. These samples: (1) fryer breast (whole-body); (2) turkey breast (boneless); (3) fryer breast (whole-body); and, (4) turkey legs (frozen separately from carcass), were frozen and held for 48 hrs, 48 hrs, 9 mos and 17 mos, respectively, at -17.8°C (0°F) prior to thawing.

The whole body fryers and boneless turkey breast were thawed for two days at 2.2°C and the turkey legs were thawed at 2.2°C overnight. Samples for analysis were removed in the manner previously described.

2. Chill Packed Poultry

The transportation and distribution of chill packed poultry and poultry products has increased considerably in recent years. Because the temperature of this product might be in the sub-freezing range, it was investigated for potential freezing changes.

The product can be chill-packed with dry ice snow, liquid nitrogen, or a combination of the two. By these methods, the outside of the product is cooled quite rapidly and then allowed to equilibrate with the warmer internal tissue. With some of these methods a surface layer is crust hardened by the coolant. Depending on the operation, the equilibrated product ready for transportation or distribution may attain an internal temperature of -3.3°C.

Commercially handled chill packed whole body fryers and cut-up chicken breast pieces were obtained from a local distributor. The internal temperature of the fryers was 0°C and of the cut-up breasts was -1.5 to-2.0°C. Ice was evident around both products and they were both very firm to the touch.

When shipped, the whole body fryers were individually placed in plastic bags and then packed in a large cardboard box; the cut-up breasts were packed in an overlapped, or shingled, style with 4 pieces to a styrofoam container, including a plastic overwrap. These styrofoam containers were then packed in a large cardboard box.

3. Pale, Soft and Exudative (PSE) Pork

Normal and PSE pork loin samples were obtained from a local packing plant. The samples were chosen with reference to the Wisconsin Pork Standards for color, firmness and exudate. Sample preparation was performed as previously described.

METHODS

Muscle press juice was used throughout our investigation to objectively determine the differences between fresh and frozen-and-thawed meat. This section of the report will include: (1) a unique way to obtain an internal control and the basis for the ultimate fresh or frozen determination; (2) a method to obtain uniform MPJ samples; (3) a method to determine total GOT activity in the MPJ; (4) a method to electrophoretically separate the MPJ components and stain the total proteins; and (5) a method to selectively stain and determine the relative activity of the MPJ electrophoretically separated GOT isozymes. Parts of each method are used in the final recommended standardized procedure.

1. Development of An Internal Control and Basis for Method

Because of the biological variation of carcasses, and to provide a needed point of reference, the unknown meat sample was split to provide an original plus several experimental samples. The experimental samples were then frozen in the laboratory, at the previously mentioned temperatures, and thawed prior to preparing the muscle press juice. Thus, if the control sample had never been frozen a marked difference in GOT_m activity would exist between it and the laboratory frozen-and-thawed sample. If it had been frozen-and-thawed, little or no difference in GOT_m activity would exist between these two samples. This formed the basis for the objective method here described.

2. Preparation of the Muscle Press Juice

The control and frozen-and-thawed experimental samples were pressed twice in a Carver Model "B" press using the specially prepared plexiglass muscle press plates. These plates (Figure 1) were 15.2 cm (6") square with the top plate being 0.95 cm (3/8") thick and the bottom plate 1.27 cm (1/2") thick. The edges and all corners were smoothed after cutting. In addition, the bottom plate was chucked in the lathe and a 0.95 cm (3/8") wide by 0.63 cm (1/4") deep circular trough (arrow) [14.8 cm (5-13/16") outside diameter] was cut into the plate.

Also, while the plate was running in the lathe a very fine piece of emery-cloth was held against the circular portion of the plate and moved toward the center. This was done to take the smooth finish off the plexiglass and keep the piece of meat being pressed stationary in the center of the plate. The maximum pressure exerted on the meat sample was approximately 10.6 kg/cm^2 [ca. 3000 pounds total (1364 kg) over 20.1 in^2 (128.7 cm^2) area of internal island equals $\sim$ 10.6 kg/cm^2]. The exuded muscle press juice (MPJ), collected in the plate trough, was pipetted into the barrel of a 5-cc syringe previously connected to a 25-mm plastic Millipore* filter holder (Figure 1) containing a 0.45-micron filter and a fiberglass prefilter. All MPJ was filtered to remove cell debris, mitochondria, and mitochondrial pieces in an attempt to eliminate unsolubilized mitochondrial GOT isozyme. After the MPJ was transferred to the syringe, the syringe was reinserted and the contents pressed through the filter array into a labelled test tube. The filtered MPJ was then held in the refrigerator until assayed for total enzymatic and isozyme activity.

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*Millipore Corporation, Bedford, Massachusetts, 01730.

