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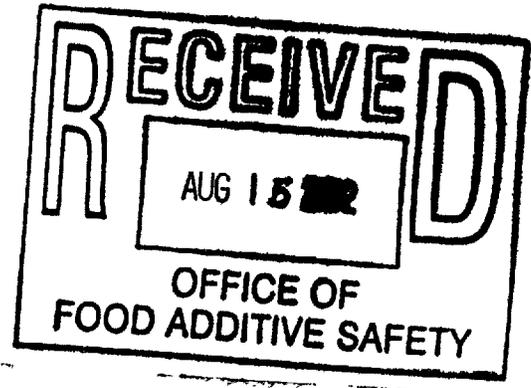
ENZYME TECHNICAL ASSOCIATION

1800 Massachusetts Avenue, NW, 2nd Floor
Washington, DC 20036-1800

Telephone (202) 778-9335
Fax (202) 778-9100
www.enzymetechnicalassoc.org

August 5, 2002

Dockets Management Branch
Office of Premarket Approval (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street SW
Washington, DC 20204



**RE: GRAS Notification – Exemption Claim for Lipase Enzyme
Preparation Produced by *Aspergillus niger* that is the Subject
of a GRAS Affirmation Petition, GRASP 3G0016**

Dear Sir or Madam:

Pursuant to proposed 21C.F.R. §§ 170.36(c)(1), 170.36(g)(2), and the Food and Drug Administration ("FDA") preamble discussion concerning the submission of a Generally Recognized As Safe ("GRAS") notification based on a previously filed GRAS affirmation petition, 62 Fed. Reg. 18938, 18953-18954 (April 17, 1997), the Enzyme Technical Association is hereby providing FDA with notice that it has determined, based on scientific procedure, that a lipase enzyme preparation from *Aspergillus niger* (*A. niger*) as a direct human food ingredient is GRAS and therefore exempt from statutory premarket approval requirements. The enzyme from the *A. niger* source organism is also the subject of a GRAS Affirmation Petition 3G0016, which was based on history of use and submitted by the Ad Hoc Enzyme Technical Committee (now known as the Enzyme Technical Association ("ETA")) to the FDA in 1973.

The following information is provided in accordance with the proposed regulation.

Proposed § 170.36(g)(2)(i): Name and address of the notifier.

Enzyme Technical Association
c/o Gary L. Yingling
1800 Massachusetts Avenue, N.W.
Second Floor
Washington, DC 20036

Proposed § 170.36(g)(2)(ii): The applicable GRAS affirmation petition number.

A GRAS Affirmation Petition for animal-derived, plant-derived, and microbially-derived enzyme preparations was originally submitted by the Ad Hoc Enzyme Technical Committee (now known as ETA) and assigned a petition number, GRASP 3G0016. The FDA filed GRASP 3G0016 on April 12, 1973 (38 Fed. Reg. 9256). The petition was amended on June 12, 1973 (38 Fed. Reg. 15471), August 29, 1984 (49 Fed. Reg. 34305), and June 23, 1987 (52 Fed. Reg. 23607) to include other plant-derived and microbially-derived enzyme preparations. This notification addresses only the lipase enzyme preparation from *A. niger* named above for which FDA action is pending.

Proposed § 170.36(g)(2)(iii): The common or usual name of the substance (i.e., the notified substance).

Lipase from *Aspergillus niger*.

Proposed § 170.36(g)(2)(iv): Applicable conditions of use.

As discussed in greater detail in GRASP 3G0016 as amended, the lipase enzyme preparation is a direct human food ingredient. The use of the enzyme preparation is for interesterification of fats and oils in the following food products: dairy based flavoring preparations, cheeses, liquid and dried egg white, bread, flour, unstandardized bakery products, modified triglycerides, hydrolyzed lecithin, edible fats and oils, and modified egg yolk.

The enzyme preparation is GRAS for use in food at levels not to exceed Good Manufacturing Practices ("GMPs").

