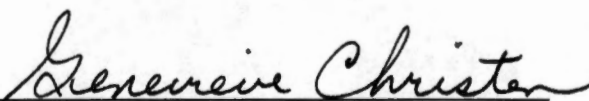
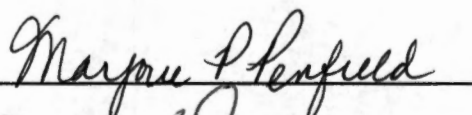
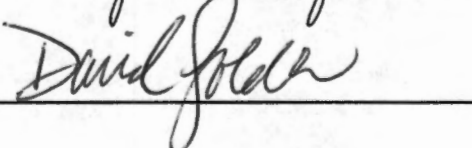
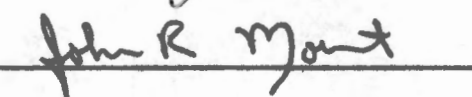


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I am submitting herewith a thesis written by Carolyn Susan Baird entitled "The Effect of Time and Temperature on Lipase Activity in Butter Oil, and Sensory Comparison of Milk Reference Samples Prepared by Three Methods." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.


Genevieve L. Christen, Major Professor

We have read this thesis and
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**The Effect of Time and Temperature on Lipase Activity in Butter
Oil, and Sensory Comparison of Milk Reference Samples
Prepared by Three Methods**

**A Thesis
Presented for the Master of Science
Degree
The University of Tennessee, Knoxville**

**Carolyn Susan Baird
May 1995**

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DEDICATION

This thesis is dedicated to my parents

Douglas D. and Nancy Ruth H. Baird

who have given me invaluable educational opportunities, love and support

and to my children

Ryan A. and Bailey M. Linderman

for their love, patience and understanding.

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ABSTRACT

Research was conducted in four phases: In Phase I, activity of four lipases at four incubation temperatures over a 60-min incubation time was evaluated; In Phase II, the same four lipases were evaluated for 60 min at an apparent optimal incubation temperature as determined in Phase I; In Phase III, milk, prepared from skim milk and butter oil hydrolyzed with the lipases, was evaluated for lipolyzed flavor by a dairy flavor expert; In Phase IV, the lipase identified in Phase III that gave the most consistent and characteristic lipolyzed flavor to butter oil was used to make recombined milk. This milk was then compared by a sensory evaluation panel to lipolyzed milk prepared using two other methods.

In Phases I and II, butter oil was hydrolyzed with four lipases at four incubation temperatures and analyzed using two methods: extraction-titration and gas chromatography (GC). The temperature needed to achieve a specific level of free fatty acids (FFA) was evaluated for the four lipases. After evaluating all lipases at 35, 40, 45 and 50°C, one apparently optimal incubation temperature was chosen for each lipase. These were 45°C for porcine pancreas, 35°C for *P. roqueforti*, 45°C for *A. niger* and 50°C for *R. miehei*. A general trend was evident for all lipases: as time increased, cumulative FFA released in butter oil by the lipases increased. The amount of activity varied with lipase and temperature. Pancreatic lipase activity was found to be reproducible at 45 and 50°C in one phase, but not in the other; *R. miehei* activity was found reproducible at 50°C only in both phases; and activity for *P. roqueforti* and *A.*

niger was not reproducible at any temperature. In GC analysis, pancreatic lipase and *P. roqueforti* were found to release more short-chain fatty acids (C4 - C12) ($P < 0.05$), while *A. niger* and *R. miehei* released more long-chain fatty acids (C14 - C18:3).

In Phases III and IV, recombined, 2% milk was prepared by combining skim milk and butter oil that had been hydrolyzed with lipases from porcine pancreas, *P. roqueforti*, *A. niger* and *R. miehei*. These milk samples were evaluated by a dairy flavor expert for characteristic lipolyzed flavor. Chemical analyses (extraction-titration and GC) were conducted on the milk samples. Pancreatic lipase was found to give the most consistent and characteristic lipolyzed flavor of all the lipases evaluated.

Lipolyzed milk, prepared by the three methods, was then evaluated by an experienced sensory panel using a paired comparison test. The methods included the current standard method (Bodyfelt et al., 1988), a modified version of the standard method (combining raw and recombined milk), and an experimental method (recombined milk consisting of skim and hydrolyzed butter oil). Panelists found a significant difference ($P < 0.05$) between samples from the standard method and the other two methods, however no significant difference ($P > 0.05$) was found between the samples from the methods using recombined milk. The differences the panelists detected were most likely off-flavors other than lipolyzed that were inherent to the recombined milk. When those inherent flavor differences were eliminated, panelists found that the standard method and experimental method had similar lipolyzed flavor. It is recommended that a substrate other than butter oil be used for hydrolysis to improve the repeatability of the experimental method for preparing lipolyzed milk reference samples.

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1. INTRODUCTION

Milk and other dairy products are consumed in many different forms world-wide. Off-flavors associated with dairy products can affect consumer acceptance and consumption rates. One flavor defect commonly associated with milk and butter is lipolyzed or rancid flavor. Many decades of research have been dedicated to determining the causes of and methods for prevention of lipolysis, yet the problem still exists.

Previous research conducted at The University of Tennessee dairy chemistry laboratory has shown that it is difficult to produce lipolyzed milk with consistent intensity and flavor using the current standard method for preparing sensory evaluation reference samples (Lee, 1992). The overall objective of this research was to develop a consistent laboratory method for producing lipolyzed milk samples with characteristic lipolyzed flavor. To achieve this objective, butter oil was hydrolyzed with lipase and combined with skim milk to make a lipolyzed milk sample. This research was subdivided into phases and various objectives were set for each phase.

In Phases I and II, the objectives included: (1) determining the optimum temperature for lipase activity in butter oil for each of four lipases researched, (2) developing a curve for lipase activity in butter oil using extraction-titration analysis for each lipase researched, (3) determining reproducibility of lipase activity for microequivalents of free fatty acids per milliliter of butter oil ($\mu\text{eq FFA/mL}$) and (4) developing a consistent method for hydrolyzing butter oil using each of four lipases.

In Phase III, the objectives of the study were to: (1) develop a consistent method for preparing laboratory-prepared lipolyzed milk using hydrolyzed butter oil and skim milk, and (2) determine which of the four lipases evaluated gave a characteristic lipolyzed milk flavor.

In Phase IV, the objectives were to: (1) determine if there was a perceivable difference among laboratory-prepared lipolyzed milk samples using three methods as analyzed by sensory evaluation and (2) compare extraction-titration and gas chromatography methods in measuring $\mu\text{eq FFA/mL}$ lipolyzed milk samples.

2. REVIEW OF LITERATURE

MILK

Raw cow milk is composed of water (85.5 - 89.5%), fat (2.5 - 6%), proteins (2.5 - 5%), lactose (3.6 - 5.5%) and minerals (0.6 - 0.9%). The amounts of these components vary among breeds, as well as individual cows (Alfa-Laval, undated). The fat portion is made up of fat globules that are covered by a thin membrane known as the milk fat globule membrane (MFGM). The MFGM contains protein, phospholipids, glycoproteins, triglycerides, cholesterol and enzymes (McPherson and Kitchen, 1983; Olivecrona, 1980). The MFGM protects the fat globule from enzymatic breakdown in raw milk and acts as a natural emulsifying agent to keep the fat globules dispersed throughout the milk (Alfa-Laval, undated; Christen et al., 1993; Kanno, 1989; McPherson and Kitchen, 1983; Oehlmann et al., 1994). Fat globules in milk range in size from 0.1 to 20 μm with an average particle size of 3 to 5 μm (Alfa-Laval, undated; Oehlmann et al., 1994). Three basic factors affect the MFGM: animal factors, processing and the environment (McPherson and Kitchen, 1983). Composition of the MFGM is affected by the diet of the cows, especially with the change between winter and summer feeds (Deeth and Fitz-Gerald, 1976; Jellema, 1980; McPherson and Kitchen, 1983). Larger fat globules are easier to remove from milk and give better yields in butter-making (Alfa-Laval, undated). Fat is less dense than skim milk, therefore a cream layer forms in non-homogenized milk (Caudill, 1993).

Milkfat is composed of different fatty acid esters (triglycerides) that are made up of glycerol and various fatty acids (Alfa-Laval, undated). A fatty acid molecule consists of a hydrocarbon chain and a carboxyl group. The hydrocarbon chain can be saturated (connected by single bonds) or unsaturated (connected by double bonds). Three fatty acids are connected to each glycerol molecule to form triglycerides (Alfa-Laval, undated). The fatty acids most abundant in milkfat are: butyric (C4), caproic (C6), caprylic (C8), capric (C10), lauric (C12), myristic (C14), palmitic (C16), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) (with the C referring to carbon chain length and the colon referring to the number of unsaturated bonds) (Alfa-Laval, undated; Chandan et al., 1975; Scanlan et al., 1965).

Protein in milk can be divided into five groups: casein, whey proteins, MFGM proteins, minor proteins and enzymes (Goff and Hill, 1993). Indigenous milk enzymes (lipases and esterases) are proteins and can be denatured by heat and acidic conditions (Alfa-Laval, undated; Bell and Parsons, 1976; Deeth and Fitz-Gerald, 1976; Shamsuzzaman et al., 1987).

Lipases are specific for the fatty acid(s) they release (Chandan et al., 1975; Kwak et al., 1989). Lipases are able to break down triglycerides when the MFGM has been damaged or altered (most commonly by agitation, homogenization, pumping, freezing or churning) (Alfa-Laval, undated; Christen et al., 1993; McPherson and Kitchen, 1983). This is known as induced lipolysis (Downey, 1980; Jellema, 1980; Jensen, 1964).

LIPOLYSIS

Lipolysis, or hydrolytic rancidity, is defined as the release of free fatty acids (FFA) and mono- and diglycerides from triglycerides through the catalytic action of lipase (Alfa-Laval, undated; Bigalke, 1985; Deeth and Fitz-Gerald, 1976; Duncan et al., 1990; Jensen, 1964; McNeill et al., 1986). Lipolysis in dairy products is affected by a variety of factors. Storage temperature has been linked to amount of FFA released in milk. Ultra high temperature (UHT) pasteurized milk stored at 18 - 25°C and 40°C had significantly higher ($P < 0.05$) levels of FFA than freshly pasteurized UHT milk (Shahin and Fahmy, 1988).

Time of year also influences proclivity for lipolysis. McNeill et al. (1986), determined that butter produced during winter months has higher levels of short-chain fatty acids than butter produced in summer months, indicating a link between diet, fatty acid production and rate of lipolyzed flavor formation during storage. Deeth and Fitz-Gerald (1976) found a similar correlation between diet, fatty acid production and lipolysis in milk in Australian cows.

Some researchers suggest that breed may affect the rate of lipolysis in milk. The fat globules in milk from Jersey cows are larger and rupture more easily than small fat globules. Deeth and Fitz-Gerald (1976) concluded that Jersey cows produce milk more susceptible to hydrolytic rancidity than other breeds. Since FFA are distributed in the fat globules and the MFGM, milk with high fat content contains greater total FFA (Kintner and Day, 1965). Cows with mastitis are also known to give milk that is more

susceptible to lipolysis than cows without mastitis cows (Christen and Lee, 1994; Deeth and Fitz-Gerald, 1976).

LIPOLYZED FLAVOR

For many years, the term "rancid" had been used to indicate hydrolytic rancidity in dairy products, but this term is misleading. In most food products the term "rancid" indicates lipid oxidation, whereas in dairy products there are two types of rancidity: oxidative and hydrolytic rancidity (Deeth and Fitz-Gerald, 1976). Oxidative rancidity is caused by the reaction of milk and oxygen in the air, which generates off-flavors described as oily, tallowy, cardboard, coconut or oxidized (Deeth and Fitz-Gerald, 1976). These off-flavors should not be confused with hydrolytic rancidity (lipolysis).

Hydrolytic rancidity (or lipolyzed flavor) is a common flavor defect that is economically destructive to the dairy industry (Bell and Parsons, 1976; Downey, 1980; Fleming, 1980; Jensen, 1964; McNeill, et al., 1986; Woo and Lindsay, 1983). Lipolyzed flavor is caused by excessive amounts of even-numbered short-chain FFA (C4 - C12) in milk, butter and cheese (Bell and Parsons, 1976; Bills et al., 1969; Fleming, 1980; McDaniel et al., 1969; McNeill et al., 1986; Scanlan et al., 1965; Woo and Lindsay, 1983).

Lipolyzed flavor has been described as soapy, bitter, goaty, butyric acid, cowy, unclean and rancid (Al-Shabibi et al., 1964; Deeth and Fitz-Gerald, 1976; Duncan et al., 1990;). Willey and Duthie (1969) determined that there are two types of lipolyzed or rancid flavor: sickening and unclean. The sickening-type lipolyzed flavor occurs when

raw and homogenized milks are combined, from churning or agitation, when proteolytic organisms produce heat stable enzymes and from temperature fluctuations between warm and cold milk (Bigalke, 1985; McPherson and Kitchen, 1983; Shipe et al., 1978; Willey and Duthie, 1969). The unclean-type lipolyzed flavor occurs from foaming milk and spontaneous lipolyzed milk (Tarassuk and Frankel, 1955; Willey and Duthie, 1969). The current standard method for creating laboratory-lipolyzed milk (Bodyfelt et al., 1988) targets the sickening-type lipolyzed flavor. This current standard method for preparing laboratory lipolyzed milk combines, with blending, raw and pasteurized homogenized milk, which is then aged for 24 hr prior to re-pasteurization. The lipolyzed milk is then diluted with homogenized milk and used in sensory evaluation of lipolyzed milk (Bodyfelt et al., 1988). Duncan et al. (1990) found that laboratory-prepared lipolyzed milk, using the method developed by Bodyfelt et al. (1988), had higher intensity scores than farm-lipolyzed samples. This indicates that prepared lipolyzed samples (Bodyfelt et al., 1988) may not represent the lipolyzed flavor found in naturally occurring lipolyzed milk samples (Duncan et al., 1990).

Sensory evaluation of milk has been identified as the best method for determining lipolyzed flavor in milk samples (Deeth and Fitz-Gerald, 1976; Duncan et al., 1990), although panelists vary in their sensitivity to lipolyzed flavor (Bell and Parsons, 1975; Christen, 1992; Woo and Lindsay, 1983). Threshold detection amounts of FFA in milk are chain-length dependent. Butyric acid (C4) has the lowest threshold level. The average threshold level for fatty acids increases as chain-length increases (McDaniel et al., 1969). Thresholds for some short-chain FFA are masked by higher concentrations

of long-chain FFA (Duncan and Christen, 1991; Woo and Lindsay, 1983). This could explain sensory panelist inconsistencies in accurately identifying lipolyzed samples. Generally, the concentration of long-chain FFA is higher than the concentration of flavorful, volatile short-chain FFA in butter (McNeill et al., 1986).

The type of dairy product affects panelist reaction to hydrolytic rancidity. Panelists can detect hydrolytic rancidity in milk after only mild lipolysis, whereas in ripened cheese, moderate lipolysis gives desirable flavor characteristics (Bills et al., 1969). It is only with extensive lipolysis that panelists detect hydrolytic rancidity in ripened cheese (Najim and White, 1990). Lipolyzed flavor in milk can be detected at a level between 1.5 and 2.0 μeq FFA/mL (Deeth and Fitz-Gerald, 1983; Lee, 1992).

Much research has been conducted to determine the correlation between FFA and lipolyzed flavor. McNeill et al. (1986), in evaluating lipolyzed flavor in butter, found a moderately high correlation ($R = 0.70$) between short-chain FFA (C4 - C10) concentration and sensory scores, whereas the long-chain FFA (C14 - C18:1) correlated poorly ($R = 0.47$) with sensory scores. Lee (1992) found good correlation between sensory scores and FFA in milk. Bell and Parsons (1976), on the other hand, found no correlation between sensory scores and FFA in butter and milk.

Opinions differ among researchers on which FFA influence lipolyzed flavor in dairy products. Many researchers have reported that even-numbered fatty acids (C4 - C12) contribute to lipolyzed flavor in milk (with no single dominate fatty acid), while fatty acids above C12 contribute little, if any, to lipolyzed flavor (Al-Shabibi et al., 1964; Bills et al., 1969; McNeill et al., 1986; Scanlan et al., 1965). Woo and Lindsay

(1983) found only butyric (C4), caproic (C6), capric (C10), and lauric (C12) acids to strongly influence lipolyzed flavor in butter.

Storage temperature can affect the rate of release of FFA, thereby affecting lipolyzed flavor (Bell and Parsons, 1975; Connolly et al., 1980). Short-chain fatty acids are released more quickly from butter during storage than long-chain fatty acids (McNeill et al., 1986). Butter stored at -18°C showed no increase in FFA released during 9 months storage, whereas butter held at 4 and 15°C released low levels of short-chain fatty acids (Connolly et al., 1980; McNeill et al., 1986).

LIPASES

There are many types of food grade lipase: milk lipase, those produced by microorganisms and those of other animal or plant origins. Each has its own characteristic properties, uses and specificities. As stated earlier, lipase acts to split triglycerides into glycerol, FFA and mono- and diglycerides and works at the fat-water interface (Alfa-Laval, undated; Bigalke, 1985; Bills et al., 1969; Downey, 1980). FFA contribute to both desirable (e.g., cheeses) and undesirable flavors in dairy products (Bills et al., 1969; Farag et al., 1992; Fernandez-Garcia et al., 1988; McNeill et al., 1986). The amount of lipase present in dairy products directly affects the rate of lipolysis (Kanawjia and Singh, 1990). Recent research evaluates the addition of lipase, in conjunction with other compounds, to accelerate the ripening of cheeses (Farag et al., 1992; Fernandez-Garcia et al., 1988).

Milk indigenously contains lipase, known as milk lipoprotein lipase (LPL). LPL is reported to be non-specific for individual fatty acids released during hydrolysis, although more short-chain than long-chain fatty acids are released (Kwak et al., 1989). LPL is the cause of lipolysis in milk and cream (Deeth and Fitz-Gerald, 1983). There are two types of lipolysis: spontaneous and induced. Spontaneous lipolysis is rare, occurs in the absence of physical agitation and has been associated with cows in advanced stages of lactation and with lower quality feed (e.g., dry or non-pasture feed) (Downey, 1980; Herrington, 1954; Jellema, 1980; Jensen, 1964).

Induced lipolysis can be easily activated by homogenization, vigorous agitation and foaming or temperature fluctuations (Bell and Parsons, 1976; Deeth and Fitz-Gerald, 1983; Herrington, 1954; Jensen, 1964, Tarassuk and Frankel, 1955). This treatment disrupts the MFGM and leaves the triglyceride open to action by LPL. The incorporation of air into milk is a major component of lipolysis (Deeth and Fitz-Gerald, 1983). Storage temperature and time directly affect the degree of lipolysis. Rapid cooling and storage at low temperatures can reduce the rate of lipolysis. Lipolysis can be reactivated by activation treatments (Bell and Parsons, 1976; Deeth and Fitz-Gerald, 1983).

LPL is relatively unstable and is inactivated by salt, acid, light, oxidation or heat (Bell and Parsons, 1976; Deeth and Fitz-Gerald, 1976; Shamsuzzaman et al., 1987). Pasteurization denatures LPL, but the flavor imparted prior to pasteurization is not removed by the heat treatment (Alfa-Laval, undated; Bell and Parsons, 1976; Deeth and Fitz-Gerald, 1976; McNeill et al., 1986; Shamsuzzaman et al., 1987).

Lipases derived from animal sources such as pancreatic, calf, lamb and kid goat are used widely in the dairy industry for their ability to produce specific flavors (Farang et al., 1992). Animal-source lipases are generally specific for release of short-chain fatty acids (Garcia et al., 1991). Bovine pancreatic lipase reportedly releases more short-chain than long-chain fatty acids, but with no absolute specificity for individual fatty acids (Chandan et al., 1975).

Lipases produced by bacteria, yeasts and molds are also known to cause hydrolytic rancidity, as well as desirable flavors in dairy products (Farang et al., 1992). This type lipase causes the majority of lipolysis in stored dairy products (Deeth and Fitzgerald, 1983). Psychrotrophic bacteria have long been associated with lipolytic flavor defects due to their ability to grow at common refrigerator temperatures (Deeth and Fitzgerald, 1976; McNeill et al., 1986). These bacteria (known as lipolytic bacteria) are present in raw milk and produce lipase during extended refrigerated storage prior to heat treatment (Cogan, 1980; McNeill et al., 1986). While the organisms are destroyed during pasteurization, the enzymes they produce are extremely heat stable (Cogan, 1980; McNeill et al., 1986). Christen and Lee (1994) determined that higher somatic cell counts and lipolytic bacteria populations yielded higher amounts of total FFA.

Lipases generated from bacterial and fungal sources vary in FFA specificity. Lipases from psychrotrophic bacteria release more C4 - C8 even-chain fatty acids than milk lipase (Breeding, 1989). Various species of *Aspergillus*, *Pseudomonas*, *Penicillium* and *Achromobacter* are known to release more short-chain than long-chain fatty acids. Fungal species of *Candida*, *Rhizomucor* and *Rhizopus* are similar in specificity to the

above mentioned bacterial lipases (Amano, 1993; Kwak et al., 1989). Bacterial lipases are more heat stable than milk lipase and may not be deactivated during pasteurization (Alfa-Laval, undated). Psychrotrophic bacteria therefore could be responsible for FFA production and lipolyzed flavor in dairy products during storage (Breeding, 1989; McNeill et al., 1986).

DETECTION METHODS FOR FFA

Through the years, several methods have been used to determine amounts of FFA in dairy products. The methods most commonly used are: acid degree value (ADV), extraction-titration, gas chromatography (GC) analysis and the copper soap method. These procedures vary in their reliability, correlation with other methods and length of time required to conduct the test (Ikens et al., 1988). ADV has traditionally been the accepted method for detecting hydrolytic rancidity in dairy products (Christen et al., 1993). ADV can be described as milliliters of 1N base required to neutralize the acids in 100 g fat (Bradley et al., 1992). Non-lipolyzed raw milk has an ADV of 0.25 - 0.40, whereas an ADV of greater than 1.2 is considered lipolyzed (Bradley et al., 1992). ADV uses detergent, heat and centrifugation to de-emulsify and separate FFA and titration with alcoholic potassium hydroxide to determine lipolysis (Duncan et al., 1990). A majority of the FFA recovered by the ADV method are hydrophobic (medium- and long-chain) fatty acids, so the hydrophilic (short-chain) fatty acids that contribute to lipolyzed flavor are not accurately quantified (Duncan and Christen, 1991; Ikens et al., 1988). Research has shown that there is no correlation between ADV and lipolyzed

flavor scores (Bell and Parsons, 1975; Duncan and Christen, 1991; Duncan et al., 1990, 1991). Milk samples can have identical ADV scores and different intensities of lipolyzed flavor, ranging from no detection to unpalatable. Conversely, samples with different ADV scores can have the same lipolyzed flavor scores (Duncan et al., 1990, 1991). There is a low correlation between both ADV scores and GC analysis for short-chain fatty acids (Ikens et al. 1988).

Extraction-titration, another technique for separating FFA from dairy products, has been extensively modified by researchers. Christen et al. (1993), developed an extraction-titration method as a modification of methods by Dole (1956) and Noble (1966). This method uses a mixture of isopropyl alcohol, hexane and sulfuric acid to extract the FFA. Potassium hydroxide and ethanol are used to titrate the mixture to quantitate total FFA (Christen, 1992; Christen et al., 1993; Christen and Lee, 1994). The extraction-titration method does not completely recover capric (C10) or lauric acids (C12), but does partially recover short-chain fatty acids (C4 - C12) (Christen et al., 1993). Extraction-titration has been found to be a simple and reliable method that correlates well with short-chain fatty acid concentration and lipolyzed flavor in milk (Christen and Lee, 1994; Christen et al., 1993; Ikens et al., 1988).

GC analysis is considered to be the most accurate, yet time consuming, of all methods for determining FFA concentration in dairy products (Christen et al., 1993; Ikens et al., 1988; Shantha and Napolitano, 1992). Four steps are involved in GC analysis: sampling, homogenization, sample preparation and analytical technique (Lichon, 1992). A GC method developed by Deeth et al. (1983) incorporates the extraction of

FFA with hexane and diethyl ether, separates the fat by passing the extracted sample through deactivated alumina and eluting the FFA from the alumina into 6% formic acid. Only small quantities of samples are needed for GC analysis and all major fatty acids are quantified (Deeth et al., 1983; Shantha and Napolitano, 1992).

The copper soap method extracts the copper salts of fatty acids with a solvent and then measures the soaps spectrophotometrically (Ikens et al. 1988). The copper soap method, while rapid and capable of automation, does not recover short-chain fatty acids (Christen et al., 1993; Ikens et al., 1988). The copper soap method does correlate well with GC analysis on medium- and long-chain fatty acid concentration (Ikens et al., 1988). Ikens et al. (1988) found the copper soap method to be more complex and less dependable than the extraction-titration method.

Since dairy products are consumed, sensory evaluation remains a common method for evaluating flavor defects in these products (Woo and Lindsay, 1983). Threshold levels for all the fatty acids have been determined using trained sensory panelists in water and in butter (McDaniel et al., 1969). Methods that quantitate FFA are usually correlated with sensory evaluation (Duncan et al., 1990). Various descriptive terms have been developed to describe the flavor defect in dairy products (Duncan et al., 1990). Terms such as soapy, bitter, unpleasant, goaty, rancid and butyric are used by sensory panels to describe the lipolyzed flavor defect (Duncan et al., 1990; Shipe et al., 1978). While flavor evaluation is commonly used to evaluate dairy products, the method is subject to panelist errors and variability and cannot detect nearly-rancid flavors (Bell and Parsons, 1975; Christen, 1992; Woo and Lindsay, 1983).

HOMOGENIZATION

Homogenization is a process where milk fat globules are reduced in size to an average diameter of less than 1 μm and therefore are uniformly distributed and stay in suspension in milk (Caudill, 1993). Homogenizers generate high pressure through a series of one to seven plungers (up to 68.95 MPa) to create a shearing force that distorts, stretches and fragments the fat globule (Alfa-Laval, undated; Caudill, 1993). This high pressure is created by a set of plungers. The tendency towards uniform fat globule size increases with the number of plungers (Caudill, 1993). Homogenized milk is an oil-in-water emulsion where the milkfat globules are dispersed throughout the water phase (Alfa-Laval, undated). The natural emulsifying properties of the MFGM keep the fat globules from recombining following homogenization (Alfa-Laval, undated; Kanno, 1989; Oehlmann et al., 1994). The normal melting point of butter fat is 33°C (Caudill, 1993). Homogenization effectiveness increases as the temperature of the milk and milk fat increases between 43 and 72°C and levels off between 72 and 75°C (Caudill, 1993). Care should be taken to prevent heating milk to a temperature above 78°C, which causes heated or cooked flavor defects (Bodyfelt et al., 1988; Shipe et al., 1978).

ANHYDROUS BUTTER OIL

Anhydrous butter oil or milk fat is produced from milk, cream or butter and has essentially all the water and proteins removed (Alfa-Laval, undated). Butter oil contains a minimum of 99.8% milk-fat and a maximum 0.1% water (Bassette and Acosta, 1988). Anhydrous butter oil is shelf-stable for several months in tropical climates and for 1 yr

at refrigerator temperatures (Alfa-Laval, undated). In many countries this butter oil is used in cooking as an alternative to vegetable oils, to reconstitute non-fat dry milk or butter, as an additive to baby foods and ready-cooked meals and in the production of ice cream and chocolate (Alfa-Laval, undated; Kebrary and Morris, 1990). Anhydrous butter oil is easy to store and transport and is an alternative to the seasonal variations associated with milk production (Alfa-Laval, undated). Processing includes separating the oil and water/protein fractions, washing the butter oil, centrifugation, homogenizing to break up the fat globules, vacuum drying to remove free water and fractionation (Alfa-Laval, undated; Bassette and Acosta, 1988). All MFGM are disrupted during processing making anhydrous butter oil an ideal medium for lipolysis. All existing FFA are removed from the butter or cream prior to processing (Alfa-Laval, undated). The fractionation process used during processing separates the butter oil into two fractions: one with a high and one with a low melting point. Food products requiring a fat with a high melting point are bread, chocolate, butter in tropical climates and ice cream (Alfa-Laval, undated).

The purpose of this present research is to determine the activity of commercial lipases in butter oil at various incubation temperatures and to evaluate the flavor of milk from the recombination of butter oil (hydrolyzed with lipases) and skim milk as analyzed by extraction-titration, gas chromatography and sensory evaluation.

3. THE EFFECT OF TIME AND TEMPERATURE ON LIPASE ACTIVITY IN BUTTER OIL

ABSTRACT

This research was divided into two phases. In Phase I, butter oil was hydrolyzed with four lipases at four incubation temperatures and analyzed using extraction-titration. After evaluating lipase activity over 60 min for all four lipases at 35, 40, 45, and 50°C, one incubation temperature was chosen for each lipase for Phase II. These were 45°C for porcine pancreas, 35°C for *Penicillium roqueforti*, 45°C for *Aspergillus niger*, and 50°C for *Rhizomucor miehei*. A general trend was evident for all lipases: as time increased, the cumulative free fatty acids (FFA) released by each lipase increased. The activity level varied with lipase and temperature. Pancreatic lipase activity was found to be reproducible at 45 and 50°C in Phase I, but not in Phase II; *R. miehei* lipase activity was found to be reproducible at 50°C only in Phase I, but not in Phase II; and the activity by *P. roqueforti* and *A. niger* was not reproducible at any temperature in either phase of the research. With GC analyses in Phase II, pancreatic and *P. roqueforti* lipases were found to release more short-chain fatty acids (C4 - C12) ($P < 0.05$), while *A. niger* and *R. miehei* released more medium- and long-chain fatty acids (C14 - C18:3).

