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(54) Title: LIPID ACYLTRANSFERASE

(57) Abrégé/Abstract:

A lipid acyltransferase is described. The lipid acyltransferase comprises the amino acid sequence shown as SEQ ID No. 90, or an amino acid sequence which has 95% or more identity with SEQ ID No. 90.



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# **DEMANDES OU BREVETS VOLUMINEUX**

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# **JUMBO APPLICATIONS / PATENTS**

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE  
THAN ONE VOLUME.**

**THIS IS VOLUME \_\_1\_\_ OF \_\_2\_\_**

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**LIPID ACYLTRANSFERASE**

**FIELD OF INVENTION**

- 10 The present invention relates to a lipid acyltransferase.

The present invention relates to a method for the *in situ* production of an emulsifier within a foodstuff by use of a lipid acyltransferase.

- 15 The present invention further relates to a method for the *in situ* production of an emulsifier within a foodstuff by use of a lipid acyltransferase, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff.

- 20 The present invention yet further relates to a method for the *in situ* production of at least two emulsifiers within a foodstuff by use of a lipid acyltransferase.



The present invention also relates to a method for the *in situ* production of a carbohydrate ester and/or a sterol ester and/or a stanol ester and/or a protein ester and/or glycerol ester and/or a hydroxy acid ester within a foodstuff by use of a lipid  
5 acyltransferase.

The present invention relates to a food enzyme composition and/or a feed enzyme composition, which contains a lipid acyltransferase, and the use of such a composition in the methods of the present invention.

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The present invention further relates to a method of identifying suitable lipid acyltransferases in accordance with the present invention and to lipid acyltransferases so identified.

15 The present invention yet further relates to an immobilised lipid acyltransferase.

## TECHNICAL BACKGROUND

The beneficial use of phospholipases and lipases (referred to as lipolytic enzymes,  
20 (EC. 3.1.1.x) used in food and/or feed industrial applications has been known for many years.

For instance, in EP 0 585 988 it is claimed that lipase addition to dough resulted in an improvement in the antistaling effect. It is suggested that a lipase obtained from  
25 *Rhizopus arrhizus* when added to dough can improve the quality of the resultant bread when used in combination with shortening/fat. WO94/04035 teaches that an improved softness can be obtained by adding a lipase to dough without the addition of any additional fat/oil to the dough. Castello, P. ESEGP 89-10 Dec. 1999 Helsinki, shows that exogenous lipases can modify bread volume.

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Lipolytic enzymes hydrolyse one or more of the fatty acids from lipids present in the food which can result in the formation of powerful emulsifier molecules within the



foodstuff which provide commercially valuable functionality. The molecules which contribute the most significant emulsifier characteristics are the partial hydrolysis products, such as lyso-phospholipids, lyso-glycolipids, and mono-glyceride molecules. The polar lipid hydrolysis products, such as lyso-phospholipids and lyso-glycolipids are particularly advantageous. In bread making, such *in situ* derived emulsifiers can give equivalent functionality as emulsifiers, such as DATEM.

However, the activity of lipolytic enzymes also results in accumulation of free fatty acids, which can lead to detrimental functionality in the foodstuff. This inherent activity of lipolytic enzymes limits their functionality.

Numerous solutions to this problem have been attempted in the art. However, these result in a significant increase in free fatty acids in the foodstuff, particularly food stuffs with high water content, including, but not limited to bread doughs and egg yolk.

Phospholipases, particularly phospholipase A2 (E.C. 3.1.1.4), have been used for many years for the treatment of egg or egg-based products (see US 4,034,124 and Dutihl & Groger 1981 J. Sci. Food Agric. 32, 451-458 for example). The phospholipase activity during the treatment of egg or egg-based products results in the accumulation of polar lysolecithin, which can act as an emulsifier. Phospholipase treatment of egg or egg-based products can improve the stability, thermal stability under heat treatment such as pasteurisation and result in substantial thickening. Egg-based products may include, but are not limited to cake, mayonnaise, salad dressings, sauces, ice creams and the like. Use of phospholipases results in the accumulation of free fatty acids. The accumulation of free fatty acids can result in significant off-flavour. In addition, the accumulation of free fatty acids can result in enhanced susceptibility to oxidation, and hence result in poor shelf-life, product discoloration and alteration of other critical food characteristics such as flavour and texture. Recently, lipolytic enzymes with broader substrate specificity have been marketed for treatment of egg yolk and related food products. These have the advantage that, unlike most of the phospholipase A2s, they do not originate from a mammalian source. However, they result in significant



accumulation of free fatty acids, not only due to the hydrolysis of phospholipids, but also triglycerides.

As mentioned above, another area where lipases have been extensively used is in the bakery industry. The use of phospholipases in baking dates back to the early 1980s.

The substrate for lipases in wheat flour is 1.5-3% endogenous wheat lipids, which are a complex mixture of polar and non-polar lipids. The polar lipids can be divided into glycolipids and phospholipids. These lipids are built up of glycerol esterified with two fatty acids and a polar group. The polar group contributes to surface activity of these lipids. Enzymatic cleavage of one of the fatty acids in these lipids leads to lipids with a much higher surface activity. It is well known that emulsifiers, such as DATEM, with high surface activity are very functional when added to dough.

However, the use of lipases (E.C. 3.1.1.X) in dough products may have a detrimental impact on yeast activity, and/or a negative effect on bread volume. The negative effect on bread volume is often explained by overdosing. Overdosing can lead to a decrease in gluten elasticity which results in a dough which is too stiff and thus results in reduced bread volumes. In addition, or alternatively, such lipases can degrade shortening, oil or milk fat added to the dough, resulting in off-flavour in the dough and baked product. Overdosing and off flavour have been attributed to the accumulation of free fatty acids in the dough.

In EP 1 193 314, EP 0 977 869 and also in WO 01/39602, the use of lipolytic enzymes active on glycolipids was reported to be particularly beneficial in application in bread making as the partial hydrolysis products the lyso-glycolipids were found to have very high emulsifier functionality, apparently resulting in a higher proportion of positive emulsifier functionality compared to the detrimental accumulation of free fatty acids. However, the enzymes were also found to have significant non selective activity on triglyceride which resulted in unnecessarily high free fatty acid.



The same finding was reported in WO 00/32758, which disclosed lipolytic enzyme variants with enhanced activity on phospholipids and/or glycolipids, in addition to variants which had a preference for long rather than short chain fatty acids. This latter feature, also disclosed in WO 01/39602, was deemed of particular importance to prevent the off-flavours associated with the accumulation of free short chain fatty acids. However, significant free fatty acids are produced.

The problem of high triglyceride activity was addressed in WO02/094123, where the use of lipolytic enzymes active on the polar lipids (i.e. glycolipids and phospholipids) in a dough, but substantially not active on triglycerides or 1-mono-glycerides is taught. However, significant free fatty acids are produced.

Some lipolytic enzymes have low or no activity on the lyso form of polar lipids (e.g. glycolipids/phospholipids). The use of such enzymes has been deemed preferable as they ensure the accumulation of the highly polar lyso-lipids, resulting in optimal functionality. Free fatty acids do however accumulate. This selective functionality is characteristic of phospholipase A2 enzymes, and the glycolipases disclosed in EP 0 977 869, EP 1 193 314, and WO01/39602. Variant enzymes of less selective lipolytic enzymes have been produced which have a lower activity on the lyso-polar lipids when compared to the parent enzyme (WO03/060112). However, significant free fatty acids are produced.

WO00/05396 teaches a process for preparing a foodstuff comprising an emulsifier, wherein food material is contacted with an enzyme such that an emulsifier is generated by the enzyme from a fatty acid ester and a second functional ingredient is generated from a second constituent. WO00/05396 teaches the use of in particular a lipase or esterase enzyme. Nowhere in WO00/05396 is the specific use of a lipid acyltransferase taught. In addition, in foodstuffs with high water content, the use of the esterases and lipases as taught in WO00/05396 would result in significant accumulation of free fatty acids.



A disadvantage associated with the use of lipases, including phospholipases and glycolipases, may be caused by the build-up of free fatty acids released from the lipids. Over the past couple of decades the use of lipolytic enzymes in foodstuffs has been limited due to the balance between the detrimental accumulation of free fatty acids and the production of the lyso-lipids which provide positive functionality. Although numerous strategies in the art have been attempted, some of which provided significant improvements in functionality, none have completely addressed and solved the fundamental problem in the art, i.e. the significant accumulation of free fatty acids in foodstuffs prepared using lipolytic enzymes *in situ*.

The presence of high levels of free fatty acids (FFA) in raw materials or food products is generally recognised as a quality defect and food processors and customers will usually include a maximum FFA level in the food specifications. The resulting effects of excess FFA levels can be in organoleptic and/or functional defects.

A result of lipolysis is hydrolytic rancidity, with the formation of characteristic "soapy" flavour. This "soapy" taste is especially acute with fatty acids of intermediate chain length (C8-C12) which, although not present in high concentrations, may be important constituents of, for example, dairy products or vegetable oils. A more common organoleptic defect is due to the combined effects of lipolytic enzymes and oxidation processes. Unsaturated fatty acids are more susceptible to enzymatic oxidation when unesterified than when esterified in acyl lipids.

Functional defects in food due to high FFA levels are recognised, but less readily explained. Without wishing to be bound by theory, the hydrolysis of unchanged lipids to carboxylic acids will increase  $[H^+]$  and produce carbonyl groups that can combine with other compounds or metal ions. Free fatty acids also combine proteins by hydrophobic interactions and can complex with starch during cooking. FFA may also interfere with the action of surface-active agents, such as polar lipids and emulsifiers.

(Lipid in Cereal Technology, P.J. Barnes, Academic Press 1983.)



WO03/100044 discloses a class of acyl transferases known as PDATs (or ATWAX). These enzymes use a monoglyceride or a diglyceride as the acceptor molecule, and phosphatidylcholine (PC) as the donor molecule to produce the following products: lyso phosphatidylcholine and triacylglycerol and/or diacylglycerol.

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In one embodiment, the present invention relates to improvements in the incorporation of whey proteins into food products, providing for improved yields without impairing the qualities – such as the texture – of the food compositions and products.

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Cheese compositions are typically prepared from dairy liquids by processes that include treating the liquid with a coagulating or clotting agent. The coagulating agent may be a curdling enzyme, an acid or a suitable bacterial culture, or it may include such a culture. The curd that results generally incorporates transformed casein, fats including natural butter fat, and flavourings that arise especially when a bacterial culture is used. The curd may be separated from the liquid whey, which contains soluble proteins not affected by the coagulation and that therefore are not incorporated into the curd.

20 Whey is thus a by-product of manufacturing in commercial processes that produce food products - such as cheeses. Traditionally, whey is disposed of as unused waste or used as fertiliser or animal feed or processed into a food ingredient.

25 The inability of whey proteins to be substantially retained in the curd is an important factor contributing to a lack of efficiency in the conventional production of dairy products – such as cheese curds - and to a reduction in overall yield relating to the incorporation of all the protein solids that are present in the starting dairy liquids into resulting cheese curds.

30 There have been numerous attempts to include whey proteins in cheese e.g. by heat treatment of the milk, heat treatment of whey, or by filtration – such as ultrafiltration.



There are also several descriptions of the use of specific proteases to induce aggregation of whey proteins. A serine protease derived from *Bacillus licheniformis* has been shown to have the ability to induce aggregation of whey proteins (US 5,523,237).

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However, there remains many difficulties associated with adding whey proteins in processes such as the manufacture of cheeses. For example, incorporation of whey protein into cheeses is associated with a deterioration in the taste and mouth-feel of the product, and furthermore tends to interfere with curding and subsequent processing of the product. Proteases that have been previously reported that can be added to cheese milk for hydrolysis of whey proteins result in significant hydrolysis of the caseins as described by Madsen, J.S. & Qvist, K.B. (1997) Hydrolysis of milk protein by a *Bacillus licheniformis* protease specific for acidic amino acid residues. *J. Food Sci.* 62, 579-582.

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Thus, there is a need in the art for methods and compositions that provide for the improved incorporation of whey protein into food products while maintaining organoleptic and other desirable properties. Such optimisation would result in increased efficiency, higher yields of food products, and reduced overall material costs.

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Lipase:cholesterol acyltransferases have been known for some time (see for example Buckley – *Biochemistry* 1983, 22, 5490-5493). In particular, glycerophospholipid:cholesterol acyl transferases (GCATs) have been found, which like the plant and/or mammalian lecithin:cholesterol acyltransferases (LCATs), will catalyse fatty acid transfer between phosphatidylcholine and cholesterol.

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Upton and Buckley (*TIBS* 20, May 1995 p 178-179) and Brumlik and Buckley (*J. of Bacteriology* Apr. 1996 p 2060-2064) teach a lipase/acyltransferase from *Aeromonas hydrophila* which has the ability to carry out acyl transfer to alcohol acceptors in aqueous media.

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## SUMMARY ASPECTS OF THE PRESENT INVENTION

According to a first aspect of the present invention there is provided a method of *in situ* production of an emulsifier in a foodstuff, wherein the method comprises the step  
5 of adding to the foodstuff a lipid acyltransferase as defined herein.

In a further aspect, the present invention provides a method of *in situ* production of an emulsifier in a foodstuff, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the  
10 foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of *in situ* production of an emulsifier and either a sterol ester and/or a stanol ester in a foodstuff, wherein the  
15 method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of *in situ* production of an emulsifier and either a sterol ester and/or a stanol ester in a foodstuff, wherein the  
20 method comprises the step of adding a lipid acyltransferase to the foodstuff.

According to another aspect of the present invention there is provided a method for the *in situ* production of at least two emulsifiers in a foodstuff, wherein the method  
25 comprises the step of adding to the foodstuff a lipid acyltransferase.

According to a further aspect of the present invention there is provided a method of *in situ* production of at least two emulsifiers and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method is such that the emulsifiers are produced without  
30 increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.



According to a further aspect of the present invention there is provided a method of *in situ* production of at least two emulsifiers and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In a further aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester together with an emulsifier in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of *in situ* production of an emulsifier, and one or more of a carbohydrate ester; a sterol ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride in a foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

According to a further aspect of the present invention there is provided a method of production of a foodstuff comprising an emulsifier, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase as defined herein.

In a further aspect, the present invention provides a method of production of a foodstuff comprising an emulsifier, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the method is such that the emulsifier is produced without increasing or



without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

5 In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

10 According to a further aspect of the present invention there is provided a method for the production of a foodstuff comprising at least two emulsifiers, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase.

15 According to a further aspect of the present invention there is provided a method of the production of a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein the method is such that the emulsifiers are produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

20 According to a further aspect of the present invention there is provided a method of the production of a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

25 In a further aspect, the present invention provides a method for the production of a foodstuff comprising a carbohydrate ester, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

30 In another aspect, the present invention provides a method for the production of a foodstuff comprising a carbohydrate ester and an emulsifier, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.



In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and one or more of a carbohydrate ester; a sterol ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

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In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier, wherein the emulsifier is generated from constituents of the food material by the lipid acyltransferase.

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In a further aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier, wherein the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the emulsifier is generated from constituents

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of the food material by the lipid acyltransferase.

In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the emulsifier is produced without increasing or

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without substantially increasing the free fatty acids in the foodstuff, and wherein the emulsifier and/or sterol ester and/or stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

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In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the emulsifier and/or sterol ester and/or stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

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In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers, wherein the two emulsifiers are generated from constituents of the food material by the lipid acyltransferase.



According to a further aspect of the present invention there is provided use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein the emulsifiers are  
5 produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein one or both of the emulsifiers and/or the sterol ester and/or the stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

10 According to a further aspect of the present invention there is provided use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein one or both of the emulsifiers and/or the sterol ester and/or the stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

15 In a further aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising a carbohydrate ester, wherein the carbohydrate ester is generated from constituents of the food material by the lipid acyltransferase.

