Japan Customs Analysis Methods

No. 124

Double Staining Test Method of Protein and Fat in Cheese

(Issued in May, 2002)

(Updated in May 2004)

1. Scope

This test method is applied to products which are required to confirm the coagulation states of their proteins by comparing with the protein-fat structure of standard cheese, due to questions regarding their classifications as cheese of heading 04.06 in the Customs Tariff Law (Appendix Table–Customs Tariff Schedule).

However, this method is not applied to products clearly classified in other headings such as butter, dairy spreads, etc. (water-in-oil emulsions) listed in heading 04.05 in the Customs Tariff Law (Appendix Table–Customs Tariff Schedule).

2. Outline of Test Method

In this method, a microscopic observation for the protein/fat structure of a sample is carried out to be compared with those of standard cheese. The procedure is as follows.

(1) Freeze and enclose sample into resin.
(2) Prepare frozen sections of the sample with a freezing-microtome.
(3) Perform a double staining for protein (blue) and fat (red) in the prepared sections.
(4) Observe the structure of protein and fat in the sections with a microscope.

3. Apparatuses

(1) Slide glass
Silane coated (APS coated).

(2) Freezing-microtome
Housing for slicing sections can be cooled down to -30 ºC.

(3) Transmission optical microscope
With magnification of 100–400 times.

4. Reagents

(1) Baker's calcium-formalin solution (10% formalin/1% calcium chloride aqueous solution)
Mix 15 mL of formalin (36% formaldehyde aqueous solution), 1.5 g of calcium chloride dehydrate and 135 mL of deionized water [(1)].

(2) 10% acrolein solution
Mix acrolein with 0.1 M phosphoric acid buffer (pH7.4) with a ratio of 1:9 and stir it using a glass rod. [(2)]

(3) Thionin-Schiff reagent (protein staining solution)
Add 0.2 g of thionin acetate to about 100 mL of deionized water [(1)] and boil it for 5 min. After cooling down to room temperature, add deionized water [(1)] equivalent to the amount of water evaporated. Add 100 mL of 2-methyl-2-propanol (*tert*-butyl alcohol), 30 mL of 1 mol/L-hydrochloric acid and 2 g of sodium disulfite to the thionin solution and stir it sufficiently. [(2)]

Tightly plug the container and leave it in a dark place at room temperature for a night and filtrate it with a paper filter (JIS No.1). It can be preserved in a dark and cool place for about one week. Return the solution in room temperature before use.

(4) Oil red O reagent (fat staining solution)
Prepare oil red O saturated solution by adding 2-propyl alcohol to oil red O so that the

concentration of oil red O becomes 0.3% or more, mixing and leaving it at 60 ºC for one night. Prepare 1% (w/w) dextrin aqueous solution by heating it at 60 ºC for one night for complete dissolution. Mix the oil red O saturate solution and the 1% dextrin solution with the volume ratio of 3:2, and filtrate it with a paper filter (JIS No.131) while the solution keeps warm. Prepare the reagent one hour before use.

(5) 60% (v/v) 2-propyl alcohol aqueous solution.

(6) Water soluble resin encapsulant

Without affinity with thionin (blue) and oil red O (red).

(7) Tissue freezing medium (compound for embedding sample in a freezing temperature)

Use commercial compound for freezing-microtome.

(8) Coolant for freeze-embedding

Liquid nitrogen or dry ice in acetone.

Note 1) Use deionized water but not distilled water when specified to use deionized water.

Note 2) Prepare this solution in a draft chamber.

5. Procedure

5.1. Preparation of samples for microscopic observation

After freezing and fixing samples in a freezing medium, prepare samples for microscopic observation by making sections with a microtome and conducting double staining of them with the two staining reagents. Detailed experimental procedures are described below.

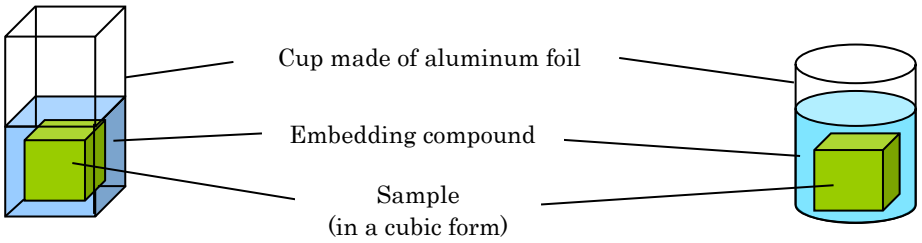
5.1.1. Freezing and fixation of samples

Make a cup of about 1 cm × 1 cm × 2 cm with aluminum foil.[3] Add tissue freezing medium into the cup up to about 2 mm from the bottom. Then put a sample of a 5-8 mm cube (which is prepared by cutting a central part of the bulk sample out) into the cup and add more tissue freezing medium so that the inside sample cube is fully covered (see the figure).[4] After cooling rapidly with the coolant for freeze embedding,[5] preserve it in a freezer at -20ºC or below.