INTRODUCTION

A common flavor defect in dairy products is lipolyzed or rancid flavor (hydrolytic rancidity). This flavor defect can cause serious economic damage to the dairy industry (Bell and Parsons, 1976; Downey, 1980). Lipolysis in dairy products is defined as the

chemical breakdown of milk fat into free fatty acids (FFA) and mono- and diglycerides by lipase (Alpha-Laval, undated; Deeth and Fitz-Gerald, 1976; Jensen, 1964). Lipase, an enzyme, is present in all milk, but is activated only under certain conditions (Deeth and Fitz-Gerald, 1976). There are two kinds of lipolysis: spontaneous and induced. Spontaneous lipolysis occurs without apparent physical cause and is rare. Induced lipolysis is the major type of lipolysis and is caused by: agitation, temperature changes, homogenization or combining raw and homogenized milk (Christen et al., 1993; Deeth and Fitz-Gerald, 1976; Downey, 1980). Induced lipolysis occurs when the membrane surrounding the milk fat globule is damaged, allowing lipase access to the triglycerides in the fat molecules (Alpha-Laval, undated; Anderson et al., 1991; Tarassuk and Frankel, 1955).

Lipases can come from a variety of sources including milk, bacteria, animal and fungus (Kwak et al., 1989). Lipases are commonly used commercially in the food industry to develop flavors and experimentally to reduce aging time in cheeses (Fernandez-Garcia et al., 1988; Kanawjia and Singh, 1990). Some lipases release specific FFA during lipolysis. Porcine pancreatic lipase (depending on the source) has been reported to be both nonspecific and to release short-chain FFA (C4 - C12) (Kwak et al., 1989); *Aspergillus niger* and *Rhizomucor miehei* are selective for short-chain FFA (Garcia et al., 1991; Kwak et al., 1989), and *Penicillium roqueforti* is reported to release more butyric acid (C4) than other FFA. Unique flavors and aromas accompany the lipases when different FFA are released.

Lipase activity is dependent on temperature, pH, quantity of built-up FFA at the oil/water interface and presence of salts in the substrate (Deeth and Fitz-Gerald, 1976; Downey, 1980). In this study, lipase activity can be described as the amount of microequivalents of free fatty acids released per milliliter of butter oil ($\mu\text{eq FFA/mL}$) over a determined incubation time at a determined incubation temperature. Initial agitation of milk (or butter oil) directly affects the rate and duration of lipolysis. Lipolysis peaks with time due to the build-up of FFA at the triglyceride oil/water interface, but can be reinitialized by re-homogenizing milk (Downey, 1980). Milk lipase is generally inactivated at pasteurization temperature (Bell and Parsons, 1976). Bacterial lipases are more heat resistant than milk lipase. The bacteria are destroyed during pasteurization, but the lipase produced is usually undamaged at that temperature (Bell and Parsons, 1976; Bigalke, 1985; Downey, 1980).

FFA are monocarboxylic acids that are released through the enzymatic cleaving of triglycerides by lipase. Each fatty acid has a distinct site on the triglyceride and is released only when a site-specific lipase attaches at the appropriate site at the oil/water interface of the triglyceride. FFA are classified according to their carbon chain-length (between 4 and 28 carbon atoms) and number and location of double bonds (Anderson et al., 1991). Even-numbered short-chain FFA (C4 - C12) are generally believed to cause off-flavors associated with rancidity (Anderson et al., 1991; Salih et al., 1977), although there are considerable differences of opinion as to which FFA or combination of FFA impart the lipolyzed flavor to dairy products (Bills et al., 1969; Woo and Lindsay, 1983).

Several methods have been used for determining levels of FFA in dairy products. While a reference method for determining content of FFA in dairy products is being sought, no one method can accurately be used for all dairy products (Anderson et al., 1991). Acid degree value (ADV) can be described as milliliters of 1N base required to neutralize the acids in 100 g fat (Bradley et al., 1992). ADV has long been used as the industry standard for determining levels of FFA in milk, but has its limitations. ADV recovers more fat-soluble FFA, including long-chain FFA (C14 - C18). The long-chain FFA are not believed to contribute to off-flavors associated with lipolyzed or rancid milk (Christen et al., 1993; Duncan and Christen, 1991). ADV correlates poorly with gas chromatographic (GC) analysis (Ikens et al., 1988). GC analysis, while tedious and time consuming, has been found to recover more short-chain FFA (C4 - C12) than any other known method (Christen et al., 1993) and is the method most used in research applications (Shantha and Napolitano, 1992). Extraction-titration is a simple and fast method for determining total FFA in milk (Christen et al., 1993; Ikens et al., 1991). However, extraction-titration only measures total FFA and does not fully recover all short-chain FFA (Christen et al., 1993).

Anhydrous milk fat or butter oil is butter with the milk proteins and water removed (less than 0.1% water content) (Alpha-Laval, undated; Bassette and Acosta, 1988). Butter oil, shelf stable for several months in opaque and gas-tight containers, has a refrigerator shelf-life of up to one year (Alpha-Laval, undated). This product is suitable for recombining and reconstitution of milk, a practice used in Egypt and other countries (Alpha-Laval, undated; Kebrary and Morris, 1990).

The objectives of this research were to (1) determine the optimum temperature for activity in butter oil for each of the four lipases researched, (2) develop an activity curve (plotting microequivalents FFA per milliliter sample ($\mu\text{eq FFA/mL}$) against incubation time) for lipase in butter oil using extraction-titration analysis for the four lipases researched, (3) determine reproducibility of lipase activity for $\mu\text{eq FFA/ml}$ butter oil and (4) develop a consistent method for hydrolyzing butter oil.

MATERIALS AND METHODS

Materials

Four individual lots of anhydrous butter oil (clarified butter) were purchased from Mid America Farms Co., (Springfield, MO), divided into 450-mL portions, placed into 500-mL Nalgene® bottles, and frozen at -37°C . Each lot was tested prior to freezing using the extraction-titration procedure to determine total FFA content. The four lots of butter oil (A - D) were determined to inherently contain 2.45 ± 1.5 , 3.23 ± 1.5 , 3.79 ± 1.2 , and $3.27 \pm 1.9 \mu\text{eq FFA/mL}$, respectively.

Food grade lipases were obtained from several suppliers: *Aspergillus niger* (Lipase AP "Amano" 12, 120,000 units/g) and *Penicillium roqueforti* (Lipase R "Amano," 10,000 units/g) were donated by Amano Enzyme U.S.A. Co., Ltd (Troy, VA); *Rhizomucor miehei* (Palatase® M, 1,000 LU/g) was donated by Novo Nordisk Bioindustrials, Inc. (Danbury, CT); and porcine pancreatic lipase (Pancreatic Lipase 250, 250 units/g) was donated by Solvay Enzymes (Elkhart, IN). Two batches of each lipase were obtained from the suppliers. The lipases were stored at 4°C throughout the study.

Gum arabic was donated by TIC Gums, Inc. (Belcamp, MD). A 10% solution (w/w) was prepared and stored in 500-mL Nalgene® bottles at 4°C. Additional solvents and chemicals were purchased from Baxter Diagnostics Inc., Scientific Products Div. (McGraw Park, IL).

Methods - Phase I: Lipase activity for four lipases at four incubation temperatures

Butter oil hydrolysis. The bottles of frozen butter oil were removed from the freezer 16 - 20 hr prior to use and held at 23 - 25°C. The butter oil was melted for 2 - 3 min in a microwave oven (Kenmore, model 566-88785350, Sears, Roebuck and Co., Chicago, IL) with a measured output of 438 watt on high power (Van Zante, 1973). A 100-g portion was poured into 1-L Erlenmeyer flasks and held at one of four predetermined incubation temperatures. A 10% gum arabic solution (4 mL) was added to each flask of butter oil to facilitate development of the oil/lipase interface. Using amounts of lipase determined in preliminary research, 0.15 g *R. miehei*, 0.0075 g *A. niger*, 0.15 g *P. roqueforti* or 0.1 g porcine pancreas lipases were added to the individual flasks of butter oil. These lipase amounts yielded FFA levels equivalent to approximately 10-mL more titrant than unhydrolyzed butter oil.

Once the lipase and gum arabic was added to the butter oil, the flasks were flushed with nitrogen gas to reduce volatilization of FFA. A Nalgene® stopper, with a 1-cm tube inserted through the stopper, sealed the flask. The tube extended from below

the butter oil surface through the stopper, allowing removal of butter oil without allowing the nitrogen gas to escape. Another rubber stopper was used to close the top of the tube.

The flasks were incubated in a six-position, circulating water bath/stirrer (Labline Instruments Inc.; Melrose Park, IL) . Separate trials were conducted for each lipase at four different temperature settings: 35, 40, 45 or 50°C. To distribute the effects of differences in stir bar speed, each lipase flask was set on a different stirrer site for each replication. Incubation time for the butter oil and lipase was 60 min with 0.5-mL samples removed every 5 min. The entire procedure was repeated four times with a different lot of butter oil (randomly chosen) used for each replication.

Extraction-titration procedure. Hydrolyzed butter oil samples (0.5 mL) were placed into 25-mL, screw-cap test tubes containing 4.5 mL distilled water. Immediately following transfer to the test tubes, 15 mL extraction mixture was added to the butter oil and water. The test tubes were then capped and mixed on a vortex for 15 sec at speed 7 to stop enzymatic reaction. Following mixing on the vortex, 16.5 mL hexane and 6.5 mL distilled water were added to each test tube. All reagents were added using repeating burette dispensers fitted atop amber reagent bottles. The test tubes were inverted by hand 15 times to mix the solvents that were then allowed to separate into two layers. Using 20-ml volumetric pipets (Kimax, ± 0.001 mL accuracy), the supernatant was removed and placed into a covered, 60-mL titration cup (Mettler Instrument Corp., Hightstown, NJ). The bottom, aqueous portion was disposed as solvent waste. A Mettler DL12 automatic titrator (Hightstown, NJ) was used to titrate each sample

(containing the extracted FFA) to a final pH of 11.3. Potassium hydroxide was standardized using lauric acid (C12) in hexane. A blank (sample replaced with hexane) and standard samples were completed each day.

Calculations and equations. The quantity of titrant needed to bring the sample to pH 11.3 was recorded and used in the following formula to determine $\mu\text{eq FFA/mL}$ butter oil:

$$\text{Equation 1: } \frac{\mu\text{eq FFA}}{\text{mL}} = \frac{(\text{mL}_{\text{Sample}} - \text{mL}_{\text{Blank}}) \times N_{\text{KOH}} \times 1000}{\text{Sample size}_{\text{mL}}}$$

Where μeq = microequivalents; FFA = free fatty acid; $\text{mL}_{\text{Sample}}$ = milliliters titrant for sample; mL_{Blank} = milliliters titrant for blank; N_{KOH} = normality of potassium hydroxide; Sample size = milliliters of sample

Activity curves ($\mu\text{eq FFA/mL}$ vs. time) were plotted for each lipase at all incubation temperatures (35, 40, 45 and 50°C). From this information, the optimum incubation temperature for each lipase was chosen.

Statistical analyses. Proc GLM (SAS Institute, Inc., 1989) was performed on the results obtained for $\mu\text{eq FFA/mL}$ butter oil (dependent variable) for all lipases at all four incubation temperatures across time for four replications (independent variables). The interaction of lipase by temperature by time was evaluated. The F value resulting from

the interaction of lipase by replication by temperature over 60 min was used to determine reproducibility of lipase activity for each lipase. The PDIFF option was used to determine differences among incubation temperatures for each lipase. Simple linear regression was used to determine slopes of the activity curves for each lipase, which indicate rate of activity. The slope interaction among temperatures for each lipase was evaluated. Significance was determined at $\alpha \leq 0.05$.

Methods - Phase II: Lipase activity for four lipases at their apparent optimal incubation temperature

Butter oil hydrolysis. From the data collected in Phase I, the incubation temperature yielding the highest activity level was chosen for each lipase. Using the second lipase batch, four replications (at the selected optimum incubation temperature) were run for each lipase/butter oil sample. Preliminary milk samples (2% butter fat, w/w) were prepared using a homogenized mixture of skim milk and hydrolyzed butter oil. These preliminary samples were evaluated for lipolyzed flavor by a dairy flavor expert. Results from this evaluation indicated that lipase activity of approximately 22 mL titrant above the titrant level for unhydrolyzed butter oil was needed. Lipase amounts were adjusted to achieve this higher activity level. Through educated trial and error, the following quantities of lipase were determined to give the desired titrant quantity: 0.18 g *R. miehei*, 0.04 g *A. niger*, 0.25 g *P. roqueforti* and 0.11 g porcine pancreas.

All other conditions and methods for butter oil hydrolysis used in Phase I were duplicated in Phase II, with a few minor exceptions. In preliminary research for Phase

II, the butter oil agitation rate was found to be less in flasks where the tube was below the surface of the butter oil. In contrast, the butter oil agitation rate appeared higher in the flasks where the tubes did not enter into the butter oil. The amount of titrant needed to bring the samples to a pH of 11.3 was greater in flasks without the tube as compared to flasks with the tube in the butter oil. Therefore in Phase II, the tube was positioned above the surface of the butter oil in all samples. To reduce the effects of differences in stir bar speed, the same stir bar site was used for the *P. roqueforti* and *R. miehei* lipase flasks. Since the pancreatic and *A. niger* lipase/butter oil mixtures were incubated at the same temperature and at the same time, an additional stir bar site was added. A balanced order for stir bar site use reduced the effects of stir bar speed variability.

Gas chromatography - sample preparation. Once the final sample was removed from the flasks after 60 min of incubation, a 0.5-mL sample was removed for GC analyses. This sample was placed into a 25-mL, screw-cap test tube; 0.1 mL 0.2 N sulfuric acid, 10 mL of a 1:1 diethyl ether-hexane mixture (containing 0.005% (w/v) butylated hydroxytoluene [BHT]) and 1 mL of the C5 and C17 internal standard in hexane were added. The test tube was capped, mixed on a vortex at speed 7 for 15 sec and 8 mL supernatant were removed to a screw-cap test tube containing 1 g anhydrous sodium sulfate (to bind any water in sample). The samples were stored at -25°C until analysis could be completed (approximately 0 - 14 days).

Basic alumina was dried in a vacuum oven (GCA Corp., Chicago, IL) at 175°C, 20 psi for 2 hr and stored in a 60-ml amber-colored bottle in a desiccator until ready for

activation. The alumina was activated by the addition of deionized water (3% v/w), vortexed to break up any clumps and allowed to equilibrate for at least 2 hr.

A glass champagne column was prepared with a small plug of silanized glass wool and 1 g activated basic alumina. The GC sample was allowed to warm to room temperature, poured twice over the alumina and collected in a 50-mL Erlenmeyer flask. The alumina was washed twice with 5 mL 1:1 diethyl ether-hexane (without BHT). The solvent that drained into the Erlenmeyer flask was discarded as solvent waste. The alumina in the glass column was dried under vacuum for 1 min and transferred to a conical, 10-mL, screw-cap test tube. The alumina samples were stored at -25°C until analysis could be completed (approximately 0 - 4 days).

The FFA were removed from the alumina with 1 mL 10% (v/v) formic acid/diethyl ether solution and mixed on a vortex at speed 7 for 1 min. The test tubes were centrifuged on a table-top centrifuge (Damon/IEC Division, Needham Hts., MA) at setting 5 for 5 min. The supernatant was transferred to a GC vial using a Pasteur pipet and sealed. Samples were held at -25°C until ready for GC analysis.

Gas chromatography - analysis of free fatty acids. FFA were separated and quantified using a Hewlett Packard (HP) 5890 Series II Gas Chromatograph (GC) (Hewlett-Packard Co., Kennett Square, PA), fitted with an HP7673 Automatic sampler (Hewlett-Packard Co., Kennett Square, PA) and a flame ionization detector (FID). Samples (3 μ L) were injected into a 25-m HP-FFA capillary column (Hewlett-Packard Co., Kennett Square, PA) with an inside diameter of 0.32 mm and a film thickness of 0.52 μ m. Flow rates

for the carrier gas and combined column and make-up gas (all helium) were: 3.03 mL/min and 8.00 mL/min, respectively. The column head pressure was maintained at 20 psi. Samples were injected into the column in the splitless mode with a purge time of 0.05 min, after which the injection port switched to the split mode of operation. The GC oven was set to increase from an initial temperature of 110°C to a final temperature of 225°C in 5°C/min increments, with a final hold time of 20 min. The injector temperature was 250°C, while the FID temperature was 260°C. Individual FFA were identified and peak areas measured using HP3365 ChemStation software (Hewlett-Packard Co., Kennett Square, PA).

Fatty acid standards for the saturated fatty acids (C4 - C18) and unsaturated fatty acids (C16:1, C18:1 - C18:3) were run in the GC as external standards to determine fatty acid retention times. Amounts used for fatty acid external standards are listed in the Appendix C-2. The peak areas for the external standards were used to calculate Relative Molar Response (RMR) and the grams of fatty acid in each sample (Appendix C-3).

Calculations and equations. The following equations were used to convert FFA peak areas to μeq FFA/mL butter oil for the samples collected at the 60 min incubation time.

$$\text{Equation 2: } \text{RMR} = \frac{\text{Area}_{\text{FA}} / \mu \text{ Mole}_{\text{FA}}}{\text{Area}_{\text{IS}} / \mu \text{ Mole}_{\text{IS}}}$$

$$\text{Equation 3: } \text{Grams}_{\text{FA}} = \frac{\text{Area}_{\text{FA}} \times \text{MolecularWeight}_{\text{FA}} \times \text{grams}_{\text{IS}}}{\text{Area}_{\text{IS}} \times \text{MolecularWeight}_{\text{IS}} \times \text{RMR}_{\text{FA/IS}}}$$

$$\text{Equation 4: } \frac{\mu\text{eq}_{\text{FA}}}{\text{ml}} = \frac{\text{gram}_{\text{FA}} \times 10^6 \mu\text{mol/mol}}{\text{gram}_{\text{FA/mol}}}$$

Where RMR = Relative molar response, μmol = micromole, FA = fatty acid, IS = internal standard, μeq = microequivalent, Area_{FA} = GC peak area of fatty acid, C5 was used as the internal standard for calculating the grams_{FA} for C4 - C12, and C17 was used as the internal standard for calculating the grams_{FA} for C14 - C18:3.

Statistical analyses. Proc GLM (SAS Institute, Inc., 1989) was run on all $\mu\text{eq FFA/mL}$ (dependent variable) calculated from the GC analyses to determine differences in amounts of individual FFA for each lipase. The PDIFF function was used to determine differences in least-squares mean individual FFA among lipases for the interaction of lipase by replication. Lipase and replication were the independent variables. Significance was established at $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Extraction-titration results

From preliminary research, quantities of lipase needed to bring the amount of titrant to 10 mL above the amount needed for the unhydrolyzed butter oil were determined. These amounts were used in Phase I because they corresponded to the level of FFA necessary to detect lipolyzed flavor in milk as determined from previous research (Lee, 1992). Each lipase was evaluated at four incubation temperatures to determine

reproducibility and apparent optimum incubation temperature and time for activity. Cumulative FFA per milliliter of butter oil at each sampling time were recorded for each temperature, and the results were plotted ($\mu\text{eq FFA/mL released vs. time}$). Graphs representing lipase activity by replication for all incubation temperatures are found in Figures A-1 through A-16 (Appendix).

In Phase II, the amount of lipase used was increased over that used in Phase I. The activity of each lipase over time was evaluated at the apparent optimal incubation temperature for that lipase. Least-squares means for cumulative FFA released from butter oil by each lipase can be found in Table 2-1. Since more lipase was used in Phase II than in Phase I, variability among lots of lipase can only be estimated. Comparison of regression line slopes between phases was used to estimate lipase variability (Tables 2-2 and 2-3). Following is a discussion of the results from both phases of the study for each lipase.

Porcine pancreatic lipase. For pancreatic lipase at the 60 min incubation time, the quantity of FFA released from butter oil was the same at 40, 45 and 50°C, but less at 35°C ($P < 0.05$) (Table 2-4). Regardless of temperature, as incubation time increased, the cumulative amount of FFA released also increased (Figure 2-1). Table 2-2 shows the rate of lipase activity at four incubation temperatures in Phase I. The regression line slope at 40°C was significantly greater ($P < 0.05$) than the slopes at 35 or 45°C, but did not differ ($P > 0.05$) from the slope at 50°C. The regression line slope for 50°C was significantly greater ($P < 0.05$) than the slope at 35°C. The slope for 45°C was greater

Table 2-1 - Least-squares mean^a cumulative free fatty acids released from butter oil by four lipases as determined by extraction-titration (Phase II)

Incubation time (min)	Lipase (Temperature of incubation, °C)			
	Pancreatic (45)	<i>P. roqueforti</i> (35)	<i>A. niger</i> (45)	<i>R. miehei</i> (50)
	-----µeq FFA/mL-----			
5	11.09	10.04	15.62	12.39
10	17.23	13.15	25.43	21.66
15	22.52	18.83	32.18	26.29
20	29.01	24.05	37.72	33.57
25	38.53	25.26	42.18	40.58
30	43.10	29.56	49.62	47.95
35	53.83	32.27	58.52	54.99
40	53.76	33.27	54.45	58.69
45	58.85	36.97	59.91	67.39
50	63.29	36.48	60.83	72.45
55	67.64	39.62	67.86	78.04
60	75.67	42.42	72.63	81.88

^aCumulative means across four replications.

Table 2-2 - Least-squares mean^a slopes of regression lines constructed for release of free fatty acids in butter oil hydrolyzed with lipase at four incubation temperatures over 60 min (Phase I)

Lipase	Incubation temperature (°C)			
	35	40	45	50
Pancreatic	0.305a	0.543c	0.478b	0.536bc
<i>P. roqueforti</i>	0.351c	0.096b	0.039a	0.076ab
<i>A. niger</i>	0.219bc	0.238c	0.180ab	0.172a
<i>R. miehei</i>	0.186a	0.279b	0.324c	0.507d

^aDifferent letters in the same row indicate a significant difference ($P < 0.05$); means over four replications.

Table 2-3 - Least-squares mean^a slopes of regression lines for replications of lipases used to hydrolyze butter oil by four lipases at the apparent optimal incubation temperature (Phase II)

	Lipase			
	Pancreatic ^a	<i>P. roqueforti</i> ^b	<i>A. niger</i> ^c	<i>R. miehei</i> ^d
Slope	1.15y	0.56x	1.28z	1.31z

^a Incubated at 45°C over 60 min.

^b Incubated at 35°C over 60 min.

^c Incubated at 45°C over 60 min.

^d Incubated at 50°C over 60 min.

^aDifferent letters indicate a significant difference ($P < 0.05$); means over four replications.

Table 2-4 - Least-squares mean^a cumulative free fatty acids released from butter oil by four lipases at four incubation temperatures in 60 min incubation as analyzed by extraction-titration

Lipase	Incubation temperature (°C)			
	35	40	45	50
	-----µeq FFA/mL-----			
Pancreatic	24.35a	36.25b	35.25b	37.25b
<i>P. roqueforti</i>	28.01b	10.62a	9.54a	8.35a
<i>A. niger</i>	17.36a	15.25a	16.22a	17.95a
<i>R. miehei</i>	16.84a	19.97a	25.49b	35.22c

^aDifferent letters in the same row indicate a significant difference ($P < 0.05$); means across four replications.

($P < 0.05$) than the slope at 35°C, but less than ($P < 0.05$) that at 40°C. Slopes for the regression curves were greater in Phase II (Table 2-3) than in Phase I. Possible explanations for differences in regression line slopes could be variability between batches of pancreatic lipase, or inherent variability in porcine pancreatic lipase activity rate.

Reproducibility of pancreatic lipase activity at each incubation temperature was examined (Table A-17). The cumulative amount of FFA released by pancreatic lipase in Phase I was found to be reproducible at 45 and 50°C ($P > 0.05$). At 35 and 40°C, the replications were not reproducible ($P < 0.05$). In contrast, pancreatic lipase activity was not reproducible ($P < 0.05$) at 45°C in Phase II. This inconsistency may be related to the low amount of water present in the anhydrous butter oil. Since lipolysis occurs at the oil/water interface, a low concentration of water in the butter oil (approximately 3.5%) may cause inconsistent development of the interface and some lipases require more water to induce lipolysis than other lipases. (Christen, 1994).

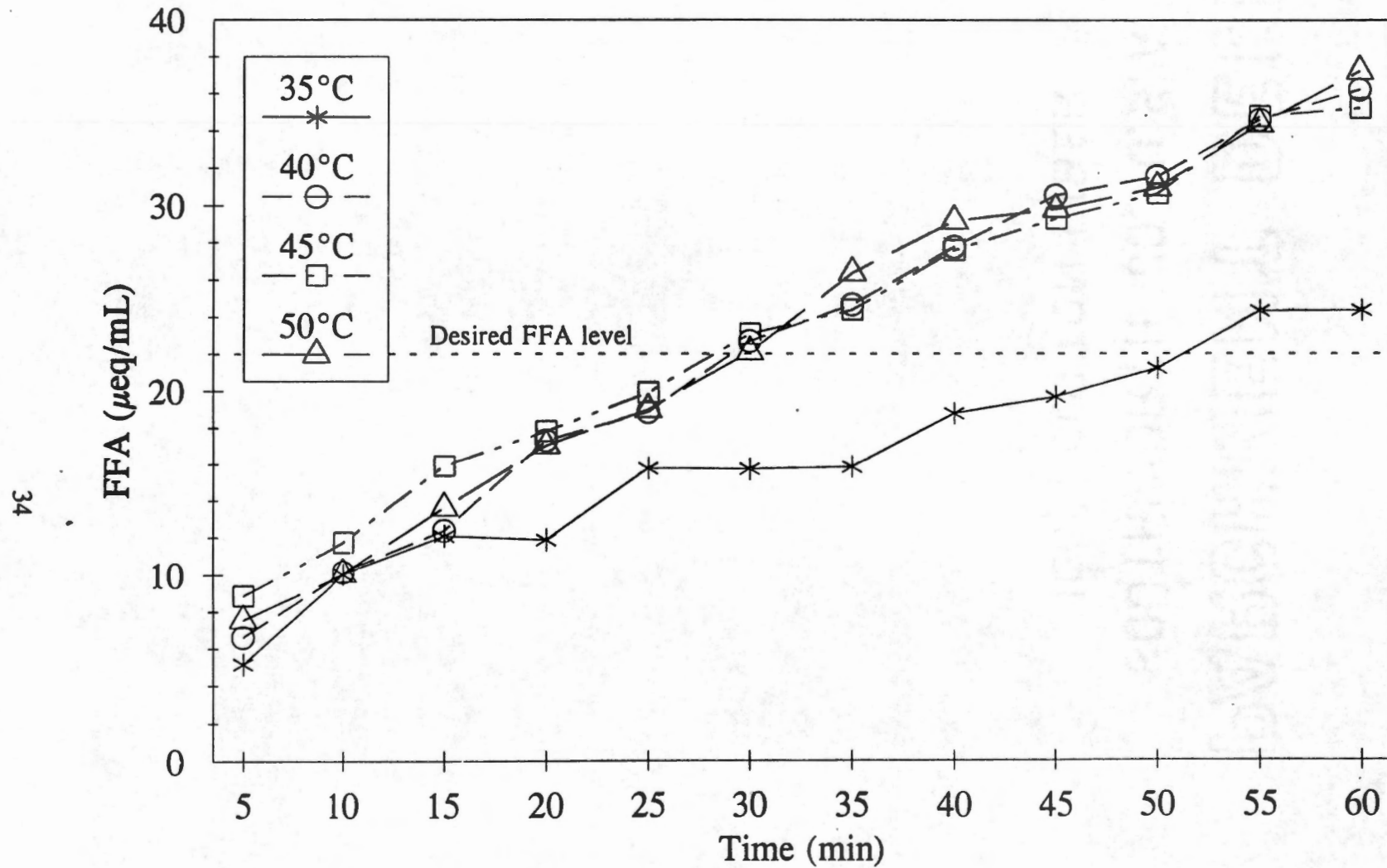


Figure 2-1 - Least-squares means cumulative free fatty acids released in butter oil hydrolyzed with porcine pancreatic lipase at four incubation temperatures over four replications as analyzed by extraction-titration

The time necessary to achieve the desired level of FFA determined in Phase I varied between 25 and 45 min at 35°C (Figure A-1); 10 and 40 min at 40°C (Figure A-2); 20 and 40 min at 45°C (Figure A-3); and approximately 30 min at 50°C (Figure A-4). In Phase II, the desired level was reached between 35 and 40 min of incubation at 45°C (Figure A-19).

Since statistically there were no differences ($P > 0.05$) among the cumulative FFA released in butter oil following 60 min incubation at 40, 45, and 50°C (Table 2-4), 45°C was arbitrarily chosen as the incubation temperature for Phase II based on perceived reproducibility of the lipase at this temperature. While the activity rate was greater at 40 than 45°C, the results were reproducible at 45, but not at 40°C. The supplier reported the optimum temperature range for pancreatic lipase was between 40 and 50°C (Solvay, 1992), and our results support this. In hindsight, 50°C may have been a better choice since the rate of activity was the same as 45°C, the cumulative FFA released at 60 min were equivalent, but desired quantity of FFA was reached in a shorter time.