The data and information to support the above uses are contained in GRASP 3G0016, as amended, and the information filed in the attached notification.

Proposed § 170.36(g)(2)(v): Basis for GRAS determination.

The basis for this GRAS determination is through experience based on scientific procedure.

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Proposed § 170.36(g)(2)(vi): Availability of information.

The complete record that supports the GRAS determination has been submitted to the agency in the above referenced GRASP 3G0016, as amended, and the attached notification. Therefore, the complete file is at FDA.

Sincerely,

Jack Harris, Chair
Enzyme Technical Association

Attachments

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A Lipase Enzyme Preparation Produced by *Aspergillus niger*

A. Introduction

The subject of this notification is a lipase enzyme preparation derived from a pure culture fermentation of a nonpathogenic, nontoxic strain of *Aspergillus niger*. This lipase may be formulated as a liquid or dry product and is intended for use as a processing aid in the following food applications: edible fats and oils, dairy based flavoring preparations, cheeses, liquid and dried egg white, bread, flour, unstandardized bakery products, hydrolyzed lecithin, and modified egg yolk.

Triacylglycerol acylhydrolases (lipases, EC 3.1.1.3) catalyze the hydrolysis of ester bonds. The lipase of *A. niger* specifically cleaves the 1- or 3- position of the glycerol component. The reaction products of the *A. niger*-derived lipase are a mixture of mono- and diglycerides and free fatty acids (MacRae, 1983). The reaction catalyzed by this lipase is reversible and, therefore, under appropriate conditions, the lipase can catalyze the synthesis of triglycerides from a mixture of glycerides and free fatty acids.

The information in the following sections is the basis for our determination of general recognition of safety of a lipase enzyme preparation produced by *A. niger*. Our safety evaluation includes an evaluation of the production strain, the enzyme, and the manufacturing process as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001). *A. niger* meets the criteria for nontoxigenicity and nonpathogenicity. *A. niger* has a long history of safe industrial use, is widely distributed in nature, and is commonly used for production of food grade enzymes (Shuster *et al.*, 2002). When the lipase enzyme preparation is manufactured, neither the source organism nor the manufacturing process will introduce impurities into the preparation that may render it unsafe. In addition, the dietary exposure to the lipase preparation does not present a basis for concern about its use.

B. Identity

1. Source
The lipase enzyme preparation is derived from a pure culture of a nonpathogenic, nontoxic strain of *Aspergillus niger*.
2. Chemical Name- Triacylglycerol acylhydrolase
EC Classification- 3.1.1.3
3. Common or usual name- Lipase

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4. CAS Registry Number – 9001-62-1
5. **Properties**

Triglycerides are fats or oils comprised of fatty acids linked by ester bonds to each of the three hydroxyl groups of glycerol. Triacylglycerol acylhydrolases (lipases) catalyze the hydrolysis of these ester bonds. The lipase of *A. niger* specifically cleaves the 1- or 3- position of the glycerol component. The reaction products of the *A. niger*-derived lipase are a mixture of mono- and diglycerides and free fatty acids (MacRae, 1983). The reaction catalyzed by this lipase is reversible and, therefore, under appropriate conditions, the lipase can catalyze the synthesis of triglycerides from a mixture of glycerides and free fatty acids.
6. **Composition**

Like most fungal-derived enzyme preparations used in food processing, the lipase enzyme preparation derived from *A. niger* is not chemically pure, but contains, in addition to the enzyme component, other compounds that derive from the production organism and the fermentation media, residual amounts of processing aids, and substances used as stabilizers, preservatives, or diluents. The *A. niger*-derived lipase is produced using fermentation ingredients and processing aids that are substances acceptable for general use in foods. The lipase enzyme preparation may be formulated as a liquid or dry product.