20 In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least a carbohydrate ester and a further emulsifier, wherein the carbohydrate ester and the emulsifier are generated from constituents of the food material by the lipid acyltransferase.

25 In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier and one or more of a carbohydrate ester; a sterol ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride, and wherein the emulsifier and/or the carbohydrate ester and/or the sterol  
30 ester and/or the stanol ester and/or the protein ester and/or the monoglyceride and/or the diglyceride is/are generated from constituents of the food material by the lipid acyltransferase.



In accordance with a further aspect of the present invention there is provided a method of the *in situ* production of an emulsifier, preferably a lysolecithin and a sterol ester in a egg based foodstuff, wherein the method is such that the emulsifier is produced  
5 without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In accordance with a further aspect of the present invention there is provided a method  
10 of the *in situ* production of an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of production of a egg  
15 based foodstuff comprising an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

20 In another aspect, the present invention provides a method of production of an egg based foodstuff comprising an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

25 In a further aspect, the present invention further provides a foodstuff obtainable by, preferably obtained by, a method according to the present invention.

In another aspect the present invention further relates to a food enzyme composition and/or a feed enzyme composition, which contains a lipid acyltransferase, and the use  
30 of such a composition in the methods of the present invention.



In accordance with a further aspect of the present invention there is provided a method of identifying a suitable lipid acyltransferase for use in accordance with the present invention, comprising the steps of testing an enzyme of interest using one or more of the "Transferase Assay in a Low Water environment", the "Transferase Assay in High  
5 Water Egg Yolk" or the "Transferase Assay in Buffered Substrate", and selecting a lipid acyltransferase if it is one which has one or more of the following characteristics: (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water  
10 Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.

The present invention yet further provides a lipid acyltransferase identified using a  
15 method according to the present invention.

In accordance with a further aspect, the present invention provides an immobilised lipid acyltransferase enzyme as defined herein.

## 20 DETAILED ASPECTS OF THE PRESENT INVENTION

The term "lipid acyltransferase" as used herein means an enzyme which as well as having lipase activity (generally classified as E.C. 3.1.1.x in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of  
25 the International Union of Biochemistry and Molecular Biology) also has acyltransferase activity (generally classified as E.C. 2.3.1.x), whereby the enzyme is capable of transferring an acyl group from a lipid to one or more acceptor substrates, such as one or more of the following: a sterol; a stanol; a carbohydrate; a protein; a protein subunit; glycerol.

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The lipid acyltransferase for use in the methods and/or uses of the present invention may be one as described in WO2004/064537 or WO2004/064987, or



PCT/IB2004/004378 or GB0513859.9, or PCT/ GB05/002823.

The lipid acyltransferase for use in the methods and/or uses of the present invention  
5 may be a natural lipid acyltransferase or may be a variant lipid acyltransferase.

Preferably, the lipid acyltransferase for use in the methods and/or uses of the present invention is capable of transferring an acyl group from a lipid (as defined herein) to one or more of the following acyl acceptor substrates: a sterol, a stanol, a carbohydrate, a protein or subunits thereof, or a glycerol.

10

For some aspects the "acyl acceptor" according to the present invention may be any compound comprising a hydroxy group (-OH), such as for example, polyvalent alcohols, including glycerol; sterol; stanols; carbohydrates; hydroxy acids including fruit acids, citric acid, tartaric acid, lactic acid and ascorbic acid; proteins or a sub-unit  
15 thereof, such as amino acids, protein hydrolysates and peptides (partly hydrolysed protein) for example; and mixtures and derivatives thereof. Preferably, the "acyl acceptor" according to the present invention is not water.

In one embodiment, the acyl acceptor is preferably not a monoglyceride and/or a  
20 diglyceride.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a sterol and/or a stanol.

25 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a carbohydrate.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a protein or a subunit thereof. Suitably the protein subunit may be one or more  
30 of the following: an amino acid, a protein hydrolysate, a peptide, a dipeptide, an oligopeptide, a polypeptide.



Suitably in the protein or protein subunit the acyl acceptor may be one or more of the following constituents of the protein or protein subunit: a serine, a threonine, a tyrosine, or a cysteine.

- 5 When the protein subunit is an amino acid, suitably the amino acid may be any suitable amino acid. Suitably the amino acid may be one or more of a serine, a threonine, a tyrosine, or a cysteine for example.

10 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to glycerol.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a hydroxy acid.

- 15 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a polyvalent alcohol.

20 In one aspect, the lipid acyltransferase may, as well as being able to transfer an acyl group from a lipid to a sterol and/or a stanol, additionally be able to transfer the acyl group from a lipid to one or more of the following: a carbohydrate, a protein, a protein subunit, glycerol.

25 Preferably, the lipid substrate upon which the lipid acyltransferase according to the present invention acts is one or more of the following lipids: a phospholipid, such as a lecithin, e.g. phosphatidylcholine, a triacylglyceride, a cardiolipin, a diglyceride, or a glycolipid, such as digalactosyldiglyceride (DGDG) for example. This lipid substrate may be referred to herein as the "lipid acyl donor". The term lecithin as used herein encompasses phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

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For some aspects, preferably the lipid substrate upon which the lipid acyltransferase acts is a phospholipid, such as lecithin, for example phosphatidylcholine.



For some aspects, preferably the lipid substrate is a glycolipid, such as DGDG for example.

- 5 Preferably the lipid substrate is a food lipid, that is to say a lipid component of a foodstuff.

For some aspects, preferably the lipid acyltransferase according to the present invention is incapable, or substantially incapable, of acting on a triglyceride and/or a 1-  
10 monoglyceride and/or 2-monoglyceride.

Suitably, the lipid substrate or lipid acyl donor may be one or more lipids present in one or more of the following substrates: fats, including lard, tallow and butter fat; oils including oils extracted from or derived from palm oil, sunflower oil, soya bean oil, safflower oil, cotton seed oil, ground nut oil, corn oil, olive oil, peanut oil, coconut oil,  
15 and rape seed oil. Lecithin from soya, rape seed or egg yolk is also a suitable lipid substrate. The lipid substrate may be an oat lipid or other plant based material containing galactolipids.

- 20 In one aspect the lipid acyl donor is preferably lecithin (such as phosphatidylcholine) in egg yolk.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 8 to 22 carbons.

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For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 16 to 22 carbons, more preferably of from 16 to 20 carbons.

- 30 For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of no greater than 14 carbons, suitably from lipids having a



fatty acid chain length of from 4 to 14 carbons, suitably 4 to 10 carbons, suitably 4 to 8 carbons.

Suitably, the lipid acyltransferase according to the present invention may exhibit one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26), triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). The term “glycolipase activity” as used herein encompasses “galactolipase activity”.

Suitably, the lipid acyltransferase according to the present invention may have at least one or more of the following activities: glycolipase activity (E.C. 3.1.1.26) and/or phospholipase A1 activity (E.C. 3.1.1.32) and/or phospholipase A2 activity (E.C. 3.1.1.4).

For some aspects, the lipid acyltransferase according to the present invention may have at least glycolipase activity (E.C. 3.1.1.26).

Suitably, for some aspects the lipid acyltransferase according to the present invention may be capable of transferring an acyl group from a glycolipid and/or a phospholipid to one or more of the following acceptor substrates: a sterol, a stanol, a carbohydrate, a protein, glycerol.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a sterol and/or a stanol to form at least a sterol ester and/or a stanol ester.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a carbohydrate to form at least a carbohydrate ester.



For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a protein to form at least protein ester (or a protein fatty acid condensate).

5

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to glycerol to form at least a diglyceride and/or a monoglyceride.

- 10 In one embodiment the acyl acceptor is glycerol. The glycerol may be naturally comprised in the foodstuff and/or food material comprising the acyl donor (i.e. the phospholipid for example) - such as butter fat, milk or cream for instance. Alternatively the glycerol may be added to the foodstuff and/or food material comprising the acyl donor (i.e. the phospholipids for example) - such as butterfat, milk  
15 or cream - either prior to, during or subsequent to the addition of lipid acyl transferase enzyme.

For some aspects, preferably the lipid acyltransferase according to the present invention does not exhibit triacylglycerol lipase activity (E.C. 3.1.1.3) or significant  
20 triacylglycerol lipase activity (E.C. 3.1.1.3).

In some aspects, the lipid acyltransferase may be capable of transferring an acyl group from a lipid to a sterol and/or a stanol. Thus, in one embodiment the "acyl acceptor" according to the present invention may be either a sterol or a stanol or a combination  
25 of both a sterol and a stanol.

In one embodiment suitably the sterol and/or stanol may comprise one or more of the following structural features:

- i) a 3-beta hydroxy group or a 3-alpha hydroxy group; and/or
- 30 ii) A:B rings in the *cis* position or A:B rings in the *trans* position or C<sub>5</sub>-C<sub>6</sub> is unsaturated.



Suitable sterol acyl acceptors include cholesterol and phytosterols, for example alpha-sitosterol, beta-sitosterol, stigmasterol, ergosterol, campesterol, 5,6-dihydrosterol, brassicasterol, alpha-spinasterol, beta-spinasterol, gamma-spinasterol, deltaspinasterol, fucosterol, dimosterol, ascosterol, serebisterol, episterol, anasterol, hyposterol, 5 chondrillasterol, desmosterol, chalinosterol, poriferasterol, clionasterol, sterol glycosides, and other natural or synthetic isomeric forms and derivatives.

In one aspect of the present invention suitably more than one sterol and/or stanol may act as the acyl acceptor, suitably more than two sterols and/or stanols may act as the 10 acyl acceptor. In other words, in one aspect of the present invention, suitably more than one sterol ester and/or stanol ester may be produced. Suitably, when cholesterol is the acyl acceptor one or more further sterols or one or more stanols may also act as the acyl acceptor. Thus, in one aspect, the present invention provides a method for the *in situ* production of both a cholesterol ester and at least one sterol or stanol ester in 15 combination. In other words, the lipid acyltransferase for some aspects of the present invention may transfer an acyl group from a lipid to both cholesterol and at least one further sterol and/or at least one stanol.

In one aspect, preferably the sterol acyl acceptor is one or more of the following: 20 alpha-sitosterol, beta-sitosterol, stigmasterol, ergosterol and campesterol.

In one aspect, preferably the sterol acyl acceptor is cholesterol. When it is the case that cholesterol is the acyl acceptor for the lipid acyltransferase, the amount of free cholesterol in the foodstuff is reduced as compared with the foodstuff prior to exposure 25 to the lipid acyltransferase and/or as compared with an equivalent foodstuff which has not been treated with the lipid acyltransferase.

Advantageously, preferably the level of cholesterol in the foodstuff (for example a dairy product, such as cheese, milk, cream, butterfat or ice cream for instance) is 30 reduced compared with a control foodstuff (for example a dairy product, such as cheese, milk, cream, butterfat or ice cream for instance), e.g. one which has not been treated with a lipid acyltransferase in accordance with the present invention).



In another embodiment the acyl acceptor is cholesterol. The cholesterol may be naturally comprised in the foodstuff and/or food material comprising the acyl donor (i.e. the phospholipid for example) - such as butter fat, milk or cream for instance.

5 Alternatively the cholesterol may be added to the foodstuff and/or food material comprising the acyl donor (i.e. the phospholipids for example) - such as butterfat, milk or cream - either prior to, during or subsequent to the addition of lipid acyl transferase enzyme.

10 Suitable stanol acyl acceptors include phytosterols, for example beta-sitosterol or stigmasterol.

In one aspect, preferably the sterol and/or stanol acyl acceptor is a sterol and/or a stanol other than cholesterol.

15

In some aspects, the foodstuff prepared in accordance with the present invention may be used to reduce blood serum cholesterol and/or to reduce low density lipoprotein. Blood serum cholesterol and low density lipoproteins have both been associated with certain diseases in humans, such as atherosclerosis and/or heart disease for example.

20 Thus, it is envisaged that the foodstuffs prepared in accordance with the present invention may be used to reduce the risk of such diseases.

Thus, in one aspect the present invention provides the use of a foodstuff according to the present invention for use in the treatment and/or prevention of atherosclerosis and/or heart disease.

25

In a further aspect, the present invention provides a medicament comprising a foodstuff according to the present invention.

30 In a further aspect, the present invention provides a method of treating and/or preventing a disease in a human or animal patient which method comprising



administering to the patient an effective amount of a foodstuff according to the present invention.

Suitably, the sterol and/or the stanol "acyl acceptor" may be found naturally within the foodstuff. Alternatively, the sterol and/or the stanol may be added to the foodstuff. When it is the case that a sterol and/or a stanol is added to the foodstuff, the sterol and/or stanol may be added before, simultaneously with, and/or after the addition of the lipid acyltransferase according to the present invention. Suitably, the present invention may encompass the addition of exogenous sterols/stanols, particularly phytosterols/phytostanols, to the foodstuff prior to or simultaneously with the addition of the enzyme according to the present invention.

For some aspects, one or more sterols present in the foodstuff may be converted to one or more stanols prior to or at the same time as the lipid acyltransferase is added according to the present invention. Any suitable method for converting sterols to stanols may be employed. For example, the conversion may be carried out by chemical hydrogenation for example. The conversion may be conducted prior to the addition of the lipid acyltransferase in accordance with the present invention or simultaneously with the addition of the lipid acyltransferase in accordance with the present invention. Suitably enzymes for the conversion of sterol to stanols are taught in WO00/061771.

Suitably the present invention may be employed to produce phytostanol esters *in situ* in a foodstuff. Phytostanol esters have increased solubility through lipid membranes, bioavailability and enhanced health benefits (see for example WO92/99640).

In some embodiments of the present invention the stanol ester and/or the sterol ester may be a flavouring and/or a texturiser. In which instances, the present invention encompasses the *in situ* production of flavourings and/or texturisers.

For some aspects of the present invention, the lipid acyltransferase according to the present invention may utilise a carbohydrate as the acyl acceptor. The carbohydrate acyl acceptor may be one or more of the following: a monosaccharide, a disaccharide,



an oligosaccharide or a polysaccharide. Preferably, the carbohydrate is one or more of the following: glucose, fructose, anhydrofructose, maltose, lactose, sucrose, galactose, xylose, xylooligosaccharides, arabinose, maltooligosaccharides, tagatose, microthecin, ascopyrone P, ascopyrone T, cortalcerone.

5

Suitably, the carbohydrate "acyl acceptor" may be found naturally within the foodstuff. Alternatively, the carbohydrate may be added to the foodstuff. When it is the case that the carbohydrate is added to the foodstuff, the carbohydrate may be added before, simultaneously with, and/or after the addition of the lipid acyltransferase according to the present invention.

10

Carbohydrate esters can function as valuable emulsifiers in foodstuffs. Thus, when it is the case that the enzyme functions to transfer the acyl group to a sugar, the invention encompasses the production of a second *in situ* emulsifier in the foodstuff.

15

In some embodiments, the lipid acyltransferase may utilise both a sterol and/or stanol and a carbohydrate as an acyl acceptor.

20

The utilisation of lipid acyltransferase which can transfer the acyl group to a carbohydrate as well as to a sterol and/or a stanol is particularly advantageous for foodstuffs comprising eggs. In particular, the presence of sugars, in particular glucose, in eggs and egg products is often seen as disadvantageous. Egg yolk may comprise up to 1% glucose. Typically, egg or egg based products may be treated with glucose oxidase to remove some or all of this glucose. However, in accordance with the present invention this unwanted sugar can be readily removed by "esterifying" the sugar to form a sugar ester.