***Penicillium roqueforti* lipase.** For the *P. roqueforti* lipase, the greatest cumulative amount of FFA released in butter oil in 60-min occurred at 35°C ($P < 0.05$) (Table 2-4). Final cumulative FFA released at 40, 45 and 50°C were significantly less ($P < 0.05$) than at 35°C. At 35°C, the increased FFA release with increasing time trend was evident (Figure 2-2). The slope of the regression line was significantly greater ($P > 0.05$) at 35°C than the slopes at 40, 45 or 50°C (Table 2-2). The rate of lipase activity did not differ between 40 and 50°C, nor between 50 and 45°C (Table 2-2). Comparison of the

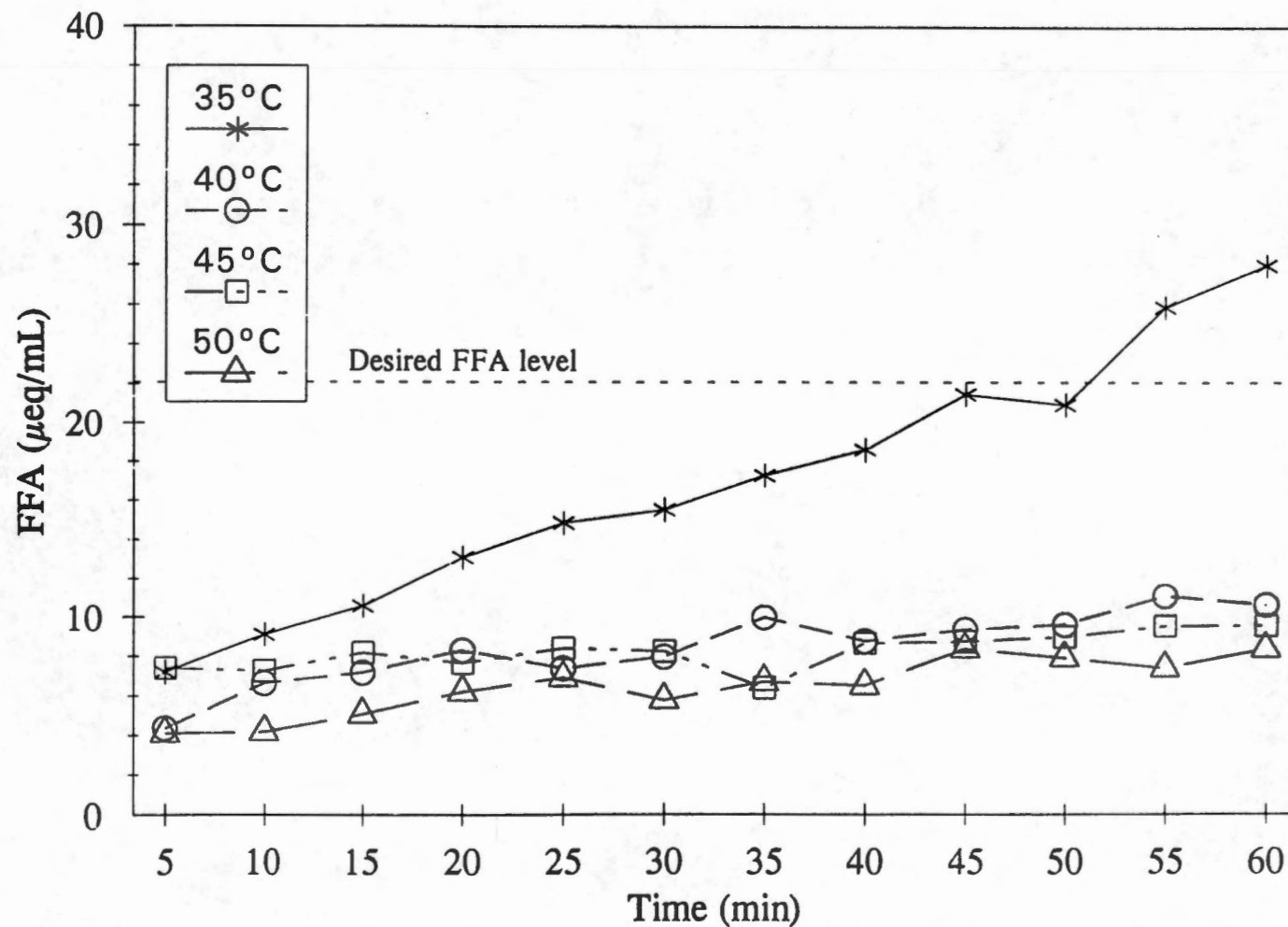


Figure 2-2 - Least-squares means cumulative free fatty acids released in butter oil hydrolyzed with lipase from *P. roqueforti* at four incubation temperatures over four replications as analyzed by extraction-titration

slopes of regression curves reveals that Phase II had a slightly greater slope than in Phase I at 35°C (Tables 2-2 and 2-3). This suggests variability between batches of lipase for *P. roqueforti*.

P. roqueforti activity was not found to be reproducible ($P > 0.05$) at any incubation temperature examined in either Phase I or Phase II (Table A-17). The 35°C incubation temperature was 2°C above the melting point for butter oil (Bassette and Acosta, 1988). Therefore butter oil at 35°C had a semi-solid consistency, which was observed to vary with the temperature of the room during incubation. This variability in butter oil consistency may have caused varied agitation rates, resulting in inability to establish the oil/water interface exactly the same with each replication.

The time necessary to achieve the desired level of activity in Phase I varied between 25 and 55 min at 35°C (Figure A-5); and did not reach the desired level at 40°C (Figure A-6), 45°C (Figure A-7) or 50°C (Figure A-8). In Phase II, the desired activity level was reached in only one replication (after 55 min incubation) (Figure A-21). An incubation temperature of 35°C was chosen for *P. roqueforti* in Phase II since the rate of activity was the greatest at this temperature, and 35°C was the only temperature where the desired level of cumulative FFA were released (Table 2-4). The supplier reports that the optimum temperature for *P. roqueforti* is 30°C, with activity rapidly decreasing at higher temperatures (Amano, 1993). Initially, an incubation temperature of 30°C was investigated, but since this was below the melting point of butter oil, accomplishing hydrolysis was unfeasible. To the degree possible, our results agree with the lipase activity estimation provided by the supplier.

***Aspergillus niger* lipase.** There was no significant difference ($P > 0.05$) in the amount of cumulative FFA released in butter oil by *A. niger* at any of the temperatures (Table 2-4). Again, the above-mentioned trend was identified at all incubation temperatures: as incubation time increased, the amount of cumulative FFA released also increased (Figure 2-3). A significantly greater rate of activity was achieved at 40°C than at 45 or 50°C (Table 2-2). Rates were the same at 35 and 40°C. Comparison of regression line slopes shows that the lipase activity rate is much less in Phase I than in Phase II (Tables 2-2 and 2-3). This suggests a greater rate of activity with greater quantities of lipase and/or variability in lipase activity between batches of *A. niger*.

A. niger was not found to be reproducible at any incubation temperature ($P > 0.05$) in either Phase I or Phase II (Table A-17). This suggests variability in activity for *A. niger* lipase in butter oil.

The time necessary to achieve the desired level of activity in Phase I varied between 40 min or not reaching the desired level at all at 35°C (Figure A-9); and 50 - 60 min (in 3 of 4 replications the desired FFA level was never achieved) at 40, 45 and 50°C (Figures A-10, A-11 and A-12, respectively). In Phase II, the desired level was achieved at 25 min in two replications, 35 min in one and > 60 min in another (Figure A-23).

An incubation temperature of 45°C was arbitrarily chosen for Phase II based on perceived reproducibility and lipase activity at this temperature. In retrospect, an incubation temperature of 40°C may have given more consistent results, based on rate of activity, and supplier recommendation. The supplier reported an optimum temperature range of 30 - 40°C (Amano, 1993).

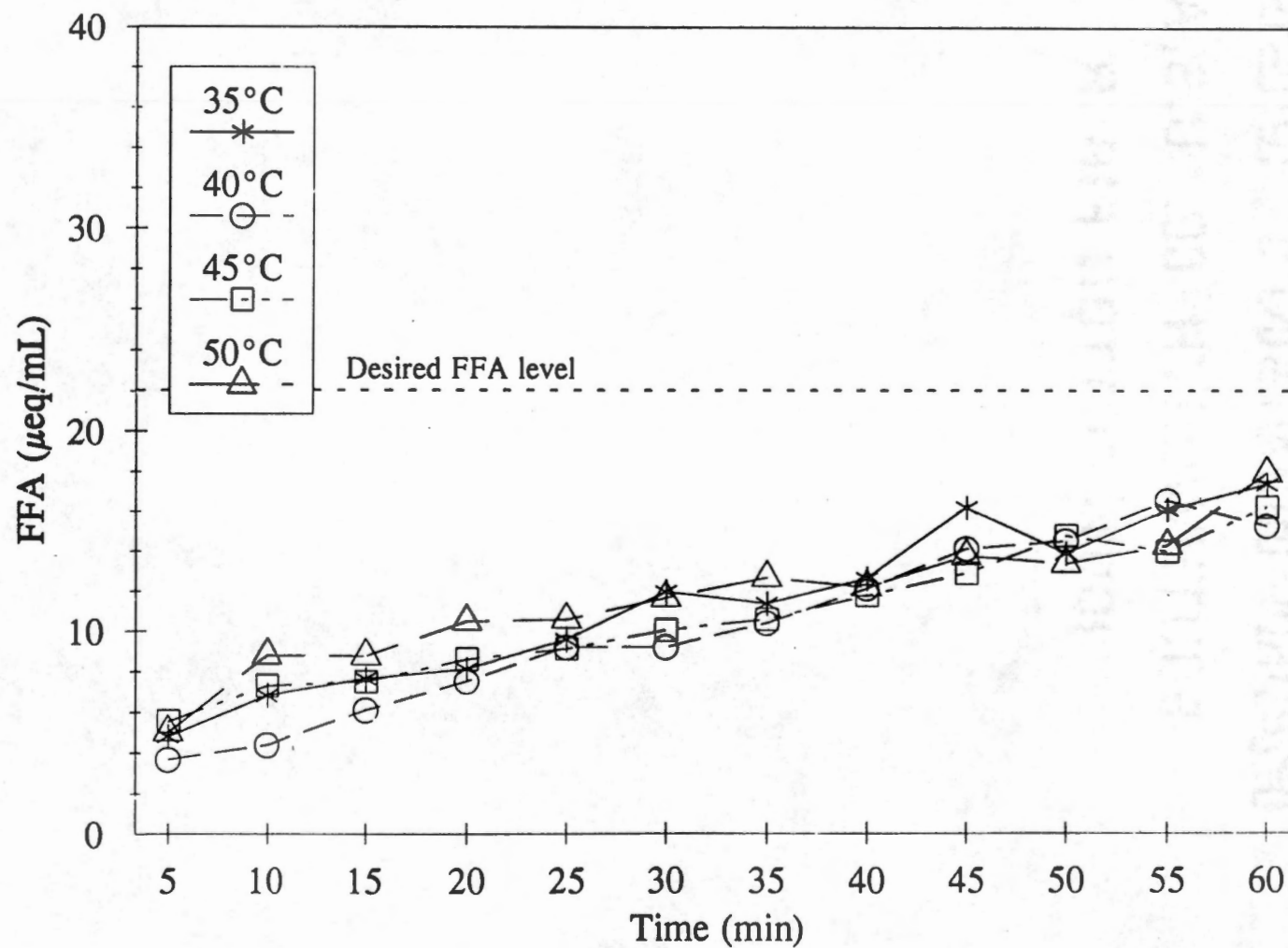


Figure 2-3 - Least-squares means cumulative free fatty acids released in butter oil hydrolyzed with lipase from *A. niger* at four incubation temperatures over four replications as analyzed by extraction-titration

***Rhizomucor miehei* lipase.** *R. miehei* was determined to release significantly more ($P < 0.05$) cumulative FFA in butter oil at 50°C at 60 min than at any of the other incubation temperatures evaluated (Table 2-4). The amount of total cumulative FFA released in butter oil did not differ ($P > 0.05$) between 35 and 40°C, but both released significantly less than at 45°C. As with the other three lipases discussed, the following trend was evident: as incubation time increased, the cumulative amount of FFA released also increased (Figure 2-4). An increasing rate of activity is shown by the slopes of the regression curves at all four incubation temperatures in Phase I where the slope at 50 > 45 > 40 > 35°C (Table 2-2). The slopes of the regression lines at 50°C are less in Phase I than in Phase II (Tables 2-2 and 2-3), indicating a greater activity rate when more lipase is present (Kanawjia and Singh, 1990) and variability between batches of lipase. Another trend was noticed for *R. miehei* that was not evident in the other three lipases evaluated: as incubation temperature increased, the amount of cumulative FFA released was higher (except between 35 and 40°C) and the rate of activity significantly increased.

In Phase I of this research, the cumulative FFA released by *R. miehei* did not significantly differ among replications ($P > 0.05$) at 50°C, but was not repeatable at any of the other incubation temperatures (Table A-17). *R. miehei* was determined to have a significant difference ($P < 0.05$) in cumulative FFA released among replications in Phase II.

The time necessary to achieve the desired level of activity in Phase I varied between 35 and greater than 60 min at 35°C for replications 2 and 3 (Figure A-13); and

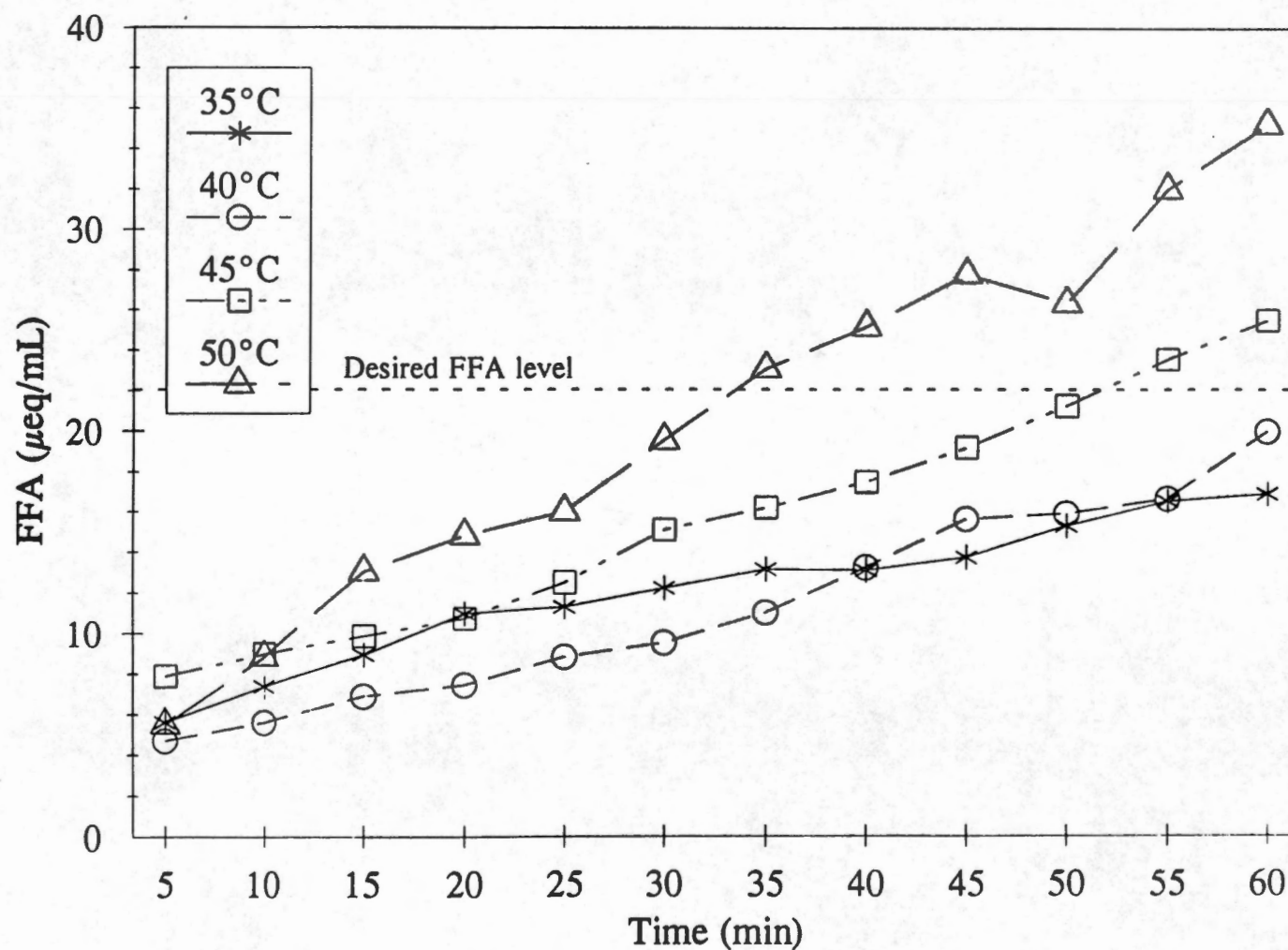


Figure 2-4 - Least-squares means cumulative free fatty acids released in butter oil hydrolyzed with lipase from *R. miehei* at four incubation temperatures over four replications as analyzed by extraction-titration.

40 min for replication 4 at 40°C (Figure A-14). The desired level was achieved for all replications between 35 and 60 min at 45°C (Figure A-15) and 25 and 55 min at 50°C (Figure A-16). In Phase II, the desired level was reached at approximately 30 min of incubation (Figure A-25).

An incubation temperature of 50°C was chosen for *R. miehei* for Phase II, based on activity at 60-min incubation, greatest rate of activity and perceived reproducibility at that temperature. The supplier reported an optimum incubation temperature of 40°C, with activity rapidly decreasing at temperatures above 40°C (Novo-Nordisk,1991). Our results indicate that *R. miehei* has a higher optimum activity temperature than indicated by the supplier.

Gas chromatography results

Through communication with each of the suppliers, Amano Enzymes U.S.A.Co., LTD, Novo-Nordisk A/S, and Solvay Enzymes, Inc., it was understood that each lipase evaluated selectively released short-chained FFA. In Phase II of this research, GC analysis was done on the samples removed at 60 min. Amounts of individual FFA (C4 - C18:3) were quantitated and the average $\mu\text{eq FFA/mL}$ for each lipase is listed in Table 2-5.

Individual free fatty acids. There was no significant difference ($P>0.05$) in the amounts of C11, C13 - 15, C16:1, and C18:0, C18:2 and C18:3 released among all four

lipases. The quantity of these fatty acids, except C14, following hydrolysis was not different from the amount in the unhydrolyzed butter oil (control).

Significantly more C4 - C10, C12, C14, and C18:1 ($P < 0.05$) was released by pancreatic lipase than was present in the unhydrolyzed butter oil (Table 2-5). Pancreatic lipase was found to release significantly more ($P < 0.05$) short-chain (C4 - C12) than medium- (C13 - C16:1) or long-chain (C18:0 - C18:3) FFA (Table 2-6). Pancreatic lipase released significantly less C16:0 than *A. niger*, but the same amount as *P. roqueforti*, *R. miehei* and the unhydrolyzed butter oil. Our research suggests that porcine pancreatic lipase is selective for releasing more short-chain FFA than the other lipases evaluated (Table 2-6). The supplier indicated that porcine pancreatic lipase is selective for releasing short-chain FFA (Solvay, 1994) and our results support this (Table 2-6).

There was not a significant difference in the distribution of short-, medium- or long-chain FFA released by *P. roqueforti* lipase (Table 2-6). *P. roqueforti* lipase however released significantly more C4 - C8, C10, C14, and C18:1 ($P < 0.05$) than was in the unhydrolyzed butter oil (Table 2-5). *P. roqueforti* released more C8 ($P < 0.05$) than *R. miehei*. *P. roqueforti* released significantly more C4 - C7, and C10 than *A. niger* and *R. miehei* (Table 2-5). Since the amount of total cumulative FFA released by *P. roqueforti* was low (Table 2-7), this lipase may require a larger quantity of water for activity in anhydrous butter oil than was present at the oil/water interface of the triglycerides.

Table 2-5 - Least-squares mean^a free fatty acids released in butter oil hydrolyzed with four lipases for 60 min and analyzed with the GC (Phase II)

Fatty Acid	Lipase (Incubation temperature °C)				
	Pancreatic (45)	<i>P.</i> <i>roqueforti</i> (35)	<i>A. niger</i> (45)	<i>R. miehei</i> (50)	Control (Butter oil)
	-----µeq FFA/mL-----				
C4	3.5726c	1.6519b	0.4568a	0.4448a	0.2482a
C6	1.4857d	0.6884c	0.2154b	0.1565ab	0.0816a
C7	0.0300c	0.0176b	0.0075a	0.0072a	0.0057a
C8	1.0172d	0.2818c	0.3038c	0.1680b	0.0224a
C9	0.0191c	0.0000a	0.0018a	0.0023ab	0.0099b
C10	1.6063d	0.5282c	0.4330b	0.3754b	0.1214a
C11	0.0249a	0.1637a	0.0031a	0.0491a	0.0131a
C12	1.4830c	0.5592a	1.1307b	1.0671b	0.2282a
C13	0.0102a	0.2583a	0.0064a	0.0809a	0.0000a
C14	1.0568b	0.7012b	1.0569b	0.7596b	0.2168a
C15	0.1361a	0.7666a	0.1747a	0.4390a	0.2277a
C16:0	2.6014ab	2.3468ab	4.6725c	3.4260bc	0.6105a
C16:1	0.1908a	0.4336a	0.1714a	1.3250a	0.1714a
C18:0	1.1608a	1.6062a	1.6342a	1.3253a	0.4395a
C18:1	2.2630b	1.8798b	2.7405b	2.6257b	0.8894a
C18:2	1.9568a	0.7782a	0.8126a	1.1606a	0.5506a
C18:3	0.2214a	0.1107a	0.1337a	0.1367a	0.0425a

^a Different letters in each row indicate a significant difference ($P < 0.05$), means over four replications.

Table 2-6 - Increase above control and percent cumulative free fatty acids released in butter oil hydrolyzed with lipase for 60 min as analyzed by GC (Phase II)

Fatty acids	Lipase				Control
	Pancreatic	<i>P. roqueforti</i>	<i>A. niger</i>	<i>R. miehei</i>	
	-----% of total within lipase ^a -----				
ΣC4 - C12	49.36e	30.34bc	18.06a	18.36ab	20.18ab
ΣC13 - C16:1	20.48ab	35.33cd	43.57de	47.05e	28.29bc
ΣC18:0 - C18:3	30.16bc	34.32cd	38.36cde	39.47cde	48.44e
	-----Increase in μeq FFA/mL above control-----				
ΣC4 - C12	11.65	4.33	2.49	2.16	
ΣC13 - C16:1	2.35	2.78	4.10	4.06	
ΣC18:0 - C18:3	1.99	1.34	1.84	1.80	
Total	3.90	2.32	2.63	2.52	

^aDifferent letters indicate a significant difference ($P < 0.05$); n = four replications.

A. niger and *R. miehei* lipases were very similar for the amounts and types of FFA released during hydrolysis. These lipases released proportionally more medium-chain FFA than pancreatic lipase (Table 2-6). *A. niger* lipase released more C8 ($P < 0.05$) than *R. miehei* lipase, but no other significant differences were found (Table 2-5). *A. niger* lipase released more C16:0 than pancreatic and *P. roqueforti* lipases (Table 2-5). These lipases were reported by Garcia et al. (1991) and Kwak et al., (1989) and confirmed by company representatives (Amano. 1994; Novo-Nordisk, 1994) to selectively release short-chain FFA. Our results indicate that more medium-chain than short-chain fatty acids were released by *A. niger* and *R. miehei* lipases (Table 2-6). Using anhydrous butter oil as a substrate, these lipases did not perform as expected.

Total free fatty acids. When quantified by GC analyses, pancreatic lipase released a significantly higher ($P < 0.05$) amount of total FFA than the other three lipases (Table 2-7). There was not a significant difference ($P > 0.05$) in the total FFA released by *P. roqueforti*, *A. niger*, and *R. miehei* lipases.

While the GC data and extraction-titration data cannot be directly compared, the trends for release of total FFA were different for the two methods. In the extraction-titration data, there was no significant difference ($P > 0.05$) in the amount of total FFA released by pancreatic, *A. niger*, and *R. miehei* (Table 2-7). This varied from the GC results where there was no significant difference ($P > 0.05$) among the FFA released by *P. roqueforti*, *A. niger*, and *R. miehei* lipases (Table 2-7). Anderson et al. (1991) determined that only 7% of the total FFA present in hydrolyzed butter are short-chained fatty acids (C4 - C12), with the remaining 93% with carbon chain-lengths greater than 12. However, results from our research indicate a much greater quantity of short-chain fatty acids released in butter oil during hydrolysis. In our research, 20.2% of the FFA in unhydrolyzed butter oil were short-chain fatty acids (Table 2-6). Of the total FFA released in hydrolyzed butter oil, pancreatic lipase released 49% short-chain fatty acids, *P. roqueforti* released 30%, *A. niger* released 18% and *R. miehei* released 18.4% (Table 2-6). Since the GC analyses only quantified fatty acids with carbon chain-lengths between 4 and 18:3, the extraction-titration analysis had the potential to quantitate all acids present. Butter oil, following hydrolysis with *P. roqueforti*, had significantly more ($P < 0.05$) total FFA than the unhydrolyzed butter oil, but significantly less ($P < 0.05$) than the other three lipases when measured by extraction-titration (Table 2-7).

Table 2-7 - Least-squares mean^a total free fatty acids released in butter oil hydrolyzed with four lipases as analyzed using two methods (Phase II)

Method ^b	Lipase				
	Pancreatic	<i>P. roqueforti</i>	<i>A. niger</i>	<i>R. miehei</i>	Control (butter oil)
	-----µeq FFA / ml-----				
Ext-titr.	75.67c	42.42b	72.63c	81.88c	3.17a
GC	18.84c	12.77b	13.96b	13.01b	3.79a

^aDifferent letters in the row indicates a significant difference ($P > 0.05$); means over four replications for 60 min incubation.

^bExt-titr. = Extraction-titration; GC = Gas chromatography.

SUMMARY AND IMPLICATIONS

Overall, the incubation temperature had little effect on the activity of the lipases evaluated. The reproducibility of the pancreatic, *P. roqueforti*, and *A. niger* lipase activities were questionable when butter oil was used as a substrate. The *R. miehei* lipase activity was reproducible at only one incubation temperature (50°C). The source of this non-reproducibility appears to lie in using anhydrous butter oil as a substrate. Water content is so low in anhydrous butter oil that an adequate oil/water interface is not available. It is noted that anhydrous butter oil is a difficult medium in which to establish an oil/water interface. These results directly contradict the belief that anhydrous butter oil is the perfect medium for lipolysis (Alfa-Laval, undated). Pancreatic lipase released even-numbered, short-chained (C4 - C12) FFA, whereas the *A. niger* and *R. miehei* lipases were found to release more medium-chained (C13 - C16:1) FFA. *P. roqueforti* released proportionally equal quantities of each of the short-, medium- and long-chain FFA.

Further work is needed to determine the specificity of various lipases in a variety of substrates. Some currently proposed work in our laboratory will involve the use of cream as a substrate using the four lipases evaluated in this research.

4. SENSORY AND CHEMICAL EVALUATION OF LIPOLYZED MILK PREPARED FROM HYDROLYZED BUTTER OIL AND SKIM MILK

ABSTRACT

Four lipases, porcine pancreatic, *Penicillium roqueforti*, *Aspergillus niger* and *Rhizomucor miehei*, were used to hydrolyze butter oil which was then recombined with skim milk to form lipolyzed 2% milk. These recombined milk samples were evaluated by a dairy flavor expert for lipolyzed flavor and were analyzed chemically by extraction-titration and GC. Of the lipases evaluated, pancreatic lipase gave the most consistent and characteristic lipolyzed flavor to the milk.

Lipolyzed milk, prepared using three methods, was evaluated by an experienced sensory panel with a paired-comparison difference test. The methods included the current standard method (Bodyfelt et al., 1988), a modified version of the standard method (combining raw and recombined milk) and an experimental method (recombined milk consisting of skim and hydrolyzed butter oil). Panelists found a difference ($P < 0.05$) between milk prepared using the standard method and the other two methods. However, no difference ($P > 0.05$) was found between the samples prepared from the methods using recombined milk. Panelists detected off-flavors, other than lipolyzed, that were inherent to the recombined milk. When those inherent differences were eliminated, panelists found the standard and experimental methods to have a similar lipolyzed flavor.

It is recommended that a substrate other than butter oil be used to improve the reproducibility of the experimental method for preparing lipolyzed milk reference samples.

INTRODUCTION

With milk and other dairy product consumption declining in recent years, interest by the dairy industry in researching and understanding flavor problems continues. A common flavor defect is lipolyzed or hydrolytic rancidity in milk and butter. This flavor defect is caused by the hydrolysis of free fatty acids (FFA) from triglycerides in milk fat by lipase (Alpha-Laval, undated; Deeth and Fitz-Gerald, 1976). The lipases responsible for lipolysis can originate in milk, or be exogenous from bacteria, animal tissue or fungus (Kwak et al., 1989). Lipolysis can occur pre-pasteurization or during product storage (Bell and Parsons, 1975; Deeth and Fitz-Gerald, 1976; Downey, 1980; Woo and Lindsay, 1983). Bacterial lipases are more heat resistant than milk lipases, which are deactivated by pasteurization. Lipolysis that occurs post-pasteurization is usually caused by bacterial lipases, even though the organisms themselves are killed during pasteurization (McNeill et al., 1986). Lipases can be specific or nonspecific for the FFA that are released during hydrolysis (Kwak et al., 1989). Porcine pancreatic lipase (depending on the source) has been reported to be both nonspecific and to release short-chain FFA (C4 - C12) (Kwak et al., 1989); *Aspergillus niger* and *Rhizomucor miehei* are selective for short-chain FFA (Garcia et al., 1991; Kwak et al., 1989); and *Penicillium*

Penicillium roqueforti is reported to release more butyric acid (C4) than any other FFA.