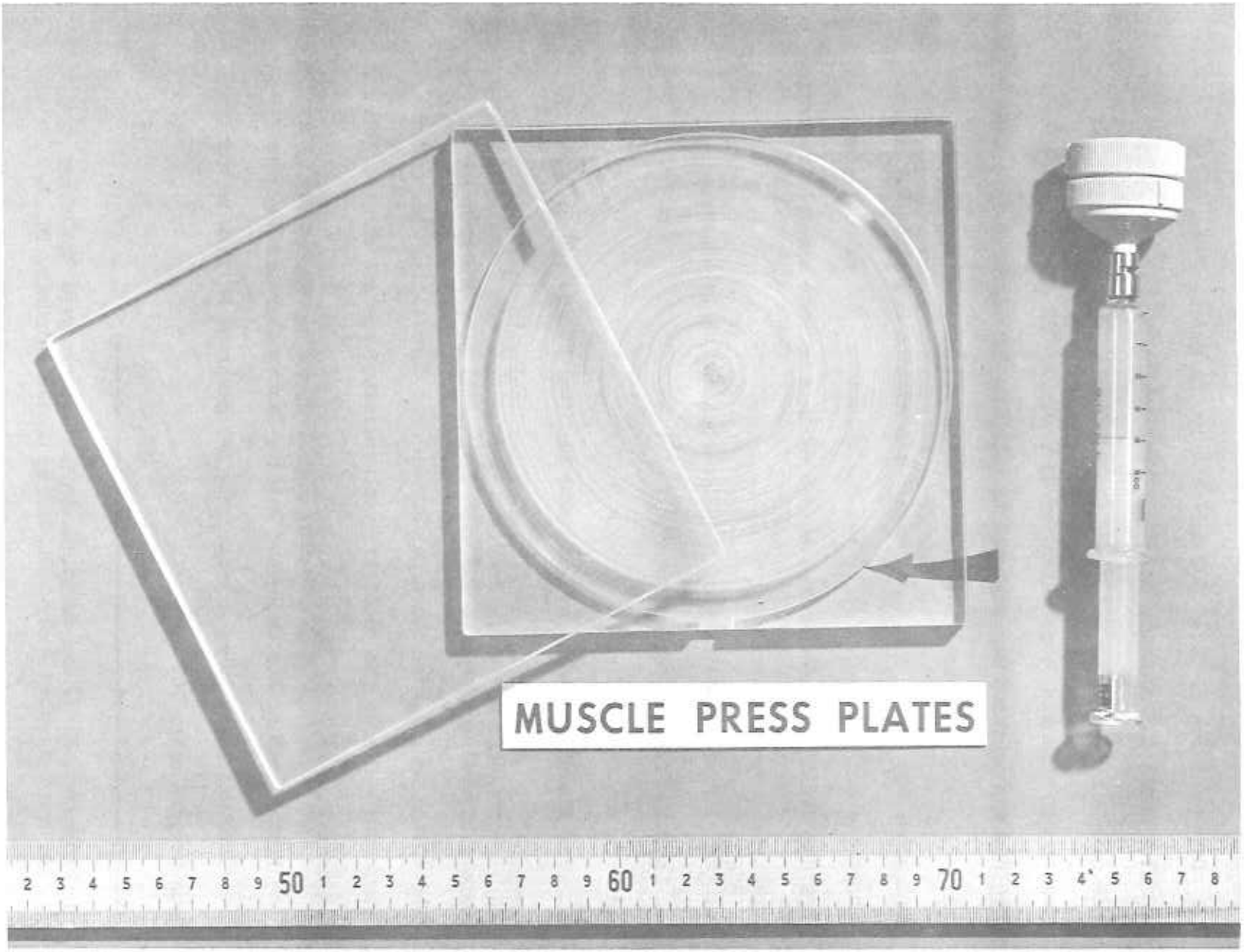

Figure 1. Apparatus for Pressing and Filtering the Muscle Press Juice

3. Determination of Total GOT Activity in the Muscle Press Juice

The total GOT activity in the MPJ was determined (Figure 2) by the method of Bergmeyer and Bernt (1970),[5] using test kit #15923 of Boehringer Mannheim Corporation for determining GOT activity in blood. The MPJ was prepared for analysis by diluting 100 microliters of MPJ with 0.1 molar phosphate buffer to a total volume of 10.0 ml. For the analysis, the 0.5 ml blood sera was replaced with 0.3 ml of 0.1 molar phosphate buffer and 0.2 ml of the MPJ solution. The rate of change in optical density was determined at 366 nm in a Beckman Model DB recording spectrophotometer maintained at 25°C.

4. Total Protein Staining of the Electrophoretically Separated Muscle Press Juice Components

(a) Preparation of Electrophoresis Buffer

For electrophoresis, a 0.05 molar barbital buffer (pH 8.6) was prepared by mixing 10.3 gms of sodium barbital with ca 900 ml of water, adjusting the pH to 8.6 with 0.1 normal HCl, and bringing the volume to 1 liter. This buffer was used for filling the buffer reservoir and also for saturating the cellulose acetate electrophoresis membrane.

(b) Preparation of the Celloluse Acetate Electrophoresis Membrane

Care was taken to evenly saturate the membrane so that no air was trapped under it.

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5 Bergmeyer, H. U. and Bernt, E. in H. U. Bergmeyer: "Methoden der Enzymatischen Analyses". Verlag Chemie Weinheim, Bd. 1, 685, 2. Aufl. 1970.

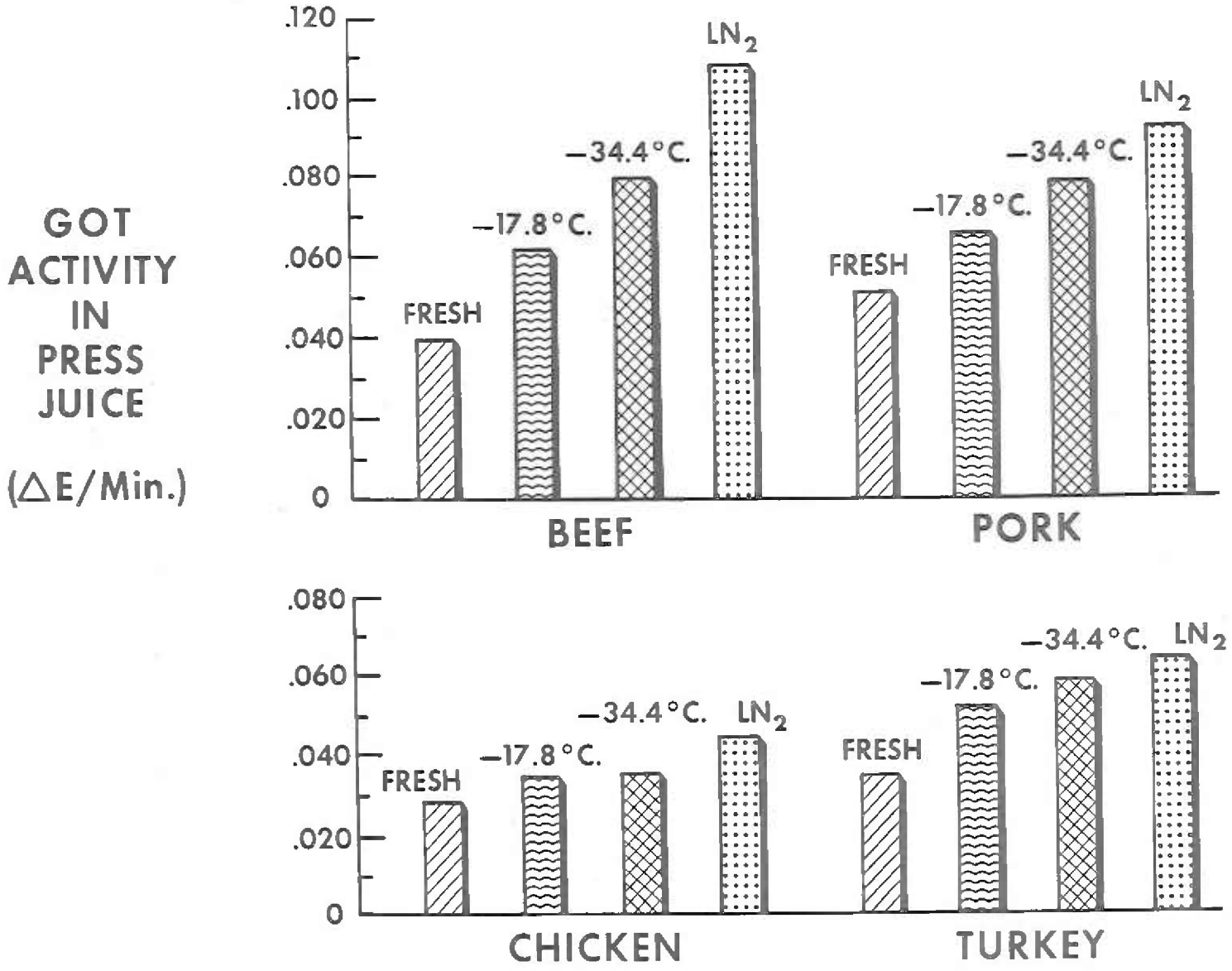

Figure 2. Total GOT Activity in Beef, Pork, Chicken and Turkey Muscle Press Juice

After the membrane was completely wetted, it was submerged, then removed and thoroughly blotted between two filter papers to remove superficial fluid. The membrane was immediately placed onto the special MicroZone* membrane holder, inserted into the MicroZone chamber and the chamber covered.