25

For some aspects of the present invention, the lipid acyltransferase according to the present invention may utilise a protein as the acyl acceptor. Suitably, the protein may be one or more of the proteins found in a food product, for example in a dairy product and/or a meat product. By way of example only, suitable proteins may be those found in curd or whey, such as lactoglobulin. Other suitable proteins include ovalbumin from

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egg, gliadin, glutenin, puroindoline, lipid transfer proteins from grains, and myosin from meat.

Thus in accordance with the present invention, one or more of the following  
5 advantageous properties can be achieved: *in situ* production of an emulsifier without an increase in free fatty acids; a reduction in the accumulation of free fatty acids in the foodstuff; a reduction in free cholesterol levels in the foodstuff; an increase in sterol esters and/or stanol esters; a reduction in blood serum cholesterol and/or low density lipoproteins; an increase in carbohydrate esters; a reduction in unwanted free  
10 carbohydrates.

An advantage of the present invention is that the emulsifier(s) is/are prepared *in situ* in the foodstuff without an increase, or a substantial, increase, in the free fatty acid content of the foodstuff. The production of free fatty acids can be detrimental to  
15 foodstuffs. In particular, free fatty acids have been linked with off-odours and/or off-flavours in foodstuffs, as well other detrimental effects, including a soapy taste in cheese for instance. Preferably, the method according to the present invention results in the *in situ* preparation of an emulsifier(s) wherein the accumulation of free fatty acids is reduced and/or eliminated. Without wishing to be bound by theory, in  
20 accordance with the present invention the fatty acid which is removed from the lipid is transferred by the lipid acyltransferase to an acyl acceptor, for example a sterol and/or a stanol. Thus, the overall level of free fatty acids in the foodstuff does not increase or increases only to an insignificant degree. This is in sharp contradistinction to the situation when lipases (E.C. 3.1.1.x) are used to produce emulsifiers *in situ*. In  
25 particular, the use of lipases can result in an increased amount of free fatty acid in the foodstuff, which can be detrimental. In accordance with the present invention, the accumulation of free fatty acids is reduced and/or eliminated when compared with the amount of free fatty acids which would have been accumulated had a lipase enzyme, in particular a phospholipase A enzyme, been used in place of the lipid acyltransferase in  
30 accordance with the present invention.



The utilisation of a lipid acyltransferase which can transfer the acyl group to a sterol and/or stanol may be particularly advantageous for foodstuffs comprising eggs. In particular, it has been found that an egg-based product with significantly better properties can be obtained following treatment with a lipid acyltransferase as defined  
5 herein compared with egg-based products treated with conventional phospholipases, such as LipopanF® (Novozymes A/S, Denmark) ), Lecitase Ultra® (Novozymes A/S, Denmark) or Lipomod 22 L from Biocatalysts, for instance.

In another aspect the acyl acceptor may be ascorbic acid or comprises ascorbic acid.  
10 Therefore ascorbic acid may be added to the foodstuff and/or food material, or aqueous emulsion, possibly in combination with an appropriate level of glycerol and optionally sterol/stanols. Ascorbic ester is an antioxidant. The use of ascorbic acid may be especially preferred when used in a foodstuff as the anti-oxidant properties can act as a preservation agent, e.g. to prevent or reduce oxidation of lipids. . In this way the use  
15 of ascorbic acid in the foodstuff and/or food material of the present invention can prevent or reduce rancidity in the modified foodstuff and/or food material. Therefore the use of ascorbic acid may be particularly useful for use in dairy products where rancidity can be a problem, for example in cheese. The amount of ascorbic acid added may be very low, e.g. at a level of up to  $1/5^{\text{th}}$ , such as up to  $1/10^{\text{th}}$  or up to  $1/100^{\text{th}}$  the  
20 amounts recommended for the addition of glycerol as herein defined. Preferably, the range of ascorbic acid should be 0.02-0.5wt%. In a preferable embodiment the ascorbic acid is added in the form of an ascorbyl-palmitate, e.g. for use as an anti-oxidant in oil, and the dosage is preferably between 0.1 and 0.2wt% corresponding to preferably between 0.04-0.08wt% ascorbic acid.

25

In a preferred embodiment the modified foodstuff and/or food material treated in accordance with the present invention comprises lysophospholipid, preferably lysolecithin, preferably the foodstuff and/or food material treated in accordance with the present invention comprises at least 0.001wt%, such as 0.005wt%, including at  
30 least 0.01 wt%, lysophospholipid, preferably lysolecithin, more preferably at least 0.05 wt%, or at least 0.1 wt%, lysophospholipid, preferably lysolecithin. Higher concentrations of lysophospholipid, preferably lysolecithin, are also envisaged, such



as at least 0.5 wt%, or at least 1 wt%, lysophospholipid, preferably lysolecithin, including at least 2 wt%, or at least 5%, lysophospholipid, preferably lysolecithin.

In a preferred embodiment the food stuff and/or food material treated in accordance with the present invention comprises one or more of the following glycerophosphatylcholine/phosphatylethanolamine phosphatylinositol and phosphatylserine, preferably the foodstuff and/or the food material treated in accordance with the present invention comprises at least 0.001wt% of one or more of the following glycerophosphatylcholine/phosphatylethanolamine phosphatylinositol and phosphatylserine, such as 0.005wt%, including at least 0.01 wt%, more preferably at least 0.05 wt%, or at least 0.1 wt%, one or more of the following glycerophosphatylcholine/phosphatylethanolamine phosphatylinositol and phosphatylserine. Higher concentrations of one or more of the following glycerophosphatylcholine/phosphatylethanolamine phosphatylinositol and phosphatylserine, are also envisaged, such as at least 0.5 wt%, or at least 1 wt%, , including at least 2 wt%, or at least 5%,

It is preferable that the modified foodstuff and/or food material described in the above paragraph comprises glycerophosphatylcholine.

When the modified foodstuff and/or food material comprises glycerophosphatylcholine, the modified foodstuff and/or food material may comprise of less than 0.001wt% lysophospholipid, such as lysolecithin. This may comprise less than 0.0005wt% lysophospholipid, including the embodiment where the modified foodstuff and/or food material comprises no lysophospholipid.

In a preferred embodiment the modified foodstuff and/or food material comprises at least 0.001wt% monoglyceride such as 0.005wt%, including at least 0.01 wt% monoglyceride, more preferably at least 0.05 wt% monoglyceride, or at least 0.1 wt% monoglyceride. Higher concentrations of monoglyceride, are also envisaged, such as at least 0.5 wt% monoglyceride, or at least 1 wt% monoglyceride, including at least 2 wt% monoglyceride, or at least 5%, monoglyceride.



In a preferred embodiment the modified foodstuff and/or food material comprises at least 0.001 wt% sterol ester such as 0.005 wt%, including at least 0.01 wt% sterol ester, more preferably at least 0.05 wt% sterol ester, or at least 0.1 wt% sterol ester. Higher  
5 concentrations of sterol ester, are also envisaged, such as at least 0.5 wt% sterol ester.

In one embodiment, i.e. where the acyl acceptor is glycerol for instance, the functional ingredient of the present invention is generated by a reaction selected from alcoholysis, preferably glycerolysis.

10

A preferred temperature for the modification of the foodstuff and/or food material according to processes of the invention may depend on several factors including the temperature optima and stability of the enzyme used, the melting point and viscosity of the foodstuff and/or food material, the volume of the foodstuff and/or food material to  
15 be modified, the heat stability of the foodstuff and/or food material.

For example, in one embodiment the enzyme modification may occur between 10-70°C, such as 10 to 32°C, or 10 to 34°C including between 10-20°C, more preferably between 20-60°C, such as between 30-60°C, or 36-60°C, such as 37-60°C, including  
20 between 40-60°C.

For the enzyme modification of milk and/or cream for example it may be preferable to use a temperature of less than about 50°C, such as between about 10 to 34°C for example, or between about 36-49°C for example, or between about 40-49°C for  
25 example, or between about 40 to 45°C for example, or between about 45-49°C for example. Suitable temperatures of between 20-50°C may be used, such as between 30-40°C for example.

In some embodiments, an advantage of the use of a lipid acyltransferase herein  
30 disclosed may be that it has a high thermal stability and may therefore be used in the treatment of a foodstuff and/or food material at a temperature where the viscosity of



said foodstuff and/or food material is low. The high thermal stability may also allow lower dosages of enzyme to be used.

Suitably, for some embodiments the lipid acyltransferase may have a temperature optima of between about 50 to about 70°C for example. Suitably, for some  
5 embodiments a lipid acyltransferase may have a temperature stability, as measured using the PLU assay, wherein said acyltransferase retains at least about 25%, such as at least about 50% of its activity after 1 hour at 55°C.

10 The process for the treatment of the foodstuff and/or food material according to the invention may occur over any suitable time period. This may depend for example on the temperature used and enzyme dosage. By way of example only the time period may be between about 1 minute and about 4 hours, such as between about 5 minutes to about 2 hours, or between about 10 minutes to about 1 hour, or between about 5  
15 minutes to about 30 minutes or between about 1 minute to about 29 minutes or between about 31 minutes to about 60 minutes. Suitably the time period may be between about 5 minutes and 1 hour.

The enzyme dosage may be in any suitable dosage, for example the enzyme dosage,  
20 when added in terms of PLU activity, may be dosed between about 1-10,000 PLU/kg foodstuff and/or food material, such as between 5-5000 PLU/kg foodstuff and/or food material, such as between 100-1000 PLU/kg foodstuff and/or food material, or 1000 to 3000 PLU/kg foodstuff and/or food material. 50 to 1000 PLU/kg foodstuff and/or food material may be preferable in some embodiments for a lipid acyl transferase.

25

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

- (i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of  
30 a lipid acyl donor is transferred to an acyl acceptor to form a new ester; and



- (ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

- 5 Preferably, X of the GDSX motif is L or Y. More preferably, X of the GDSX motif is L. Thus, preferably the enzyme according to the present invention comprises the amino acid sequence motif GS<sub>1</sub>DL.

10 The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology Apr. 1996, Vol. 178, No. 7, p 2060-2064).

- 15 To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database.

20 Pfam is a database of protein domain families. Pfam contains curated multiple sequence alignments for each family as well as profile hidden Markov models (profile HMMs) for identifying these domains in new sequences. An introduction to Pfam can be found in Bateman A *et al.* (2002) Nucleic Acids Res. 30; 276-280. Hidden Markov models are used in a number of databases that aim at classifying proteins, for review see Bateman A and Haft DH (2002) Brief Bioinform 3; 236-245.

25

30

For a detailed explanation of hidden Markov models and how they are applied in the Pfam database see Durbin R, Eddy S, and Krogh A (1998) Biological sequence



analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4. The Hammer software package can be obtained from Washington University, St Louis, USA.

- 5 Alternatively, the GDSX motif can be identified using the Hammer software package, the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2 profile provided within this specification.

10

The PFAM database can be accessed, for example, through several servers which are currently located on publicly available websites.

20

The database offers a search facility where one can enter a protein sequence. Using the default parameters of the database the protein sequence will then be analysed for the presence of Pfam domains. The GDSX domain is an established domain in the database and as such its presence in any query sequence will be recognised. The database will return the alignment of the Pfam00657 consensus sequence to the query sequence.

25

A multiple alignment, including *Aeromonas salmonicida* or *Aeromonas hydrophila* can be obtained by:

- a) manual

30

obtain an alignment of the protein of interest with the Pfam00657 consensus sequence and obtain an alignment of P10480 with the Pfam00657 consensus sequence following the procedure described above;

or



b) through the database

After identification of the Pfam00657 consensus sequence the database offers the option to show an alignment of the query sequence to the seed alignment of the Pfam00657 consensus sequence. P10480 is part of this seed alignment and is indicated by GCAT\_AERHY. Both the query sequence and P10480 will be displayed in the same window.

The *Aeromonas hydrophila* reference sequence:

The residues of *Aeromonas hydrophila* GDSX lipase are numbered in the NCBI file P10480, the numbers in this text refer to the numbers given in that file which in the present invention is used to determine specific amino acids residues which, in a preferred embodiment are present in the lipid acyltransferase enzymes of the invention.

The Pfam alignment was performed (Figure 33 and 34):

The following conserved residues can be recognised and in a preferable embodiment may be present in the enzymes for use in the compositions and methods of the invention;

Block 1 - GDSX block

hid hid hid hid Gly Asp Ser hid  
28 29 30 31 32 33 34 35

Block 2 - GANDY block

hid Gly hid Asn Asp hid  
130 131 132 133 134 135

Block 3 - HPT block

His  
309



Where 'hid' means a hydrophobic residue selected from Met, Ile, Leu, Val, Ala, Gly, Cys, His, Lys, Trp, Tyr, Phe.

Preferably the lipid acyltransferase enzyme for use in the compositions/methods of the invention can be aligned using the Pfam00657 consensus sequence.

Preferably, a positive match with the hidden markov model profile (HMM profile) of the pfam00657 domain family indicates the presence of the GDSL or GDSX domain according to the present invention.

10

Preferably when aligned with the Pfam00657 consensus sequence the lipid acyltransferase for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, of the following, a GDSx block, a GANDY block, a HPT block. Suitably, the lipid acyltransferase may have a GDSx block and a GANDY block. Alternatively, the enzyme may have a GDSx block and a HPT block. Preferably the enzyme comprises at least a GDSx block.

15

Preferably, residues of the GANDY motif are selected from GANDY, GGND A, GGNDL, most preferably GANDY.

20

Preferably, when aligned with the Pfam00657 consensus sequence the enzyme for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, preferably more than three, preferably more than four, preferably more than five, preferably more than six, preferably more than seven, preferably more than eight, preferably more than nine, preferably more than ten, preferably more than eleven, preferably more than twelve, preferably more than thirteen, preferably more than fourteen, of the following amino acid residues when compared to the reference *A. hydrophilia* polypeptide sequence, namely SEQ ID No. 32: 28hid, 29hid, 30hid, 31hid, 32gly, 33Asp, 34Ser, 35hid, 130hid, 131Gly, 132Hid, 133Asn, 134Asp, 135hid, 309His

25

30



The pfam00657 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

5 The pfam00657 consensus sequence is presented in Figure 1 as SEQ ID No. 1. This is derived from the identification of the pfam family 00657, database version 6, which may also be referred to as pfam00657.6 herein.

The consensus sequence may be updated by using further releases of the pfam database.

10

For example, Figures 33 and 34 show the pfam alignment of family 00657, from database version 11, which may also be referred to as pfam00657.11 herein.

15 The presence of the GDSx, GANDY and HPT blocks are found in the pfam family 00657 from both releases of the database. Future releases of the pfam database can be used to identify the pfam family 00657.

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

- 20 (i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to an acyl acceptor to form a new ester;
- (ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.;
- 25 (iii) the enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2 or SEQ ID No. 32).

30 Preferably, the amino acid residue of the GDSX motif is L.



In SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. His-309 of the full length sequence, that is the protein including the signal sequence, equates to His-291 of the mature part of the protein, i.e. the sequence without the signal sequence.

5

Preferably, the lipid acyltransferase enzyme according to the present invention comprises the following catalytic triad: Ser-34, Asp-306 and His-309 or comprises a serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-306 and His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32). As stated above, in the sequence shown in SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-306 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-288 and His-291 of the mature part of the protein, i.e. the sequence without the signal sequence. In the pfam00657 consensus sequence, as given in Figure 1 (SEQ ID No. 1) the active site residues correspond to Ser-7, Asp-345 and His-348.

10

15

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

20

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a first lipid acyl donor is transferred to an acyl acceptor to form a new ester; and

25

(ii) the enzyme comprises at least Gly-32, Asp-33, Ser-34, Asp-306 and His-309 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-32, Asp-33, Ser-34, Asp-306 and His-309, respectively, in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32).

30

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following



genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.