Unique flavors and aromas accompany the lipases when different FFA are released.

Lipolysis can be induced (hence the name induced lipolysis) through excessive agitation, temperature fluctuations, homogenization or the addition of raw to homogenized milk (Christen et al., 1993; Deeth and Fitz-Gerald, 1976). These mechanisms for lipolysis result in the "sickening" type lipolysis, the most common type, as reported by Willey and Duthie (1969). The second type of lipolysis, "unclean," which is rare, is the result of foaming or spontaneous lipolysis (Willey and Duthie, 1969). While lipolyzed flavor can be considered a defect under some circumstances, the food industry uses controlled lipolysis to create certain dairy flavors in cheeses and processed foods (Kwak et al., 1989; Shipe et al., 1978).

FFA responsible for lipolyzed flavor are the hydrophilic, even-numbered short-chain fatty acids with carbon chain-lengths between 4 and 12 (Scanlan et al., 1965). Researchers differ as to if one FFA has a dominate effect on lipolyzed flavor (Bills et al., 1969; Woo and Lindsay, 1983). Lipolyzed flavor has been described as "goaty," "bitter," "soapy" and "butyric" (Shipe et al., 1978).

Lipolysis can be evaluated with chemical and sensory methods. Some chemical analyses for evaluating lipolysis include acid degree value (ADV), extraction-titration and gas chromatography (GC). ADV has been found to recover more long-chain FFA (C14 - C18), that are hydrophobic in nature and are released into the fat recovered by the ADV procedure. Extraction-titration is a fast and simple method for measuring FFA, but only measures total FFA and only partially recovers all the short-chain FFA (Christen et al.,

1993). GC analyses, although more reliable and quantitative, presently involves a long and tedious recovery procedure (Christen et al., 1993). ADV has been found to correlate poorly with sensory scores on lipolyzed milk samples (Duncan et al., 1990, 1991). Sensory evaluation remains the best method for evaluating lipolyzed flavor in dairy products (Duncan et al., 1990).

To aid in research on lipolyzed milk, several methods for preparing lipolyzed milk samples were developed. The most common of these involves combining raw and homogenized milk, agitating in a blender, incubating for 24 to 48 hr under refrigeration, pasteurizing, extending to a larger quantity with homogenized milk and incubating overnight again (at refrigerator temperatures) to allow lipolyzed flavors to develop (Bodyfelt et al., 1988). Lee (1992) discovered inconsistencies in lipolyzed flavor of milk samples when the method developed by Bodyfelt et al. (1988) was used. Duncan et al. (1990) found that laboratory-prepared lipolyzed milk samples had intensities higher than farm-lipolyzed milk samples.

Recombination of butter oil and nonfat dry milk (NFDM) is a common alternative to fresh milk in countries such as Egypt and other countries, where raw milk is in short supply (Shahin and Fahmy, 1988). In the United States, NFDM is associated with a distinct flavor that varies from that of fresh milk. Using the concept of recombined milk used by other countries, butter oil can be homogenized with fresh skim milk to the desired fat level with a less objectionable taste than NFDM.

The objectives of this research were to: (1) devise a consistent method for producing laboratory-prepared lipolyzed milk using hydrolyzed butter oil and skim milk,

(2) determine which of the four lipases evaluated gave a characteristic lipolyzed milk flavor, (3) determine if there is a perceivable difference between laboratory-prepared lipolyzed milk samples using the standard method developed by Bodyfelt et al. (1988), a modified version of this method using recombined milk and an experimental method using hydrolyzed butter oil homogenized with skim milk, and (4) to see how extraction-titration and GC data relate for $\mu\text{eq FFA/ml}$ lipolyzed milk samples.

MATERIALS AND METHODS

Materials

Four individual lots of anhydrous butter oil (clarified butter) were purchased from Mid America Farms Co. (Springfield, MO). They were divided into 450-mL portions, placed into 500-mL Nalgene® bottles and frozen at -37°C . Each lot was tested for total FFA content prior to freezing using the extraction-titration procedure. The four lots of butter oil (A - D) were determined to inherently contain 2.45 ± 1.5 , 3.23 ± 1.5 , 3.79 ± 1.2 , and $3.27 \pm 1.9 \mu\text{eq FFA/mL}$, respectively.

Food grade lipases were obtained from several suppliers: *Aspergillus niger* and *Penicillium roqueforti* were donated by Amano Enzyme U.S.A. Co., Ltd (Troy, VA); *Rhizomucor meihei* was donated by by Novo Nordisk Bioindustrials, Inc. (Danbury, CT); and porcine pancreatic lipase was donated by Solvay Enzymes (Elhart, IN). Lipase storage temperature was 4°C throughout the study.

A 10% gum arabic (TIC Gums, Inc., Belcamp, MD) solution (w/w) was prepared and stored in 500-mL Nalgene® bottles at 4°C . Additional solvents and chemicals were

purchased from Baxter Diagnostics Inc., Scientific Products Division (McGraw Park, IL).

Raw cows milk was obtained from The University of Tennessee Experiment Station dairy farm. Samples (40 mL) were frozen at -70°C in 60-mL, amber colored bottles and stored at -30°C . Just prior to use, the raw milk samples were thawed in a 25°C water bath.

Commercial 2% milk in light impervious containers, used prior to the expiration code, was purchased locally and stored at 4°C for less than 36 hr prior to use. A different supply of 2% milk was used for each replication.

Commercial skim milk in light impervious containers, used prior to the expiration code, was purchased locally and divided into 700-g portions and frozen at -40°C in resealable plastic freezer bags. The frozen skim milk samples were stored at -35°C for less than 10 days. The skim milk was (1) partially thawed at 25°C for 0.5 to 2 hr and (2) fully thawed for 2 to 3 min in a microwave oven (Kenmore, model 566-88785350) with a measured output of 438 watt on high power (Van Zante, 1973).

Methods - Phase III: Flavor description by dairy flavor expert

Butter oil hydrolysis. The frozen butter oil was removed from the freezer 16 - 20 hr prior to use and held at $23 - 25^{\circ}\text{C}$. It was then melted for 2- 3 min in a microwave oven with a measured output of 438 watts (Van Zante, 1973). One-hundred-gram portions

were poured into 1-L Erlenmeyer flasks and held at a prescribed incubation temperature. A 10% gum arabic solution (4 mL) was added to each flask to facilitate production of the oil/water interface. Amounts of lipase added to each flask to hydrolyze the butter oil were: 0.18 g *R. meihei*, 0.04 g *A. niger*, 0.23 g *P. roqueforti*, or 0.11 g pancreatic lipase. These amounts were determined in Phase II of the research and yielded FFA levels equivalent to 22 mL titrant more than unhydrolyzed butter oil.

Once the lipase and gum arabic had been added to the butter oil, the flasks were flushed with nitrogen gas to reduce volatilization of FFA. A Nalgene® stopper was used to seal the flask. A 1-cm tube was inserted through the stopper, and another stopper was used to close the top of the tube.

The flasks were incubated in a six-position, Labline Instruments Inc. (Melrose Park, IL) circulating waterbath/stirrer set at 35°C for *P. roqueforti*, 45°C for pancreatic and *A. niger*, and 50°C for *R. miehei*. The butter oil was agitated using a 34-mm round finned stir bar at a circulating speed of 980 ± 40 rpm (35°C), 1070 ± 30 rpm (45°C) and 1085 ± 20 rpm (50°C) (Walker, 1994). To neutralize any differences in stir bar speed, the same three stirrer sites were used for all lipases on all replications. Incubation time for the butter oil and lipase combination was 1 hr.

For each lipase, 4-mL samples were removed from each flask after 30, 45 and 60 min of incubation time and used to form composite samples. Two samples were removed from the composite sample for GC and extraction-titration analysis. The entire procedure was replicated four times.

Extraction-titration procedure. Hydrolyzed butter oil samples (0.5 mL) from each time period were placed into 25-mL screw-cap test tubes containing 4.5 mL distilled water. Immediately following removal from the flasks, 15 mL extraction mixture were added to the butter oil/water mixture. The test tubes were capped and vortexed for 15 sec at speed setting 7. Following vortexing, 16.5 mL hexane and 6.5 mL distilled water were added to each test tube. All reagents were added using repeating burette dispensers fitted atop amber reagent bottles. The test tubes were inverted by hand 15 times to mix the solvents, placed upright and allowed to separate into two layers.

Using 20-mL volumetric pipets (Kimax, ± 0.001 mL), the supernatant was removed and placed into a covered, 60-mL titration cup (Mettler Instrument Corp, Hightstown, NJ). The bottom, aqueous solution was disposed of as solvent waste. A Mettler DL12 automatic titrator (Hightstown, NJ) was used to titrate the sample (containing the extracted FFA) to a final pH of 11.3. Potassium hydroxide was standardized using lauric acid (C12) in hexane. A blank (sample replaced with hexane) and three standard samples were completed each day. The milliliters of titrant needed to bring the sample to pH 11.3 was recorded and used in the following formula to determine microequivalents of FFA per milliliter of sample ($\mu\text{eq/mL}$):

$$\text{Equation 1: } \frac{\mu\text{eq FFA}}{\text{mL}} = \frac{(\text{mL}_{\text{Sample}} - \text{mL}_{\text{Blank}}) \times N_{\text{KOH}} \times 1000}{\text{mL}_{\text{sample}}}$$

Where μeq = microequivalents; FFA = free fatty acid; $\text{mL}_{\text{Sample}}$ = milliliters titrant for sample; mL_{Blank} = milliliters titrant for blank; N_{KOH} = normality of potassium hydroxide; Sample size = milliliters of sample.

Milk samples prepared from hydrolyzed butter oil samples. Skim milk, purchased locally, was placed into resealable plastic freezer bags in 850-g portions and frozen at -40°C , then stored at -35°C until needed for sample preparation. The skim milk was thawed in a microwave oven (438-watt), then 196-g portions were placed into 500-mL Nalgene® screwcap bottles and heated in a 55°C waterbath.

Hydrolyzed butter oil portions acquired at 30, 45, and 60 min incubation times for each lipase were heated in a microwave oven (438 watt) to 85°C and held at least 10 seconds to deactivate the lipases. The microwave-heated butter oil (4 g) was added to the warm skim milk (196 g) and tempered to 50°C . Milk/butter oil samples were homogenized in a Virtishear Cyclone IQ² homogenizer (The Virtis Co., Gardiner, NY) at 15,000 rpm for 3.5 min. Preliminary tests showed these conditions produced milk with minimal fat separation. Milk samples were held at 10°C for 18 - 22 hr and filtered through sterilized cheese cloth to remove any fat clumps.

Sensory evaluation of milk samples by dairy expert. The dairy-flavor expert received 3 or 4 lipolyzed milk samples at a session to be evaluated for characteristic lipolyzed milk flavor and flavor intensity. Seven sampling sessions, spaced 30 min apart, were

held each day. The samples tested in each of the seven sessions are shown in Table 3-1. Milk samples were presented to the dairy-flavor expert in randomized order in opaque, 60-mL plastic cups labeled with randomly chosen 3-digit codes. Samples were evaluated at 10°C and were expectorated following sampling. Saltine crackers (unsalted) were used to clear the palate after each session. Four replications (4 days) of sensory evaluation were conducted.

Extraction-titration procedure for milk samples. Milk samples (5 mL) from each incubation time for each lipase were evaluated with the extraction-titration procedure as described previously. The extraction-titration procedure was as described previously.

Statistical analyses. Proc GLM (SAS Institute, Inc., 1989) was performed on the μeq FFA/mL milk (dependent variable) for all lipases. The PDIFF function was used to determine differences among cumulative FFA at the three incubation times for all the lipases (independent variables) and to determine reproducibility of the lipase action.

Table 3-1 - Sampling sets for dairy expert flavor description per replication

Incubation time (min)	Lipases			
	Pancreatic	<i>P. roqueforti</i>	<i>A. niger</i>	<i>R. miehei</i>
30	1,5 ^a	2,5	3,5	4,5
45	1,6	2,6	3,6	4,6
60	1,7	2,7	3,7	4,7

^aEach number represents an evaluation session.

Gas chromatography - sample preparation. Milk samples prepared from skim milk and hydrolyzed butter oil samples at 30-, 45-, and 60-min incubation times were analyzed using GC. A 10-mL milk sample was placed into a 40-ml screw-cap Nalgene® centrifuge tube, and 3 mL 37% hydrochloric acid (Fisher Scientific,) 25 mL of a 1:1 (v/v) diethyl ether-hexane mixture (containing 0.005% (w/v) BHT) and 1 mL of the C5 and C17 internal standard in hexane were added. The test tube was capped, vortexed at speed setting 7 for 15 sec, and 11 mL of supernatant transferred to a test tube containing 1 g anhydrous sodium sulfate (to bind any water in sample). The samples were then stored at -10°C until the extraction procedure could be completed.

Basic alumina was dried in a vacuum oven at 175°C, at 20 psi for 2 hr and stored in a 60-mL amber-colored bottle in a dessicator until ready for activation. The alumina was activated by the addition of deionized water (3% v/w), vortexed to break up clumps and allowed to equilibrate for at least 2 hr.

A glass champagne column was prepared with a small plug of silanized glass wool and 1 g activated basic alumina. The sample was poured twice over the alumina and collected in a 50-ml Erlenmeyer flask. The alumina was washed twice with 5 mL 1:1 (v/v) diethyl ether-hexane (without BHT). The drained solution in the Erlenmeyer flask was discarded as solvent waste. The alumina was dried under vacuum for 1 min and transferred to a conical, 10-mL screw-cap test tube. The sample-impregnated alumina was stored frozen at -25°C until analysis could be completed.

The FFA were removed from the alumina with 1 mL 10% (v/v) formic acid/diethyl ether solution and vortexed (speed setting 7) for 1 min. The test tubes were

centrifuged on a table-top centrifuge (Damon/IEC Division, Needham Hts., MA) at setting 5 for 5 min. The supernatant was transferred to a GC vial using a Pasteur pipet and sealed. Samples were held at -25°C until GC analysis was completed.

Gas chromatography - analysis of free fatty acids. Free fatty acids were separated and quantified using a Hewlett Packard (HP) 5890 Series II Gas Chromatograph (GC) (Hewlett-Packard Co., Kennett Square, PA), fitted with an HP7673 automatic sampler (Hewlett-Packard Co., Kennett Square, PA) and a flame ionization detector (FID). A 3- μ L sample was injected into a 25-m HP-FFA capillary column (Hewlett-Packard Co., Kennett Square, PA) with an inside diameter of 0.32 mm and a film thickness of 0.52 μ m. Helium was used for the carrier gas at a flow rate of 3.03 mL/min and also for the combined column and make-up gas at a flow rate of 8.00 mL/min. The column head pressure was maintained at 20 psi. The splitless mode, with a purge time of 0.05 min, was used when the samples were injected into the column, after which the injection port switched to the split mode of operation. The GC oven was set to increase from an initial temperature of 110°C to a final temperature of 225°C, increasing at 5°C/min increments with a final hold time of 20 min. The injector temperature was 250°C, while the FID temperature was 260°C. Individual FFA were identified and peak areas measured using HP3365 ChemStation software (Hewlett-Packard Co., Kennett Square, PA).

Fatty acid standards for the saturated fatty acids (C4 - C18) and unsaturated fatty acids (C16:1 and C18:1 - C18:3) served as external standards to determine fatty acid retention times and to quantify FFA. Amounts used for fatty acid external standards are

listed in Appendix C-2. The peak areas for the external standards were used to calculate the Relative Molar Response (RMR) (Appendix C-3) and the grams of fatty acid in each sample.

Calculations and equations. The following equations were used to convert FFA retention times and peak areas to μeq FFA/mL milk for the samples evaluated by sensory evaluation:

$$\text{Equation 2: } \text{RMR} = \frac{\text{Area}_{\text{FA}} / \text{Mole}_{\text{FA}}}{\text{Area}_{\text{IS}} / \text{Mole}_{\text{IS}}}$$

$$\text{Equation 3: } \text{grams}_{\text{FA}} = \frac{\text{Area}_{\text{FA}} \times \text{MolecularWeight}_{\text{FA}} \times \text{grams}_{\text{IS}}}{\text{Area}_{\text{IS}} \times \text{MolecularWeight}_{\text{IS}} \times \text{RMR}_{\text{FA/IS}}}$$

$$\text{Equation 4: } \frac{\mu\text{eq}_{\text{FA}}}{\text{ml}} = \frac{\text{gram}_{\text{FA}} \times 10^6 \mu\text{mol} / \text{mol}}{\text{gram}_{\text{FA/mol}}}$$

Where RMR = relative molar response, FA = fatty acid, IS = internal standard, μmol = micromole, μeq = microequivalent, Area_{FA} = GC peak area of fatty acid, C5 was used as the internal standard for calculating the grams_{FA} for C4 - C12, C17 was used as the internal standard for calculating the grams_{FA} for C14 - C18:3.

Statistical analyses. Proc GLM (SAS Institute, Inc., 1989) was performed on the calculated FFA data (dependent variable) to determine differences in quantities of

individual FFA for each lipase. Lipase, time and replication were the independent variables. The PDIFF option was used to determine differences in least-squares means of individual FFA for the interaction of lipase by time and to determine reproducibility of the hydrolysis method through analysis of the interaction of lipase by time by replication.

Methods - Phase IV: Sensory evaluation by experienced panel

Sample preparation. Three methods for preparing lipolyzed milk samples were evaluated. The current standard method for preparing "rancid" milk (Bodyfelt et al., 1988) was used with the following modification: a microwave oven (Kenmore, model 566-88785350, Sears, Roebuck and Co., Chicago, IL) with a measured output of 53 watts on low power (Van Zante, 1973) was used to pasteurize the raw and 2% milk mixture instead of a waterbath. A temperature probe attached to the microwave was used to monitor the milk until a temperature of 74°C ($165 \pm 3^{\circ}\text{F}$) was obtained. The milk sample was agitated manually by swirling the bottle at each 5°C temperature increase. The milk was held at 74°C for 1 min to insure proper pasteurization. The pasteurized milk was immediately cooled in an icebath to quickly bring the milk temperature to below 20°C . One sample was pasteurized at a time.

In the second method, 2% milk (w/w) was prepared by combining thawed, 35°C skim milk (392 g) and melted anhydrous butter oil (8 g) in 1-L Erlenmeyer flasks. The

recombined milk was heated to between 50 and 53°C in a 55°C waterbath and homogenized using a Virtishear Cyclone IQ² homogenizer (Virtis Co., Gardiner, NY) at 15,000 rpm for 3.5 min. Once homogenization was complete, the milk samples were cooled in an icebath to below 20°C. Raw milk (20 mL) was added to 100 mL recombined milk and allowed to incubate for 48 hr at 4°C. This rancid milk mixture was pasteurized in a microwave oven as previously described. Following cooling, the pasteurized milk was extended to 600 mL with recombined 2% milk and held at 4°C overnight to further develop the lipolyzed flavor.

The third method for preparing lipolyzed milk used butter oil hydrolyzed with pancreatic lipase as reported in Phase II. The method for recombining 2% milk, as described above, was utilized with the following modification: hydrolyzed butter oil (HBO) was used instead of plain anhydrous butter oil. A dual purpose was served by heating the HBO to 85°C in a microwave oven (438 watt) on high power: inactivation of the lipase and melting the butter oil. As with other lipolyzed milk samples, the homogenized HBO/skim milk samples were refrigerated at 4°C and held 15 - 18 hr to further develop the lipolyzed flavor.

All glassware and equipment used for butter oil hydrolysis and milk sample preparation was food-grade and kept separate from any glassware used for chemical analysis.

Sensory evaluation. Lipolyzed milk samples prepared using three methods were evaluated by an experienced panel consisting of 30 students, staff and faculty from the Institute of Agriculture at The University of Tennessee - Knoxville. A paired-comparison difference test was used to determine if there was a difference in milk flavor between pairs of samples prepared by the three methods. A sample scorecard can be found in Appendix C-5. Each panelist received three sets of two samples per replication. If a difference was perceived between each set of samples, an explanation of the differences was requested. A balanced order of presentation (Evolutionary Software, Inc., 1991) was used for samples to eliminate panelist bias. The samples (40-mL) were presented to the panelists in 60-mL, clear-glass, screw-top bottles labeled with randomly chosen 3-digit numbers under white lighting. All sensory evaluation by the panelists was conducted in the Department of Food Science and Technology Sensory Evaluation Laboratory at The University of Tennessee between the hours of 9:30 am and 1:00 pm. Panelists were asked to expectorate all milk samples. They were instructed to rinse their mouths with water and eat three unsalted oyster crackers between sets of samples.

Gas chromatography. The same procedures for GC sample preparation, analysis of FFA, calculations and equations that were used in Phase III were also used in Phase IV.

Statistical analyses. Simple difference test tables (Roessler et al., 1978) were used to determine significance in the sensory panel data. Proc GLM (SAS Institute, 1989) (PDIFF function of least-squares means) was used to determine differences among

RESULTS AND DISCUSSION

Phase III: Sensory and chemical evaluation of milk using all four lipases

Each of the four lipases was used to hydrolyze butter oil for three different incubation periods. The hydrolyzed butter oil was recombined with skim milk to make lipolyzed 2% milk. These milk samples were evaluated by a dairy flavor expert for characteristic lipolyzed flavor. Comments are summarized in Table 3-2. This table is divided into two sections: (1) flavor description by the dairy flavor expert for the three incubation periods within each lipase and (2) flavor description by the dairy flavor expert for each of the incubation periods among the four lipases evaluated. Following this evaluation, extraction-titration and GC analysis were conducted on each of the samples. Sensory comments and total FFA in the samples for each lipase by replication can be found in Tables B-1 through B-4. These tables are similar to Table 3-2 with the following exception: the extraction-titration results are listed for each replication. Following is a discussion of the results from samples prepared by hydrolyzing butter oil by each individual lipase.

Porcine pancreatic lipase. Based on evaluation by the dairy flavor expert, this lipase was found to give a characteristically lipolyzed flavor to the milk which was consistent

Table 3-2 - Summary of flavor descriptions^a for milk samples made by combining hydrolyzed butter oil and skim milk, for four lipases and three incubation times (Phase III)

Evaluation	Lipase	Incubation time (min)		
		30	45	60
Flavor descriptions within lipases ^b (across incubation times)	Pancreatic	Slightly rancid	Rancid	Very rancid
	<i>P. roqueforti</i>	Very slight to not rancid	Slightly rancid	Slight to rancid
	<i>A. niger</i>	Not rancid, bad milk	Slightly rancid	Rancid
	<i>R. miehei</i>	Very weak to not rancid	Very weak to not rancid	Very weak to not rancid
Flavor descriptions among lipases ^b (within incubation times)	Pancreatic	Strong char. rancid	Very rancid	Good example of rancid
	<i>P. roqueforti</i>	Chemical taste, not char.	Slight chemical or strange taste	Weakest rancid
	<i>A. niger</i>	Chemical taste, not char.	Slight chemical, slight rancid	Weak rancid
	<i>R. miehei</i>	Rancid, less than Pancreatic	Chemical taste, slight rancid	Low rancidity

^a Flavor description by dairy flavor expert.

^bn = four replications.

throughout the four replications. When compared to the other three lipases ("among"), pancreatic consistently had the highest, and most consistent description of lipolyzed flavor, regardless of incubation time. When evaluating "within" samples, the intensity of perceived rancidity for the samples increased with incubation time of butter oil (Table 3-2).

The trend for perceived rancidity in milk followed the same trend as the extraction-titration and GC results predicted: perceived rancidity increased as total FFA present in the milk increased. In extraction-titration analysis, fewer FFA were released after 30 min of incubation ($P < 0.05$) than after 45 and 60 min (Table 3-3). In GC analysis, total FFA following 30 min incubation were significantly less than at 60 min. Samples produced by using pancreatic lipase did not differ ($P > 0.05$) in total FFA as determined by extraction-titration analysis in 3 of 4 replications (Table B-5). Pancreatic lipase, incubated at 45°C for 45 min, was chosen for further evaluation.

***Penicillium roqueforti* lipase.** When skim milk and butter oil hydrolyzed with *P. roqueforti* were recombined, the resulting milk flavor was described as "bad milk," "slight chemical taste" and "not rancid" to "slightly rancid" (Table 3-2 and Tables B-1 and B-4). The lipolyzed flavor intensity did not increase with incubation time (Table 3-2). When milk prepared from *P. roqueforti* was compared to milk prepared from the other lipases, the flavor was described as "chemical" and was not characteristic lipolyzed (Table 3-2).

The extraction-titration results for milk (Table 3-3) did not follow the trend observed in butter oil reported previously (Table 2-1). Similarly, total FFA in milk determined by GC were the same ($P > 0.05$) regardless of incubation time (Table 3-3). Extraction-titration values for total FFA present in milk prepared using butter oil hydrolyzed with *P. roqueforti* lipase across time was not found to be reproducible ($P < 0.05$) (Table B-5).

Table 3-3 - Least-squares mean^a total free fatty acids present in lipolyzed milk samples prepared from hydrolyzed butter oil and skim milk as analyzed by two methods (Phase III)

Lipase	Method of analysis					
	Extraction-titration			Gas chromatography		
	Butter oil incubation time (min)					
	30	45	60	30	45	60
	----- $\mu\text{eq FFA/mL}$ -----					
Pancreatic	3.54a	4.21b	4.70b	1.69x	3.16xy	4.23y
<i>P. roqueforti</i>	2.82a	2.61a	2.86a	2.51x	1.52x	1.95x
<i>A. niger</i>	2.84a	3.36ab	3.85b	1.57x	1.20x	1.27x
<i>R. miehei</i>	4.35a	4.76a	4.71a	0.59x	0.61x	0.43x

^aDifferent letters in the same row comparing a similar method indicates a significant difference ($P < 0.05$); cumulative means over four replications.

***Aspergillus niger* lipase.** This lipase gave an increasingly lipolyzed flavor to milk as incubation time increased (Table 3-2), with 60 min having a clearly identifiable lipolyzed flavor. The flavor descriptors for milk prepared from butter oil hydrolyzed by *A. niger* lipase within the three incubation periods were: "bad milk," "slightly rancid" to "rancid" and "nice rancid" (Table 3-2 and Tables B-1 through B-4). When milk prepared from butter oil hydrolyzed by *A. niger* lipase at all incubation periods was compared to milk from the other lipases ("among"), the flavor was perceived as "chemical" instead of "rancid" (Table 3-2). However following 60 min incubation, a weak rancid flavor was detected in the milk (Table 3-2).

The extraction-titration results in milk followed the trend: total FFA increased as incubation time increased; significantly less ($P < 0.05$) total FFA were present at 30 min than at 60 min (Table 3-3). The extraction-titration results correspond to description of flavor intensities over time, in that total cumulative FFA present in milk increase as the flavor intensity increases (Table 3-2). The GC results however, indicated variability in FFA present over time, and did not follow the same trend as the extraction-titration and sensory evaluation. There was no significant difference ($P > 0.05$) in amount of FFA released over time by *A. niger* lipase as analyzed by GC (Table 3-3). Total FFA present in the milk prepared using butter oil hydrolyzed with *A. niger* lipase across time was not found to be reproducible using extraction-titration analysis (Table B-5).

***Rhizomucor miehei* lipase.** When the milk prepared from *R. miehei* lipase was compared over the three incubation times, the flavor imparted to the milk was described as "not rancid" to "very weak rancid" (Table 3-2). The lipolyzed flavor intensity did not increase with increased incubation time. When *R. miehei* was compared to the other three lipases, the milk flavor was described as "slight chemical taste" or "rancid," but at a lower intensity than pancreatic (Table 3-2).

Total FFA present in milk, as analyzed by extraction-titration, were not different ($P > 0.05$) among times (Table 3-3), confirming the sensory response reported above. Similarly, there is no difference ($P > 0.05$) in total FFA present in the milk at any incubation time as analyzed by GC. Total FFA present in the milk prepared using butter

miehei lipase across time was not found to be reproducible using extraction-titration analysis (Table B-5).

Phase IV: Sensory and chemical evaluation using porcine pancreatic lipase only

The sensory panelists were asked whether there was a difference between pairs of samples presented for evaluation. Several panelists were members of The University of Tennessee Dairy Products Evaluation Team and were familiar with milk flavor defects. All panelists had previous experience in sensory evaluation, and several admitted to having a pre-conceived idea that one of the three pairs presented must contain "like" samples. This idea may have biased panelist perception of the samples. A summary of panelist detection responses is listed in Table 3-4. A compilation of panelist remarks on differences detected is listed in the Appendix C-6a - 6c.

In this section, the standard method for preparing lipolyzed milk will be referred to as 2% standard method, the modified standard method (where recombined 2% milk was used instead of retail 2% milk) will be referred to as recombined standard method, and the samples where 2% milk was prepared from the recombination of hydrolyzed butter oil and skim milk will be referred to as the experimental method.

Sensory panel results. In 3 of 4 replications, panelists were significantly ($P < 0.05$) able to identify a difference between the 2% standard method and both the recombined standard method and the experimental method (Table 3-4). Panelists were not able to identify a difference ($P > 0.05$) between the milk samples made from the recombined

standard method and the experimental method. Comments from panelists indicated that "buttery," "clarified butter" and "oxidized" were flavors detected in both the recombined standard method samples and the experimental method samples. These flavors may have over-powered or masked the lipolyzed flavor, or been the only differences that were perceived. Other comments indicated that differences in milk "body" (whole vs. skim milk) were the differences detected. Panelists were able to identify the lipolyzed flavor in both the recombined milk samples by using such terms as: "soapy," "rancid" and "bitter," which are common descriptors for lipolyzed milk (Duncan et al., 1990; Shipe et al., 1978; Woo and Lindsay, 1983). Since the panelists were only asked if there was a difference between samples, the differences perceived may not have been related only to lipolyzed flavor, but to other characteristics inherent to recombined milk. When those inherent differences were eliminated (i.e. comparison of the standard method and experimental method), panelists could not differentiate between the samples. Since there was no significant difference ($P > 0.05$) between the recombined standard method and the experimental method, it can be speculated that the nature and degree of rancidity for the two methods is the same.