(c) MPJ Electrophoresis

The MPJ (ca. 0. 5µl) was applied directly to the prepared cellulose acetate membrane and electrophoresed at 250 volts for one-half hour.

(d) Staining

Following electrophoresis, the MPJ components were stained with a dye solution as outlined in the MicroZone manual. The dye consisted of 0.2% Ponceau-S stain, 3.0% trichloroacetic acid and 3.0% sulfosalicylic acid (all on a weight basis) and mixed with distilled water to 100 ml. After staining, the membrane was rinsed in 5% acetic acid bath, transferred to 95% alcohol rinse, and then cleared in a mixture of 25% acetic acid and 75% ethanol. The membrane was transferred to a glass plate and dried in a 100°C oven for 15 minutes.

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*Beckman Instruments, Inc., Fullerton, California

5. Determination of GOT_s and GOT_m Activity.

Previous Investigators (Kőrmendy et al., 1965;[6] Romel and LaMancusa, 1965[7]) have determined that sarcoplasmic and mitochondrial isozymes of GOT exist in muscle and that they differ in several chemical properties including electrophoretic methods.

(a) Electrophoresis and Isozyme Staining.

Electrophoresis was performed using the MicroZone system and 0.05 molar barbital buffer (Ph 8.6) as described in Section 4, above. The buffer saturated membrane was spotted with ca. 0.5µl (using special applicator twice) of undiluted MPJ at each sample location. Following application, the system was operated at 250 volts for one-half hour. After electrophoresis, the membrane was transferred and placed, application side down, against a specially prepared substrate/dye agar plate as described in 5 (b) and (c). The plate was inverted and the enzymatic reaction allowed to proceed for one hour in a 37°C incubator. To complete the staining procedure the membrane was washed in water to remove any adhering agar (MicroZone Method), rinsed in a 5% acetic acid solution to fix the color, and air dried.

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6 Kőrmendy, L., Gantner, G. and Hamm, R. 1965. Isozyme der Glutamat-Oxalacetat-Transaminase im Skeletmuskel von Schwein und Rind. Biochem. Zeitschr. 342:31.

7 Romel, W. C., LaMancusa, S. J. 1965. Electrophoresis of Glutamic Oxalacetic Transaminase in Serum, Beef Heart, and Liver Homogenates on Cellulose Acetate. Clinical Chemistry 11:131.

(b) Preparation of the Substrate/Dye Agar Plate.

The substrate/dye agar plate (Figure 3) was individually prepared by placing 0.05 gm of L-aspartate 0.03 gm α ketogluturate and 0.1 gm Noble agar into a clean 10-cm-diameter Petri dish, and thenmixing these components with 9.0 ml of "TransAc"* substrate buffer solution as described in 5 (c). The lid was replaced and the mixture heated to boiling on a hot plate. After the mixture had cooled slightly, 1.0 ml of the 0.005% pyridoxal phosphate (coenzyme in transaminations) and 2.0 ml of TransAc dye, 6-benzamido-4-methoxy-m-toluidine diazonium chloride, solution (50 mg dye/5 ml H_2O) were added. The agar mixture was swirled and allowed to solidify in the dark at room temperature.

The color of the plate should be a light straw-colored yellow. If the plate is a light reddish brown it should be discarded as it will not react properly with the isozymes.

(c) Buffered Substrate Solution pH 7.40

Components per 100 ml TransAc solution (Babson et al. 1962):[8] α ketoglutaric acid, 5 milli molar (73.1 mg); L-aspartic acid, 20 milli molar (266.2 mg); dibasic potassium phosphate (K_2HPO_4) 3.35 gm; monobasic potassium (KH_2PO_4), 100 mg; polyvinylpyrrolidone (PVP),

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*Products of the General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N.J.

8 Babson, A. L., Shapiro, P. O., Williams, P. A. R., and Phillips, G. E. 1962. The use of a Diazonium Salt for the Determination of Glutamic-Oxalacetic Transaminase in Serum. Clin. Chim. Acta 7:199.

1.0 gm; and ethylenediamine tertaacetic acid (EDTA) tetrasodium salt, 0.1 gm. Although this could be made, it was purchased locally each time either separately or as part of the TransAc kit.

(d) Densitometry

Densitometric measurements of the separated and stained GOT_m and GOT_s spots were made with a Clifford Model Densitometer and integrator system. The wavelength was 520 nm with a slit opening of 3.0 mm by 0.70 mm.

RESULTS AND DISCUSSION

The possibility was examined that the staining patterns of MPJ from fresh and frozen-and-thawed meat on an electrophoretic membrane would be sufficiently different to indicate whether the sample had been frozen, as reported by Howard et al. (1960).[9] Using the Micro-Zone procedure described in the methods section the patterns for MPJ obtained from fresh and frozen-and-thawed meat appeared practically identical and no attempt was made to increase the sensitivity of the protein staining test. Enzyme activities, however, do show marked differences as previously noted and received most of our attention.

The increased GOT activity in the MPJ provides a sensitive index to determine if meat has been frozen-and-thawed. As originally planned, much of the work in this project has been completed with poultry, as its availability in fresh form was much greater than mammalian meat.

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9 Howard, A., Lawrie, R. A. and Lee, C. A. 1960. in Lawrie, R. A. 1968. The interaction of Freezing and Post-Mortem Changes in Muscle. "Recent Advances in Food Science" vol. 4, p. 375. ed. Hawthorn, J. and Rolfe, E. J. Pergamon Press. London

However, adequate experimental results have been obtained with mammalian skeletal muscle to realize that its behavior, both in the fresh and frozen-and-thawed forms, parallels that of avian skeletal muscle.

Figure 2 shows the total GOT activity of the MPJ from fresh and frozen-and-thawed meats. The changes between the fresh and -17.8°C frozen samples of these four species range from a 25-60% increase in total GOT activity, with larger changes occurring at the lower freezing temperatures.

Originally, the investigation was directed at determining the total GOT activity in the MPJ as a simple and rapid objective method to differentiate between fresh and frozen-and-thawed muscle. However, in the analysis of nearly 500 individual and duplicate mammalian and avian skeletal muscle samples, the results for some samples indicate that the increase in total GOT activity, although consistent, was not sufficient to make this method reliable. A better method of objective differentiation was needed. The value of using GOT activity lies in the increased activity which comes from the solubilization or release of the mitochondrial isozyme. Since these isozymes can be easily separated, the quantitative increase in GOT_m can be measured.

A method of quantitating the isozymes was successfully developed. The MicroZone electrophoresis system was used to separate the isozymes and agar embedded with substrate and dye (Figure 3) was used for staining.