C. Manufacturing Process

The lipase enzyme preparation from *A. niger* is manufactured according to procedures outlined by Pariza and Foster (1983) using standard microbial enzyme production technology (Aunstrup, 1979; Aunstrup *et al.*, 1979; Enzyme Applications, 1994) and according to current good manufacturing practice (cGMP).

1. **Raw materials**

The fermentation ingredients used in the manufacture of lipase enzyme preparations are substances that are acceptable for general use in food.
2. **Fermentation process**

The large-scale growth of *A. niger* for the production of lipase can be performed using a liquid medium in a submerged fermentation or the microorganism may be grown on a solid or semi-solid medium held in large trays or drums. With either production method, environmental factors such as pH, temperature, and aeration are controlled. During growth, fermentors are routinely sampled and tested for possible contamination. Should evidence of a significant contamination exist, the batch is rejected.

3. Recovery process
The recovery process is a multi-step operation that begins immediately following the fermentation step and involves both the purification and concentration of the product.
4. Formulation
Stabilizers, diluents, and/or preservatives that are suitable for general use in food may be added to the lipase enzyme preparation from *A. niger*. The lipase enzyme preparation may be formulated as a liquid or dry product. The product is standardized according to the product specifications.

D. Specifications

The *A. niger*-derived lipase preparation meets the general and additional requirements for enzyme preparations in the monograph on Enzyme Preparations in the *Food Chemicals Codex* (National Research Council, 1996). The lipase assay can be performed using the method attached in Appendix 1 or by any appropriate validated method.

E. Application

1. Mode of action
The lipase derived from *A. niger* catalyzes the interesterification of fats and oils at the 1- and 3- positions of triglycerides to produce a mixture of mono- and diglycerides and free fatty acids (MacRae, 1983) .
2. Foods in which used
A niger-derived lipase enzyme preparations can be used for the interesterification of fats and oils in the following food products: dairy based flavoring preparations, cheeses, liquid and dried egg white, bread, flour, unstandardized bakery products, modified triglycerides, hydrolyzed lecithin, edible fats and oils, and modified egg yolk.
3. Level of use
The lipase enzyme preparation is used in food at minimum levels necessary to achieve the desired effect and according to cGMP.
4. Enzyme residues in final food
Lipase enzyme preparations are generally used as processing aids and are not added directly to food. Residues in food of the lipase derived from *A. niger* will be similar to that of the *Rhizopus niveus* lipase, which has been affirmed as GRAS by the FDA. In some applications, such as interesterification of fats and oils, the enzyme will be inactivated and removed from the final product during oil processing. In other applications such as baking, the enzyme will be inactivated during processing and some residual enzyme protein may remain in the final product.

F. Safety Evaluation

1. Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001). If the organism is nontoxic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (IFBC, 1990). Pariza and Foster (1983) define a nontoxic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. niger* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. niger* is not considered pathogenic by JECFA (1987).

Shuster *et al.* (2002) reviewed the safety of *A. niger* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes. *A. niger* is generally regarded as a nonpathogenic species (Shuster *et al.*, 2002). *A. niger* has been used since 1919 to produce citric acid (Shuster *et al.*, 2002; IFBC, 1990), a commodity chemical, widely used in both the food and pharmaceutical industries. The Food and Drug Administration (FDA) lists *A. niger* as a safe source of citric acid (21 CFR §173.280). In GRN #89, ETA determined non-pathogenic, non-toxicogenic strains of *A. niger* to be safe hosts for the production of carbohydrase, pectinase, protease, glucose oxidase and catalase based on common use in food. Gluconic acid and fumaric acids, though of less economic importance, have also been produced using *A. niger*.