Chemical analysis results. Extraction-titration and GC results for lipolyzed milk samples are presented in Table 3-5. While the experimental method milk appeared to have more total FFA than either of the other methods in both extraction-titration and GC analysis, these differences were not significant ($P > 0.05$). There was no difference ($P > 0.05$) in the individual FFA present in milk samples by any of the methods as

Table 3-4 - Sensory panel responses^d distinguishing differences among three methods for preparing lipolyzed milk samples (Phase IV)

Replications ^b	Panelist responses	Method comparisons ^c		
		2%SM vs. Recom. SM	Recom. SM vs. Exp M	2% SM vs. Exp M
1	Different	23 ^a	11	22 ^a
	Alike	7	19	8
2	Different	22 ^a	11	16
	Alike	8	19	14
3	Different	19	11	26 ^a
	Alike	11	19	4
4	Different	24 ^a	11	27 ^a
	Alike	6	19	3
Total	Different	88 ^a	44	91 ^a
	Alike	32	76	29

^a indicates samples are significantly different ($P < 0.05$);

^bn = 30 experienced panelists per replication.

^c2%SM = 2% standard method; RecSM = Recombined milk standard method; ExpM = Recombined milk made with hydrolyzed butter oil method.

^dSignificance determined with significance tables (Roessler et al., 1978).

analyzed by GC (Table B-10). Sensory evaluation confirmed the lack of significant difference ($P > 0.05$) in total FFA released in milk prepared using either of the recombined milk methods (Tables 3-4 and 3-5). Christen and Lee (1994), found a similar relationship between sensory evaluation and extraction-titration values. In the current research, chemical analyses support the hypothesis that the experimental method can produce a lipolyzed milk sample that is similar in flavor to the method developed by Bodyfelt et al. (1988).

Table 3-5 - Least-squares means^a total free fatty acids present in lipolyzed milk samples prepared by three methods as analyzed by two methods (Phase IV)

Analysis method	Sample ^b		
	2%SM	RecSM	ExpM
	----- $\mu\text{eq FFA/mL}$ -----		
Ext-Titr.	1.47a	1.28a	3.82a
GC	4.52a	4.97a	9.09a

^aDifferent letters in each row indicate a significant difference ($P < 0.05$); means over four replications.

^b2%SM = 2% Standard method; RecSM = Recombined milk Standard method; ExpM = Recombined milk made with hydrolyzed butter oil method.

SUMMARY AND IMPLICATIONS

Porcine pancreatic lipase gave a consistent, characteristic lipolyzed flavor to milk at all of the three butter oil incubation times evaluated. The flavor descriptions indicated increased intensity as incubation time increased. *P. roqueforti*, *A. niger*, and *R. miehei* did not give consistent flavor descriptions, nor a characteristic lipolyzed flavor. The

chemical analyses followed the same trend as the sensory description trends for pancreatic, but not for the other lipases.

The sensory evaluation panel found a difference between samples from the standard method for preparing lipolyzed milk (Bodyfelt et al., 1988) and samples from the recombined standard method and the experimental method. However, it appears that the differences perceived by the panelists were differences inherent to the milk, not brought about by lipolysis. Since the panelists did not perceive a difference between the two methods made with recombined milk, it can be concluded that there are no lipolyzed flavor differences between the recombined standard method and the experimental method.

It is clear that butter oil is not the best substrate for hydrolysis since it may not allow for development of a good oil/water interface at which lipolysis can occur and it has an inherent oxidized or clarified butter flavor that is transferred to the milk and can mask other flavors. The procedure described for preparing a consistent lipolyzed milk sample looks promising, but butter oil is not the substrate that should be used. Further research needed in this area includes: using cream as the substrate for lipolysis, using a trained sensory panel to evaluate the differences between the three methods for preparing lipolyzed milk samples, doing more replications of the experiment to confirm reproducibility of pancreatic lipase activity and to determine what other factors (besides agitation, temperature and amount of water present) affect lipase activity.

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APPENDIXES

APPENDIX A.

LIPASE ACTIVITY BY REPLICATION AS ANALYZED BY EXTRACTION-TITRATION

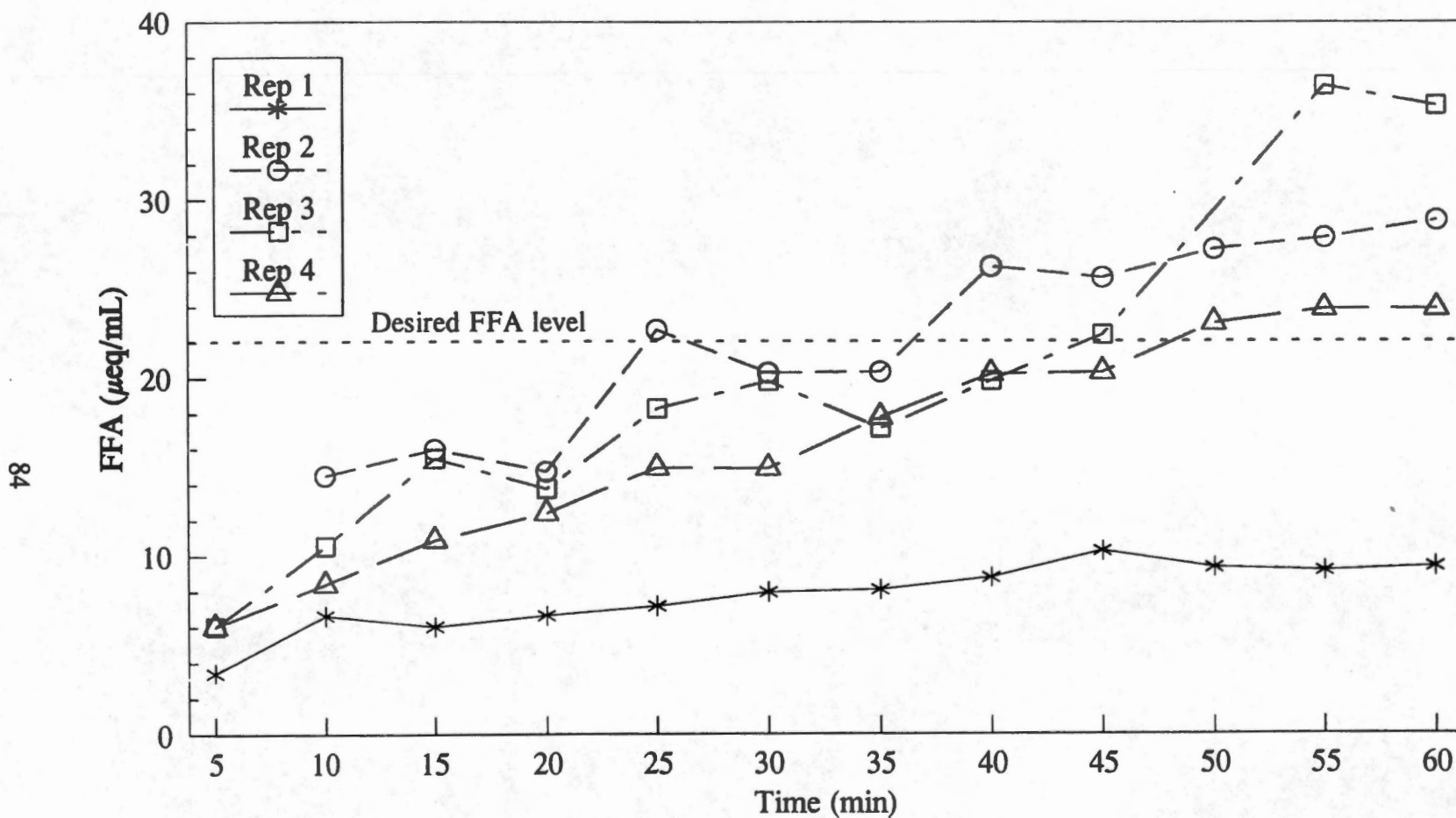


Figure A-1 - Free fatty acids (FFA) released in butter oil hydrolyzed with porcine pancreatic lipase at an incubation temperature of 35°C (in Phase I).

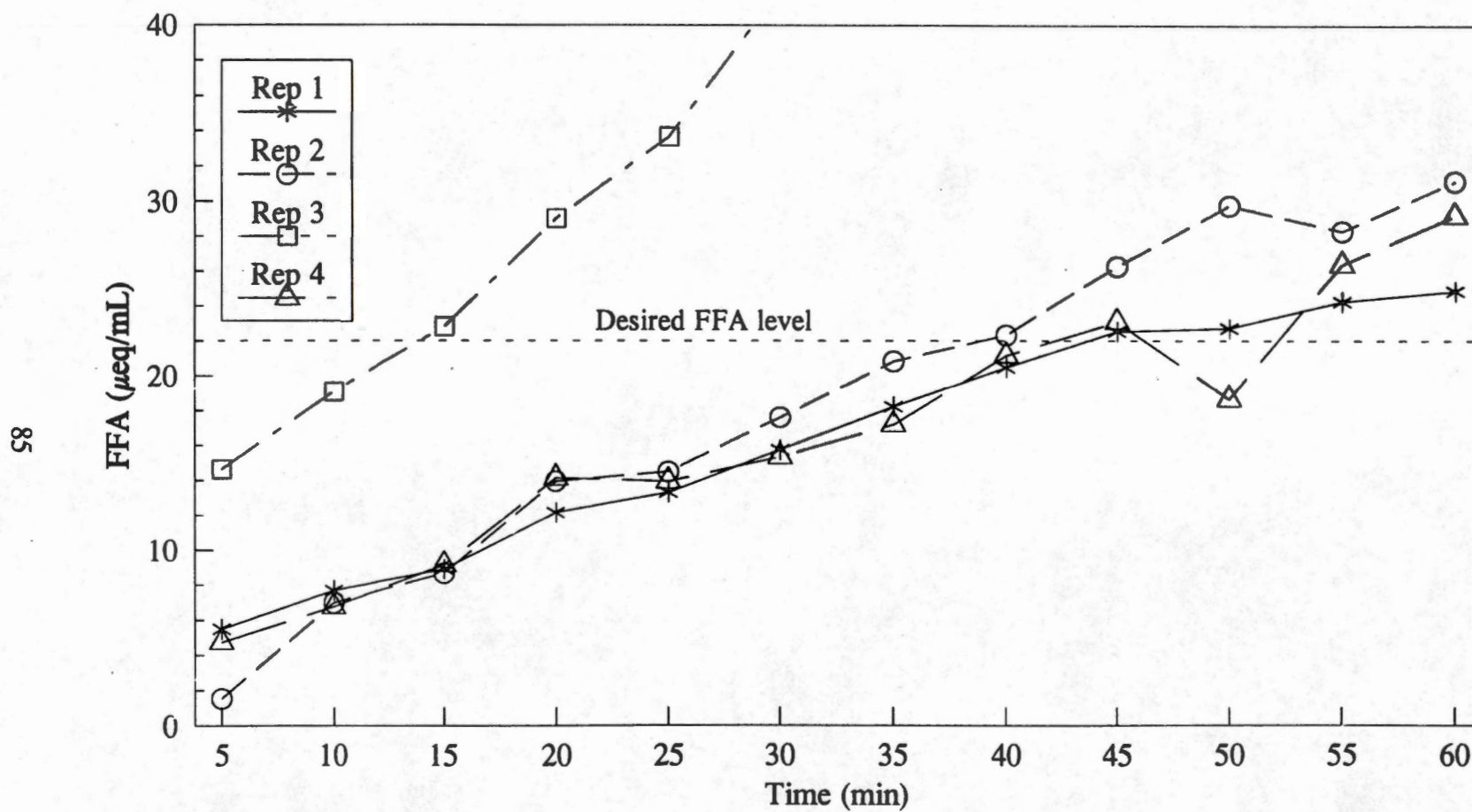


Figure A-2 - Free fatty acids (FFA) released in butter oil hydrolyzed with porcine pancreatic lipase at an incubation temperature of 40°C (in Phase I).

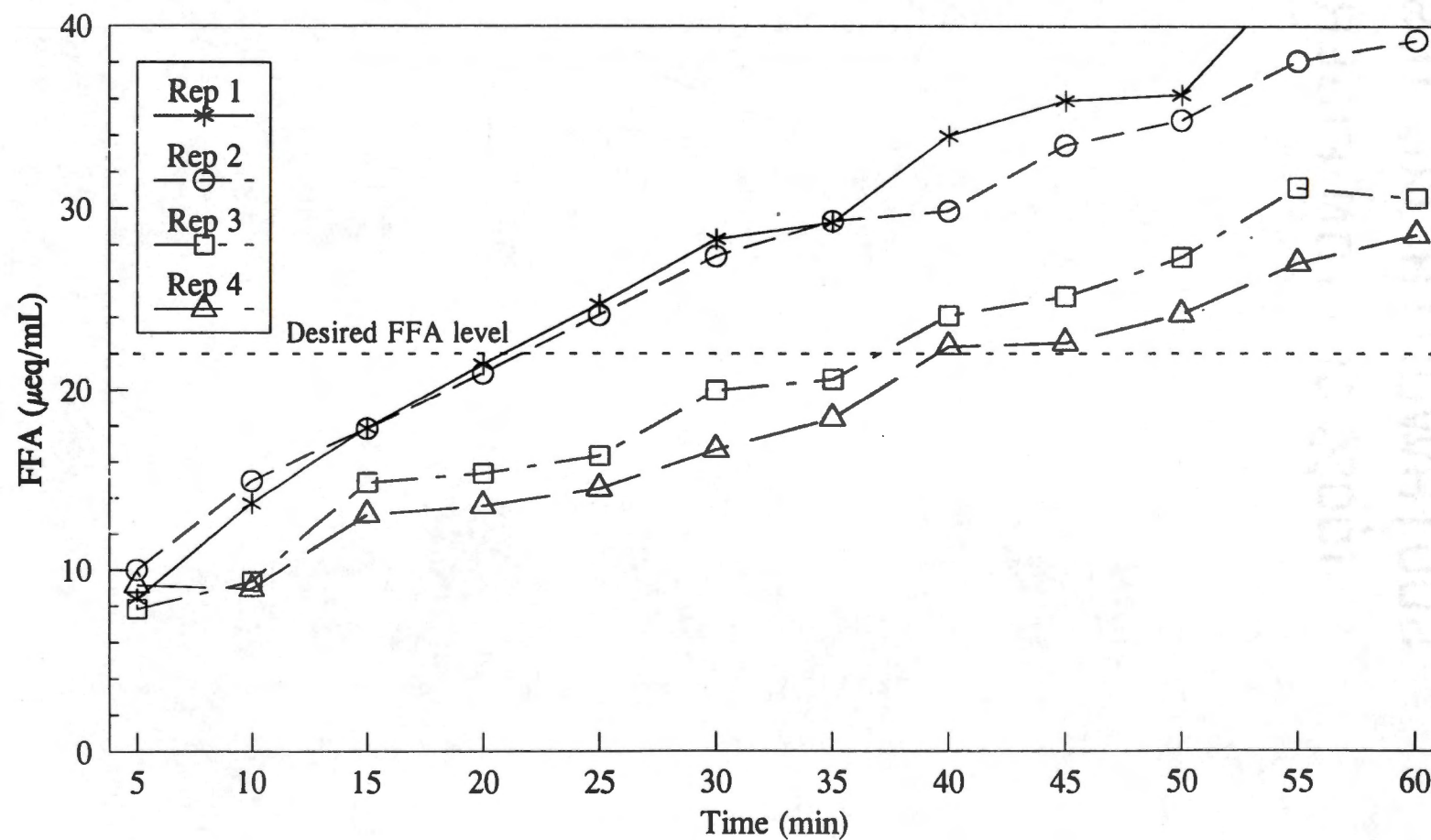


Figure A-3 - Free fatty acids (FFA) released in butter oil hydrolyzed with porcine pancreatic lipase at an incubation temperature of 45°C (in Phase I).

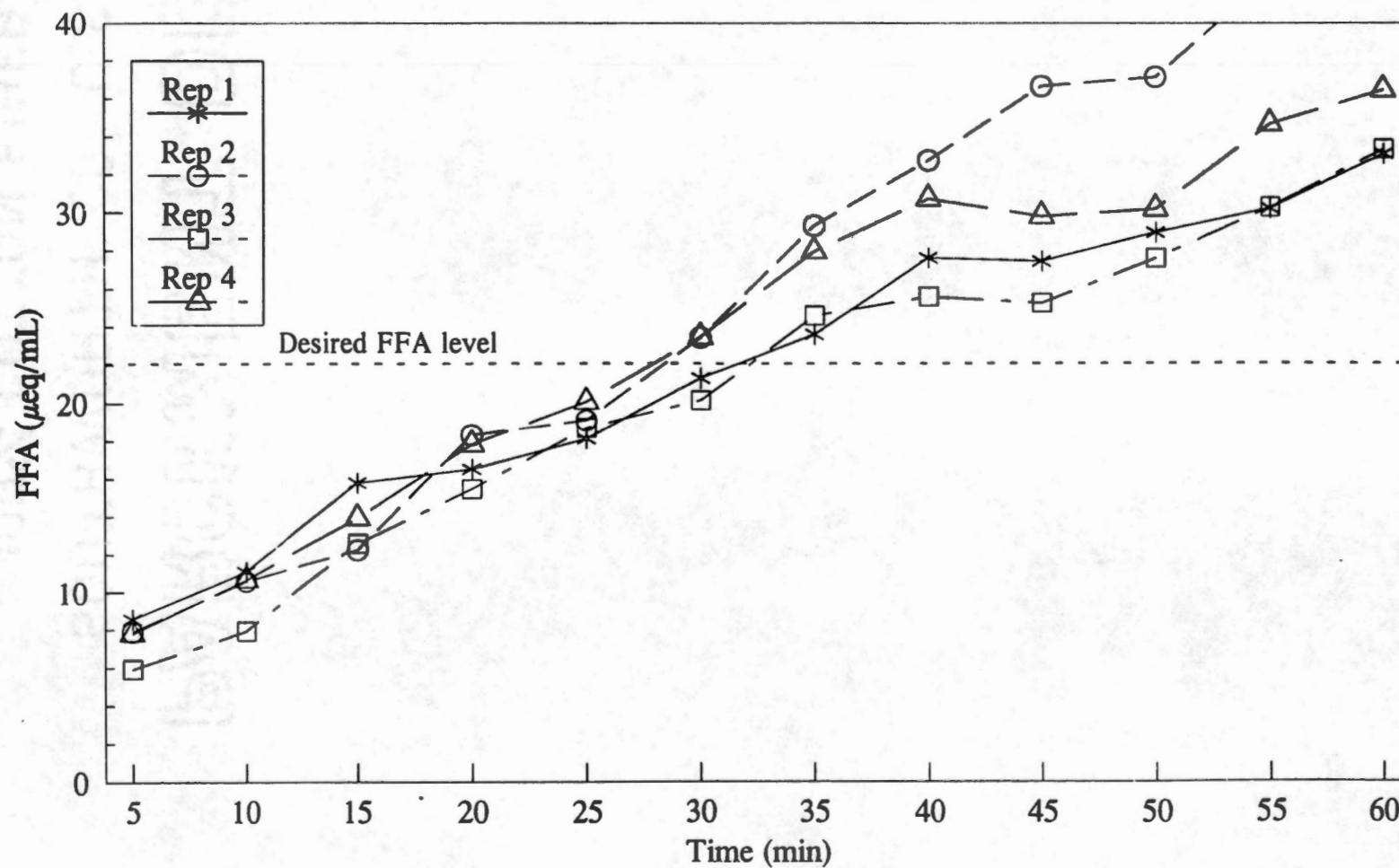


Figure A-4 - Free fatty acids (FFA) released in butter oil hydrolyzed with porcine pancreatic lipase at an incubation temperature of 50°C (in Phase I).

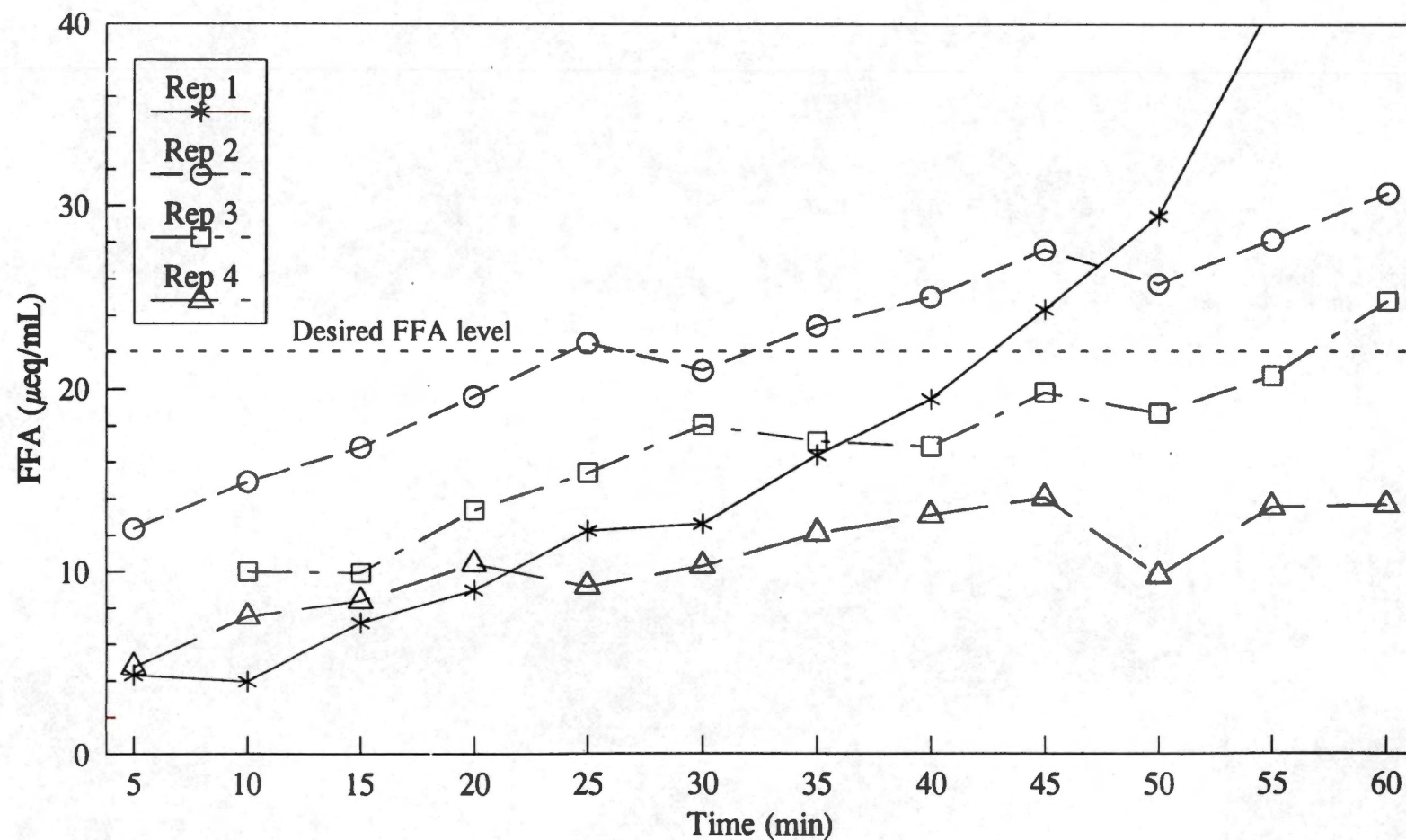


Figure A-5 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *P. roqueforti* at an incubation temperature of 35°C (in Phase I).

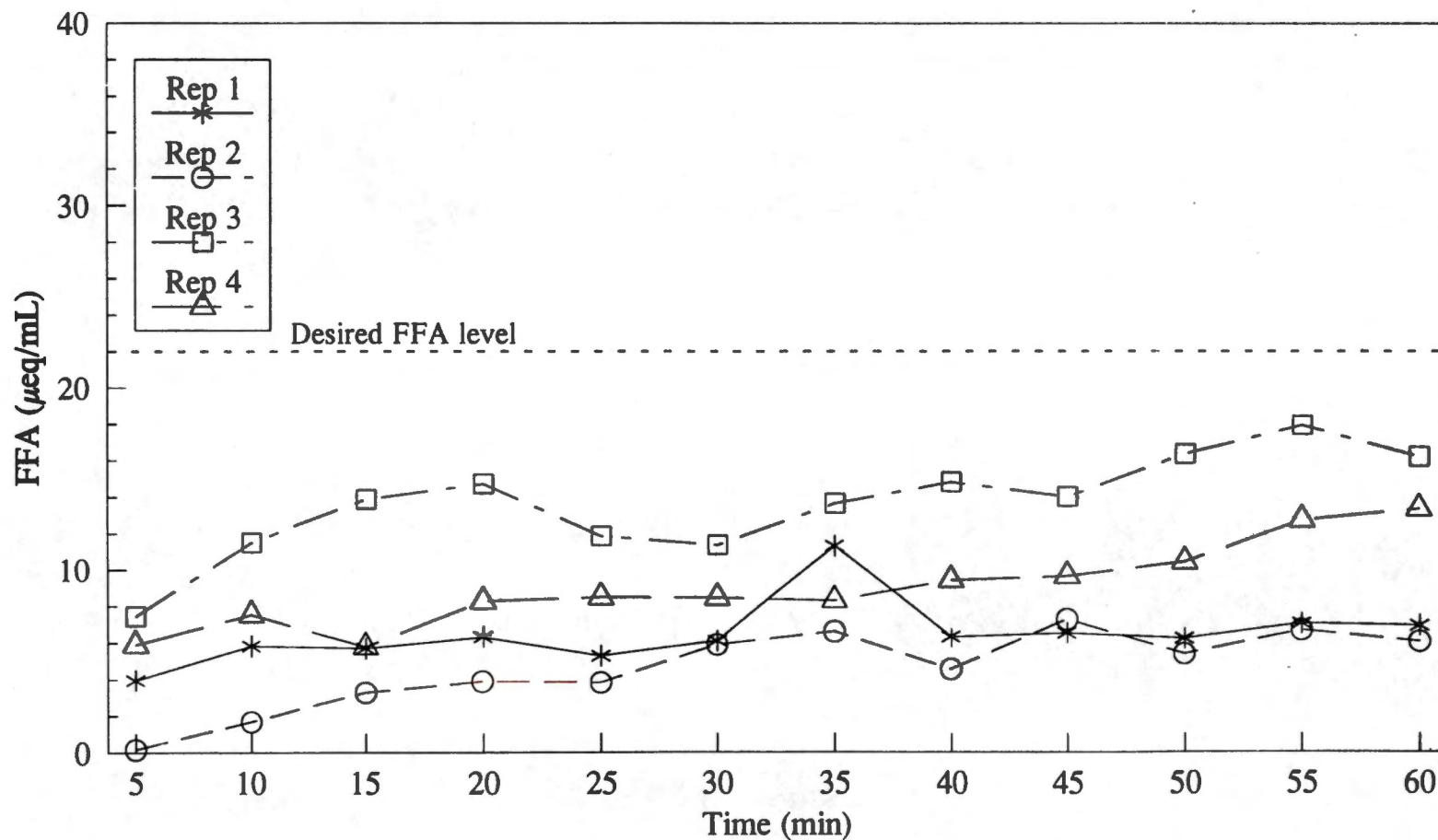


Figure A-6 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *P. roqueforti* at an incubation temperature of 40°C (in Phase I).

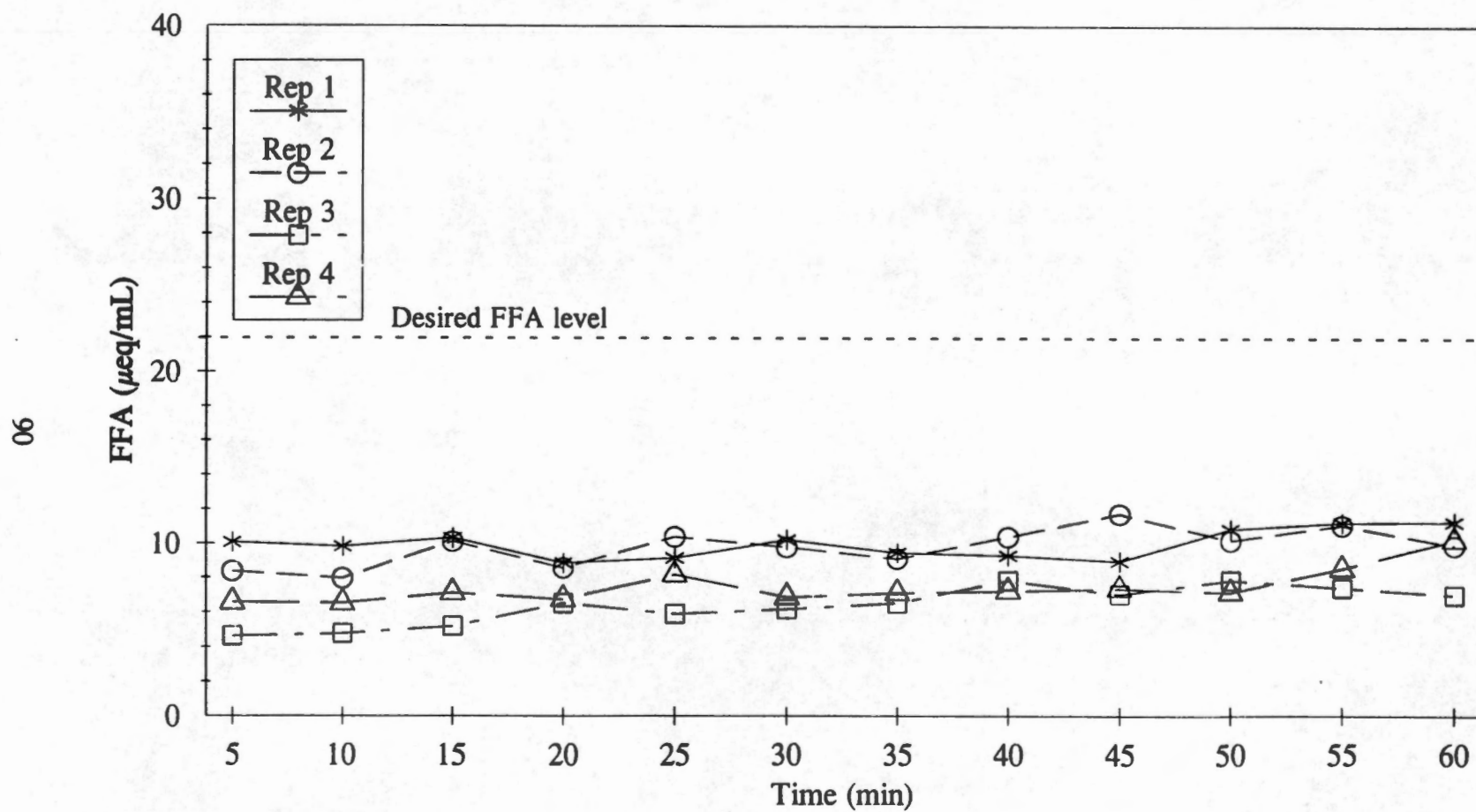


Figure A-7 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *P. roqueforti* at an incubation temperature of 45°C (in Phase I).