INCUBATING AGAR FOR GOT ISOZYME ANALYSIS

9.0 ml "TRANSAC" SUBSTRATE BUFFER

0.1 gm NOBLE AGAR

0.05 gm L-ASPARTATE

0.03 gm α-KETOGLUTARATE

1.0 ml 0.005% PYRIDOXAL PHOSPHATE SOLUTION

2.0 ml "TRANSAC" DYE (50 mg/5 ml H_2O)

Figure 3. Incubating Agar for GOT Isozyme Analysis

The dye in this system reacts selectively with the oxalacetic acid produced from the transamination reaction that occurs while the MicroZone membrane is in contact with the agar plate. The azure color formed is proportional to the amount of oxalacetate produced. Densitometric scanning gives relative GOT isozyme content values. Tables I through V, show the results for beef, pork, lamb, chicken and turkey with a summary in Table VI. The GOT_m content of the MPJ increases from 54% to 214% of the fresh value, when meat is frozen at -17.8°C, and increases considerably more when it is frozen at lower temperatures. This striking increase was readily observed by visual examination of the incubated electrophoretic membrane (Figure 4) and by densitometry (Figure 5).

It was interesting, though expected, that the size of the GOT_s peak was nearly constant (Figure 5) throughout this temperature sequence, indicating that its solubility was unchanged with temperature. Nevertheless, the peak for GOT_m continuously and consistently increased as the freezing temperature was lowered. The curves in Figure 5 correspond to the values for beef in Table 1, Sample 1.

Unlike Hamm and Kŏrmendy (1969),[3] who reported finding little to no GOT_m in the MPJ of fresh muscle, our studies show approximately 10-20% GOT_m in all fresh samples.

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3 Hamm, R., Kŏrmendy, L. 1969. Transaminases of Skeletal Muscle. 3. Influence of Freezing and Thawing on the Subcellular Distribution of Glutamic-Oxaloacetic Transaminase in Bovine and Porcine Muscle. J. Food Sci. 34:452.

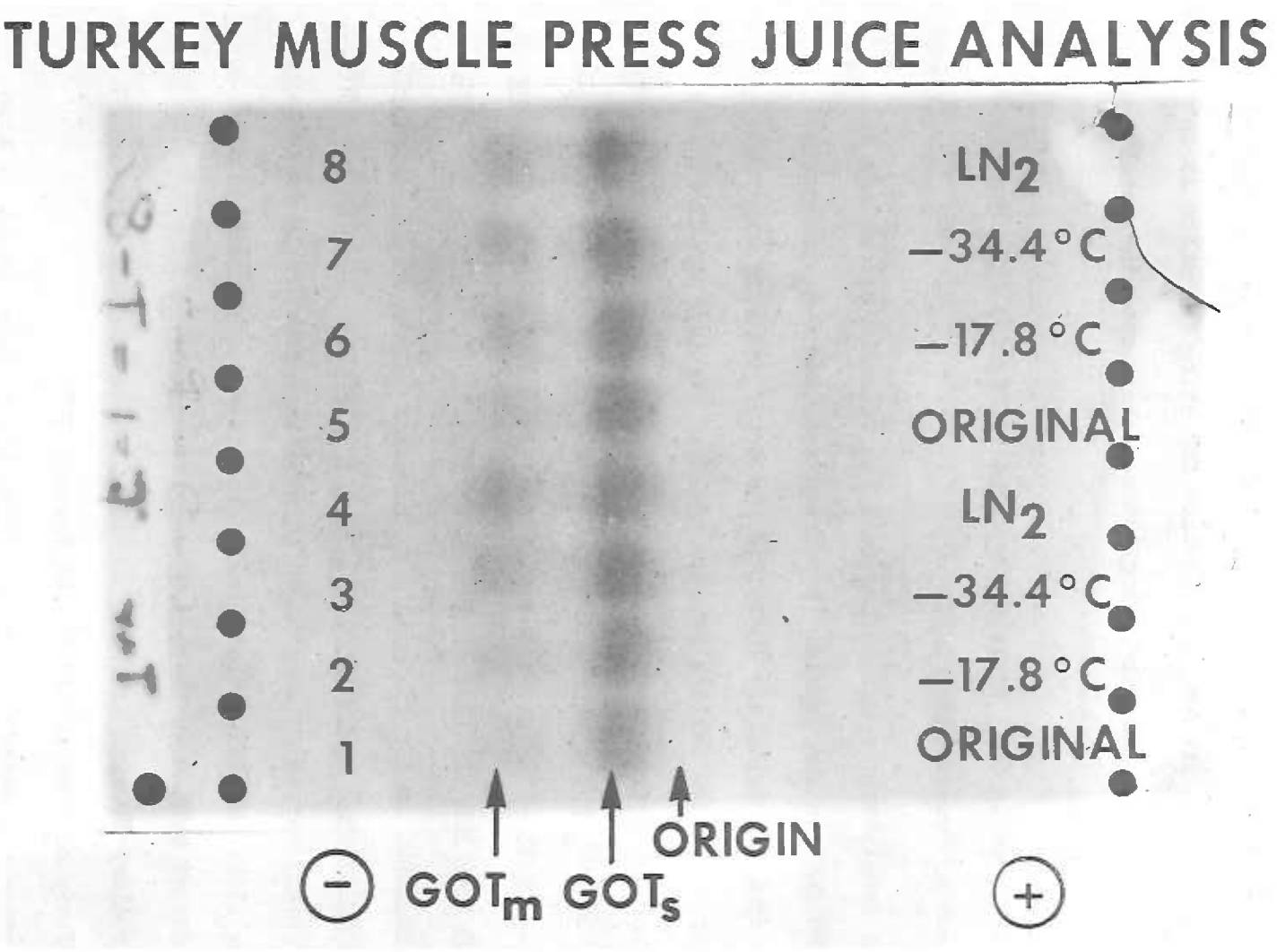

Figure 4. Incubated and Stained Electrophoretic Membrane Showing GOT_s and GOT_m spots of Fresh and Frozen-and-Thawed Turkey Samples

However, this did not interfere with the procedure as we routinely split the sample, freezing one portion and using the other as an original control. The classification was based upon the results of these two samples.

1. Difference in GOT_m Activity Required to Signify that Meat is Fresh or Frozen-and-Thawed

The densitometric values provided the relative percentage of GOT_s and GOT_m in the sample. For fresh meat the GOT_m percentage ranges from approximately 10-25% of the total activity with the majority of values falling between 15-20%. Through our experience in working with fresh meat of known thermal history a minimum increase of 50% in GOT_m has always followed intentional laboratory freezing-and-thawing at -17.8°C for 48 hours (see data in tables). Thus, we propose that the original sample be classified as fresh if at least a 50% increase in the relative GOT_m activity occurs in the laboratory frozen-and-thawed sample compared to the original. If less than 50%, the original sample is considered to have been previously frozen-and-thawed. Like any other biological determination, evaluation of a large lot of meat must include appropriate sampling.

2. Corollary Studies

(a) Effects of Time at -17.8°C on Percent GOT_m

A study was undertaken to determine the minimum amount of time at -17.8°C to effect a significant change in percent GOT_m in the MPJ.