Since at least the 1950s, *A. niger* has become the source of a number of commercially important food enzymes, including glucoamylase, pectinase, hemicellulase, protease, glucose oxidase, and catalase (Beckhorn *et al.*, 1965; Frost and Moss, 1987; Pariza and Johnson, 2002). Carbohydrase and cellulase derived from *A. niger* is approved by the FDA for use in clam and shrimp processing (21 CFR §173.120). *A. niger* is a source of GRAS carbohydrase, catalase, glucose oxidase, pectinase, and protease enzyme preparations (GRN #89). Further, FDA lists *A. niger* as a source of enzymes it recognized as GRAS in opinion letters issued in the early 1960s (www.cfsan.fda.gov/~dms/opa-enzy.html; Partial List of Enzymes Used in Foods-see compilation of microbially-derived enzymes which the FDA recognized as GRAS in opinion letters in the early 1960s; bottom of the web page).

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Several studies have been performed where the pathogenic potential of *A. niger* was evaluated. The results of these studies demonstrated that neither ingestion of large doses of spores (Nyireddy *et al.*, 1975) nor inhalation of spores (Bhatia and Mohapatra, 1969) induced mycosis in animals under the conditions of the test.

2. Enzyme

Enzyme proteins themselves do not generally raise safety concerns (FDA, 1992 and 1998; Pariza and Foster, 1983). As indicated in section B, the enzyme preparation is a triacylglycerol lipase, IUB EC 3.1.1.3, which hydrolyzes the primary ester bonds in triglycerides. Most of the lipases commonly used in food processing belong to this group (FDA, 1998).

Microbial lipases are reported to have been used in food production since 1952 (Beckhorn *et al.*, 1965; Rogers, 1977). Animal lipase is affirmed as GRAS (21 CFR 184.1415) based on its common use in food prior to 1958. Esterase-lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive (21 CFR 173.140). Lipase enzyme from *Rhizopus niveus* is affirmed as GRAS based on scientific procedures (21 CFR 184.1420). A petition was filed in 1989 proposing to affirm that insoluble esterase-lipase enzyme preparation derived from *Mucor miehei* which has been fixed by immobilization with a substance that is generally recognized as safe or an approved food additive is GRAS for use as a direct human food ingredient (54 FR 9565). Esterase lipase enzyme preparations from *Mucor miehei* have been marketed as GRAS since that time. Lipase preparations from genetically modified strains of *A. oryzae* are the subject of GRAS notifications GRN #43, #75, and #103. Finally, FDA issued an opinion letter in which the Agency recognized the GRAS status of lipase derived from *A. niger* (www.cfsan.fda.gov/~dms/opa-enzy.html; Partial List of Enzymes Used in Foods-see compilation of microbially-derived enzymes which the FDA recognized as GRAS in opinion letters in the early 1960s; bottom of the web page).

3. Manufacturing Process

The lipase enzyme preparation meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*. As described in Section C, the lipase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (Aunstrup, 1979; Aunstrup *et al.*, 1979, Enzyme Applications, 1994).

G. Analytical Methodologies

It is recommended that the *A. niger* lipase enzyme preparation be assayed using the assay provided in Appendix 1. This assay is based on the measurement of the amount of free fatty acid formed from an olive oil emulsion in the presence of sodium taurocholate over a fixed time interval. Other appropriate validated methods may also be used (National Research Council, 1996).

H. Exposure

The estimated dietary exposure to *A. niger*-derived lipase preparation for the proposed use as an enzymatic catalyst in the interesterification of fats and oils will be similar to that of the *R. niveus* lipase preparation (FDA, 1998). In the GRAS affirmation notice for *R. niveus*, the FDA stated that it determined the predominant source of potential exposure to the total organic solids in the enzyme preparation to be baked goods that use interesterified fats at levels up to 30 percent. However, since the enzyme is removed from the food product following catalysis, no detectable *R. niveus* lipase remained in the interesterified product.

In other applications, such as egg yolk modification, where the enzyme can be added directly to the food ingredient and may not be removed, there may be minimal exposure to inactivated enzyme protein.

Like other enzymes used as processing aids, the enzyme of this submission will be used at very low levels. Thus in all cases, we conclude that the dietary exposure to the *A. niger* lipase enzyme preparation is negligible and therefore does not present a basis for concern with its use.