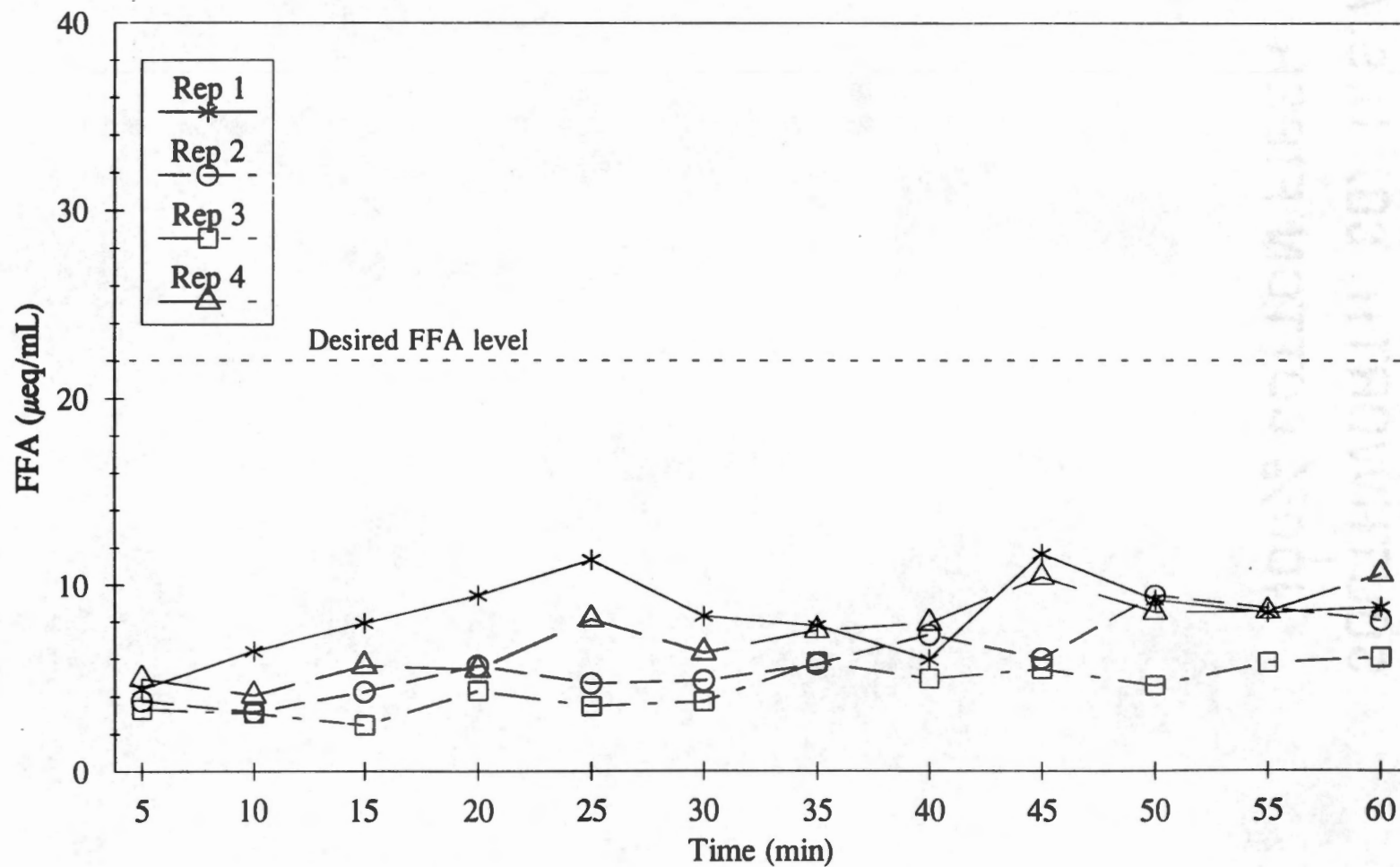


Figure A-8 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *P. roqueforti* at an incubation temperature of 50°C (in Phase I).

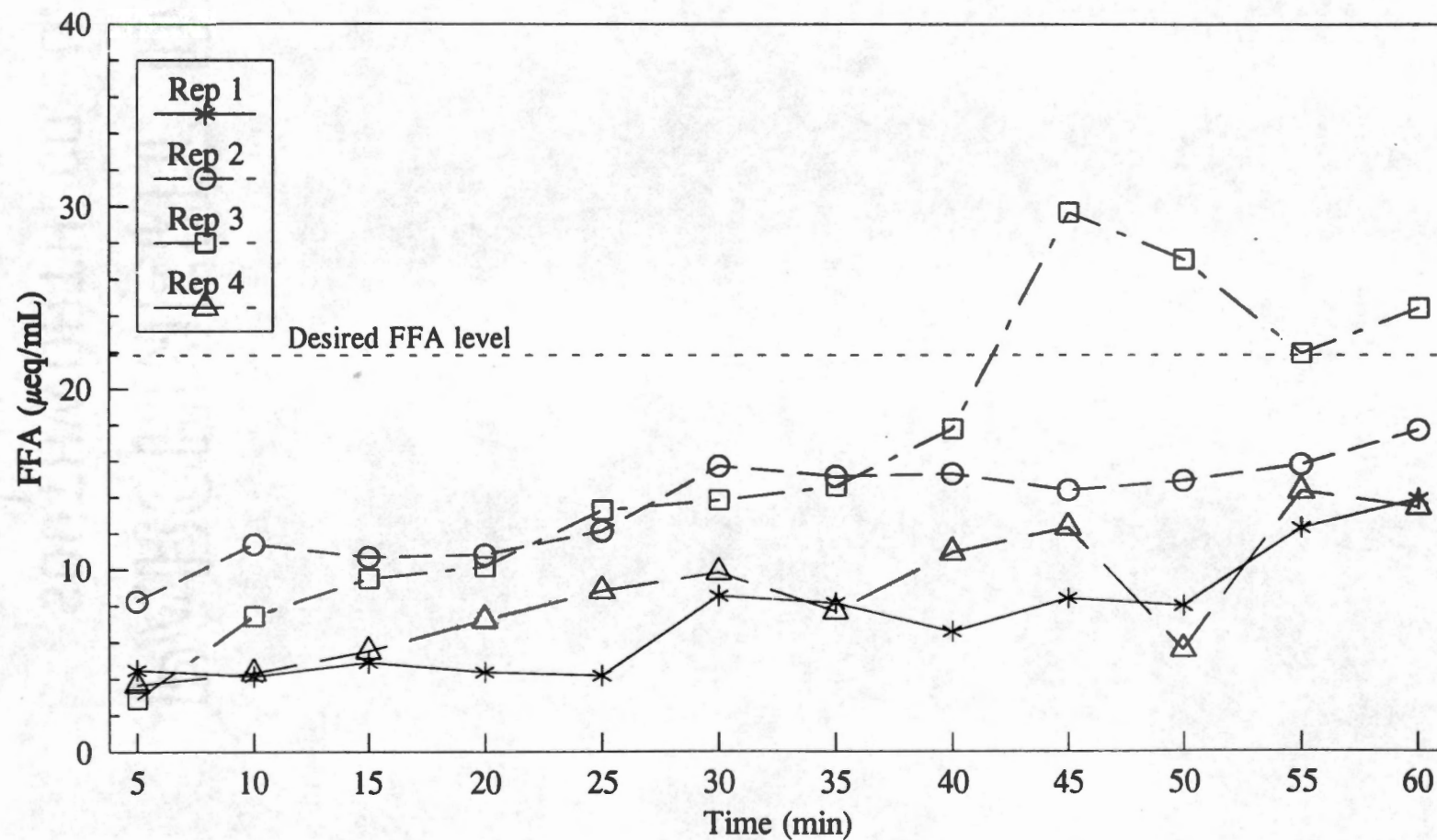


Figure A-9 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *A. niger* at an incubation temperature of 35°C (in Phase I).

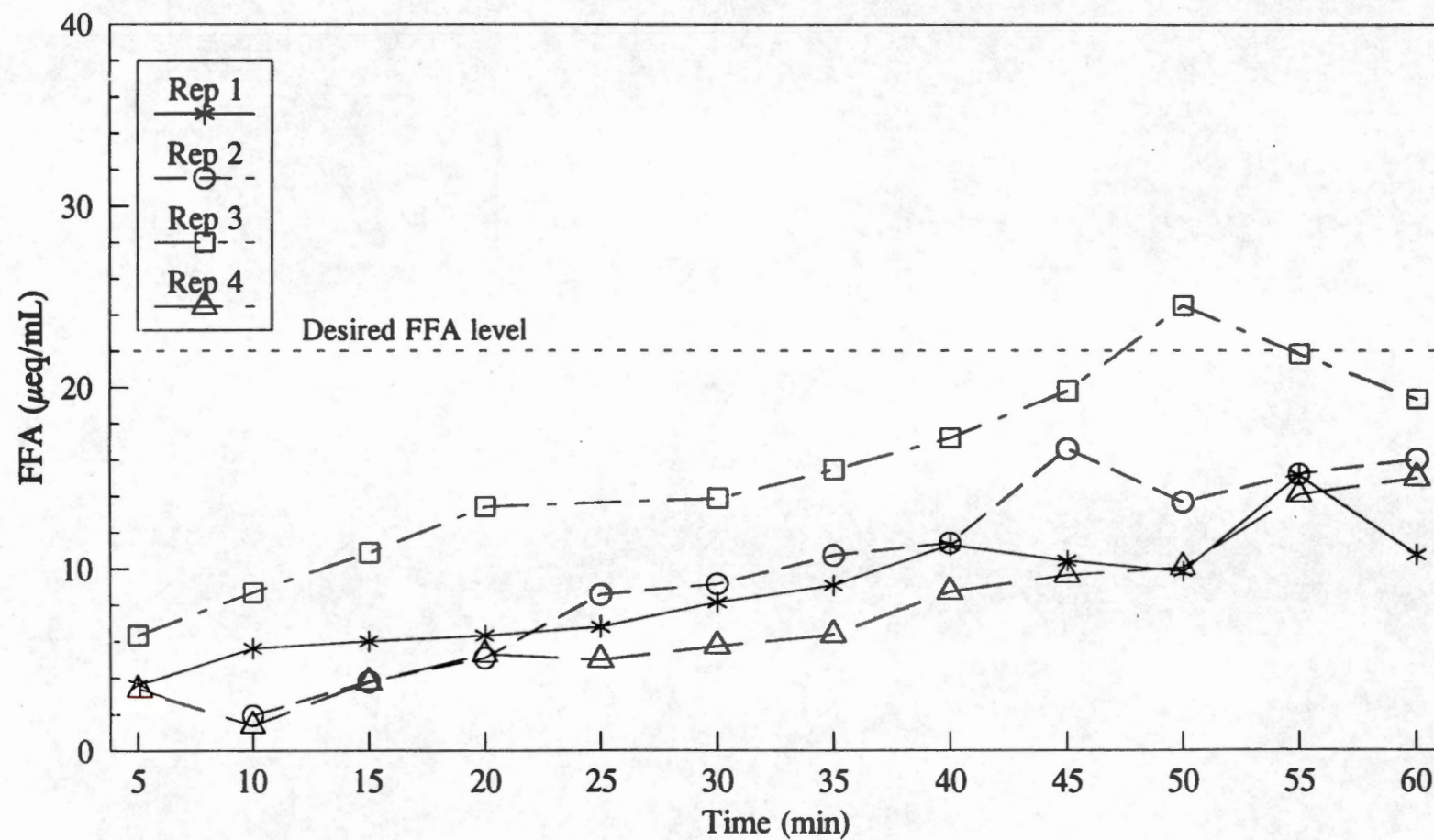


Figure A-10 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *A. niger* at an incubation temperature of 40°C (in Phase I).

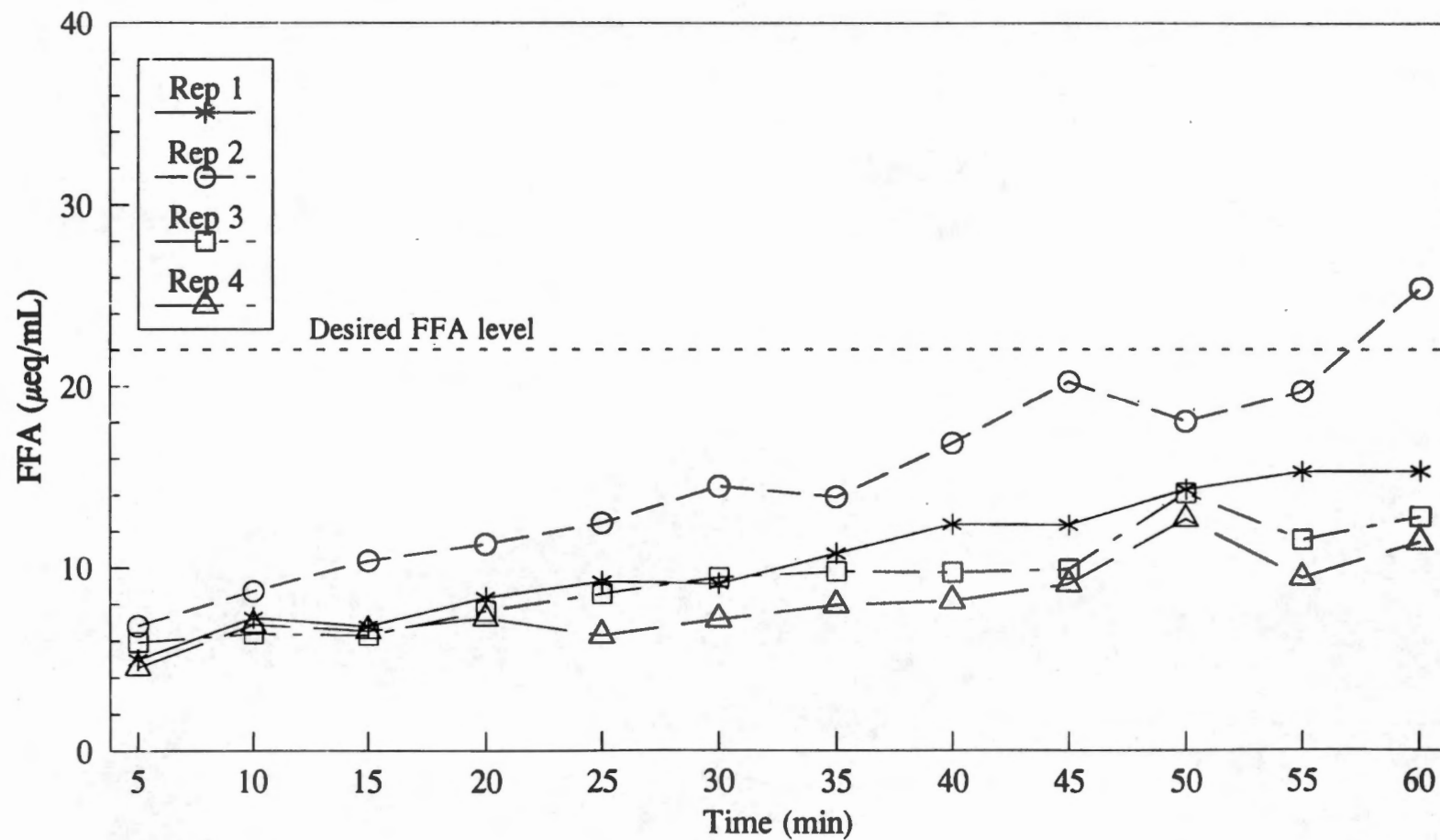


Figure A-11 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *A. niger* at an incubation temperature of 45°C (in Phase I).

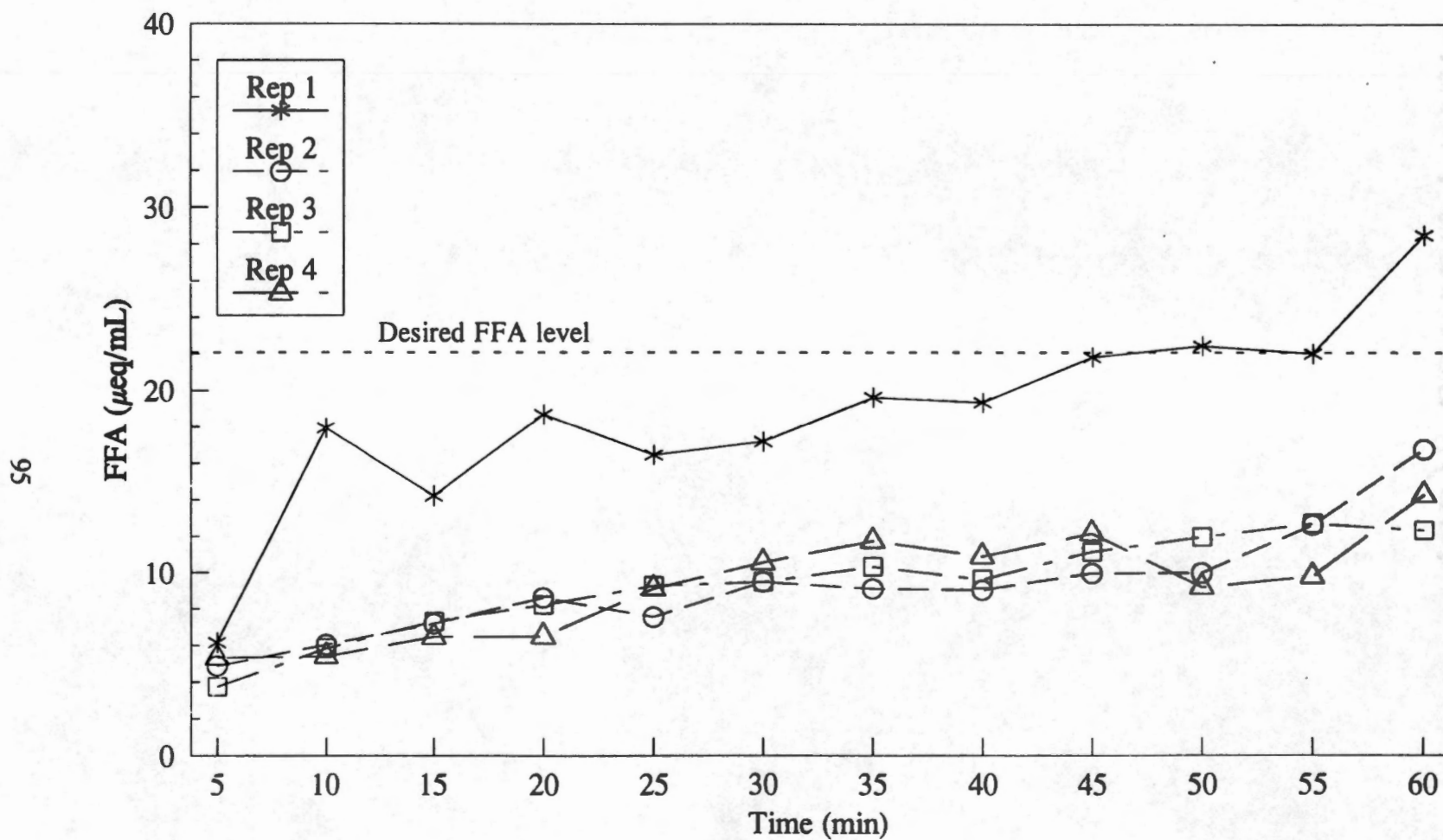


Figure A-12 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *A. niger* at an incubation temperature of 50°C (in Phase I).

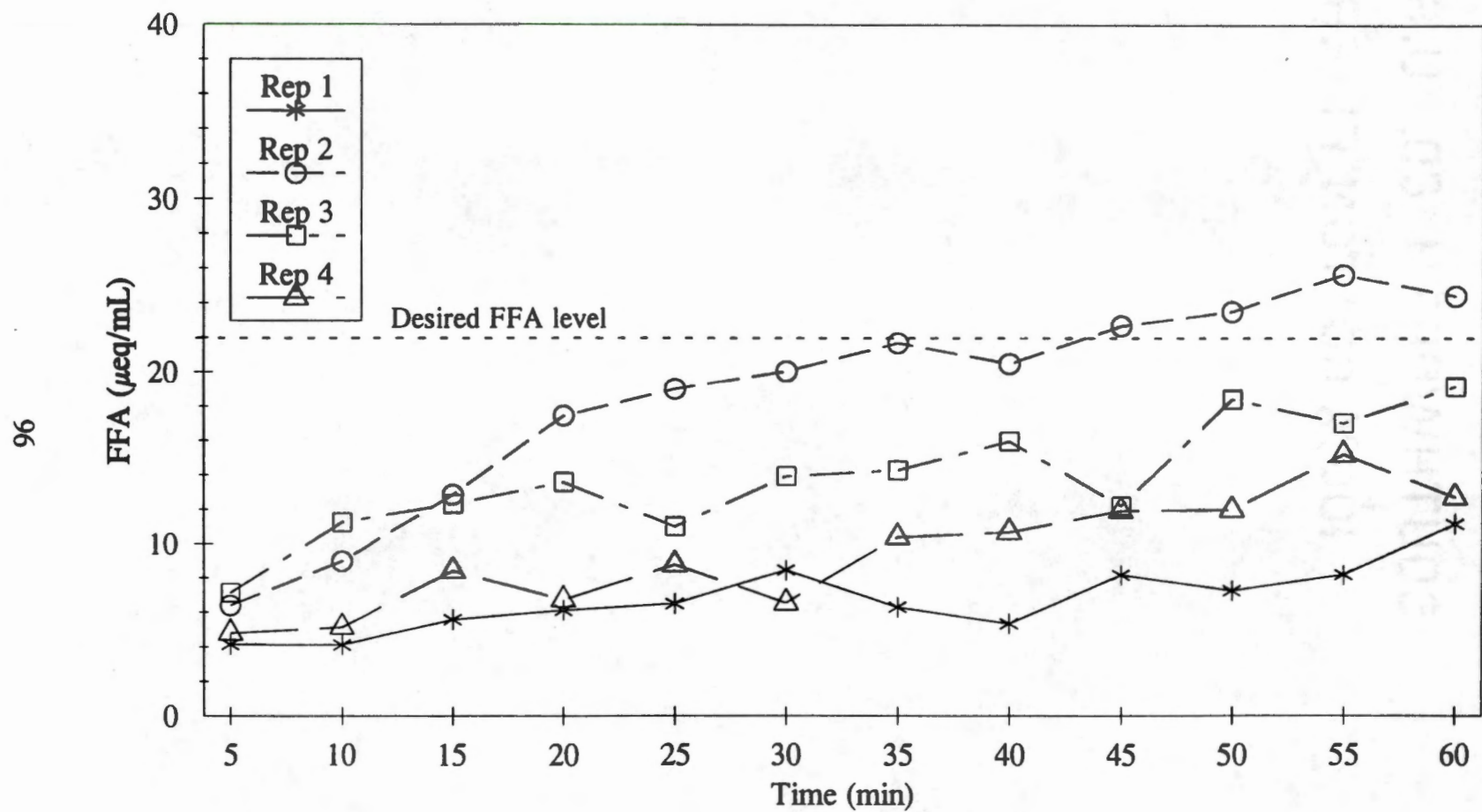


Figure A-13 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *R. miehei* at an incubation temperature of 35°C (in Phase I).

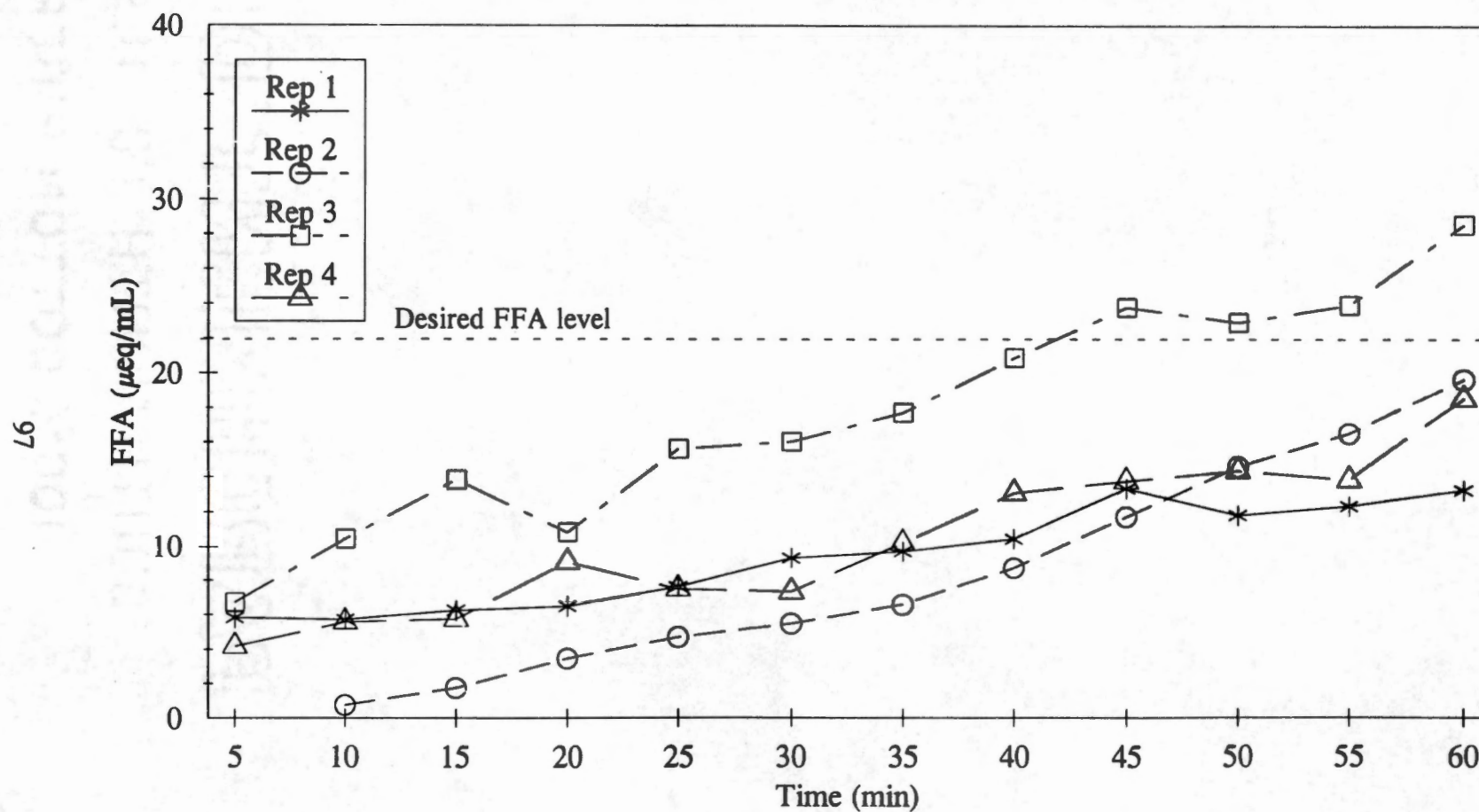


Figure A-14 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *R. miehei* at an incubation temperature of 40°C (in Phase I).