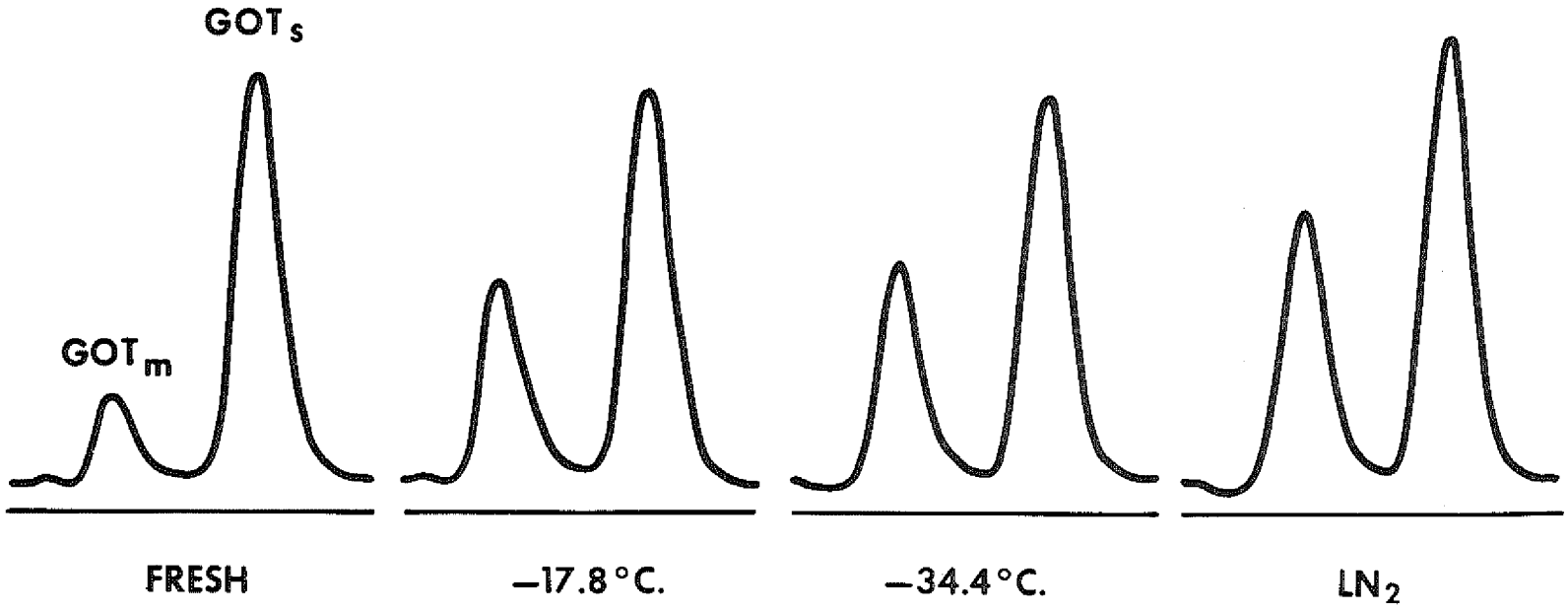

Figure 5. Densitometric Scans of Beef GOT Isozymes Showing Effects of Freezing

The results in percent GOT_m increase are shown in Table 7. In this comparison 4 hours was set as the minimum time in frozen storage needed to produce at least a 50% increase for turkey breast and fryer breast samples. It can be seen from the table that further increases in GOT_m occur by freezing for increasing lengths of time, although from 6 to 96 hrs the amount of increase is fairly small.

(b) Effect of Fresh Storage after Slaughter on Percent GOT_m in MPJ

The effect of fresh storage on the percent GOT_m was also investigated, although in our previous studies no large increase seemed to take place. The fresh samples tended to have approximately 10-20% GOT_m no matter when they were sampled. To test this observation, the right Pectoralis major muscle was removed from a fryer carcass one day after slaughter without removing the skin covering the left muscle. The left muscle was removed eleven days later and the results compared (Table 8). The slight difference between these samples would not cause any interference in the objective frozen-and-thawed determination that has been proposed.

(c) Evaluation of Previously Frozen-and-Thawed Meat

In this analysis the previously Frozen-and-thawed samples, obtained as noted in the Materials section, were evaluated.

After thawing, split samples were taken and analyzed for GOT_m by the standardized procedure. The results in Table 9 are consistent with expectation. That is, the percent GOT_m values found in the original (frozen-and-thawed) samples were much higher than the normal 10-20% GOT_m found in fresh meat. The increases in GOT_m values (0-19%) caused by laboratory freezing of the split samples were far less than the 50% or greater difference that occurs between fresh and frozen-and-thawed meat values.

The combined thaw drip obtained from the first thawing of the two 17-month frozen turkey legs contained an almost equal percentage of GOT_m (Table 9) as did the MPJ. These values for GOT_m in 9-month frozen fryer breast and 17-month frozen turkey legs indicate that GOT is stable in frozen storage, as expected, and as necessary, if the test is valid for use on meat stored for long periods of time.

(d) Evaluation of Chill-Packed Poultry

Several samples of commercially chill-packed poultry were evaluated because chill-packing may unintentionally involve some freezing (see comments in materials sections). Results of analyzing 3 samples of whole body birds and 3 samples of chicken breast are reported in Tables 10 and 11. The internal breast temperature of the chill packed fryers was $0^{\circ}C$ and of the cut-up breasts was -1.5 to $2.0^{\circ}C$. From Table 10 it can be observed, both by the percent GOT_m

in the original sample and by the percentage increase caused by laboratory freezing-and-thawing, that the whole-body fryers would be classified as fresh poultry. However, the chill packed cut up breasts (Table 11), with an internal temperature somewhat below that of the fryers, show samples number one and three as fresh but number two as previously frozen-and-thawed. This conclusion was supported by the high amount of GOT_m already solubilized in original sample number two and the lack of at least a 50% increase in the amount of GOT_m found in the laboratory frozen-and-thawed portion of sample number two. Unevenness in rapidly chilling the breast packages prior to boxing, or direct contact with the CO_2 snow in the box, could account for this frozen sample found in the same pack as the fresh samples, particularly as these breast samples were shingled in the pack, and the top piece would have been completely exposed to the coolant. The other pieces would be partially protected from the top by the first piece and from the bottom by the styrofoam container. Nevertheless, this frozen-and-thawed meat sample would not be distinguishable from meat that had been intentionally frozen.

(e) Evaluation of Pale, Soft and Exudative Pork.

Some question has been raised about the use of this test with PSE pork (Vandekerckhove, et al. 1972).[10] However, in our initial evaluation (Table 2) no difference was found between PSE and normal pork in the percent GOT_m found in the original sample, or in the percentage increase

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10 Vandekerckhove, P., Demeyer, D. and Henderickx, H. 1972. Evaluation of a Method to Differentiate Between Nonfrozen and Frozen-and-Thawed Meat. J. Food Sci. 37:636.

of GOT_m caused by freezing. Nevertheless, we agree quite closely with the percent GOT_m found in Vandekerckhove's nonfrozen and frozen pork samples.

(f) Evaluation of Muscle Exudate from Fresh and Frozen-and-Thawed Meat.