5

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms:

*Aeromonas hydrophila*, *Aeromonas salmonicida*, *Streptomyces coelicolor*, *Streptomyces rimosus*, *Mycobacterium*, *Streptococcus pyogenes*, *Lactococcus lactis*,  
 10 *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Desulfitobacterium dehalogenans*, *Bacillus sp*, *Campylobacter jejuni*, *Vibrionaceae*, *Xylella fastidiosa*, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, *Aspergillus terreus*, *Schizosaccharomyces pombe*, *Listeria innocua*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Mesorhizobium loti*, *Ralstonia solanacearum*, *Xanthomonas*  
 15 *campestris*, *Xanthomonas axonopodis* and *Candida parapsilosis*.

In one aspect, preferably the lipid acyltransferase enzyme according to the present invention is obtainable, preferably obtained, from one or more of *Aeromonas hydrophila* or *Aeromonas salmonicida*.

20

Suitably, the lipid acyltransferase enzyme according to the present invention may be encoded by any one of the following nucleotide sequences:

- (a) the nucleotide sequence shown as SEQ ID No. 7 (see Figure 9 );
- (b) the nucleotide sequence shown as SEQ ID No. 8 (see Figure 10);
- 25 (c) the nucleotide sequence shown as SEQ ID No. 9 (see Figure 11 );
- (d) the nucleotide sequence shown as SEQ ID No. 10 (see Figure 12);
- (e) the nucleotide sequence shown as SEQ ID No. 11 (see Figure 13);
- (f) the nucleotide sequence shown as SEQ ID No. 13 (see Figure 15);
- (g) the nucleotide sequence shown as SEQ ID No. 21 (see Figure 17);
- 30 (h) the nucleotide sequence shown as SEQ ID No. 23 (see Figure 19);
- (i) the nucleotide sequence shown as SEQ ID No. 25 (see Figure 21);
- (j) the nucleotide sequence shown as SEQ ID No. 27 (see Figure 23);



- (k) the nucleotide sequence shown as SEQ ID No. 29 (see Figure 25);
- (l) the nucleotide sequence shown as SEQ ID No. 31 (see Figure 27);
- (m) the nucleotide sequence shown as SEQ ID No. 33 (see Figure 29);
- (n) the nucleotide sequence shown as SEQ ID No. 35 (see Figure 31);
- 5 (o) the nucleotide sequence shown as SEQ ID No. 46 (see Figure 95);
- (p) the nucleotide sequence shown as SEQ ID No. 75 (see Figure 87);
- (q) the nucleotide sequence shown as SEQ ID No. 77 (see Figure 89);
- (r) the nucleotide sequence shown as SEQ ID No. 78 (see Figure 90);
- (s) the nucleotide sequence shown as SEQ ID No. 81 (see Figure 93);
- 10 (t) the nucleotide sequence shown as SEQ ID No. 83 (see Figure 37);
- (u) the nucleotide sequence shown as SEQ ID No. 87 (see Figure 99);
- (v) the nucleotide sequence shown as SEQ ID No. 88 (see Figure 100);
- (w) or a nucleotide sequence which has 70% or more, preferably 75% or more,  
identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8,  
15 SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21,  
SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31,  
SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 46, SEQ ID No. 75, SEQ ID No. 77,  
SEQ ID No. 78, SEQ ID No. 81, SEQ ID No. 83, SEQ ID No. 87, or SEQ ID No.  
88.<sup>^^</sup>

20

Suitably the lipid acyltransferase encoded by the nucleotide sequence of any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 46, SEQ ID No. 75, SEQ ID No. 77, SEQ ID No. 78, SEQ ID No. 81, SEQ ID No. 83, SEQ ID No. 87, or SEQ ID No. 88 or by a nucleotide sequence which has 70% or more, preferably 75% or more, identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 46, SEQ ID No. 75, SEQ ID No. 77, SEQ ID No. 78, SEQ ID No. 81, SEQ ID No. 83,



SEQ ID No.87, or SEQ ID No. 88 may be post-transcriptionally and/or post-translationally modified.

Suitably the nucleotide sequence may have 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 46, SEQ ID No. 75, SEQ ID No. 77, SEQ ID No. 78, SEQ ID No. 81, SEQ ID No. 83, SEQ ID No.87, or SEQ ID No. 88.

In one embodiment, the nucleotide sequence encoding a lipid acyltransferase enzyme for use in the methods and uses of the present invention is a nucleotide sequence which has 70% or more, preferably 75% or more, identity with any one of the sequences shown as: SEQ ID No. 88, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 33, and SEQ ID No. 34. Suitably the nucleotide sequence may have 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as: SEQ ID No. 88, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 33, and SEQ ID No. 34.

20

In one embodiment, the nucleotide sequence encoding a lipid acyltransferase enzyme for use in the methods and uses of the present invention is a nucleotide sequence which has 70% or more, 75% or more, 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity the sequence shown as SEQ ID No. 88.

25

Suitably, the lipid acyltransferase enzyme according to the present invention may comprise one or more of the following amino acid sequences:

- (i) the amino acid sequence shown as SEQ ID No. 2 (see Figure 2)
- 30 (ii) the amino acid sequence shown as SEQ ID No. 3 (see Figure 3)
- (iii) the amino acid sequence shown as SEQ ID No. 4 (see Figure 4)
- (iv) the amino acid sequence shown as SEQ ID No. 5 (see Figure 5)



- (v) the amino acid sequence shown as SEQ ID No. 6 (see Figure 6)
- (vi) the amino acid sequence shown as SEQ ID No. 12 (see Figure 14)
- (vii) the amino acid sequence shown as SEQ ID No. 20 (Figure 16)
- (viii) the amino acid sequence shown as SEQ ID No. 22 (Figure 18)
- 5 (ix) the amino acid sequence shown as SEQ ID No. 24 (Figure 20)
- (x) the amino acid sequence shown as SEQ ID No. 26 (Figure 22)
- (xi) the amino acid sequence shown as SEQ ID No. 28 (Figure 24)
- (xii) the amino acid sequence shown as SEQ ID No. 30 (Figure 26)
- (xiii) the amino acid sequence shown as SEQ ID No. 32 (Figure 28)
- 10 (xiv) the amino acid sequence shown as SEQ ID No. 34 (Figure 30)
- (xv) the amino acid sequence shown as SEQ ID No. 62 (Figure 74)
- (xvi) the amino acid sequence shown as SEQ ID No. 90 (Figure 102) or  
an amino acid sequence which has 75% or more identity with any one of the sequences  
shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6,  
15 SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26,  
SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 62 or  
SEQ ID No. 90.

Suitably, the lipid acyltransferase enzyme according to the present invention may  
20 comprise either the amino acid sequence shown as SEQ ID No. 2 or as SEQ ID No. 3  
or SEQ ID No. 32 or SEQ ID No. 34 or SEQ ID No. 62 or SEQ ID No. 90 or may  
comprise an amino acid sequence which has 75% or more, preferably 80% or more,  
preferably 85% or more, preferably 90% or more, preferably 95% or more, identity  
with the amino acid sequence shown as SEQ ID No. 2 or the amino acid sequence  
25 shown as SEQ ID No. 3 or the amino acid sequence shown as SEQ ID No. 32 or the  
amino acid sequence shown as SEQ ID No. 34 or the amino acid sequence shown as  
SEQ ID No. 62 or the amino acid sequence shown as SEQ ID No. 90.

For the purposes of the present invention, the degree of identity is based on the  
30 number of sequence elements which are the same. The degree of identity in  
accordance with the present invention for amino acid sequences may be suitably  
determined by means of computer programs known in the art, such as Vector NTI 10



(Invitrogen Corp.). For pairwise alignment the score used is preferably BLOSUM62 with Gap opening penalty of 10.0 and Gap extension penalty of 0.1.

Suitably the lipid acyltransferase enzyme according to the present invention comprises  
5 an amino acid sequence which has 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, or SEQ ID No. 34.

10

Suitably, the lipid acyltransferase enzyme according to the present invention may comprise one or more of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34 or SEQ  
15 ID No. 62 before being post-translationally modified. The present invention also encompasses the use of a lipid acyltransferase enzyme which has been post-translationally modified, wherein the originally translated enzyme or pro-enzyme comprises one or more of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, or  
20 SEQ ID No. 62.

In one embodiment the lipid acyltransferase enzyme according to the present invention may be a fragment of one or more of the amino acid sequences SEQ ID No. 2, SEQ  
25 ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 62 or SEQ ID No. 90. In one embodiment preferably the amino acid sequence fragment has 70% or more, preferably 75% or more identity with any one of the sequences shown as SEQ ID No.  
30 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 62 or SEQ ID No. 90 when



determined over the whole of the sequence shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 62 or SEQ ID No. 90 respectively.

5

In one embodiment, suitably the lipid acyl transferase in accordance with the present invention comprises (or consists of) the amino acid sequence shown in SEQ ID No. 90 or comprises (or consists of) an amino acid sequence which has at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 98% identity to SEQ ID No. 90.

10

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

15

- (a) an amino acid sequence shown as amino acid residues 1-100 of SEQ ID No. 2 or SEQ ID No. 32;
- (b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 2 or SEQ ID No. 32;
- (c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 2 or SEQ ID No. 32; or
- (d) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(c) above.

20

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

25

- (a) an amino acid sequence shown as amino acid residues 28-39 of SEQ ID No. 2 or SEQ ID No. 32;
- (b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 2 or SEQ ID No. 32;
- (c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 2 or SEQ ID No. 32;

30



- (d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 2 or SEQ ID No. 32;
- (e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 2 or SEQ ID No. 32; or
- 5 (f) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(e) above.

In one aspect, the lipid acyl transferase for use in the method and uses of the present invention may be the lipid acyl transferase from *Candida parapsilosis* as taught in EP 1 275 711. Thus in one aspect the lipid acyl transferase for use in the method and uses of the present invention may be a lipid acyl transferase comprising one of the amino acid sequences taught in SEQ ID No. 63 or SEQ ID No. 64.

- 15 Much by preference, the lipid acyltransferase for use in the method and uses of the present invention may be a lipid acyl transferase (lipid acyltransferase) comprising the amino acid sequence shown as SEQ ID No. 62, or the amino acid sequence shown as SEQ ID No. 90 or an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more
- 20 preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No. 62 or SEQ ID No. 90. This enzyme may be considered a variant enzyme.

In one aspect, the lipid acyltransferase according to the present invention may be a lecithin:cholesterol acyltransferases (LCAT) or variant thereof (for example a variant

25 made by molecular evolution)

Suitable LCATs are known in the art and may be obtainable from one or more of the following organisms for example: mammals, rat, mice, chickens, *Drosophila melanogaster*, plants, including *Arabidopsis* and *Oryza sativa*, nematodes, fungi and

30 yeast.



In one embodiment the lipid acyltransferase enzyme according to the present invention may be the lipid acyltransferase obtainable, preferably obtained, from the *E. coli* strains TOP 10 harbouring pPet12aAhydro and pPet12aASalmo deposited by Danisco A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at the National Collection of Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 22 December 2003 under accession numbers NICMB 41204 and NCIMB 41205, respectively.

- 10 Highly preferred lipid acyltransferase (in particular a phospholipid glycerol acyl transferase) for use in the methods of the invention include those isolated from *Aeromonas spp.*, preferably *Aeromonas hydrophila* or *A. salmonicida*, most preferable *A. salmonicida*. Most preferred lipid acyl transferases for use in the present invention are encoded by one of SEQ ID No.s 2, 3, 32, 34, 62 or 90. It will be recognised by the skilled person that it is preferable that the signal peptides of the acyl transferase has been cleaved during expression of the transferase. The signal peptide of SEQ ID 2, 3, 15 32, 34, 62 and 90 are amino acids 1-18. Therefore the most preferred regions are amino acids 19-335 for SEQ ID No. 32 and SEQ ID No. 2 (*A. hydrophila*) and amino acids 19-336 for SEQ ID No. 3, SEQ ID No. 34, SEQ ID No. 62 and SEQ ID No. 90. 20 (*A. salmonicida*). When used to determine the homology of identity of the amino acid sequences, it is preferred that the alignments as herein described use the mature sequence. The mature sequence may be on which has the signal peptide removed and/or may be one which has been post-translationally modified.
- 25 Therefore the most preferred regions for determining homology (identity) are amino acids 19-335 for SEQ ID No.s 32 and 2 (*A. hydrophila*) and amino acids 19-336 for SEQ ID No.s 3, 34 and 62. (*A. salmonicida*). SEQ ID No.s 73 and 74 are "mature" (i.e. without signal peptide) protein sequences of the highly preferred lipid acyl transferases from *A. hydrophila* and *A. salmonicida* respectively. SEQ ID No.s 73 and 30 74 may or may not undergo further post-translational modification.



A lipid acyl transferase for use in the invention may also be isolated from *Thermobifida*, preferably *T. fusca*, most preferably that encoded by SEQ ID No. 67.

5 A lipid acyl transferase for use in the invention may also be isolated from *Streptomyces*, preferable *S. avermitis*, most preferably that encoded by SEQ ID No. 71. Other possible enzymes for use in the present invention from *Streptomyces* include those encoded by SEQ ID No.s 4, 5, 20, 22, 24, 26, 28, 30, 70, 72.

10 An enzyme for use in the invention may also be isolated from *Corynebacterium*, preferably *C. efficiens*, most preferably that encoded by SEQ ID No. 68.

Suitably, the lipid acyltransferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising any one of the amino acid sequences shown as SEQ ID No.s 76, 77, 79, 80, 82, 84, or 86 or an amino acid  
15 sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No.s 75, 78, 81, 83, 85, or 87 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

20 In one embodiment the lipid acyltransferase for use in the methods and uses according to the present invention is preferably a lipid acyltransferase encoded by a nucleic acid selected from the group consisting of:

- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 75;
- b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 75 by  
25 the degeneration of the genetic code; and
- c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 75.

30 In one embodiment, the lipid acyltransferase for use in the methods and uses according to the present invention is preferably a lipid acyltransferase comprising an amino acid sequence as shown in SEQ ID No. 76 or an amino acid sequence which has at least 60% identity thereto.



In a further embodiment the lipid acyltransferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising any one of the amino acid sequences shown as SEQ ID No. 76, 77, 79, 80, 82, 84 or 86 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 78, 81, 83, 85 or 87 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

10 In a further embodiment the lipid acyltransferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising any one of amino sequences shown as SEQ ID No. 77, 79, 80, 84 or 86 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

15 In a further embodiment the lipid acyltransferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising any one of amino sequences shown as SEQ ID No. 77, 79, or 86 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

20 More preferably in one embodiment the lipid acyltransferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 86 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In another embodiment the lipid acyl transferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 82 or 83 or an amino acid sequence which has at least 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.



In another embodiment the lipid acyl transferase for use in the methods and uses according to the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 80 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

5

In one embodiment the lipid acyl transferase for use in the methods and uses according to the present invention may be encoded by a nucleic acid selected from the group consisting of:

- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 75;
- 10 b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 75 by the degeneration of the genetic code; and
- c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 75.

- 15 In one embodiment the lipid acyltransferase according to the present invention may be a lipid acyltransferase obtainable, preferably obtained, from the *Streptomyces* strains L130 or L131 deposited by Danisco A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of
- 20 Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 25 June 2004 under accession numbers NCIMB 41226 and NCIMB 41227, respectively.

Suitable lipid acyltransferases for use in accordance with the present invention and/or

25 in the methods of the present invention may comprise any one of the following amino acid sequences and/or be encoded by the following nucleotide sequences:

- a polynucleotide encoding a lipid acyltransferase according to the present invention (SEQ ID No. 62);
- an amino acid sequence of a lipid acyltransferase according to the present invention
- 30 (SEQ ID No. 63);
- a polynucleotide encoding a lipid acyltransferase according to the present invention (SEQ ID No. 90).