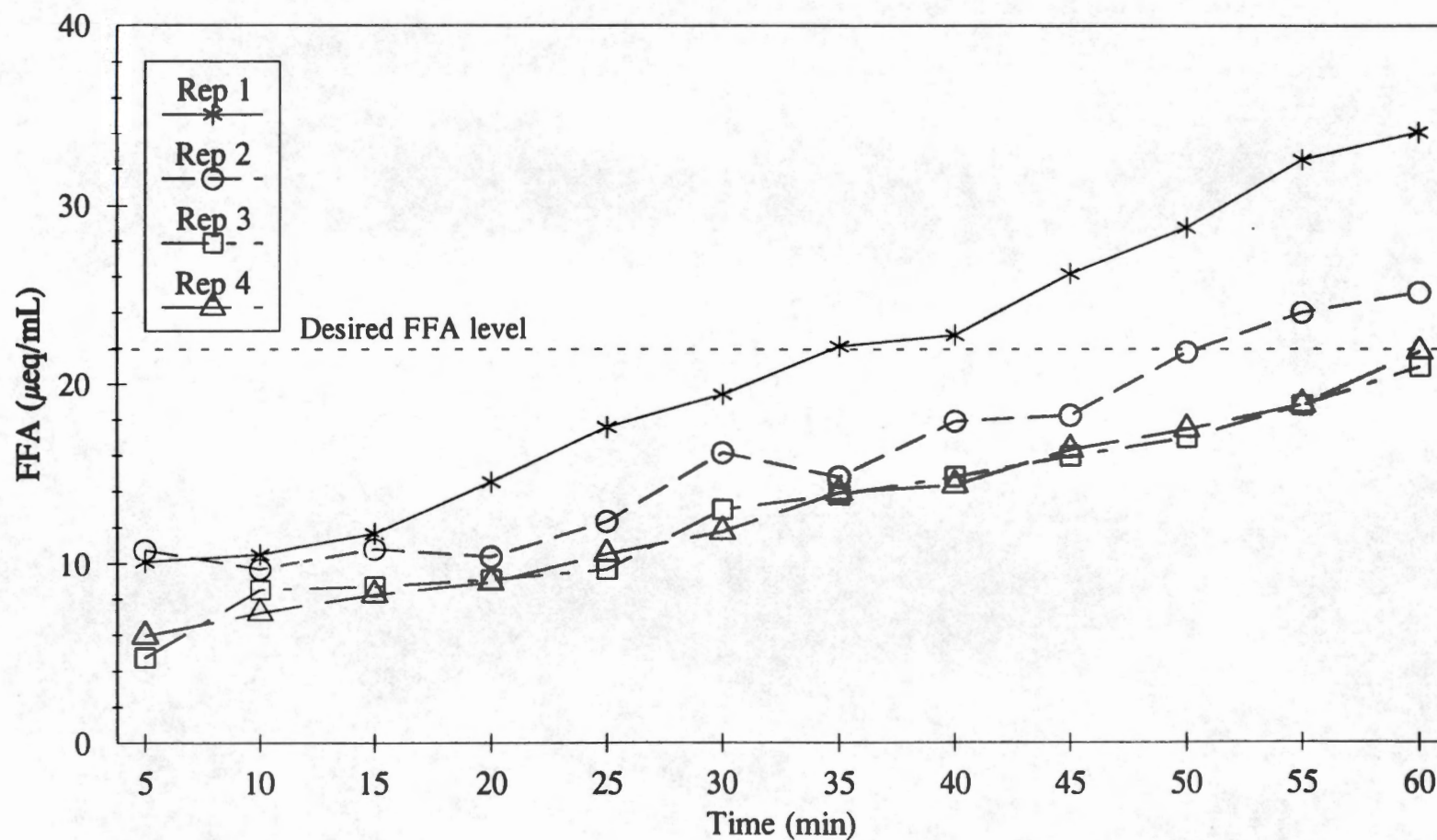


Figure A-15 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *R. miehei* at an incubation temperature of 45°C (in Phase I).

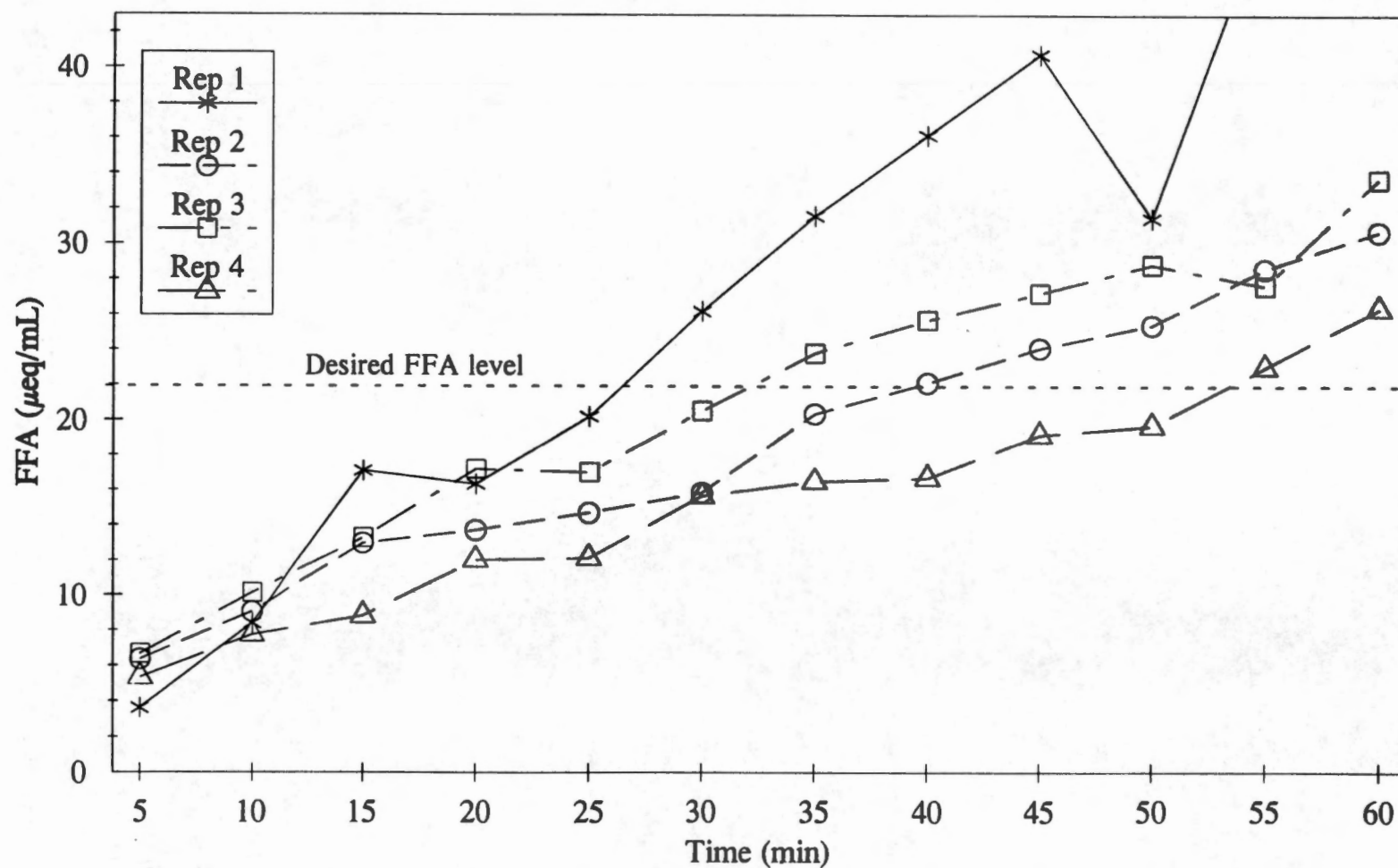


Figure A-16 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *R. miehei* at an incubation temperature of 50°C (in Phase I).

Table A-17 - Probabilities^a that replications differ for free fatty acids released in butter oil hydrolyzed with four lipases incubated for 60 min as analyzed by extraction-titration

Lipase	Incubation temperatures (°C)				
	35	40	45	50	Varied
	P values ^a				
	Phase I				Phase II
Pancreatic	0.0001	0.0001	0.1245	0.1480	0.0036
<i>P. roqueforti</i>	0.0001	0.0001	0.0001	0.0001	0.0001
<i>A. niger</i>	0.0003	0.0006	0.0002	0.0007	0.0001
<i>R. miehei</i>	0.0011	0.0001	0.0001	0.1138	0.0078

^aP>0.05 = no significant difference (and is considered reproducible); P<0.05 = significant difference (and is not considered reproducible); n = four replications.

Table A-18 - Least-squares mean^a cumulative free fatty acids released from butter oil by porcine pancreatic lipase at four incubation temperatures as analyzed by extraction-titration (in Phase I)

Incubation time (min)	Incubation temperature (°C)			
	35	40	45	50
	-----µeq FFA/mL-----			
0 ^b	3.58a	2.13a	5.30a	2.52a
5	5.15a	6.61a	8.86a	7.54a
10	10.02a	10.13a	11.74a	10.04a
15	12.08a	12.38a	15.91a	13.62a
20	11.87a	17.31b	17.8b	17.02ab
25	15.77a	18.85a	19.90a	18.98a
30	15.72a	22.69b	23.08b	22.09b
35	15.84a	24.68b	24.39b	26.37b
40	18.75a	27.77b	27.59b	29.17b
45	19.64a	30.56b	29.28b	29.79b
50	21.19a	31.57b	30.64b	30.97b
55	24.32a	34.63b	34.81b	34.40b
60	24.35a	36.25b	35.25b	37.25b

^aDifferent letters in the same row indicate a significant difference ($P < 0.05$); means across four replications.

^bValues inherent to butter oil prior to addition of lipase.

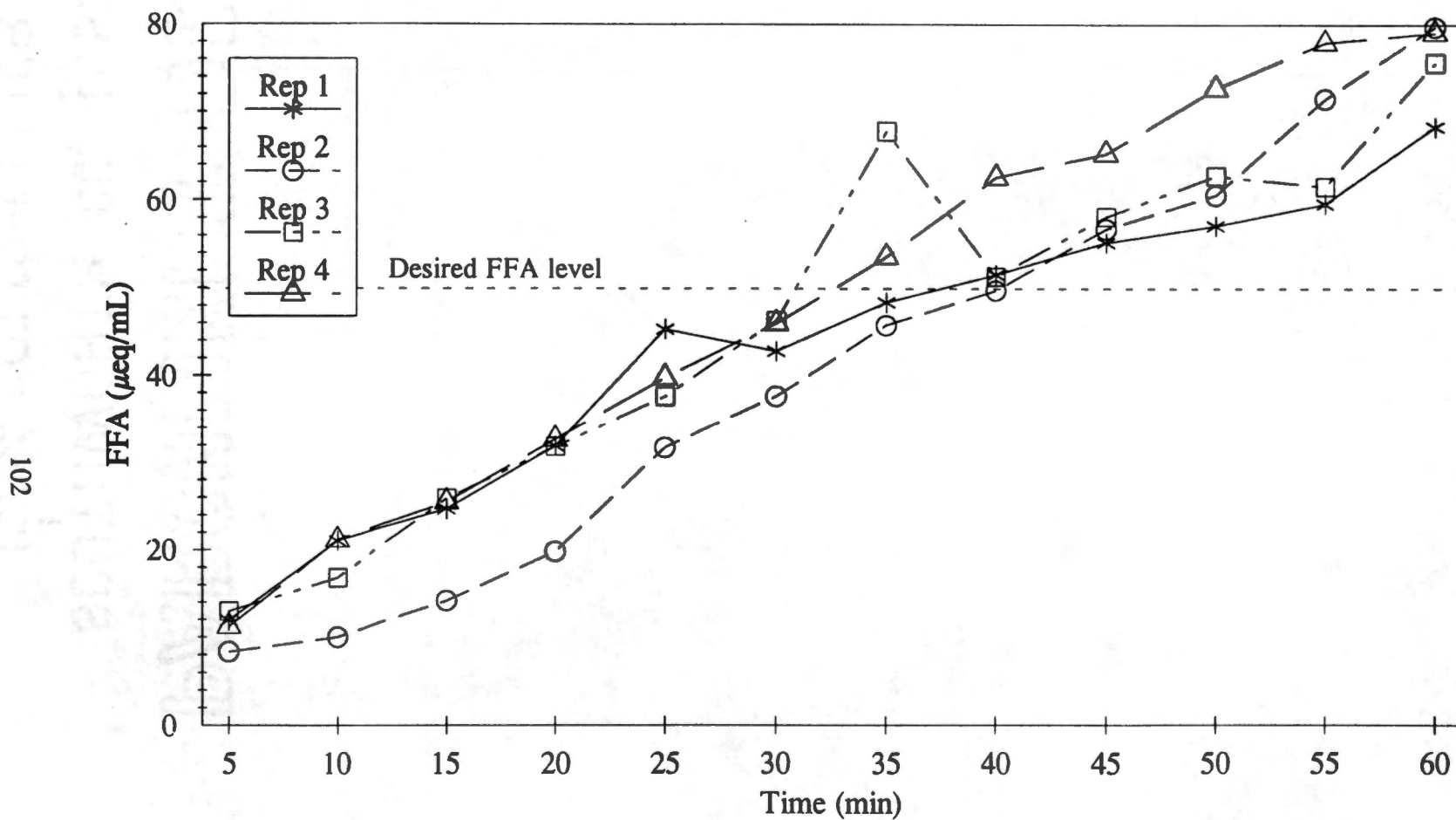


Figure A-19 - Free fatty acids (FFA) released in butter oil hydrolyzed with porcine pancreatic lipase incubated at 45°C over 4 replications as analyzed by extraction-titration (in Phase II).

Table A-20 - Least-squares mean^a cumulative free fatty acids released from butter oil by lipase from *P. roqueforti* at four incubation temperatures as analyzed by extraction-titration (in Phase I)

Incubation time (min)	Incubation temperature (°C)			
	35	40	45	50
	-----µeq FFA/mL-----			
0 ^b	3.58a	2.13a	5.30a	2.52a
5	7.20a	4.37a	7.40a	4.10a
10	9.10a	6.64a	7.25a	4.18a
15	10.57b	7.16ab	8.16ab	5.06a
20	13.07b	8.28ab	7.63a	6.18a
25	14.82b	7.37a	8.38a	6.89a
30	15.50b	7.95a	8.24a	5.79a
35	17.26b	9.96a	6.39a	6.69
40	18.59b	8.75a	8.64a	6.50a
45	21.45b	9.33a	8.72a	8.35a
50	20.91b	9.58a	8.96a	7.88a
55	25.89b	11.08a	9.52a	7.38a
60	28.01b	10.62a	9.54a	8.35a

^aDifferent letters in the same row indicate a significant difference ($P < 0.05$); means across four replications.

^bValues inherent to butter oil prior to addition of lipase.

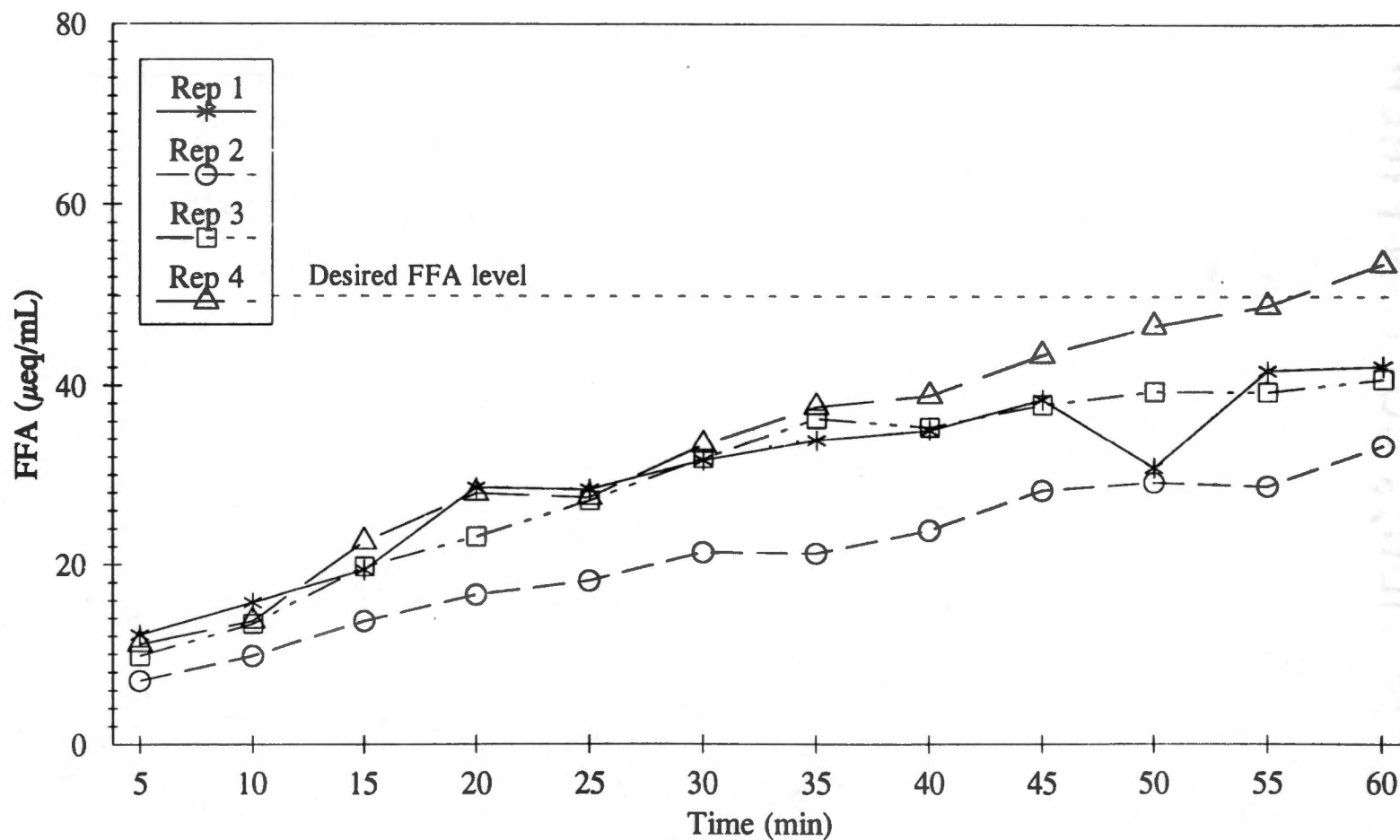


Figure A-21 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *P. roqueforti* incubated at 35°C over 4 replications as analyzed by extraction-titration (in Phase II).

Table A-22 - Least-squares mean^a cumulative free fatty acids released from butter oil by lipase from *A. niger* at four incubation temperatures analyzed by extraction-titration (in Phase I)

Incubation time (min)	Incubation temperature (°C)			
	35	40	45	50
	-----µeq FFA/mL-----			
0 ^b	3.58	2.13	5.30	2.52
5	4.83	3.68	5.57	5.03
10	6.82	4.40	7.30	8.81
15	7.65	6.10	7.50	8.77
20	8.13	7.52	8.60	10.48
25	9.58	9.20	9.12	10.57
30	11.96	9.20	10.04	11.66
35	11.38	10.39	10.59	12.67
40	12.62	12.13	11.77	12.19
45	16.17	14.10	12.88	13.74
50	13.88	14.49	14.76	13.37
55	16.08	16.54	13.97	14.27
60	17.36	15.25	16.22	17.95

^aThere were no significant differences ($P < 0.05$) among values in each row; means across four replications.

^bValues inherent to butter oil prior to addition of lipase.

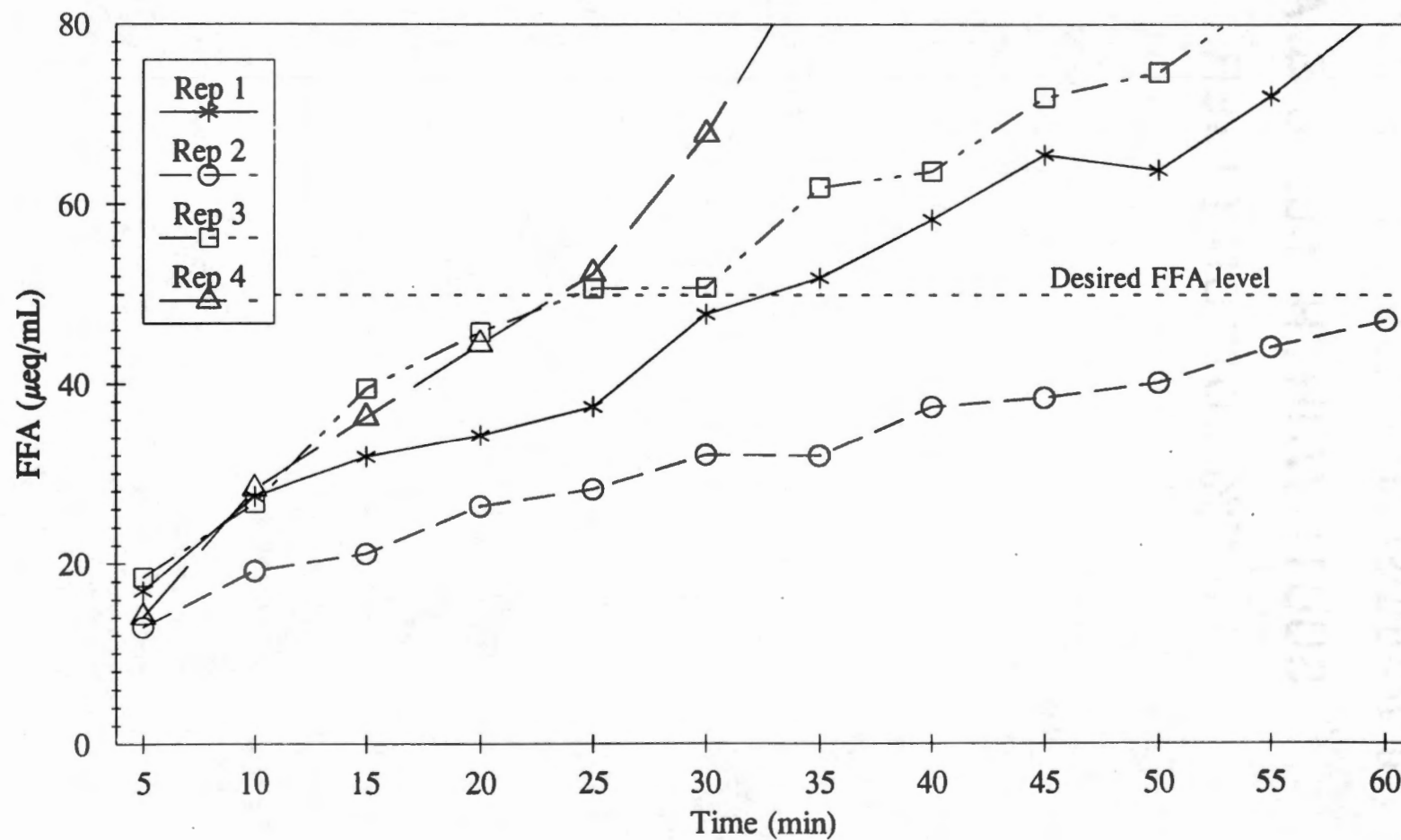


Figure A-23 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *A. niger* incubated at 45°C over 4 replications as analyzed by extraction-titration (in Phase II).

Table A-24 - Least-squares mean^a cumulative free fatty acids released from butter oil by lipase from *R. miehei* at four incubation temperatures analyzed by extraction-titration (in Phase I)

Incubation time (min)	Incubation temperature (°C)			
	35	40	45	50
	-----µeq FFA/mL-----			
0 ^b	3.58a	2.13a	5.30a	2.52a
5	5.62a	4.71a	7.88a	5.5a
10	7.37a	5.61a	8.97a	8.82a
15	8.91a	6.88a	9.83ab	13.03b
20	10.95bc	7.42a	10.71b	14.79c
25	11.29ab	8.82a	12.49ab	15.98b
30	12.22ab	9.50a	15.07bc	19.54c
35	13.14ab	11.00a	16.17b	23.06c
40	13.09a	13.23a	17.45a	25.16a
45	13.71a	15.61ab	19.16b	27.80c
50	15.26a	15.88a	21.22b	26.35c
55	16.50a	16.62a	23.53b	32.03c
60	16.84a	19.97a	25.49b	35.22c

^aDifferent letters in the same row indicate a significant difference ($P < 0.05$); means across four replications.

^bValues inherent to butter oil prior to addition of lipase.

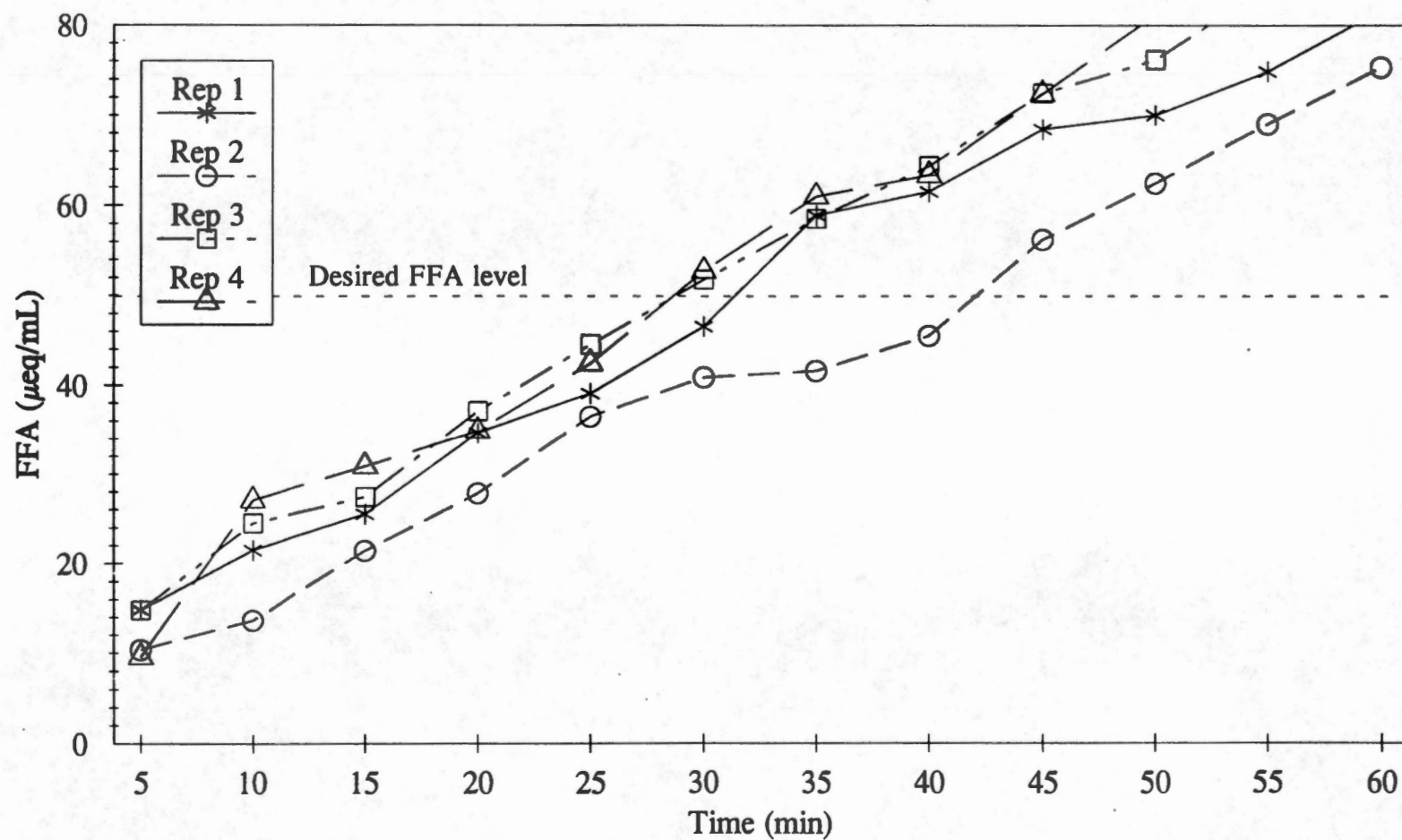


Figure A-25 - Free fatty acids (FFA) released in butter oil hydrolyzed with lipase from *R. miehei* incubated at 50°C over 4 replications as analyzed by extraction-titration (in Phase II).

APPENDIX B

FLAVOR DESCRIPTION AND CHEMICAL ANALYSES

Table B-1 - Flavor descriptions^a and extraction-titration analyses for lipolyzed milk samples made from hydrolyzed butter oil and skim milk using four lipases and three incubation times in replication 1

Evaluation	Lipase	Incubation time (min)		
		30	45	60
Flavor descriptions within lipases (across incubation times)	Pancreatic	Plenty rancid	Too intensely rancid	Too intensely rancid
	<i>P. roqueforti</i>	Not rancid, Bad milk	Not rancid, Bad milk	Rancid, diff. pan.
	<i>A. niger</i>	Not rancid, Bad milk	Not rancid, Bad milk	Rancid, diff. pan.
	<i>R. miehei</i>	Not rancid	Not rancid	Not rancid
Flavor descriptions among lipases (within incubation times)	Pancreatic	Ext. strong rancid (char.)	Very intensely rancid	Very intensely rancid (odor)
	<i>P. roqueforti</i>	Chemical taste, not char.	Slight chemical, strange taste	Weakest rancid
	<i>A. niger</i>	Chemical taste, not char.	Slight chemical, strange taste	Weakest rancid
	<i>R. miehei</i>	Rancid, less than pancreatic	Chemical taste, not rancid	Rancid, less pancreatic
Extraction-titration ^b (μeq FFA/mL)	Pancreatic	4.05	4.51	4.45
	<i>P. roqueforti</i>	4.60	3.77	3.86
	<i>A. niger</i>	3.98	5.05	5.72
	<i>R. miehei</i>	5.48	5.59	6.55

^a Flavor description by dairy flavor expert.

^bn = two samples per lipase for extraction-titration analyses.