A potentially faster way of analyzing meat to determine if it is fresh or frozen-and-thawed is to evaluate the muscle exudate (weep) obtained during storage or after thawing. Exudate samples were filtered and run exactly as the MPJ. The relative GOT_m activities in the muscle exudates (Table 12) from fresh and frozen-and-thawed fryer breast pieces were similar to those from the MPJ. The GOT_m activity in the fresh exudate was within the range of 15-20% of the total GOT activity and the GOT_m activity of the exudate from the thawed sample was at least 50% higher than that of the fresh exudate. These composite exudates were obtained from the individual plastic bags holding breast samples from 7 fryers. The total GOT activity in the exudate was slightly less than that of MPJ.

The exudate from the whole-body fryer (Table 12) was obtained after freezing (48 hrs, -17.8°C)-and-thawing, and prior to removing the breast samples for the analysis reported in Table 11, sample 1. The 37.1% GOT_m of the exudate parallels the value for the MPJ from the same fryer.

Although by using the exudate a potentially faster or simpler method of analysis could be achieved, further evaluation would be needed to ensure that exudate from various meats consistently reflect the enzyme composition of the corresponding MPJ.

3. Modifications of Standardized Procedure

(a) Electrophoretic Membranes

Although the electrophoretic separation was routinely run with the MicroZone system, it should be performed with equal success using 2.54-cm-wide cellulose acetate strips normally used in clinical laboratories or by making two applications with a 2.54-cm-wide applicator on the MicroZone membrane (Figure 6). In fact, by using wider strips or applications it should be easier to visually quantitate the differences between the original and the frozen-and-thawed samples.

(b) A Potential Method of Evaluation Without Densitometer

The test is based on objective methodology, except for the means of quantitation. It should be possible to compare the original to the experimental frozen-and-thawed sample by conducting electrophoresis and staining steps on serial dilutions of the frozen-and-thawed MPJ (25%, 50%, and 100%), and comparing the color intensity visually to that of the original. Interpretation of whether the original sample was fresh or frozen-and-thawed would be based on the guidelines previously described. That is, if the color intensity corresponding to the GOT_m isozyme in the 50% dilution sample of the frozen-and-thawed MPJ is equal to or greater than that of the GOT_m of the original undiluted MPJ, then the original sample was fresh. Lack of time prevented evaluation of this potential procedure.

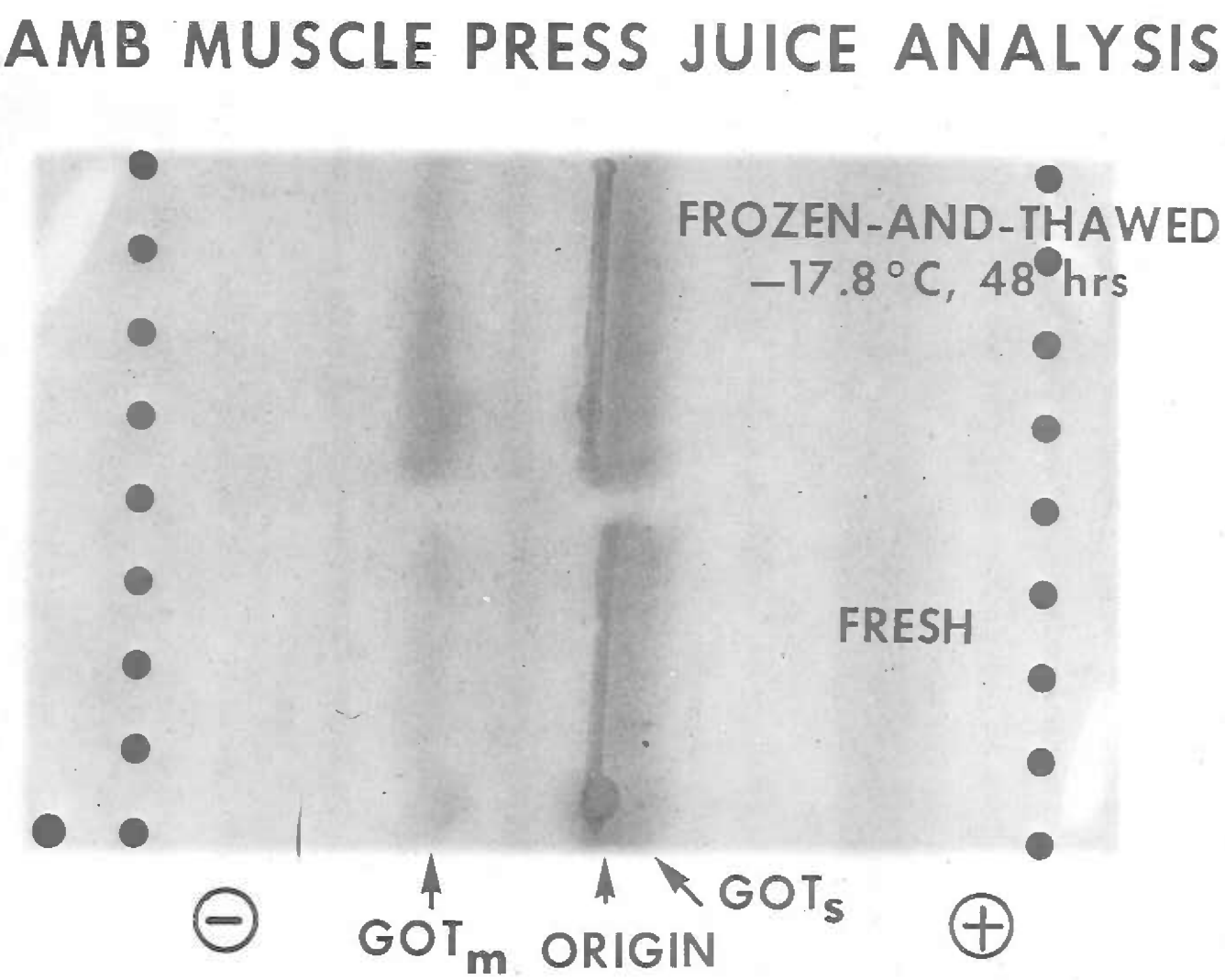


Figure 6. Separation of Fresh and Frozen-and-Thawed Lamb MPJ Using 2.54 cm Wide Applicator on MicroZone Membrane

4. Outline of Standaradized Procedure (Figure 7)

(a) Obtain representative samples (enough for statistical evaluation).

(b) From each original sample remove two 2.5-cm^3 (ca. 15 gms) pieces. Place in plastic bags and freeze one at -17.8°C for 4-24 hours and refrigerate the other.

(c) Thaw the frozen piece at 2.2°C, or under observation at room temperature.

(d) Press each sample twice between plexiglass plates in Carver Press (10.6 kg/cm^2) and collect MPJ (2-5 ml).

(e) Filter MPJ through 0.45 μ Millipore filter.