I. Conclusions

The evidence provided in this document shows that the lipase enzyme preparation derived from *A. niger* will achieve its intended technical effect and that the enzyme component of the lipase enzyme product is similar in function to other lipases that are used in food to catalyze the hydrolysis of ester bonds at the 1- or 3- positions of the glycerol component of a triglyceride. Further, the basic conclusions and guidelines for determining the safety of enzymes used in food and food processing as stated by Pariza and Foster (1983), reiterated by FDA (1998), and updated by Pariza and Johnson (2001) have been applied to this lipase enzyme preparation. When the lipase enzyme preparation is manufactured, neither the source organism (a nonpathogenic, nontoxigenic strain of *A. niger*) nor the manufacturing process will introduce impurities into the preparation that may render it unsafe. In addition, the dietary exposure to the lipase preparation does not present a basis for concern about its use.

We conclude, based upon the evaluation of published and unpublished data and information, and based upon scientific procedures (21CFR§170.30(b)), that the lipase enzyme preparation derived from *A. niger* is GRAS for its use as an enzymatic catalyst in the interesterification of fats and oils and as a processing aid in other food applications.

J. References

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K. List of Appendices

1. Method for Determining Lipase Activity

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**Institute of Medicine
Food and Nutrition Board
Committee on Food Chemicals Codex**

New General Method – Lipase (Microbial) Activity for Medium- and Long-Chain Fatty Acids

Please send comments to the Committee on Food Chemicals Codex/FO3042, Institute of Medicine, 2101 Constitution Avenue, N.W., Washington, DC 20418 or email them to fcc@nas.edu. All comments must be received by November 24, 2000, for consideration for the Third Supplement.

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July 3, 2000

Insert the following new general procedure in *Appendix V: Enzyme Assays*

Lipase (Microbial) Activity for Medium- and Long-Chain Fatty Acids

Application and Principle

This procedure is used to determine the lipase activity in preparations derived from microbial sources. The assay is based on the measurement of the amount of free fatty acids formed from an olive oil emulsion in the presence of sodium taurocholate over a fixed time interval. This assay is particularly used for measuring lipase activity in foods.

Reagents and Solutions

Acacia (Gum Arabic) Solution Dissolve 110 g of Acacia (gum arabic)(Sigma, Catalog No. G-9752, or equivalent) and 12.5 g of analytical-grade calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in 800 mL of water in a 1000-mL volumetric flask, and dilute to volume with water. Shake or stir for 30 min at room temperature to dissolve completely. Centrifuge at $4000 \times g$ for 20 min or filter through a Büchner funnel using Celite as a filter aid. Store the supernatant or filtrate at 4° . Divide into single-use, 24-mL aliquots. The solution is stable for 6 months at -20° .

Substrate Emulsion Place 130 mL of olive oil (Sigma, Catalog No. O-1500, or equivalent) and 400 mL of *Gum Arabic Solution* in a mixer bowl, and cool the mixture to 5° to 10° on ice.

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Emulsify the mixture with a Waring Blendor, or equivalent, operated at high speed for 30 min, keeping the temperature below 30° by repeatedly mixing at high speed for 5 min and turning the blender off for 1 min. Check the quality of the emulsion microscopically: 90% of the droplets should have a diameter equal to or less than 3 μm , and the remaining 10% should not exceed 10 μm . The emulsion is stable for 3 days at 4°.

Reference Standard Solution Dissolve an aliquot of Fungi Lipase-International FIP Standard (International Commission on Pharmaceutical Enzymes F.I.P., Center for Standards of the Federation Internationale Pharmaceutique, Harelbekestraat 72, B-9000 Gent, Belgium) in a 1% sodium chloride solution and dilute it to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per mL. Prepare this solution fresh.

0.02 N Sodium Hydroxide Solution Prepare daily by diluting 10 mL of analytical-grade 1 *N* sodium hydroxide to 500 mL with recently boiled water.