A suitable lipid acyl-transferase enzyme for use in the methods of the invention may also be identified by alignment to the L131 (SEQ ID No. 76) sequence using Align X, the Clustal W pairwise alignment algorithm of VectorNTI using default settings.

5

An alignment of the L131 and homologues from *S. avermitilis* and *T. fusca* illustrates that the conservation of the GDSx motif (GDSY in L131 and *S. avermitilis* and *T. fusca*), the GANDY box, which is either GGND A or GGNDL, and the HPT block (considered to be the conserved catalytic histadine). These three conserved blocks are highlighted in Figure 103.

10

When aligned to either the pfam Pfam00657 consensus sequence and/ or the L131 sequence herein disclosed (SEQ ID No 76) it is possible to identify three conserved regions, the GDSx block, the GANDY block and the HTP block.

15

When aligned to either the pfam Pfam00657 consensus sequence and/ or the L131 sequence herein disclosed (SEQ ID No 76)

- i) The lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GDSx motif, more preferably a GDSx motif selected from GDSL or GDSY motif.

20

and/or

- ii) The lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GANDY block, more preferably a GANDY block comprising amino GGNDx, more preferably GGND A or GGNDL.

25

and/or

- iii) The enzyme of the invention, or for use in methods of the invention, has preferable an HTP block.

and preferably

- iv) The lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GDSx or GDSY motif, and a GANDY block comprising amino GGNDx, preferably GGND A or GGNDL, and a HTP block (conserved histadine).

30



**Variant lipid acyl transferase**

In a preferred embodiment the lipid acyl transferase is a variant lipid acyl transferase.

5 Suitable methods for the production of lipid acyl transferases for use in the invention are disclosed in WO2005/066347. Variants which have an increased activity on phospholipids, such as increased hydrolytic activity and/ or increased transferase, preferably increased transferase activity on phospholipids.

10 Preferably the variant lipid acyltransferase is prepared by one or more amino acid modifications of the lipid acyl transferases as herein defined.

Suitably, when the lipid acyltransferase for use in the methods or uses of the present invention, may be a variant lipid acyltransferase, in which case the enzyme may be  
15 characterised in that the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S, and wherein the variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 (as defined WO2005/066347 and  
20 hereinbelow).

For instance the variant lipid acyltransferase enzyme for use in the methods or uses of the present invention may be characterised in that the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid  
25 residues L, A, V, I, F, Y, H, Q, T, N, M or S, and wherein the variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues detailed in set 2 or set 4 or set 6 or set 7 (as defined in WO2005/066347 and hereinbelow) identified by said parent sequence being structurally aligned with the structural model of P10480 defined herein, which is  
30 preferably obtained by structural alignment of P10480 crystal structure coordinates with 1IVN.PDB and/or 1DEO.PDB as defined WO2005/066347 and hereinbelow.



In a further embodiment the variant lipid acyltransferase enzyme for use in the methods or uses of the present invention may be characterised in that the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S, and wherein the  
5 variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues taught in set 2 identified when said parent sequence is aligned to the pfam consensus sequence (SEQ ID No. 1 – Figure 2) and modified according to a structural model of P10480 to ensure best fit overlap as defined WO2005/066347 and hereinbelow.

10

Suitably the variant lipid acyltransferase enzyme may comprise an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 73, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 65, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No.  
15 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 89, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No. 68, SEQ ID No. 69, SEQ ID No. 71, or SEQ ID No. 72 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 (as defined WO2005/066347 and hereinbelow) identified by sequence alignment with SEQ ID No. 73.

20

Alternatively the variant lipid acyltransferase enzyme may be a variant enzyme comprising an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 73, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 65, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26,  
25 SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 89, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No. 68, SEQ ID No. 69, SEQ ID No. 71, or SEQ ID No. 72 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 as defined WO2005/066347 and hereinbelow, identified by said parent sequence being  
30 structurally aligned with the structural model of P10480 defined herein, which is preferably obtained by structural alignment of P10480 crystal structure coordinates



with 1IVN.PDB and/or 1DEO.PDB as taught within WO2005/066347 and hereinbelow.

Alternatively, the variant lipid acyltransferase enzyme may be a variant enzyme  
 5 comprising an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 73, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 89, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 89, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No. 68, SEQ ID No. 69,  
 10 SEQ ID No. 71, or SEQ ID No. 72 except for one or more amino acid modifications at any one or more of the amino acid residues taught in set 2 identified when said parent sequence is aligned to the pfam consensus sequence (SEQ ID No. 1) and modified according to a structural model of P10480 to ensure best fit overlap as taught within WO2005/066347 and hereinbelow.

15

Preferably, the parent enzyme is an enzyme which comprises, or is homologous to, the amino acid sequence shown as SEQ ID No. 73 and/or SEQ ID No. 34 and/or SEQ ID No. 74.

20 Preferably, the variant enzyme is an enzyme which comprises an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 73 or SEQ ID No. 74 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 as defined in WO2005/066347 and hereinbelow.

25

## DEFINITION OF SETS

Amino acid set 1:

30 Amino acid set 1 (note that these are amino acids in 1IVN – Figure 53 and Figure 54)  
Gly8, Asp9, Ser10, Leu11, Ser12, Tyr15, Gly44, Asp45, Thr46, Glu69, Leu70, Gly71, Gly72, Asn73, Asp74, Gly75, Leu76, Gln106, Ile107, Arg108, Leu109, Pro110,



Tyr113, Phe121, Phe139, Phe140, Met141, Tyr145, Met151, Asp154, His157, Gly155, Ile156, Pro158

The highly conserved motifs, such as GDSx and catalytic residues, were deselected from set 1 (residues underlined). For the avoidance of doubt, set 1 defines the amino acid residues within 10Å of the central carbon atom of a glycerol in the active site of the 1IVN model.

Amino acid set 2:

10

Amino acid set 2 (note that the numbering of the amino acids refers to the amino acids in the P10480 mature sequence)

Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn87, Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161, Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171, Ala172, Tyr179, His180, Asn181, Met209, Leu210, Arg211, Asn215, Lys284, Met285, Gln289 and Val290.

Table of selected residues in Set 1 compared with Set 2:

| IVN model |                 |           | P10480                            |
|-----------|-----------------|-----------|-----------------------------------|
| IVN       | A.hyd homologue |           | Mature sequence Residue<br>Number |
|           | PFAM            | Structure |                                   |
| Gly8      | Gly32           |           |                                   |
| Asp9      | Asp33           |           |                                   |
| Ser10     | Ser34           |           |                                   |
| Leu11     | Leu35           |           | Leu17                             |
| Ser12     | Ser36           |           | Ser18                             |
|           |                 |           | Lys22                             |
|           |                 |           | Met23                             |
| Tyr15     | Gly58           |           | Gly40                             |
| Gly44     | Asn98           |           | Asn80                             |



|        |        |            |        |
|--------|--------|------------|--------|
| Asp45  | Pro99  |            | Pro81  |
| Thr46  | Lys100 |            | Lys82  |
|        |        |            | Asn87  |
|        |        |            | Asn88  |
| Glu69  | Trp129 |            | Trp111 |
| Leu70  | Val130 |            | Val112 |
| Gly71  | Gly131 |            |        |
| Gly72  | Ala132 |            | Ala114 |
| Asn73  | Asn133 |            |        |
| Asp74  | Asp134 |            |        |
| Gly75  | Tyr135 |            | Tyr117 |
| Leu76  | Leu136 |            | Leu118 |
| Gln106 |        | Pro174     | Pro156 |
| Ile107 |        | Gly177     | Gly159 |
| Arg108 |        | Gln178     | Gln160 |
| Leu109 |        | Asn179     | Asn161 |
| Pro110 |        | 180 to 190 | Pro162 |
| Tyr113 |        |            | Ser163 |
|        |        |            | Ala164 |
|        |        |            | Arg165 |
|        |        |            | Ser166 |
|        |        |            | Gln167 |
|        |        |            | Lys168 |
|        |        |            | Val169 |
|        |        |            | Val170 |
|        |        |            | Glu171 |
|        |        |            | Ala172 |
| Phe121 | His198 | Tyr197     | Tyr179 |
|        |        | His198     | His180 |
|        |        | Asn199     | Asn181 |
| Phe139 | Met227 |            | Met209 |



|        |        |  |        |
|--------|--------|--|--------|
| Phe140 | Leu228 |  | Leu210 |
| Met141 | Arg229 |  | Arg211 |
| Tyr145 | Asn233 |  | Asn215 |
|        |        |  | Lys284 |
| Met151 | Met303 |  | Met285 |
| Asp154 | Asp306 |  |        |
| Gly155 | Gln307 |  | Gln289 |
| Ile156 | Val308 |  | Val290 |
| His157 | His309 |  |        |
| Pro158 | Pro310 |  |        |

#### Amino acid set 3:

- 5 Amino acid set 3 is identical to set 2 but refers to the *Aeromonas salmonicida* (SEQ ID No. 3) coding sequence, i.e. the amino acid residue numbers are 18 higher in set 3 as this reflects the difference between the amino acid numbering in the mature protein (SEQ ID No. 73) compared with the protein including a signal sequence (SEQ ID No. 36).

10

The mature proteins of *Aeromonas salmonicida* GDSX (SEQ ID No. 3) and *Aeromonas hydrophila* GDSX (SEQ ID No. 73) differ in five amino acids. These are Thr3Ser, Gln182Lys, Glu309Ala, Ser310Asn, Gly318-, where the *salmonicida* residue is listed first and the *hydrophila* residue is listed last. The *hydrophila* protein is only  
 15 317 amino acids long and lacks a residue in position 318. The *Aeromonas salmonicidae* GDSX has considerably high activity on polar lipids such as galactolipid substrates than the *Aeromonas hydrophila* protein. Site scanning was performed on all five amino acid positions.

20

#### Amino acid set 4:



Amino acid set 4 is S3, Q182, E309, S310, and -318.

Amino acid set 5:

- 5 F13S, D15N, S18G, S18V, Y30F, D116N, D116E, D157 N, Y226F, D228N Y230F.

Amino acid set 6:

- Amino acid set 6 is Ser3, Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn 87,  
 10 Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161,  
 Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171,  
 Ala172, Tyr179, His180, Asn181, Gln182, Met209, Leu210, Arg211, Asn215,  
 Lys284, Met285, Gln289, Val290, Glu309, Ser310, -318.

- 15 The numbering of the amino acids in set 6 refers to the amino acids residues in P10480  
 (SEQ ID No. 36) – corresponding amino acids in other sequence backbones can be  
 determined by homology alignment and/or structural alignment to P10480 and/or  
 1IVN.

- 20 Amino acid set 7:

- Amino acid set 7 is Ser3, Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn 87,  
 Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161,  
 Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171,  
 25 Ala172, Tyr179, His180, Asn181, Gln182, Met209, Leu210, Arg211, Asn215,  
 Lys284, Met285, Gln289, Val290, Glu309, Ser310, -318, Y30X (where X is selected  
 from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), Y226X (where X is  
 selected from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), Y230X (where  
 X is selected from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), S18X  
 30 (where X is selected from A, C, D, E, F, H, I, K, L, M, N, P, Q, R, T, W or Y), D157X  
 (where X is selected from A, C, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y).



The numbering of the amino acids in set 7 refers to the amino acids residues in P10480 (SEQ ID No. 36) – corresponding amino acids in other sequence backbones can be determined by homology alignment and/or structural alignment to P10480 and/or 1IVN).

5

Suitably, the variant enzyme comprises one or more of the following amino acid modifications compared with the parent enzyme:

S3E, A, G, K, M, Y, R, P, N, T or G

E309Q, R or A, preferably Q or R

10 -318Y, H, S or Y, preferably Y.

Preferably, X of the GDSX motif is L. Thus, preferably the parent enzyme comprises the amino acid motif GDSL.

15 Suitably, said first parent lipid acyltransferase may comprise any one of the following amino acid sequences: SEQ ID No. 73, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 65, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 89, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No.  
20 68, SEQ ID No. 69, SEQ ID No. 71 or SEQ ID No. 72.

Suitably, said second related lipid acyltransferase may comprise any one of the following amino acid sequences: SEQ ID No. 2, SEQ ID No. 73, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 65, SEQ ID No.  
25 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 89, SEQ ID No. 66, SEQ ID No. 67, SEQ ID No. 68, SEQ ID No. 69, SEQ ID No. 71 or SEQ ID No. 72.

The variant enzyme must comprise at least one amino acid modification compared  
30 with the parent enzyme. In some embodiments, the variant enzyme may comprise at least 2, preferably at least 3, preferably at least 4, preferably at least 5, preferably at



least 6, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10 amino acid modifications compared with the parent enzyme.

When referring to specific amino acid residues herein the numbering is that obtained from alignment of the variant sequence with the reference sequence shown as SEQ ID No. 73 or SEQ ID No. 74.

In one aspect preferably the variant enzyme comprises one or more of the following amino acid substitutions:

10

S3A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or  
 L17A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 S18A, C, D, E, F, H, I, K, L, M, N, P, Q, R, T, W, or Y; and/or  
 K22A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 15 M23A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or  
 Y30A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or  
 G40A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 N80A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 P81A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or  
 20 K82A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 N87A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 N88A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 W111A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; and/or  
 V112A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or  
 25 A114C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 Y117A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or  
 L118A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 P156A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or  
 D157A, C, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 30 G159A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 Q160A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or  
 N161A, C, D, E, F, G, H, I, K, L, M P, Q, R, S, T, V, W, or Y; and/or



P162A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or  
 S163A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or  
 A164C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 R165A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; and/or  
 5 S166A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or  
 Q167A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or  
 K168A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 V169A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or  
 V170A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or  
 10 E171A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 A172C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 Y179A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or  
 H180A, C, D, E, F, G, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 N181A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or  
 15 Q182A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y, preferably K; and/or  
 M209A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or  
 L210 A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 R211 A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 N215 A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 20 Y226A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or  
 Y230A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V or W; and/or  
 K284A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 M285A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or  
 Q289A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or  
 25 V290A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or  
 E309A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or  
 S310A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y.

In addition or alternatively thereto there may be one or more C-terminal extensions.

30 Preferably the additional C-terminal extension is comprised of one or more aliphatic  
 amino acids, preferably a non-polar amino acid, more preferably of I, L, V or G. Thus,



the present invention further provides for a variant enzyme comprising one or more of the following C-terminal extensions: 318I, 318L, 318V, 318G.

Preferred variant enzymes may have a decreased hydrolytic activity against a phospholipid, such as phosphatidylcholine (PC), may also have an increased transferase activity from a phospholipid.

Preferred variant enzymes may have an increased transferase activity from a phospholipid, such as phosphatidylcholine (PC), these may also have an increased hydrolytic activity against a phospholipid.

Modification of one or more of the following residues may result in a variant enzyme having an increased absolute transferase activity against phospholipid:

S3, D157, S310, E309, Y179, N215, K22, Q289, M23, H180, M209, L210, R211, P81, V112, N80, L82, N88; N87

Specific preferred modifications which may provide a variant enzyme having an improved transferase activity from a phospholipid may be selected from one or more of the following:

S3A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably N, E, K, R, A, P or M, most preferably S3A

D157A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; preferably D157S, R, E, N, G, T, V, Q, K or C

S310A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably S310T-318 E

E309A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably E309 R, E, L, R or A

Y179A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V or W; preferably Y179 D, T, E, R, N, V, K, Q or S, more preferably E, R, N, V, K or Q

N215A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N215 S, L, R or Y



K22A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W or Y; preferably K22 E, R, C or A

Q289A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W or Y; preferably Q289 R, E, G, P or N

5 M23A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; preferably M23 K, Q, L, G, T or S

H180A, C, D, E, F, G, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably H180 Q, R or K

M209 A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; preferably M209 Q, S, R, A, N, Y, E, V or L

10 L210A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W or Y; preferably L210 R, A, V, S, T, I, W or M

R211A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W or Y; preferably R211T

P81A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W or Y; preferably P81G

V112A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W or Y; preferably V112C

15 N80A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N80 R, G, N, D, P, T, E, V, A or G

L82A, C, D, E, F, G, H, I, M, N, P, Q, R, S, T, V, W or Y; preferably L82N, S or E

N88A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N88C

N87A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N87M or G

20

Preferred modification of one or more of the following residues results in a variant enzyme having an increased absolute transferase activity against phospholipid:

S3 N, R, A, G

25 M23 K, Q, L, G, T, S

H180 R

L82 G

Y179 E, R, N, V, K or Q

E309 R, S, L or A

30

One preferred modification is N80D. This is particularly the case when using the reference sequence SEQ ID No. 74. Therefore in a preferred embodiment of the



present invention the lipid acyltransferase according to the present invention comprises SEQ ID No. 74. or an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No.  
5 74.