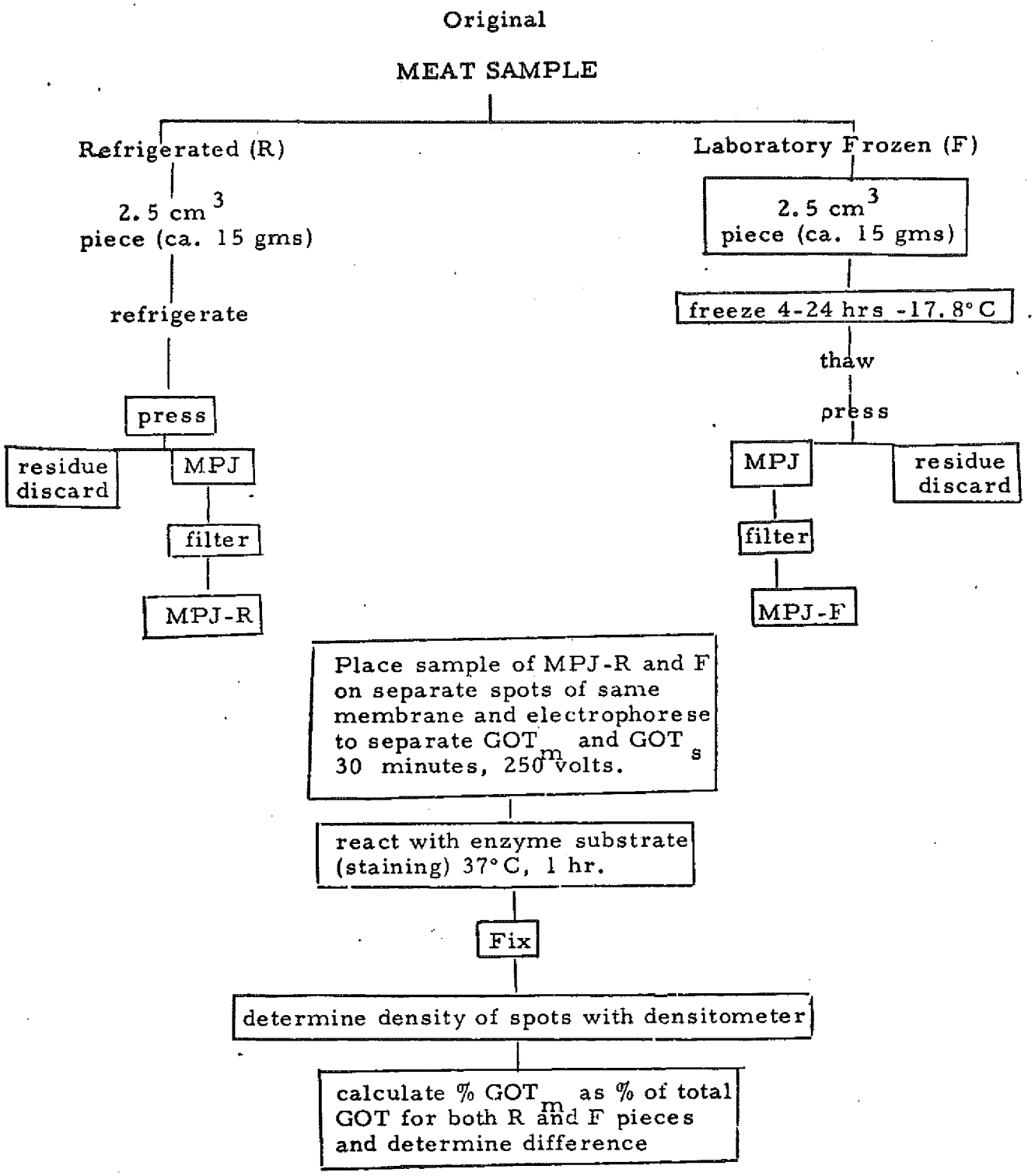
(f) Spot MicroZone membrane with ca. 0. 5μl each of the two MPJs and electrophorese for 30 minutes at 250 volts to separate GOT_m and GOT_s.

(g) Remove membrane, cut off excess ends, invert and place on top of the prepared substrate/agar/dye plate being careful to remove any air bubbles.

(h) Invert plate and place in 37°C incubator for one hour to allow the isozymes to react and produce color proportional to the amount of each isozyme present.

(i) Remove membrane, wash with distilled water to remove any adhering agar, fix color in 5% acetic acid and dry between two layers of filter paper.

(j) Use densitometer to measure color developed corresponding to the amount of GOT_m and GOT_s in the two MPJs and record the results in Table 13.



Interpretation: if the difference in GOT_m between the original (R) and the frozen-and-thawed (F) sample is greater than 50%, the original unknown sample is fresh, if less than 50%, it is to be considered as having been previously frozen.

Figure 7. Scheme of standardized procedure to determine if original meat sample has been frozen-and-thawed.

(k) If MPJ from the frozen-and-thawed sample has at least 50% more GOT_m activity than MPJ from the original sample, then the meat had not been previously frozen.

RECOMMENDATIONS FOR TESTING

As the meat samples would need to be evaluated near the point of initial procurement, it is recommended that a clinical laboratory be used for testing.

SUGGESTIONS FOR FURTHER WORK

1. The 50% increase in GOT_m from fresh meat following laboratory freezing may need further refinement, after full-scale field testing.
2. In the future, if a specific inhibitor of GOT_s is found, the GOT_m activity could be determined using the standard totoal GOT assay, thus eliminating the time-consuming electrophoretic, staining and densitometric procedures.
3. An objective definition of frozen meat should be established. The minimum time and temperature below $0^{o}C$ that will cause at least a 50% increase in GOT_m is presently unknown. This should be known to adequately classify chill-packed meat.
4. With refinement in the method and accumulated field data including: species and muscle variations and ranges of GOT_m for both fresh and frozen-and-thawed meat, it should be possible to classify meat on the basis of only the original sample without the necessity of freezing-and-thawing a sample in the laboratory. Therefore, by knowing the species, muscle, and percent GOT_m it should be possible to fit the sample into either the fresh or the frozen-and-thawed established range. Of course, if a conflict should arise, retesting would be warrented.

Table 1. Showing Percent GOT_m in Fresh Muscle Samples and the Effects of Freezing at Different Temperatures Upon Percentage Increase in GOT_m Activity.

Beef Round Samples	GOT_m Activity in Fresh Sample as Percent of Total GOT Activity	Percent Increase in GOT_m Activity (Column 2) Caused by Freezing		
		-17.8°C	-34.4°C	LN_2
1 [1/]	21.3	73	54	78
2 [2/]	21.4	68	78	89

[1/] Frozen for 4 hours at -17.8°C and -34.4°C.

[2/] Frozen for 48 hours at -17.8°C and -34.4°C.

Table 2. Showing Percent GOT_m in Fresh Muscle Samples and the Effects of Freezing at Different Temperatures Upon Percentage Increase in GOT_m Activity.

Pork Loin Samples	GOT_m Activity in Fresh Sample as Percent of Total GOT Activity	Percent Increase in GOT_m Activity (Column 2) Caused by Freezing		
		-17.8°C	-34.4°C	LN_2
1 [1/]	20.0	69	60	97
2	18.8	65	75	96
3 [2/]	22.5	95	140	138

[1/] Frozen 48 hours.