0.5% Sodium Taurocholate Solution Dissolve 0.5 g of sodium taurocholate (DIFCO, Catalog No. 0278-15-8) in 100 mL of water. Prepare this solution fresh.

Sample Preparation Dissolve an accurately weighed amount of the enzyme preparation in a 1% sodium chloride solution, and dilute to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per mL. Prepare this solution fresh.

Procedure (Note: Assay the Fungi Lipase-International FIP Standard as an internal standard each time.)

Automatic Titration Use an automatic titration device with a 25 mL \pm 0.02 mL buret, a pH meter giving a resolution to 0.01, and a reaction vessel with a capacity of 100 mL. Add 24 mL of *Substrate Emulsion*, 9 mL of water, and 2 mL of *0.5% Sodium Taurocholate Solution* to the reaction vessel. Place the reaction vessel in a water bath preheated to 37° \pm 0.5° over a hot plate provided with magnetic stirring, and add a magnet to the reaction vessel. Pre-incubate the reaction vessel at 37° \pm 0.5° for 10 to 15 min while stirring at about 300 rpm. Immerse a pH-electrode and the tip of the buret into the solution. If desired, gently blow nitrogen gas onto the solution. Adjust the pH of the solution to 7.0 with *0.02 N Sodium Hydroxide Solution*. Set the automatic buret to zero. Add 5.0 mL of the enzyme solution while simultaneously starting a timer. Maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (within 30 s) bring the pH to 9.0 by manually adding additional *0.02 N Sodium Hydroxide Solution*. Record the volume of

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0.02 N Sodium Hydroxide Solution consumed as N_1 . Run the test with a blank by setting up the titration in the same manner, except after adjusting the pH to 7.0 with 0.02 N Sodium Hydroxide Solution, set the automatic buret to zero, and maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (^{new} within 30 s) bring the pH to 9.0 (^{new} as before), and then add 5.0 mL of enzyme solution. Because the enzyme lowers the pH, return the pH to 9.0 by adding 0.02 N Sodium Hydroxide Solution. Record the volume of 0.02 N Sodium Hydroxide Solution consumed as N_2 . removed
↓
(exactly)

Manual Titration Follow the same procedure as with *Automatic Titration*, but keep the pH at 7.0 with 0.02 N sodium hydroxide from a 25-mL buret, demarked in 0.02 mL.

Calculation One unit of enzyme activity (FIP Unit) is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1 μ mol of fatty acid per min from the *Substrate Emulsion* under the described assay conditions. The specific activity is expressed in international FIP units per mg of the *Sample Preparation*.

The use of an enzyme reference standard of known activity, controlled by the Center for Standards of the Commission, eliminates difficulties from interlaboratory differences in quality of reagents such as the *Gum Arabic Solution*, olive oil, or *Substrate Emulsion* or in the set-up of the experiment. The activity (FIP U/mg) using an enzyme reference standard is calculated by the formula

$$(A \times C)/B,$$

in which A is the specific activity, in units/mg, of the test sample (measured); B is the specific activity, in units/mg, of Fungi Lipase-International FIP Standard (measured); and C is the number of FIP units/mg of Fungi Lipase-International FIP Standard as indicated on the container.

One mL of the 0.02 N Sodium Hydroxide Solution corresponds with the neutralization of 20 μ mol of fatty acids. Five mL of enzyme solution liberates $(N_1 - N_2)$ mL \times 20 μ mol of fatty acids over a 10-min time interval. If the enzyme solution contains W mg of enzyme preparation per mL, the specific activity, in units/mg, is calculated as follows:

$$[(N_1 - N_2) \times 20]/(10 \times 5 \times W),$$

in which $(N_1 - N_2)$ is the volume, in mL, of the *0.02 N Sodium Hydroxide Solution* used for the titration.

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SUBMISSION END

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