Note 3) A UV/VIS spectrophotometer cell is suitable for molding. Pens such as felt-tip pens with a diameter of 1.5 cm are also available to form a cylindrical shape cup.

Note 4) Place sample cube at the center and do not let it touch against the interior wall.

Note 5) First, soak the lower half of the cup in the coolant for freeze-embedding. Then, just before complete solidification of the embedding compound, sink the cup down into the coolant for 2-3 seconds. Soak the cup completely in the coolant for a few seconds repeatedly. In case of samples having many cracks, leave them for 20-30 min before soaking the cup in the coolant in order to help the embedding compound penetrating into the sample.



Figure

5.1.2. Preparation of sections

Remove the aluminum foil from the sample prepared in 5.1.1, fix it with a sample holder and cut out most of the frozen embedding-compound with a knife. Then, cut off sections with a thickness of 10 µm with a freezing-microtome[6] set at -20 ºC.[7] Adhere the sections

onto a glass slide, and then leave it at room temperature for 15 min or more to air dry.[8]

Note 6) The cutting angle should be adjusted from 0 to 5 °

Note 7) . For samples with higher fat content, set the freezing-microtome at 5-10 ºC lower.

Note 8) Air dried for about 2 hours would be allowed.

5.1.3. Double staining

After dipping the glass slide adhering sections prepared in 5.1.2 in Baker's calcium-formalin solution for 30 min at room temperature, wash it with running water for 5 min. Fixate the sections' structures by dipping the glass slide in 10% acrolein solution at room temperature for 60 min[9] and then dip it in deionized water[1] for 5 min. After replacing the deionized water to a new one[1], repeat the same procedure twice.[10]

Dip the glass slide in the thionin-Schiff reagent (protein staining solution) at room temperature for 30 min to stain protein blue. Immediately after taking the glass slide off, wash it with running water for 20 min and rinse it with deionized water. [1]

Next dip the glass slide in 60% (v/v) 2-propyl alcohol aqueous solution which has been pre-heated at 37 ºC for 1 min. Then dip it in the oil red O reagent (fat staining solution) at 37 ºC for 15 min to stain fat red. Dip the glass slide in 60% (v/v) 2-propyl alcohol aqueous solution at 37 ºC again for 2 min. Then, wash it with running water for 5 min and rinse it with deionized water [1].

Drop water-soluble sealing resin on the stained sections, put a cover glass on the sections for microscopic observation.

Note 9) Handle it in a draft chamber.

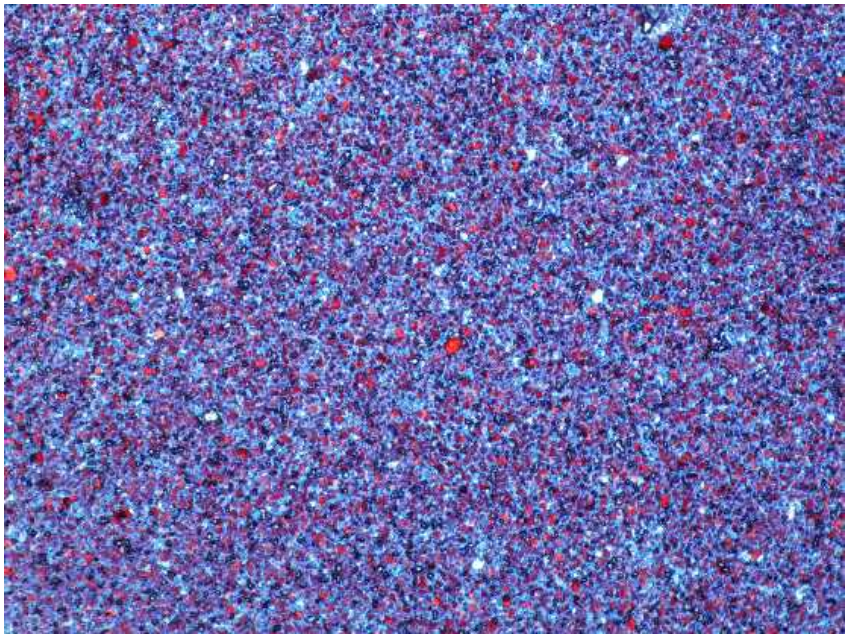
Note 10) Wash out and remove the acrolein solution completely.

5.2. Observation with a microscope

Observe the stained sections prepared in 5.1 with a microscope under low and high magnifications. Using sections of standard cheese (Gouda, Camembert, cream, etc.) prepared in the same manner as the reference, compare the structures and confirm whether the observed sample shows characteristics of cheese structures.