Table B-2 - Flavor descriptions^a and extraction-titration analyses for lipolyzed milk samples made from hydrolyzed butter oil and skim milk using four lipases in replication 2

Evaluation	Lipase	Incubation time (min)		
		30	45	60
Flavor descriptions within lipases (across incubation times)	Pancreatic	Weakly rancid	Rancid	Very rancid
	<i>P. roqueforti</i>	Very slightly rancid, no diff.	Very slightly rancid, no diff.	Very slightly rancid, no diff.
	<i>A. niger</i>	Very slightly rancid, no diff.	Very slightly rancid, no diff.	Very slightly rancid, no diff.
	<i>R. miehei</i>	Very weak rancid, no diff.	Very weak rancid, no diff.	Very weak rancid, no diff.
Flavor descriptions among lipases (within incubation times)	Pancreatic	Rancid, but not strongly	Rancid	Good example of rancid
	<i>P. roqueforti</i>	Chemical taste	Slightly rancid	Low rancidity, similar to <i>R. miehei</i>
	<i>A. niger</i>	Chemical taste	Slightly rancid	Low rancidity, similar to <i>R. miehei</i>
	<i>R. miehei</i>	Slightly rancid	Slightly rancid	Low rancidity
Extraction-titration ^b (μ eq FFA/mL)	Pancreatic	3.10	3.36	4.16
	<i>P. roqueforti</i>	1.84	2.07	2.19
	<i>A. niger</i>	1.67	1.86	2.49
	<i>R. miehei</i>	4.65	4.72	4.77

^a Flavor descriptions by dairy flavor expert.

^bn = two samples per lipase for extraction-titration analyses.

Table B-3 - Flavor descriptions^a and extraction-titration analyses for lipolyzed milk samples made from hydrolyzed butter oil and skim milk using four lipases and three incubation times in replication 3

Evaluation	Lipase	Incubation time (min)		
		30	45	60
Flavor descriptions within lipases (across incubation times)	Pancreatic	Least rancid	Rancid	Extremely rancid
	<i>P. roqueforti</i>	Lowest rancidity	Rancid, no diff.	Rancid, no diff.
	<i>A. niger</i>	Hardly rancid	Intensity 30 > 60	Rancid
	<i>R. miehei</i>	Very weak rancid	Weak rancid, no diff.	Weak rancid, no diff.
Flavor descriptions among lipases (within incubation times)	Pancreatic	Rancid flavor and odor	Strongly rancid	Extremely rancid
	<i>P. roqueforti</i>	Rancid, less Pan.	Not rancid, lacks freshn.	Chemical, no diff.
	<i>A. niger</i>	Chemical taste	Not rancid, lacks freshn.	Chemical, no diff.
	<i>R. miehei</i>	Not rancid, lacks freshn.	Not rancid, lacks freshn.	Rancid, no diff.
Extraction-titration ^b (μeq FFA/mL)	Pancreatic	3.31	4.72	5.34
	<i>P. roqueforti</i>	2.01	1.66	1.72
	<i>A. niger</i>	1.73	2.29	1.94
	<i>R. miehei</i>	2.35	3.36	3.40

^a Flavor description by dairy flavor expert.

^bn = two samples per lipase for extraction-titration analyses.

Table B-4 - Flavor descriptions^a and extraction-titration analyses for lipolyzed milk samples made from hydrolyzed butter oil and skim milk using four lipases and three incubation times in replication 4

Evaluation	Lipase	Incubation time (min)		
		30	45	60
Flavor descriptions within lipases (across incubation times)	Pancreatic	Slightly rancid	Rancid	Very rancid
	<i>P. roqueforti</i>	Slightly chemical, lowest int.	Slightly rancid, no diff. w/ 60 min	Slightly rancid, no diff. w/ 45 min
	<i>A. niger</i>	Least intense rancid	Less intense rancid	"Nice" rancid
	<i>R. miehei</i>	Not rancid, no diff.	Not rancid, no diff.	Not rancid, no diff.
Flavor descriptions among lipases (within incubation times)	Pancreatic	Pronounced rancid flavor	Very rancid	Not rancid
	<i>P. roqueforti</i>	Chemical, not rancid	Weak rancid, no diff. w/ <i>A. niger</i> & <i>R. miehei</i>	Not rancid
	<i>A. niger</i>	Slightly rancid	Weak rancid, no diff. w/ <i>P. roqueforti</i> & <i>R. miehei</i>	Most rancid sample
	<i>R. miehei</i>	Definitely rancid	Weak rancid, no diff. w/ <i>P. roqueforti</i> & <i>A. niger</i>	Not rancid
Extraction-titration ^b ($\mu\text{eq FFA/mL}$)	Pancreatic	3.70	4.25	4.84
	<i>P. roqueforti</i>	2.84	2.95	3.67
	<i>A. niger</i>	3.99	4.24	5.27
	<i>R. miehei</i>	4.94	5.39	4.33

^a Flavor descriptions by dairy flavor expert.

^bn = two samples per lipase for extraction-titration analyses.

Table B-5 - Least-squares mean^a cumulative free fatty acids present in milk prepared from skim milk and butter oil hydrolyzed with four lipases as analyzed by extraction-titration

Lipase	Replication			
	1	2	3	4
	-----μeq FFA/mL-----			
Pancreatic	4.33b	3.53a	4.45b	4.26b
<i>P. roqueforti</i>	4.07b	2.03a	1.79a	3.15b
<i>A. niger</i>	4.91b	2.01a	1.98a	4.49b
<i>R. miehei</i>	5.87c	4.64b	3.03a	4.88b

^aDifferent letters in each row indicate a significant difference ($P < 0.05$); cumulative means over three samples (from 30, 45 and 60 min incubation time samples).

Table B-6 - Least-squares mean^a free fatty acids present in lipolyzed milk prepared using skim milk and butter oil hydrolyzed with pancreatic lipase as analyzed by GC

Fatty Acid	Incubation time (min)		
	30	45	60
	----- μ eq FFA/mL-----		
C4	1.69a	3.17ab	4.24b
C6	0.83a	1.60ab	2.03b
C7	0.01a	0.03ab	0.04b
C8	0.50a	0.96ab	1.19b
C9	0.03a	0.02a	0.03a
C10	0.41a	0.88ab	1.14b
C11	0.03a	0.02a	0.07a
C12	0.23a	0.43a	0.60a
C13	0.02a	0.00a	0.00a
C14	0.13a	0.28a	0.37a
C15	0.07a	0.03a	0.04a
C16:0	0.43a	0.93a	1.19a
C16:1	0.10a	0.04a	0.05a
C18:0	0.31a	0.52a	0.63a
C18:1	0.24a	0.65a	0.89a
C18:2	0.00a	0.09a	0.09a
C18:3	0.00a	0.00a	0.01a

^aDifferent letters in each row indicates a significant difference ($P < 0.05$); means across four replications.

Table B-7 - Least-squares mean^a free fatty acids present in lipolyzed milk prepared using skim milk and butter oil hydrolyzed with lipase from *P. roqueforti*, as analyzed by GC

Fatty Acid	Incubation time (min)		
	30	45	60
	----- $\mu\text{eq FFA/mL}$ -----		
C4	2.51	1.52	1.95
C6	1.23	0.79	1.01
C7	0.03	0.02	0.02
C8	0.58	0.41	0.50
C9	0.04	0.04	0.04
C10	0.73	0.47	0.55
C11	0.03	0.03	0.04
C12	0.59	0.35	0.41
C13	0.03	0.02	0.02
C14	0.47	0.29	0.32
C15	0.12	0.10	0.10
C16:0	0.47	0.94	1.03
C16:1	1.06	0.12	0.14
C18:0	0.39	0.55	0.60
C18:1	0.78	0.62	0.67
C18:2	0.87	0.03	0.01
C18:3	0.01	0.00	0.03

^aThere was no significant difference ($P > 0.05$) among samples in each row; means across four replications.

Table B-8 - Least-squares mean^a free fatty acids present in lipolyzed milk, prepared using skim milk and butter oil hydrolyzed with lipase from *A. niger*, analyzed by GC

Fatty Acid	Incubation time (min)		
	30	45	60
	----- μ eq FFA/mL-----		
C4	1.58	1.20	1.28
C6	0.79	0.68	0.71
C7	0.03	0.02	0.02
C8	0.51	.045	0.58
C9	0.03	0.04	0.08
C10	0.55	0.42	0.48
C11	0.03	0.03	0.05
C12	0.52	0.35	0.42
C13	0.03	0.02	0.19
C14	0.28	0.19	0.13
C15	0.10	0.09	0.58
C16:0	0.37	0.69	0.43
C16:1	0.33	0.08	0.31
C18:0	0.71	0.23	0.59
C18:1	0.25	0.37	0.30
C18:2	0.03	0.30	0.01
C18:3	0.00	0.02	0.02

^aThere was no significant difference ($P > 0.05$) among samples in each row; means across four replications.

Table B-9 - Least-squares mean^a free fatty acids in lipolyzed milk prepared from skim milk and butter oil hydrolyzed using lipase from *R. miehei*, as analyzed by GC

Fatty Acid	Incubation time (min)		
	30	45	60
	----- $\mu\text{eq FFA/mL}$ -----		
C4	0.59	0.61	0.44
C6	0.32	0.32	0.21
C7	0.04	0.01	0.01
C8	0.30	0.24	0.09
C9	0.04	0.02	0.14
C10	0.21	0.22	0.19
C11	0.11	0.03	0.01
C12	0.22	0.22	0.22
C13	0.08	0.02	0.06
C14	0.22	0.42	0.19
C15	0.18	0.08	0.18
C16:0	0.88	0.57	0.79
C16:1	0.10	1.04	0.08
C18:0	0.69	0.36	0.52
C18:1	0.57	0.78	0.47
C18:2	0.58	0.70	0.01
C18:3	0.03	0.07	0.00

^aThere was no significant difference ($P > 0.05$) among samples in each row; means across four replications.

Table B-10 - Least-squares mean^a free fatty acids present in lipolyzed milk prepared using three methods and butter oil hydrolyzed using pancreatic lipase as analyzed by GC

Fatty Acid	Methods for preparing lipolyzed milk ^b			
	2 %SM	RecSM	ExpM	HBO
	----- μ eq FFA/mL-----			-- μ eq FFA/g--
C4	0.90	1.10	3.29	3.81
C6	0.51	0.60	1.67	1.6
C7	0.02	0.10	0.03	0.11
C8	0.24	0.23	0.92	1.06
C9	0.02	0.09	0.03	0.2
C10	0.23	0.23	0.76	1.87
C11	0.05	0.09	0.02	0.23
C12	0.19	0.17	0.40	1.91
C13	0	0	0.09	0.22
C14	1.17	1.21	1.10	2.65
C15	0	0	0.30	0.97
C16:0	4.32	4.31	3.56	8.72
C16:1	0.10	0.10	0.49	0.84
C18:0	2.14	2.10	1.95	5.19
C18:1	3.91	3.90	3.33	7.74
C18:2	0.29	0.33	0.52	0.66
C18:3	0.13	0.11	0.13	0.11
Total FFA	4.52	4.98	9.09	35.64

^aThere was no significant difference between samples in each row ($P > 0.05$); means over four replications.

^b2%SM = 2% standard method; RecSM = Recombined milk standard method; ExpM = Recombined milk made with hydrolyzed butter oil method; HBO = hydrolyzed butter oil (45°C for 45 min). n = four replications.

APPENDIX C

ADDITIONAL INFORMATION

APPENDIX C-1

Extraction-titration reagents

Extraction Reagent

Isopropyl alcohol	(40 parts)	800 mL
Hexane	(10 parts)	200 mL
0.1 N Sulfuric acid	(1 part)	20 mL
(0.1 N Sulfuric acid = 1.06 mL H ₂ SO ₄ per 100 mL H ₂ O)		

Titration Reagent

Stock solution	(1 part)	10 mL
Ethanol	(99 parts)	990 mL

Stock solution = 0.4986 g KOH per 100 mL Ethanol (ETOH)

Titration reagent must be used before it is 1 week old. Keep tightly covered.

C12 Standard Solution

0.1999 g Lauric acid per 100 mL hexane

Solution must be kept refrigerated. Stable for 2 months.

APPENDIX C-2

Concentrations of Gas Chromatography External Standards

Fatty acid		Amount
C4	Butyric (Butanoic)	0.0615 g
C5	n-Valeric (Pentanoic)	0.0656 g
C6	n-Caproic (Hexanoic)	0.0622 g
C7	Heptanoic	0.0654 g
C8	Caprylic (Octanoic)	0.0748 g
C9	Nonanoic	0.0742 g
C10	Capric (n-Decanoic)	0.0955 g
C11	Undecanoic	0.0970 g
C12	Lauric (dodecanoic)	0.1062 g
C13*	Tridecanoic	0.0468 g
C14	Myristic (tetradecanoic)	0.1199 g
C15	Pentadecanoic	0.1288 g
C16:0	Palmitic (hexadecanoic)	0.1265 g
C16:1	Palmitoleic (cis-9-hexadecanoic)	0.1148 g
C17	Heptadecanoic	0.1369 g
C18:0	Stearic (Octadecanoic)	0.1425 g
C18:1	Oleic (cis-9-Octadecanoic)	0.1454 g
C18:2	Linoleic (cis-9-, cis-12-octadecanoic)	0.1445 g
C18:3	Linoleic	0.1260 g

All fatty acids were combined with hexane to a total volume of 100 mL

*C13 amount should have been 0.1032 g

Concentrations of Gas Chromatography Internal Standards

C5	n-Valeric (Pentanoic)	0.0488 g
C17	Heptadecanoic	0.0488 g

These fatty acids were combined with hexane to a total volume of 100 mL

This concentrated C5 and C17 internal standard was then diluted 1:10 prior to adding to the GC samples (1 part internal standard : 10 parts hexane)

Gas Chromatography reagents

1:1 Diethyl ether - hexane (with 0.005% BHT, w/v)

Diethyl ether	450 mL
Hexane	450 mL
Butylated hydroxytoluene (BHT)	0.05g

(Keep solution covered tightly to prevent evaporation)

0.2 N Sulfuric acid

4.6 g H_2SO_4 into 500 mL H_2O

1:1 Diethyl ether - hexane (no BHT)

Diethyl ether	450 mL
Hexane	450 mL

(Keep solution covered tightly to prevent evaporation)

10% Formic acid

Formic acid	1.14 mL
Diethyl ether	8.86 mL

APPENDIX C-3**RMR values for GC calculations**

Fatty acid	Retention time (min)	Formula weight	Wt. injected (μ g)	FFA μ moles	Peak area	RMR
C4	4.404	88.10	1.845	0.02094	79846	0.7109
C5	5.976	102.13	1.968	0.01927	103349	None
C6	7.739	116.16	1.866	0.01606	94442	1.09619
C7	9.621	130.19	1.962	0.01507	15539	1.13273
C8	11.562	144.21	2.244	0.01556	104317	1.24998
C9	13.497	158.20	2.226	0.01407	90184	1.19505
C10	15.397	172.26	2.865	0.01663	118054	1.32348
C11	17.249	186.30	2.910	0.01562	103714	1.23803
C12	19.041	200.31	3.186	0.01591	101393	1.18861
C13	20.778	214.30	1.404	0.00655	29612	1.12000
C14	22.450	228.36	3.597	0.01575	85537	1.34564
C15	24.155	242.40	3.864	0.01594	78715	1.22362
C16:0	26.244	256.42	3.795	0.01480	68210	1.14204
C16:1	27.119	254.4	3.444	0.01354	62799	1.14948
C17	28.888	270.5	4.107	0.01518	61260	None
C18:0	32.281	284.47	4.275	0.01503	57195	0.94309
C18:1	33.447	282.45	4.362	0.01544	64504	1.03499
C18:2	35.779	282.4	4.335	0.01535	57295	0.92488
C18:3	39.38	278.4	3.78	0.01358	40538	0.73983

APPENDIX C-4 Analysis of Variance Tables

	Source	DF	Type III SS	FValue	Pr > F
ANOVA for Phase I - Extraction-titration for LSMean Lipase*Temp*Time					
All lipases	Lipase	4	33323.437	627.19	0.0001
	Time	11	14643.677	100.22	0.0001
	Temp	3	510.009	12.8	0.0001
	Rep	3	1599.317	40.14	0.0001
	Lipase*Temp*Time	165	2464.505	1.12	0.1603
	Lipase*Time	44	7432.353	12.72	0.0001
	Lipase*Temp	12	7226.281	45.34	0.0001
	Lipase*Rep	12	1180.527	7.41	0.0001
	Temp*Rep	9	6302.287	52.72	0.0001
ANOVA for Phase I - Slopes for regression lines per lipase					
<i>A. niger</i>	Temp	3	66.8478	7.21	0.3327
	Rep(Temp)	12	212.6131	5.73	0.0001
	Time	1	2297.6631	743.5	0.0001
	Time*Temp	3	41.4223	4.47	0.0048
	Time*Rep*Temp	12	337.5904	9.1	0.0001
Pancreatic	Temp	3	40.67	0.58	0.6395
	Rep(Temp)	12	280.708	8.65	0.0001
	Time	1	12078.514	4467	0.0001
	Time*Temp	3	491.089	60.54	0.0001
	Time*Rep*Temp	12	1088.037	33.53	0.0001
<i>P. roqueforti</i>	Temp	3	51.032684	0.37	0.7793
	Rep(Temp)	12	558.6882	18.75	0.0001
	Time	1	1096.843	441.64	0.0001
	Time*Temp	3	824.3565	110.64	0.0001
	Time*Rep*Temp	12	689.2686	23.13	0.0001
<i>R. miehei</i>	Temp	3	84.2061	1.51	0.2615
	Rep(Temp)	12	222.6657	6.91	0.0001
	Time	1	5858.8924	2180.78	0.0001
	Time*Temp	3	769.1628	95.43	0.0001
	Time*Rep*Temp	12	738.7930	22.92	0.0001

Source		DF	Type III SS	FValue	Pr > F
ANOVA for Phase I - Extraction-titration for LSMean for reproducibility					
<i>A. niger</i> at 35°C	Rep	3	7.70335	5.47	0.003
	Time	1	25.7332	54.78	0.0001
	Time*Rep	3	3.50754	2.49	0.0742
Pancreatic at 35°C	Rep	3	2.65542	9.85	0.0001
	Time	1	10.162	114.43	0.0001
	Time*Rep	3	0.32597	1.22	0.3144
<i>R. miehei</i> at 35°C	Rep	3	3.05987	6.51	0.0011
	Time	1	12.3078	78.54	0.0001
	Time*Rep	3	1.23843	2.63	0.0633
<i>P. roqueforti</i> at 35°C	Rep	3	5.17279	22.59	0.0001
	Time	1	13.46501	176.39	0.0001
	Time*Rep	3	3.95722	17.28	0.0001
<i>A. niger</i> at 40°C	Rep	3	2.05375	7.37	0.0006
	Time	1	12.11843	130.46	0.0001
	Time*Rep	3	0.8013	2.88	0.0495
Pancreatic at 40°C	Rep	3	2.40000	18.80	0.0001
	Time	1	14.58626	342.70	0.0001
	Time*Rep	3	0.26679	2.09	0.1165
<i>R. miehei</i> at 40°C	Rep	3	5.90705	60.98	0.0001
	Time	1	14.05286	435.21	0.0001
	Time*Rep	3	3.45772	35.69	0.0001
<i>P. roqueforti</i> at 40°C	Rep	3	20.5444	21.00	0.0001
	Time	1	9.90239	30.37	0.0001
	Time*Rep	3	7.30038	7.46	0.0005
<i>A. niger</i> at 45°C	Rep	3	1.83852	8.27	0.0002
	Time	1	18.141998	244.72	0.0001
	Time*Rep	3	0.396332	1.78	0.1670
Pancreatic at 45°C	Rep	3	0.45754	2.04	0.1245
	Time	1	12.66870	169.48	0.0001
	Time*Rep	3	0.28683	1.28	0.2954

Source		DF	Type III SS	FValue	Pr > F
ANOVA for Phase I - Extraction-titration for LSMean for reproducibility					
<i>R. miehei</i> at 45°C	Rep	3	1.6943	17.54	0.0001
	Time	1	17.05382	529.55	0.0001
	Time*Rep	3	0.53101	5.5	0.0030
<i>P. roqueforti</i> at 45°C	Rep	3	5.35244	17.69	0.0001
	Time	1	3.39310	33.81	0.0001
	Time*Rep	3	1.53777	5.11	0.0047
<i>A. niger</i> at 50°C	Rep	3	1.78021	6.91	0.0007
	Time	1	7.99813	93.16	0.0001
	Time*Rep	3	0.13705	0.53	0.6629
Pancreatic at 50°C	Rep	3	0.27435	1.88	0.1480
	Time	1	14.66778	301.99	0.0001
	Time*Rep	3	0.20918	1.44	0.2467
<i>R. miehei</i> at 50°C	Rep	3	1.24030	2.11	0.1138
	Time	1	20.30376	103.75	0.0001
	Time*Rep	3	2.10474	3.58	0.0219
<i>P. roqueforti</i> at 50°C	Rep	3	4.78973	11.97	0.0001
	Time	1	8.76865	65.71	0.0001
	Time*Rep	3	0.96886	2.42	0.0810
ANOVA for Phase II - Extraction-titration for LSMean for Lipase*Time					
All lipases	Lipase	4	71471.869	497.77	0.0001
	Time	11	42688.652	108.11	0.0001
	Lipase*Time	44	15466.946	9.79	0.0001
	Rep	3	4657.658	43.25	0.0001
ANOVA for Phase II - Slopes for regression lines for all lipase and reproducibility					
All lipases	Lipase	3	111.6784	3.42	0.7074
	Rep(Lipase)	12	946.1963	7.25	0.0001
	Time	1	51193.2840	4708.35	0.0001
	Time*Lipase	3	4680.5771	143.49	0.0001

Source	DF	Type III SS	FValue	Pr > F
ANOVA for Phase II - Total FFA for GC for all lipases				
Lipase	4	473.52729	39.71	0.0001
Rep	3	25.06027	2.80	0.0852
ANOVA for Phase III - LSMeans of lipase*rep for reproducibility for extraction-titration				
Lipase	4	95.37675	170.94	0.0001
Time	2	2.63746	9.45	0.0007
Rep	3	129.59999417	309.69	0.0001
Lipase*Time	8	2.63803	2.36	0.0419
Lipase*Rep	12	123.41624	73.73	0.0001
ANOVA for Phase III - Total FFA for GC for all lipases				
Lipase	3	409.75178	1.78	0.2291
Rep	3	530.84623	2.30	0.1537
ANOVA for Phase IV - Extraction-titration for all method				
Method	6	11583.014	154.48	0.0001
Rep	3	65.018	1.73	0.1958
ANOVA for Phase IV - GC for all methods				
Method	3	2650.9371	3.45	0.0647
Rep	3	939.1339	1.22	0.3564

APPENDIX C-5

Sample of Sensory Scorecard

Milk Sensory Evaluation Panel

Panelist _____

1. Please rinse your mouth with water and taste your first milk sample.
2. DO NOT SWALLOW the milk samples - a cup is provided for you to expectorate the sample.
3. Taste the samples in the order indicated and mark the appropriate statement.

Sample 193 Sample 439

_____ The samples are alike

_____ The samples are different

4. Describe the difference _____

5. Be sure to rinse your mouth after each set of samples and eat the crackers provided.

Thank you for participating in this panel!

APPENDIX B-6a

Sensory panelist comments comparing lipolysed milk samples prepared by the normal standard method (2% + raw) and modified standard method (recombined 2% + raw).

- 2% Standard method milk has a smoother feel and less of an aftertaste
- 2% Standard method milk is good
- 2% Standard method milk sweet compared to recombined
- 2% Standard method milk watery
- 2% Standard method milk has no creamy taste
- 2% Standard method milk has a fresher taste
- 2% Standard method milk not sweet
- Recombined milk is sweeter
- Recombined milk is thinner
- Recombined milk tastes rancid
- Recombined milk tastes buttery
- Recombined milk tastes slightly bad
- Recombined milk was flat; 2% left bad taste in mouth
- Recombined milk was oxidized
- Recombined milk creamier; more sweet milk flavor
- Recombined milk has cooked flavor
- Recombined milk has drawn butter flavor
- Recombined milk more free fat and more rancid
- Recombined milk oxidized and/or metallic
- Recombined milk is bitter
- Recombined milk is a little sour
- Recombined milk has a real off-flavor
- Recombined milk is thinner
- Recombined milk has a buttery, soapy flavor
- Recombined milk has a butter aftertaste
- Recombined milk tastes like it has hydrogen peroxide added to it
- Recombined milk had a plastic taste; 2% tasted like skim milk- not a strong taste
- Recombined milk tastes richer
- Recombined milk is stronger
- Recombined milk has a grassy flavor; 2% more fatty flavor - not fresh
- Recombined milk has a sour milk taste
- Recombined milk acidic aftertaste
- Recombined milk tastes spoiled
- Recombined milk was much stronger, tasted very thick; 2% tasted much better
- Recombined milk has a slight feed taste
- Recombined milk tastes and smells rancid
- Recombined milk is full milk; 2% is skim or low-fat milk
- Recombined milk slightly metallic; less mouthfeel

APPENDIX C-6b

Sensory panelist comments comparing lipolyzed milk samples prepared by the normal standard method (2% + raw) and an experimental method (hydrolyzed butter oil added to skim milk).

- 2% Standard method milk tastes like whole milk; experimental method milk has hydrogen peroxide in it
- 2% Standard method milk tastes cooked; experimental method milk tastes oxidized
- 2% Standard method milk is tastier, milder
- 2% Standard method milk less aftertaste, more creamy & not as acidic
- 2% Standard method milk tastes fresher
- 2% Standard method milk sweeter
- 2% Standard method milk has none of the creamy, strong fatty flavor
- 2% Standard method milk seemed very sl. sweeter than experimental method milk
- 2% Standard method milk fresher than experimental method milk
- 2% Standard method milk not as creamy as experimental method milk
- Did not pick up any off-flavor on 2%; experimental method milk had a real fatty taste
- Both lacked freshness, experimental method milk more so
- Both samples are cooked, but experimental method milk more so
- Experimental method milk is stronger, sweeter
- Experimental method milk tastes buttery
- Experimental method milk denser than 2% Standard method milk
- Experimental method milk too strong a taste
- Experimental method milk tastes like it has hydrogen peroxide in it
- Experimental method milk has a stronger oxidized flavor
- Experimental method milk has a strong plastic flavor; 2% Standard method milk did not have much taste
- Experimental method milk tastes flat
- Experimental method milk has metallic off-flavor
- Experimental method milk tasted spoiled, oily aftertaste; 2% tasted less dense
- Experimental method milk has a slightly soapy flavor
- Experimental method milk had a stronger milk flavor
- Experimental method milk tastes older, sour
- Experimental method milk had a slightly feed taste
- Experimental method milk more rancid and has rancid odor
- Experimental method milk has an aftertaste that remains after sample is expectorated
- Experimental method milk tasted sour; did not like at all!
- Experimental method milk more bouquet
- Experimental method milk more oxidized & metallic
- Experimental method milk has a cottage cheese flavor
- Experimental method milk had a stronger painty flavor
- Experimental method milk too strong/thick
- One is more roughage tasting

APPENDIX C-6c

Sensory panelist comments comparing lipolysed milk samples prepared by the modified standard method (recombined 2% + raw) and an experimental method (hydrolyzed butter oil added to skim milk).

- Experimental method milk feels more creamy, has more fat and more rancid
- Experimental method milk has a thicker mouthfeel
- Experimental method milk more watery and less sweet than recombined
- Experimental method milk tastes like has hydrogen peroxide in it
- Experimental method milk had more aftertaste; recombined milk seemed smoother
- Experimental method milk is more rich
- Experimental method milk stronger rancid
- Experimental method milk tasted fresher, recombined milk tasted more creamy
- Experimental method milk tastes flat; recombined milk cooked
- Experimental method milk sweeter and creamier
- Experimental method milk less milky
- Experimental method milk denser and sweeter than recombined milk
- Experimental method milk was oxidized and recombined milk cooked was rancid
- Experimental method milk tastes like hydrogen peroxide
- Experimental method milk had less aftertaste, less acidic and a smoother/creamier feel
- Experimental method milk is soured, bitter
- Experimental method milk has stronger milky flavor
- Experimental method milk tastes almost spoiled
- Experimental method milk tastes like baby milk; Recombined milk tastes like 2%
- Experimental method milk has a stronger flavor
- Experimental method milk has a sharper flavor
- Experimental method milk tastes fresher
- Both samples were oxidized, experimental method milk more so than 2%
- Both taste oxidized, but recombined milk more so and also cooked
- Both taste rancid
- Both had buttery and creamy taste, both tasted tallowy
- Both were very sour/spoiled tasting. Experimental method milk more so.
- Both had off-flavor, but recombined milk was stronger
- Recombined milk is heavier in body, salty
- Recombined milk tastes cooked, experimental method milk tastes oxidized
- Recombined milk has strong rancid taste, it tastes bad!
- Recombined milk is sweeter and has a more pleasing flavor
- Recombined milk seemed a little sweeter
- Recombined milk watery and off-taste; experimental method milk sweeter
- Recombined milk watery; experimental method milk rancid
- Recombined milk is sweeter, experimental method milk tastes oily

VITA

Carolyn Susan Baird was born in Knoxville, Tennessee on May 4, 1958. She attended elementary schools in Blacksburg, Virginia, St. Louis, Missouri, and Knoxville, Tennessee. She attended Junior high schools in St. Louis, Missouri and Hilton, New York. She attended high school in Hilton, New York and graduated from Parkway West High School in Chesterfield, Missouri in June, 1976. The following September she entered The University of Tennessee, Knoxville and received the degree Bachelor of Science in Agriculture in Food Technology and Science in March, 1980. She worked in Spartanburg, SC at Spartan Food Systems, Inc. in food product development for four years. For the next eight years she devoted herself to full-time motherhood, as well as working part-time as a Jazzercise® instructor in Jacksonville, FL. In January, 1993 she re-entered The University of Tennessee, Knoxville and received a Master of Science degree in Food Science and Technology in May, 1995. She is a member of the Institute of Food Technologists, American Dairy Science Association, Gamma Sigma Delta Agricultural Honor Society, and Alpha Xi Delta sorority.