As noted above, when referring to specific amino acid residues herein the numbering is that obtained from alignment of the variant sequence with the reference sequence shown as SEQ ID No. 73 or SEQ ID No. 74.

10

Much by preference, the lipid acyltransferase for use in the method and uses of the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 62 or the amino acid sequence shown as SEQ ID No. 90, or an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No. 62 and/or  
15 SEQ ID No. 90. This enzyme may be considered a variant enzyme.

20

For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US53711) (Needleman & Wunsch  
25 (1970), J. of Molecular Biology 48, 443-45) using the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. Suitably, the degree of identity with regard to an amino acid sequence is determined over at least 20 contiguous amino acids, preferably over at least 30 contiguous amino acids, preferably over at least 40 contiguous amino acids, preferably  
30 over at least 50 contiguous amino acids, preferably over at least 60 contiguous amino acids.



Suitably, the lipid acyltransferase/ lipid acyl transferaseenzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*,  
5 *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas*, *Candida*, *Thermobifida* and *Corynebacterium*.

Suitably, the lipid acyltransferase / lipid acyl transferaseenzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms: *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Streptomyces coelicolor*, *Streptomyces rimosus*, *Mycobacterium*, *Streptococcus pyogenes*, *Lactococcus lactis*, *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Streptomyces thermosacchari*, *Streptomyces avermitilis* *Lactobacillus helveticus*,  
10 *Desulfitobacterium dehalogenans*, *Bacillus sp*, *Campylobacter jejuni*, *Vibrionaceae*, *Xylella fastidiosa*, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, *Aspergillus terreus*, *Schizosaccharomyces pombe*, *Listeria innocua*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Mesorhizobium loti*, *Ralstonia solanacearum*, *Xanthomonas campestris*, *Xanthomonas axonopodis* , *Candida parapsilosis* *Thermobifida fusca* and  
15 *Corynebacterium efficiens*.

In one aspect, preferably the lipid acyl transferaseenzyme according to the present invention is obtainable, preferably obtained or derived from one or more of *Aeromonas spp.*, *Aeromonas hydrophila* or *Aeromonas salmonicida*.

25

Preferably, when carrying out a method according to the present invention the product is produced without increasing or substantially increasing the free fatty acids in the foodstuff.

30 The term “transferase” as used herein is interchangeable with the term “lipid acyltransferase”.



Suitably, the lipid acyltransferase as defined herein catalyses one or more of the following reactions: interesterification, transesterification, alcoholysis, hydrolysis.

5 The term “interesterification” refers to the enzymatic catalysed transfer of acyl groups between a lipid donor and lipid acceptor, wherein the lipid donor is not a free acyl group.

10 The term “transesterification” as used herein means the enzymatic catalysed transfer of an acyl group from a lipid donor (other than a free fatty acid) to an acyl acceptor (other than water).

15 As used herein, the term “alcoholysis” refers to the enzymatic cleavage of a covalent bond of an acid derivative by reaction with an alcohol ROH so that one of the products combines with the H of the alcohol and the other product combines with the OR group of the alcohol.

As used herein, the term “alcohol” refers to an alkyl compound containing a hydroxyl group.

20 As used herein, the term “hydrolysis” refers to the enzymatic catalysed transfer of an acyl group from a lipid to the OH group of a water molecule. Acyl transfer which results from hydrolysis requires the separation of the water molecule.

25 The term “without increasing or without substantially increasing the free fatty acids” as used herein means that preferably the lipid acyl transferase according to the present invention has 100% transferase activity (i.e. transfers 100% of the acyl groups from an acyl donor onto the acyl acceptor, with no hydrolytic activity); however, the enzyme may transfer less than 100% of the acyl groups present in the lipid acyl donor to the acyl acceptor. In which case, preferably the acyltransferase activity accounts for at  
30 least 5%, more preferably at least 10%, more preferably at least 20%, more preferably at least 30%, more preferably at least 40%, more preferably 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more



preferably at least 90% and more preferably at least 98% of the total enzyme activity. The % transferase activity (i.e. the transferase activity as a percentage of the total enzymatic activity) may be determined by the following protocol:

5

Protocol for the determination of % acyltransferase activity:

- 10 A foodstuff to which a lipid acyltransferase according to the present invention has been added may be extracted following the enzymatic reaction with  $\text{CHCl}_3:\text{CH}_3\text{OH}$  2:1 and the organic phase containing the lipid material is isolated and analysed by GLC and HPLC according to the procedure detailed hereinbelow. From the GLC and HPLC analyses the amount of free fatty acids and one or more of sterol/stanol esters;
- 15 carbohydrate esters, protein esters; diglycerides; or monoglycerides are determined. A control foodstuff to which no enzyme according to the present invention has been added, is analysed in the same way.

*Calculation:*

- From the results of the GLC and HPLC analyses the increase in free fatty acids and
- 20 sterol/stanol esters and/or carbohydrate esters and/or protein esters and/or diglycerides and/or monoglycerides can be calculated:

$\Delta \% \text{ fatty acid} = \% \text{ Fatty acid(enzyme)} - \% \text{ fatty acid(control)}$ ; Mv fatty acid = average molecular weight of the fatty acids;

- A =  $\Delta \% \text{ sterol ester/Mv sterol ester}$  (where  $\Delta \% \text{ sterol ester} = \% \text{ sterol/stanol ester(enzyme)} - \% \text{ sterol/stanol ester(control)}$  and Mv sterol ester = average molecular weight of the sterol/stanol esters) – applicable where the acyl acceptor is a sterol
- 25 and/or stanol;

- B =  $\Delta \% \text{ carbohydrate ester/Mv carbohydrate ester}$  (where  $\Delta \% \text{ carbohydrate ester} = \% \text{ carbohydrate ester(enzyme)} - \% \text{ carbohydrate ester(control)}$  and Mv carbohydrate ester
- 30 = average molecular weight of the carbohydrate ester) - applicable where the acyl acceptor is a carbohydrate;



$C = \Delta \% \text{ protein ester} / Mv \text{ protein ester}$  (where  $\Delta \% \text{ protein ester} = \% \text{ protein ester(enzyme)} - \% \text{ protein ester(control)}$  and  $Mv \text{ protein ester} = \text{average molecular weight of the protein ester}$ ) – applicable where the acyl acceptor is a protein; and

$D = \text{absolute value of diglyceride and/or monoglyceride} / Mv \text{ di/monoglyceride}$  (where  $\Delta\% \text{ diglyceride and/or monoglyceride} = \% \text{ diglyceride and/or monoglyceride (enzyme)} - \% \text{ diglyceride and/or monoglyceride (control)}$  and  $Mv \text{ di/monoglyceride} = \text{average molecular weight of the diglyceride and/or monoglyceride}$ ) – applicable where the acyl acceptor is glycerol.

10 The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = \frac{A^* + B^* + C^* + D^* \times 100}{A^* + B^* + C^* + D^* + \Delta \% \text{ fatty acid} / (Mv \text{ fatty acid})}$$

\* - delete as appropriate.

15

If the free fatty acids are increased in the foodstuff they are preferably not increased substantially, i.e. to a significant degree. By this we mean, that the increase in free fatty acid does not adversely affect the quality of the foodstuff.

20 In some aspects of the present invention, the term “without substantially increasing free fatty acids” as used herein means that the amount of free fatty acid in a foodstuff or composition treated with an lipid acyltransferase according to the present invention is less than the amount of free fatty acid produced in the foodstuff or composition when an enzyme other than a lipid acyltransferase according to the present invention  
25 had been used, such as for example as compared with the amount of free fatty acid produced when a conventional phospholipase enzyme, e.g. LipopanF® (Novozymes A/S, Denmark), had been used.

The term “*in situ*” as used herein means that the emulsifier(s) and/or the sterol/stanol esters and/or the carbohydrate esters and/or the protein esters and/or the mono- or  
30 diglycerides are produced within the foodstuff or fraction of the foodstuff. This contrasts the situation where the emulsifier(s) and/or the sterol/stanol esters and/or the



carbohydrate esters and/or the protein esters and/or the mono- or diglycerides are produced separately of the foodstuff and are added as formed products to the foodstuff during preparation of the same. In other words, the term "*in situ*" as used herein means that by the addition of the lipid acyltransferase enzyme according to the present invention to a foodstuff, or to the food ingredients/materials constituting the foodstuff, an emulsifier and/or a sterol ester and/or a stanol ester and/or a carbohydrate ester and/or a protein ester and/or a mono- or diglyceride may be produced from components of the foodstuff. Suitably, the components of the foodstuff may be the substrate(s) for the enzyme. If necessary, the components of the foodstuff may be supplemented by addition of one or more further components which further components may be the same as those present in the foodstuff or may be additional to those components already present in the foodstuff. For the avoidance of doubt, in one embodiment, the method according to the present invention may be a method for the production of an emulsifier and/or a sterol ester and/or a stanol ester and/or a carbohydrate ester and/or a protein ester and/or a mono- or diglyceride *in situ* in a foodstuff and is not a method for preparing an emulsifier and/or a sterol ester and/or a stanol ester (for example is an isolated and/or purified form) for subsequent addition to a foodstuff.

20 In another embodiment the lipase acyl-transferase may be used during the food processing, but not remain in the foodstuff. For example, the lipase acyl transferase may be immobilised, allowing it to be reused.

Preferably, the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a lipid to a sterol and/or stanol and/or a carbohydrate and/or a protein and/or glycerol when present in a polar environment, preferably in an aqueous environment, preferably a water containing foodstuff. Suitably, the aqueous environment may be an aqueous buffer or may be the aqueous phase in a foodstuff. The term "aqueous environment" as used herein preferably means an environment which is absent an organic solvent, preferably absent a polar organic solvent, more preferably absent a non-edible organic solvent. In particular, the term "aqueous environment" may refer to an environment to which no exogenous organic solvents,



preferably no polar organic solvents, have been added. The term organic solvent as used herein does not encompass food oils, preferably does not encompass food oils that are high in non-polar lipids. In one embodiment the term organic solvent may exclude edible organic solvents, such as ethanol, propylene glycol and/or glycerol.

5 Suitably, the aqueous environment according to the present invention may comprise less than 80% by volume organic solvents, less than 70% by volume organic solvents, less than 50% by volume organic solvents, less than 30% by volume organic solvents, more preferably less than 15% by volume organic solvents, more preferably less than 5%. Suitably the foodstuff may comprise between 1 and 5% organic solvent, for  
10 example ethanol. However, when the foodstuff comprises such an organic solvent, preferably it is produced endogenously within the foodstuff. That is to say, when the foodstuff comprises such an organic solvent, preferably the organic solvent is not an exogenous organic solvent.

15 The term "foodstuff" as used herein means a substance which is suitable for human and/or animal consumption.

Suitably, the term "foodstuff" as used herein may mean a foodstuff in a form which is ready for consumption. Alternatively or in addition, however, the term foodstuff as  
20 used herein may mean one or more food materials which are used in the preparation of a foodstuff. By way of example only, the term foodstuff encompasses both baked goods produced from dough as well as the dough used in the preparation of said baked goods. By way of further example, the term foodstuff encompasses both the final product, i.e. for example the final dairy product such as cheese, as well as the milk  
25 (e.g. cheese milk), the cream and/or the butterfat for example used in the preparation of the said dairy product (e.g. the cheese).

The term "food material" as used herein means one or more materials used in the preparation of a foodstuff. The term foodstuff may be used herein to mean food  
30 material and vice versa. In some embodiments for example the food material may be the final foodstuff. By way of example only the final foodstuff may be an edible oil (such as a cooking oil); in such instances the food material may also be the edible oil.



In some embodiments for example the food material may be one constituent of the final foodstuff. By way of example only the final foodstuff may be a dairy product, such as cheese for instance; in such instances the food material may be milk (e.g. cheese milk), cream and/or butterfat for example used in the preparation of said dairy product (e.g. the cheese).

When the food material forms only one constituent of the final foodstuff for instance in some embodiments the final foodstuff may be comprised of less than 10 wt % of the food material, such as less than 5wt%.

In some embodiments, suitably the final foodstuff may be comprised of from 0.01 to 4 wt % of the food material.

In some embodiments, suitably the final foodstuff may be comprised of from 0.01 to 2 wt % of the food material.

In some embodiments, suitably the final foodstuff may be comprised of from 0.01 to 1 wt % of the food material.

In some embodiments, suitably the final foodstuff may be comprised of from 0.01 to 0.5 wt % of the food material.

In some embodiments, suitably the final foodstuff may be comprised of from 0.01 to 0.3 wt % of the food material.

In a preferred aspect the present invention provides a foodstuff as defined above wherein the foodstuff is selected from one or more of the following: eggs, egg-based products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom; baked goods, including breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and cookies; confectionery, including chocolate, candies, caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum,



soft bubble gum, chewing gum and puddings; frozen products including sorbets, preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk drinks and yoghurts; mousses, whipped vegetable creams, meat products, including  
5 processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and spreads including low fat and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions and sauces.

10 Suitably the foodstuff in accordance with the present invention may be a "fine foods", including cakes, pastry, confectionery, chocolates, fudge and the like.

In one aspect the foodstuff in accordance with the present invention may be a dough product or a baked product, such as a bread, a fried product, a snack, cakes, pies,  
15 brownies, cookies, noodles, snack items such as crackers, graham crackers, pretzels, and potato chips, and pasta.

In a further aspect, the foodstuff in accordance with the present invention may be a plant derived food product such as flours, pre-mixes, oils, fats, cocoa butter, coffee  
20 whitener, salad dressings, margarine, spreads, peanut butter, shortenings, ice cream, cooking oils.

In another aspect, the foodstuff in accordance with the present invention may be a dairy product, including butter, milk, cream, cheese such as natural, processed, and  
25 imitation cheeses in a variety of forms (including shredded, block, slices or grated), cream cheese, ice cream, frozen desserts, yoghurt, yoghurt drinks, butter fat, anhydrous milk fat, other dairy products. The enzyme according to the present invention may improve fat stability in dairy products.

30 As used herein the term 'milk' may comprise milk from either animal or vegetable origin. It is possible to use milk from animal sources such as buffalo, (traditional) cow, sheep, goat etc. either individually or combined. Vegetable milks such as soya



milk may also be used. The vegetable milk may be used in combination with the animal milk, for example at a low percentage (of vegetable milk) say below 15%, or below 20%, or below 25% v/v. The term milk may also comprise cheese milk and cream. One advantage of the present invention is that it may assist the incorporation  
5 of soy milk into cheese production at a higher concentration when blended with milk from an animal source. Without wishing to be bound by theory, this may be due to the emulsification properties of soy milk treated in accordance with the present invention.

In one aspect the foodstuff in accordance with the present invention may be ice cream.  
10

In one aspect the foodstuff in accordance with the present invention may be or may comprise cheese or a cheese analogue.