(Reference) the structural characteristics of "cheese": Proteins (stained blue) form a network structure without isolation and are distributed surrounding fat globules (stained red).

(Examples of Pictures)



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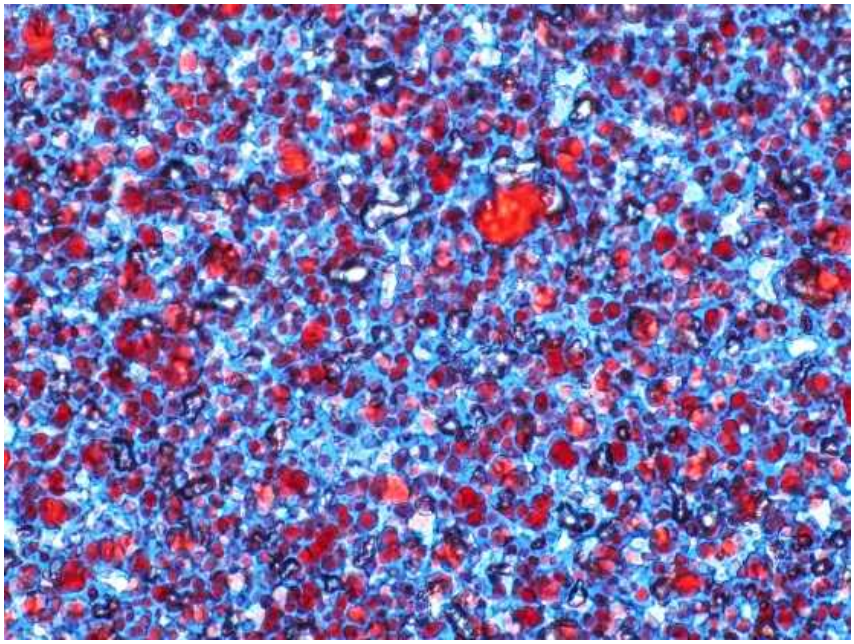

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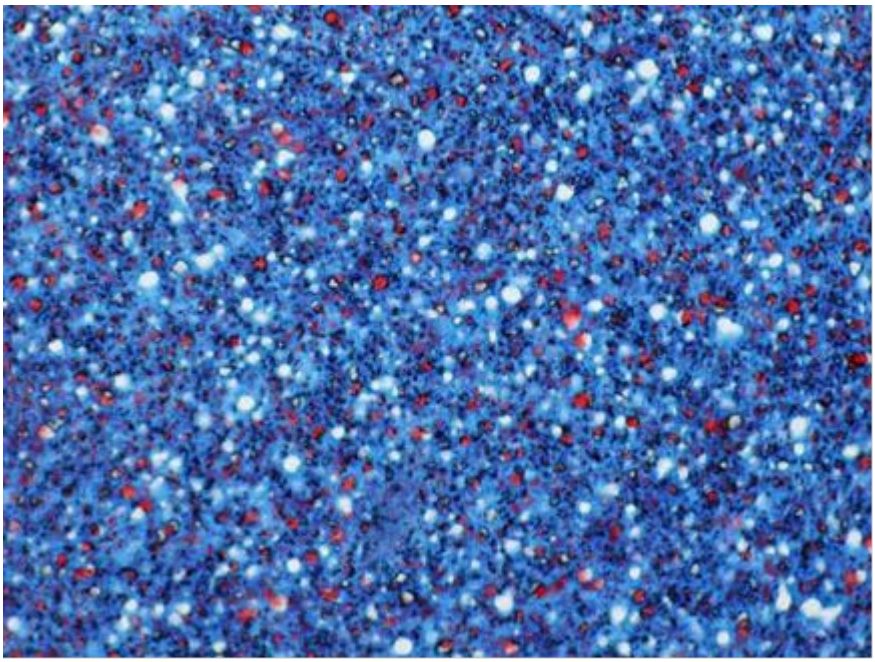
Figure 1 Photomicrographs of Gouda Cheese