[2/] Frozen 4 hours, Pale, Soft, and Exudative Quality.

Table 3. Showing Percent GOT_m in Fresh Muscle Samples and the Effects of Freezing at Different Temperatures Upon Percentage Increase in GOT_m Activity.

Lamb Loin Samples	GOT_m Activity in Fresh Sample as Percent of Total GOT Activity	Percent Increase in GOT_m Activity (Column 2) Caused by Freezing	
		-17.8°C.	LN_2
1 1/	19.1	63	90
2	21.4	80	
3	19.4	90	76

1/ Frozen 48 hours.

Table 4. Showing Percent GOT_m in Fresh Muscle Samples and the Effects of Freezing at Different Temperatures upon Percentage Increase in GOT_m Activity.

Chicken Breast Samples	GOT_m Activity in Fresh Sample as Percent of Total GOT Activity	Percent Increase in GOT_m Activity (Column 2) Caused by Freezing		
		-17.8°C	-34.4°C	LN_2
1 [1/]	16.4	74	81	124
2	9.5	144	160	301
3 [2/]	13.1	144	173	218
4 [3/] a	11.1	214		
b	12.9	150		
5	11.3	185		
6	11.9	203		
7	11.7	180		
8	12.0	204		

[1/] Birds 1 and 2 held 48 hours at -17.8°C and -34.4°C.

[2/] Bird 3 held 4 hours at -17.8°C and -34.4°C.

[3/] Birds 4-8 held at -17.8°C.

Table 5. Showing Percent GOT_m in Fresh Muscle Samples and the Effects of Freezing at Different Temperatures Upon Percentage Increase in GOT_m Activity.

Turkey Breast Samples	GOT_m Activity in Fresh Sample as Percent of Total GOT Activity	Percent Increase in GOT_m Activity (Column 2) Caused by Freezing		
		-17.8°C	-34.4°C	LN_2
1 1/	19.2	60	81	113
2	21.8	57	47	67
3	23.1	65	62	93
4 2/ a	17.7	78		
b	16.0	100		
5 a	9.5	174		
b	11.7	109		
6	13.7	78		
7	13.4	104		
8 3/	15.5	83		
9 4/	15.5	137		

1/ Samples 1-3--Frozen 48 hours.

2/ Samples 4-7--Frozen 36 hours.

3/ Sample 8--Frozen 4 hours.

4/ Sample 9--Frozen 6 hours.

Table 6. Summary of Results on GOT_m Values for Fresh and Frozen-and-Thawed Meat.

Species	Number of Samples	Range for GOT_m as Percent of Total GOT		Average Percent Percent	Minimum Percent increase observed for any fresh sample frozen at -17.8°C [1]
		Fresh	Frozen		
Beef	2	21	36-37	71	68
Pork	3	19-23	31-44	76	65
Lamb	3	19-21	33-39	81	73
Chicken	9	10-16	23-36	166	74
Turkey	11	10-23	24-38	95	57

[1] The minimum percent increase observed is the critical finding. The nonfrozen and frozen range and the average percent increase only shows that the meat responds similarly to freezing.

Table 7. Percent Increase in GOT_m Following Freezing at -17.8°C for Varying Periods of Time

	Original	4 hr	6 hr	36 hr	72 hr	96 hr
1. Turkey Breast	15.5 [1]	83	137		148	154
	15.4					
2. Turkey Breast	13.4			104		
3. Whole Fryer	19.3	63			115	

[1] Percent GOT_m in control (original) sample.

Table 8. Effect of Time After Slaughter at 2.2°C Upon Percent GOT_m in MPJ

A.*	1 day	11.7
B.	11 days	10.2, 9.2

*Right and left Pectoralis major muscles of same fryer.

Table 9. Percent GOT_m in Previously Frozen-and-Thawed (Original) and Refrozen[1/] Poultry Samples

	Original	Refrozen	Exudate
Fryer Breast[2/]	37	44 (19)[6/]	
Turkey Breast[2/]	27	29 (7)	
Fryer Breast[3/]	25	28 (11)	
Turkey Leg[4/]	40	43 (8)	
Turkey Leg[4/]	44	44 (0)	42[5/]

[1/] Refrozen 24 hrs -17.8°C

[2/] Frozen 48 hrs -17.8°C

[3/] Frozen 9 mos -17.8°C

[4/] Frozen 17 mos -17.8°C

[5/] Composite exudate obtained from package after thawing the above two leg samples following 17 mos storage.

[6/] Percent increase in GOT_m

Table 10. Percent GOT$_m$ Activity in Low Temperature Transported Fryers (Chill-Packed)[1/] from Texas

Bird No.	Original	Frozen-and-Thawed[2/]	Percent Increase
1	12.7[3/]	29.1	129
2	17.2	31.8	85
3	13.5	24.8	84

1/ Temperature 0°C at Laboratory.

2/ Frozen -17.8°C for 24 hours.

3/ Average of two muscle samples except for the frozen-and-thawed Sample for Number 2.

Table 11. Percent GOT$_m$ Activity in Low Temperature Transported Chicken Breasts (Chill-Packed)[1/] from Texas

Breast No.	Original	Frozen-and-Thawed	Percent Increase
1	15.8[3/]	32.0	103
2	26.5	30.9	17
3	8.9	23.2	161

1/ Temperature -1.5 to -2.0°C at Laboratory.

2/ Frozen -17.8°C for 24 hours.

3/ Average of two muscle samples.

Table 12. Percent GOT_m in Exudate from Fresh and Frozen-and-Thawed Chicken

Fresh[1/]	16.2
Frozen-and-thawed[1/]	31.9
Frozen-and-thawed whole body fryer	37.1

[1/] Composite exudate from breast pieces of 7 chickens.

Table 13. Example of Data Sheet That Could Be Used in Sample Evaluation.

Sample	Code	Integrator reading GOT_s	Integrator reading GOT_m	Integrator reading Total	% GOT_m	% Increase GOT_m	Circle Classification
1. Orig.	C-1[1/]	18,867	2,563	21,430	12.0 [2/]	XXXXXXXXXXXXXXXXXXXXXXXXX	
F + T	C-2	18,851	10,818	29,669	36.5	204.2%	(Fresh,) F + T
2. Orig.	t-1	34,089	12,476	46,565	26.8	XXXXXXXXXXXXXXXXXXXXXXXXX	
F + T	t-2	34,717	14,616	49,333	29.6	10.4%	Fresh, (F + T)
3. Orig.						XXXXXXXXXXXXXXXXXXXXXXXXX	
F + T							Fresh, F + T

[1/] Actual values for fresh chicken and previously frozen-and-thawed turkey.

[2/] Calculation:

$$\frac{\left(\frac{GOT_m}{GOT_s + GOT_m}\right)^{F+T} - \left(\frac{GOT_m}{GOT_s + GOT_m}\right)^{Fresh}}{\left(\frac{GOT_m}{GOT_s + GOT_m}\right)^{Fresh}} \times 100 = \%\ GOT_m \text{ increase between fresh and frozen-and-thawed samples}$$

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TABLES