In one embodiment the present invention relates to a method for the production of  
15 chesse using a lipid acyltransferase and/or the use of a lipid acyltransferase for the production of cheese. Preferably, the use leads to one or more of the technical effects in the cheese taught herein.

Suitably, in some embodiments the foodstuff may be a derivative of the foodstuff in  
20 accordance with the present invention. By way of example only the foodstuff may be a pizza comprising cheese produced in accordance with the present invention.

In the present appliaction, the term cheese "refers to any kind of cheese, such as natural cheese, cheese analogues and processed cheese for example. The cheese may  
25 be obtained by any suitable process known in the art, such as, e.g. by enzymatic coagulation of the cheese milk and/or cream with rennet, or by acidic coagulation of the cheese milk and/or cream with food grade acid or acid produced by lactic acid bacteria growth.

30 In one embodiment, the cheese manufactured by the process of the invention is rennet-curd cheese. Rennet is commercially available, e.g. as Naturene (animal rennet), Chymaxe (fermentation produced chymosin), Microlane (Microbial coagulant produced



by fermentation), all from Chr. Hansen A/S, Denmark). The cheese milk and/or cream may be subjected to a conventional cheese-making process.

5 A preferable coagulant is Marzyme®, a pure, microbial coagulant, provides the benefits of fermentation-produced chymosin (FPC) without affecting yield or taste.

10 Processed cheese is preferably manufactured from natural cheese or cheese analogues by cooking and emulsifying the cheese, such as, with emulsifying salts (e. g. phosphates and citrate). The process may further include the addition of spices/condiments.

15 The term "cheese analogues" refers to cheese-like products which contain fat (such as, e. g., milk fat (e.g. cream)) as a part of the composition, and, in which further contain, as part of the composition, a non-milk constituents, such as, e.g. vegetable oil. An example of a cheese analogue is cheese base. Cheese analogues may comprise soya milk or soya protein.

20 The cheeses produced by the process of the present invention comprise all varieties of cheese, such as, e. g. Campesino, Chester, Danbo, Drabant, Herregård, Manchego, Primativo, Provolone, Saint Paulin, Soft cheese, Svecia, Taleggio, White cheese, including rennet-curd cheese produced by rennet-coagulation of the cheese curd; ripened cheeses such as Cheddar, Colby, Edam, Muenster, Gryere, Emmenthal, Camembert, Parmesan and Romano; fresh cheeses such as Mozzarella and Feta; acid coagulated cheeses such as cream cheese, Neufchatel, Quarg, Cottage Cheese and  
25 Queso Blanco; and pasta filata cheese.

One embodiment relates to the production of pizza cheese by the process of the invention.

30 In cheese manufacturing, the coagulation of the casein in milk is preferably performed in two ways: the so-called rennet-curd and acid-curd cheese. In cheese production these two types of curds makes up two major groups of cheese types. Fresh acid-curd



cheeses refer to those varieties of cheese produced by the coagulation of milk, cream or whey via acidification or a combination of acid and heat, and which are ready for consumption once the manufacturing without ripening are completed. Fresh acid-curd cheeses generally differ from rennet-curd cheese varieties (e. g. Camembert, Cheddar, 5 Emmenthal) where coagulation normally is induced by the action of rennet at pH values 6.4-6. 6, in that coagulation normally occur close to the isoelectric point of casein, i.e. e.g. at pH 4.6 or at higher values when elevated temperatures are used, e. g. in Ricotta pH 6.0 and 80 C.

10 In one embodiment of the invention, the cheese belongs to the class of rennet curd cheeses.

Mozzarella is a member of the so-called pasta filata, or stretched curd, cheeses which are normally distinguished by a unique plasticising and kneading treatment of the fresh 15 curd in hot water, which imparts the finished cheese its characteristic fibrous structure and melting and stretching properties, cf. e. g. "Mozzarella and Pizza cheese" by Paul S. Kindstedt, Cheese: Chemistry, physics and microbiology, Volume 2: Major Cheese groups, second edition, page 337-341, Chapman & Hall. Pizza cheese as used herein includes cheeses suitable for pizzas and they are usually pasta filata/stretched curd 20 cheeses. In one embodiment, the process of the invention further comprises a heat/stretching treatment as for pasta filata cheeses, such as for the manufacturing of Mozzarella.

In one embodiment preferably the cheese according to the present invention is 25 Mozzarella.

In further embodiments of the invention, the cheese milk is prepared, totally or in part, from dried milk fractions, such as, e.g., whole milk powder, skim milk powder, casein, caseinate, total milk protein or buttermilk powder, or any combination thereof. 30

In one embodiment, preferably the foodstuff and/or the food material in accordance with the present invention is butterfat.



In one embodiment, particularly when the foodstuff and/or the food material treated with the lipid acyltransferase in accordance with the present invention is butterfat, the enzyme treated butterfat may be then used to produce a further dairy product  
5 (particularly cheese) and/or margarine or spreads (including low fat and very low fat spreads).

In one embodiment, the enzyme treated butterfat in accordance with the present invention may be added to milk (e.g. cheese milk) and/or cream which may  
10 subsequently be used to prepare a further dairy product, such as cheese for example.

In another embodiment, the foodstuff and/or the food material in accordance with the present invention may be milk and/or cream.

15 In one embodiment, particularly when the foodstuff and/or the food material treated with the lipid acyltransferase in accordance with the present invention is milk (preferably cheese milk) and/or cream, the enzyme treated milk and/or cream may be then used to produce a further dairy product (such as one or more of cheese, ice cream, frozen desserts, yoghurt, yoghurt drinks for instance, particularly cheese and/or ice  
20 cream).

In one embodiment the foodstuff consists of or comprises a cheese foodstuff which is heated to above the melting temperature of the cheese. The use of cheese prepared in accordance with the invention in foodstuffs which are heated can lead to a reduced  
25 oiling off effect from the cheese. There may also be beneficial texture and flavour benefits in using cheese or cheese products prepared according to the present invention.

The present invention further relates to use of the cheese produced by the process of  
30 the present invention in pizza, ready-to-eat dishes, such as lasagne or processed cheese, or as an ingredient in other food products. Accordingly, the cheese produced according to the process of the invention may be used in further processed food



products like processed cheese, pizza, burgers, toast, sauces, dressings, cheese powder, or cheese flavours.

In further embodiments, the process of the invention further comprises the step of  
5   subjecting the cheese, or foodstuff comprising the cheese, prepared in accordance with  
the present invention to a heating treatment, such as for example in the range of about  
150-350 °C, or in the range of about 155-345 °C, or in the range of about 160-340 °C  
or in the range of about 170-330 °C or in the range of about 180-320 °C or in the  
range of about 200-300 °C. Suitably the heating treatment may be for at least 2  
10   minutes such as at least 5 minutes, including at least 10 minutes.

In one aspect of the present invention the cheese produced in accordance with the  
present invention has a melting temperature which does not significantly differ from  
that of a control cheese (i.e. one which has not been produced using a lipid  
15   acyltransferase).

In another aspect of the present invention the cheese produced in accordance with the  
present invention has a texture and consistency which is similar to (if not better than)  
that of a control cheese (i.e. one which has not been produced using a lipid  
20   acyltransferase).

It is particularly advantageous to utilise the present invention in cheese as the  
production of free fatty acids in cheese is associated with a "soapy" taste. Thus, the  
use of a lipid acyltransferase in accordance with the present invention advantageously  
25   produces cheese without a "soapy" taste.

The reduced "soapy" taste and/or reduced off-flavours and off-taste associated with the  
use of a lipid acyltransferase in accordance with the present invention provides a  
significant advantage compared with the use of a standard lipase and/or phospholipase  
30   (such as Lecitase™ for example). The reduced off-flavours and off-taste may  
advantageously be the result of a reduction in the production of free fatty acids during  
the enzyme reactions. Fatty acids enzymatically removed by the lipid acyltransferase



from the acyl donor are transferred to an acyl acceptor molecule, and thus do not accumulate in the cheese.

In another aspect, the foodstuff in accordance with the present invention may be a food product containing animal derived ingredients, such as processed meat products, cooking oils, shortenings.

In a further aspect, the foodstuff in accordance with the present invention may be a beverage, a fruit, mixed fruit, a vegetable or wine. In some cases the beverage may contain up to 20 g/l of added phytosterols.

In another aspect, the foodstuff in accordance with the present invention may be an animal feed. The animal feed may be enriched with phytosterol and/or phytostanols, preferably with beta-sitosterol/stanol. Suitably, the animal feed may be a poultry feed. When the foodstuff is poultry feed, the present invention may be used to lower the cholesterol content of eggs produced by poultry fed on the foodstuff according to the present invention.

In one aspect preferably the foodstuff is selected from one or more of the following: eggs, egg-based products, including mayonnaise, salad dressings, sauces, ice cream, egg powder, modified egg yolk and products made therefrom.

Preferably the foodstuff according to the present invention is a water containing foodstuff. Suitably the foodstuff may be comprised of 10-98% water, suitably 14-98%, suitably of 18-98% water, suitably of 20-98%, suitably of 40-98%, suitably of 50-98%, suitably of 70-98%, suitably of 75-98%.

For some aspects, preferably the foodstuff in accordance with the present invention is not a pure plant derived oil, such as olive oil, sunflower oil, peanut oil, rapeseed oil for instance. For the avoidance of doubt, in some aspects of the present invention the foodstuff according to the present invention may comprise an oil, but preferably the foodstuff is not primarily composed of oil or mixtures of oil. For some aspects,



preferably the foodstuff comprises less than 95% lipids, preferably less than 90% lipids, preferably less than 85%, preferably less than 80% lipids. Thus, for some aspects of the present invention oil may be a component of the foodstuff, but preferably the foodstuff is not an oil *per se*.

5

The claims of the present invention are to be construed to include each of the foodstuffs listed above.

When it is the case that a carbohydrate ester is produced in accordance with the present invention, the carbohydrate ester is preferably an oligosaccharide ester, a monosaccharide ester or a disaccharide ester.

Suitably, the carbohydrate ester when produced in accordance with the present invention may be one or more of the following: glucose ester, fructose ester, anhydrofructose ester, maltose ester, lactose ester, galactose ester, xylose ester, xylooligosaccharide ester, arabinose ester, maltooligosaccharide ester, tagatose ester, sucrose ester, microthecin ester, ascopyrone P ester, ascopyrone T ester or cortalcerone ester.

Preferably, the carbohydrate ester when produced in accordance with the present invention is one or more of the following: a carbohydrate mono-ester, a sugar mono-ester, an oligosaccharide mono-ester, a trisaccharide mono-ester, a disaccharide mono-ester, a monosaccharide mono-ester, a glucose mono-ester, a fructose mono-ester, anhydrofructose mono-ester, maltose mono-ester, lactose mono-ester, galactose mono-ester, xylose mono-ester, xylooligosacchride mono-ester, arabinose mono-ester, maltooligosaccharide mono-ester, tagatose mono-ester, sucrose mono-ester, microthecin ester, ascopyrone P ester, ascopyrone T ester or cortalcerone ester.

In one embodiment, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as an antimicrobial agent. Alternatively or in addition thereto, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as one or both of an antioxidant and/or emulsifier.



Preferably, the formation of the carbohydrate ester (if any) in accordance with the present invention is independent of UDP-glucose.

- 5 Preferably, the foodstuff according to the present invention does not comprise UDP-glucose, or only comprises UDP-glucose in insignificant amounts.

Suitably, the emulsifier in accordance with the present invention may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-  
10 monoglyceride or a lysolecithin, such as lysophosphatidylcholine for example, a digalactosyl monoglyceride (DGMG). The emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysolecithin as used herein encompasses lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol, lysophosphatidylserine and  
15 lysophosphatidylglycerol

- Where one of the emulsifiers is a carbohydrate ester, the second emulsifier may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-monoglyceride, lysophosphatidylcholine, or digalactosyl monoglyceride (DGMG).  
20 The second emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.
- 25 Preferably the second emulsifier is DGMG. Suitably, the DGMG is produced *in situ* by the removal of an acyl group from DGDG with the transfer of the removed acyl group onto a carbohydrate to form a carbohydrate ester.

- Where one of the emulsifiers is a protein ester and/or a diglyceride and/or a  
30 monoglyceride, the second emulsifier may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-monoglyceride, lysophosphatidylcholine, or digalactosyl monoglyceride (DGMG). The second



emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.

5

In one embodiment the lipid acyl transferase of the invention can be used in a process for the preparation of a foodstuff such as a cooking (e.g. edible) oil, margarine or spread, butterfat (e.g. for subsequent use in cheese and/or margarine and/or spreads), whereby the foodstuff naturally contains, or has been supplemented with, glycerol  
10 and/or has been supplemented with at least one phospholipid (for example lecithin) and/or glycolipid (for example digalactosyl-diglyceride), and optionally a phytosterol or phytostanol.

In one embodiment the lipid acyl transferase of the invention can be used in a process  
15 for the preparation of a foodstuff such as margarine or spread, whereby the foodstuff naturally contains, or has been supplemented with, glycerol, at least one phospholipid (for example lecithin) and/or glycolipid (for example digalactosyl-diglyceride), and optionally a phytosterol or phytostanol.

20 In one embodiment, the present invention provides a process for the production of modified edible oil or fat (including butterfat) comprising i) lysophospholipid and/or one or more of the following, glycerophosphatylcholine, phosphatylethanolamine, phosphatylinositol and phosphatylserine, and ii) monoglyceride, said process comprising:

- 25 a) selecting at least one edible oil or fat, or combination thereof, wherein said edible oil or fat comprises at least a phospholipid,  
b) supplementing said edible oil or fat selected in step a) with exogenous glycerol and optionally b) exogenous phospholipid; wherein when the modified edible oil or fat selected in step a) essentially consists of a vegetable oil, exogenous  
30 phospholipid is added during step b),  
c) contacting the supplemented edible oil or fat of step b) with at least one lipid acyl transferase, and optionally a further enzyme,



to produce an edible oil/enzyme reaction mixture, and

d) incubating said edible oil/enzyme reaction mixture at a temperature at which said at least one lipid acyl transferase is active in order to produce a modified edible oil or fat comprising i) lysophospholipid and/or one or more of the following  
5 glycerophosphatylcholine, phosphatylethanolamine, phosphatylinositol and phosphatylserine, and ii) monoglyceride, and

e) optionally deactivating or removing said lipid acyl transferase and/or optional further enzyme.

10 When used as a cooking oil or margarine, the foodstuff may have enhanced anti-plattering properties. In addition or alternatively the foodstuff may have one or more beneficial technical properties, for example improved oxidative stability, improved emulsification properties, or health benefits.

15 In one embodiment the lipid acyl transferase of the invention can be in the preparation of low fat foodstuffs, such as low fat spreads, low fat salad dressings, low fat mayonnaise, low fat margarines etc. In such low fat food products, the fat content is typically reduced by the addition of emulsifiers and additional water compared to the higher fat equivalent.

20

The lipid acyl transferases used in the compositions and methods of the invention have been found to have unique properties when compared to lipolytic enzymes in that they have a marked preference for transfer of acyl groups from lipids to acceptors other than water, even in the presence of significant water. In a comparison with prior art  
25 enzymes, the lipid acyl transferase used in the invention were found to have a high relative transferase activity in the presence of 6% water, 54% water, 73% water, 89% water and approximately 95%. Lipolytic enzymes tested had virtually no significant relative transferase activity at these water concentrations.

30 The phospholipase activity of an enzyme may be evaluated using the following assays. In this way, a lipid acyltransferase having the enzyme characteristics defined herein may be obtained/identified.