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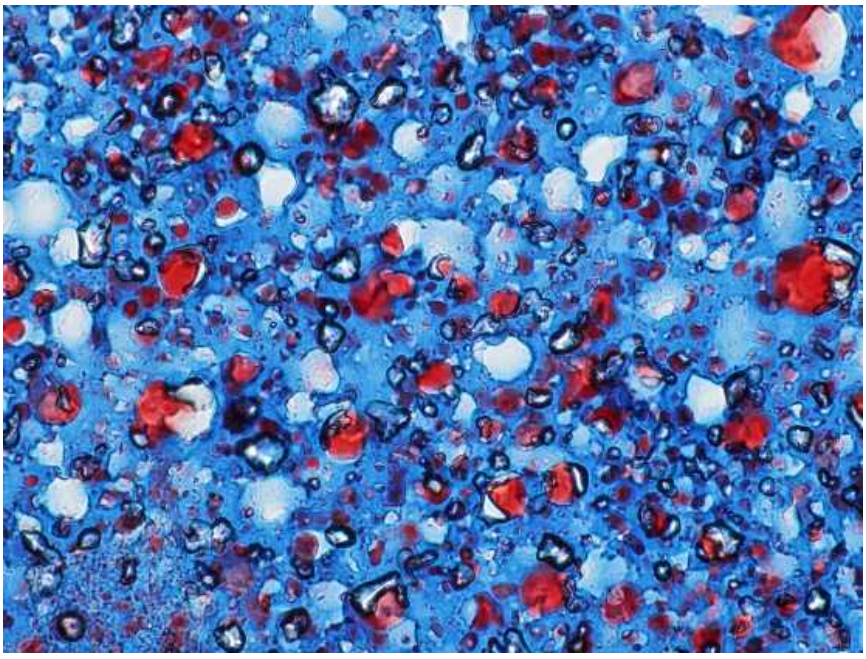

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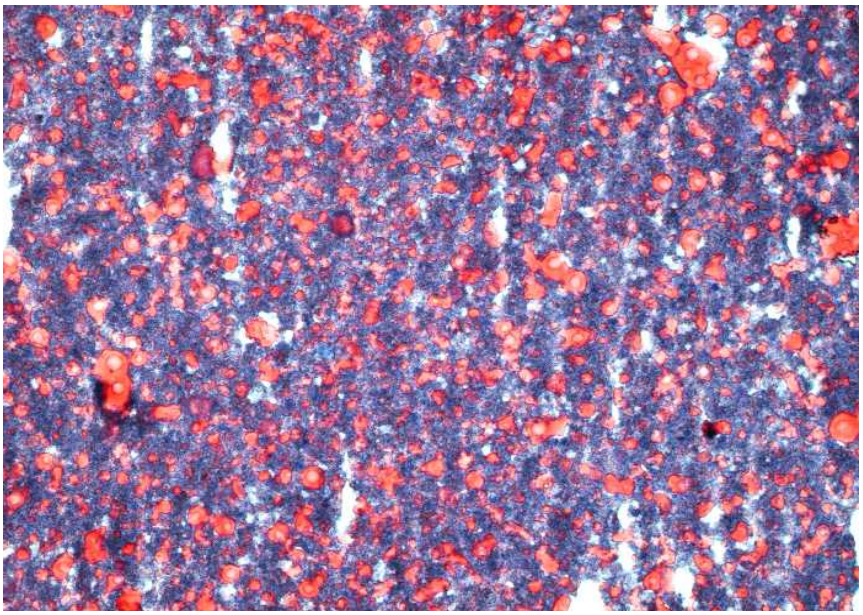
Figure 2 Photomicrographs of Camembert Cheese



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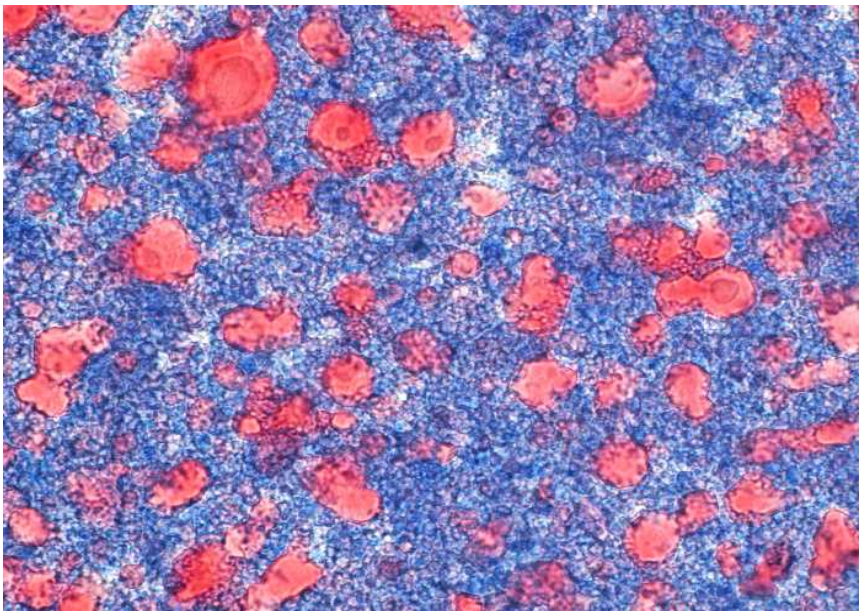

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Figure 3 Photomicrographs of Cream Cheese

Note) Numerical values under photos denote optical magnifications used.

6. References

(1) Naganawa T., Watanabe K., Kanzaki B., Ohta T., Nijikawa K., Hosono A., Yamaguchi T. (2002) Milkscience, **51**: 33 (in Japanese).