**Determination of phospholipase activity (phospholipase activity TIPU-K assay):**

## Substrate

5

1,75% L- Phosphatidylcholine 95% Plant (Avanti #441601), 6,3% Triton-X 100<sup>\*</sup> (Peroxide free) and 5 mM CaCl<sub>2</sub> is dissolved in 0.05M HEPES buffer pH 7.

## Assay procedure:

10

21 µL substrate is added to a cuvette (Kone-Lab. Robot) and incubated 30°C for 5 minutes. At time t= 0 min, 4 µL enzyme solution is added. Also a blank with water instead of enzyme was analyzed. At time t=10 min 75 µl NEFA A (Substrate A of NEFA Kit from Wako Chemicals, Germany) is added, mixed and incubated at 30 °C.

15 At time t=15 min 150 µl NEFA B (Substrate B of NEFA Kit from Wako Chemicals, Germany) is added and incubated at 30 °C. At time t=20 min the Absorbance (OD 520 nm) is measured.

A calibration curve based on oleic acid is produced and used for the calculation of free fatty acid in the samples.

20

Enzyme activity TIPU-K is calculated as micromole fatty acid produced per minute under assay conditions.

**Determination of phospholipase activity (phospholipase activity PLU-7 assay):**

25

## Substrate

0.6% L-α Phosphatidylcholine 95% Plant (Avanti #441601), 0.4% Triton-X 100<sup>\*</sup> (Sigma X-100) and 5 mM CaCl<sub>2</sub> is dispersed in 0.05M HEPES buffer pH 7.

30

## Assay procedure:

\* Trade-mark



400  $\mu$ L substrate is added to a 1.5 mL Eppendorf tube and placed in an Eppendorf Thermomixer at 37°C for 5 minutes. At time  $t=0$  min, 50  $\mu$ L enzyme solution is added. Also a blank with water instead of enzyme is analyzed. The sample is mixed at 10x100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time  $t=10$  min  
5 the Eppendorf tube is placed in another thermomixer at 99°C for 10 minutes to stop the reaction.

Free fatty acid in the samples is analyzed by using the NEFA C kit from WAKO GmbH.

10

Enzyme activity PLU-7 at pH 7 is calculated as micromole fatty acid produced per minute under assay conditions.

The lipase and acyltransferase activity of an enzyme may be evaluated using the  
15 following assays. In this way, a lipid acyltransferase having the enzyme characteristics defined herein may be obtained/identified.

#### **Transferase Assay in Buffered Substrate (see Example 12)**

20 Enzymes which function as lipid acyltransferases for use in the compositions and methods of the invention can be routinely identified using the assay taught herein in Example 12. This assay will be hereinafter referred to as the 'Transferase Assay in Buffered Substrate'. In Example 12 the lipid acyltransferase enzyme from *Aeromonas salmonicida* in accordance with the present invention was analysed and compared with  
25 a range of lipolytic enzymes not encompassed by the present invention. As can be seen, of the lipolytic enzymes only LIPOPAN<sup>®</sup> F (Novozymes, Denmark) was found to have any transferase activity and then only a very low level (1.3%).

Enzymes suitable for use in the compositions and methods of the invention can be  
30 routinely identified using the Transferase Assay in Buffered Substrate. Using this assay, in which there is a very high water content – approximately 95%, lipid acyltransferases in accordance with the present invention are those which have at least



2% acyltransferase activity (relative transferase activity), preferably at least 5% relative transferase activity, preferably at least 10% relative transferase activity, preferably at least 15%, 20%, 25%, 26%, 28%, 30%, 40%, 50%, 60% or 75% relative transferase activity. Suitably, the lipid acyltransferase in accordance with the present invention may have less than 28%, less than 30%, preferably less than 40%, 50%, 60%, 70%, 80%, 90% or 100% acyltransferase activity.

#### **Transferase Assay in high water egg yolk (see Example 11)**

- 10 As an alternative to (or in addition to) using the "Transferase Assay in Buffered Substrate" (see above), a lipid acyltransferase for use in accordance with the present invention may be identified using the "Transferase Assay in High Water Egg Yolk" taught in Example 11.
- 15 In one embodiment, the lipid acyltransferase suitable for use in the methods and/or compositions according to the present invention is one which when tested using the Transferase Assay in High Water Egg Yolk in an egg yolk with 54% water, has up to 100% relative transferase activity. Indeed, experiments in high water egg yolk have shown that at the start of the experiment the initial transferase rate was calculated to be
- 20 100% transferase activity, *i.e.* no hydrolytic activity was observed. In contrast, the lipolytic enzymes used as control, *i.e.* LIPOPAN<sup>®</sup> F and phospholipase A2, showed no detectable transferase activity in egg yolk with 54% water, or egg yolk with enriched water content (namely egg yolk with 73% water or 89% water). Preferably the increase in water content does not significantly decrease the percentage acyl
- 25 transferase activity of a lipid acyltransferase for use in the methods or compositions according to the present invention.

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 54%, a lipid acyltransferase for use in the present invention will have an initial percentage acyltransferase activity (initial relative transferase activity) measured after 10% consumption of the donor molecule (*i.e.* phospholipid) of at least 0.1% relative transferase activity, preferably at least 1%



relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 50% relative transferase activity, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, preferably at least 95%, preferably at least 99%, preferably about 100% acyl transferase activity.

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 54%, and measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the compositions and methods of the invention has detectable transferase activity, *i.e.* relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 45%, 50%, 60%, 70%, 80%, or 90% relative transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg Yolk with 54% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase activity) of less than 45%, 47%, 50%, 60%, 70%, 80%, 90% or 100%.

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 73%, measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the compositions and methods of the invention has detectable transferase activity, *i.e.* relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 45%, 50%, 58%, 60%, 70%, 80%, or 90% relative



transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg Yolk with 73% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase activity) of  
5 less than 45%, 47%, 50%, 58%, 60%, 70%, 80%, 90% or 100%.

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 89%, and measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the  
10 compositions and methods of the invention has detectable transferase activity, *i.e.* relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40%  
15 relative transferase activity, preferably at least 45%, 50%, 60%, 70%, 80%, or 90% relative transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg Yolk with 89% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase  
20 activity) of less than 45%, 47%, 50%, 60%, 70%, 80%, 90% or 100%.

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water  
25 contents), and has an equivalent relative transferase activity in egg yolk with a water content of 54% as in an egg yolk with a water content of 73%, when measured after 10% consumption of the donor molecule (i.e. phospholipid).

In a preferable embodiment, with reference to the Transferase Assay in High Water  
30 Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water contents), and has an equivalent relative transferase activity in egg yolk with a water



content of 54% as in an egg yolk with a water content of 89%, when measured after 10% consumption of the donor molecule (i.e. phospholipid).

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water contents), and has an equivalent relative transferase activity in egg yolk with a water content of 73% as in an egg yolk with a water content of 89%, when measured after 10% consumption of the donor molecule (i.e. phospholipid).

The term “equivalent relative transferase activity” as referred to herein means that the enzyme has a relative transferase activity (% acyltransferase activity) which is at least 2% lower, preferably at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% lower, in the egg yolk with the higher water content compared with that in the egg yolk with the lower water content.

### **Transferase Assay in a Low Water Environment**

As an alternative to (or in addition to) using the “Transferase Assay in High Water Egg Yolk” and/or the “Transferase Assay in Buffered Substrate”, lipid acyltransferases for use in accordance with the present invention may be identified using the “Transferase Assay in a Low Water Environment”.

In order to determine if an enzyme is a lipid acyltransferase according to the present invention, one may carry out a “Transferase Assay in a Low Water Environment”, namely in an oily environment with 6% water as taught in Example 22. This example illustrates that in an oily environment with 6% water content the lipid acyltransferase of the invention has a high relative transferase activity, where the prior art lipolytic enzymes have hydrolytic activity.

In one embodiment, the lipid acyltransferase suitable for use in the methods and/or compositions according to the present invention is one which when tested using the



“Transferase Assay in a Low Water Environment”, measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%, preferably at least 2%, preferably at least 5%, preferably at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%,  
5 preferably at least 60%, preferably at least 70%, preferably at least 75%. Suitably, the lipid acyl transferase in accordance with the present invention may have less than 30%, 40%, 50%, 60%, 70%, or 80% activity when measured after a time period of 10, 20, 30 or 120 minutes using the “Transferase Assay in a Low Water Environment”.

- 10 As described above, the lipase acyltransferase of the invention can be identified using either the “Transferase Assay in Buffered Substrate” or in the “Transferase Assay in Low Water Environment” using cholesterol as the acyl acceptor. Of course, the skilled person would be readily aware that, with obvious amendments to the analytical methods the ‘Transferase Assay in Buffered Substrate’ or the ‘Transferase Assay in  
15 Low Water Environment’ may be used to determine the lipid acyltransferase activity for any lipid acyl donor or any acyl acceptor combination. The skilled person would, if necessary, simply replace the acyl donor substrate (e.g. phospholipid) with an alternative acyl donor substrate (e.g. glycolipid, triacylglyceride) and/or replace the acyl acceptor (e.g. cholesterol) with an alternative acyl acceptor substrate (e.g. a  
20 carbohydrate, a protein, another sterol, a stanol or glycerol).

The term “high water” as used herein means any substrate or foodstuff with more than 2% water content, preferably more than 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%.

25

The term “low water” as used herein means any substrate or foodstuff with less than 6% water content, preferably less than 5%, 4%, 3%, 2%, 1% or 0.5%.

#### LUS ASSAY

30

The ability to hydrolyse triglyceride (E.C. 3.1.1.3 activity) may be determined by lipase activity is determined according to Food Chemical Codex (3rd Ed., 1981, pp 492-493)



modified to sunflower oil and pH 5.5 instead of olive oil and pH 6.5. The lipase activity is measured as LUS (lipase units sunflower) where 1 LUS is defined as the quantity of enzyme which can release 1 [mu]mol of fatty acids per minute from sunflower oil under the above assay conditions.

5

#### LUT ASSAY

Alternatively the LUT assay as defined in WO9845453 may be used.

10

The lipid acyl transferase lipid acyl transferase according to the present invention or for use in the method and/or uses of the present invention which is substantially incapable of acting on a triglyceride may have a LUS/mg of less than 1000, for example less than 500, such as less than 300, preferably less than 200, more preferably less than 100, more preferably less than 50, more preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 LUS/mg. Alternatively LUT/mg activity is less than 500, such as less than 300, preferably less than 200, more preferably less than 100, more preferably less than 50, more preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 LUT/mg.

20

The lipid acyl transferase lipid acyl transferase according to the present invention or for use in the method and/or uses of the present invention which is substantially incapable of acting on a monoglyceride may be determined by using mono-oleate(M7765 1-Oleoyl-*rac*-glycerol 99%) in place of the sunflower oil in the LUS assay. 1 MGHU is defined as the quantity of enzyme which can release 1 [mu]mol of fatty acids per minute from monoglyceride under the assay conditions.

25

The lipid acyl transferase lipid acyl transferase according to the present invention or for use in the method and/or uses of the present invention which is substantially incapable of acting on a triglyceride may have a MGHU/mg of less than 5000, for example less than 1000, for example less than 500, such as less than 300, preferably

30



less than 200, more preferably less than 100, more preferably less than 50, more preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 MGHU/mg.

5 Preferably the method and/or use according to the present invention may be carried out, for example, in foodstuff at a temperature of 15-60°C, preferably at a temperature of 20-60°C, preferably 20-50°C, preferably 20-45°C, preferably 20-40°C. For some aspects, for example in dough, preferably the temperature of the food during which the acyltransferase reaction takes place is between 20 and 40°C. For other aspects, for  
10 example with regard to dairy products, such as cheese, the temperature of the food may suitably be between 30°C and 60°C. In yet other aspects, for example with regard to mayonnaise, the temperature of the food may suitably be between 20 and 40°C, more preferably between 25 and 30°C.

15 Preferably, the emulsifier produced according to the present invention comprises less than 5 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 4 wt % of the foodstuff.

20

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 2 wt % of the foodstuff.

25 Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 1 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 0.5 wt % of the foodstuff.

30 Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 0.3 wt % of the foodstuff.



Suitably, the method according to the present invention includes inactivating or denaturing the enzyme to provide a foodstuff comprising the enzyme in an inactive or denatured form. Suitably the enzyme may be denatured by either baking or by  
5 pasteurisation.

The present invention may further encompass the use of a lipid acyltransferase as defined herein in food and/or feed enzyme compositions, and may encompass food and/or feed enzyme compositions comprising a lipid acyltransferase as defined herein.  
10 Such compositions may contain one or more further enzymes, such as those listed herein. Alternatively, the enzyme composition of the invention may be used in combination with other food ingredients/additives, such as those listed herein, including other enzyme compositions. By formulation of the lipid acyl transferase of the invention within a food and/or feed composition, the enzyme can be stabilised to  
15 allow for prolonged storage (under suitable conditions) prior to use in food and/or feed production. In addition the enzyme composition of the present invention provides the enzyme in a suitable form for safe use for the 'in situ' application in the preparation of foodstuffs and/or feedstuffs, or ingredients for use in food and/or feed preparation. Such compositions may be in either liquid, semi-liquid or solid/granular form.

20

In one embodiment the food enzyme composition may suitably be a dough improving composition. The dough improving composition may comprise other beneficial components such as an emulsifier and/or other enzymes as listed herein.

25 Food enzymes are sold as stabilised liquid concentrates or as particulate solids. Formulation into food enzyme composition minimises losses in enzymatic activity during transport, storage, and use. Enzymes are often exposed to humid, hot, or oxidative environments in food and beverage processing. Formulations enhance stability by counteracting the primary forces of deactivation: denaturation, catalytic-  
30 site deactivation, and proteolysis. Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure under thermal or chemical stress. Once an enzyme begins to unfold it becomes dramatically more vulnerable to deactivation and



proteolysis. To minimise unfolding, the formulator can alter the protein's environment so as to induce a compact protein structure; this is done most effectively by “preferential exclusion” of water from the protein surface by adding water-associating compounds such as sugars, polyhydric alcohols, and lyotropic salts. The best ways to  
5 combat active site inactivation are to ensure sufficient levels of any required cofactors, to add reversible inhibitors, and to exclude oxidising or reactive species from the formulation.

Besides enzymatic stability, a formulation should meet several key secondary  
10 requirements, including preservation against microbial contamination, avoidance of physical precipitation or haze formation, minimising the formation of sensitising dusts or aerosols, and the optimisation of aesthetic criteria such as colour and odour. Many of these problems are best addressed by focusing as far “upstream” as possible, including the choice of raw materials in the fermentation or enzyme recovery process.  
15 Downstream operations such as diafiltration, adsorption, chromatography, crystallization, and extraction can be used to remove impurities responsible for colour, odour, and precipitation. The risk of physical precipitation is minimised by formulating near the isoelectric point of the enzyme with hydrophilic solvents such as glycerol or propylene glycol. One can effectively also add moderate levels of solvating  
20 salts to avoid either salting-out or “reverse salting-in”. To prevent microbial contamination, one can use a combination of filtration, acidification, and the minimisation of free water; biocides can be effective, but the range of acceptable chemicals for controlling or killing microbes is increasingly circumscribed by health and safety regulations.

25

Two processes producing the most attrition-resistant granules to date are high-shear granulation and fluidised-bed spray coating, see for example T. Becker: “Separation and Purification Processes for Recovery of Industrial Enzymes” in R. K. Singh, S. S. H. Rizvi (eds.): *Bioseparation Processes in Foods*, Marcel Dekker, New York, pp.  
30 427 – 445. These processes use various binders, coatings, and particle morphologies to produce nonfriable particles which still protect enzymes during storage but allow for their ready release in solution during use.



Food enzyme compositions containing the lipid acyl transferase of the invention may be made using standard formulation techniques, such as spray drying or liquid formulation.

5

The lipid acyl-transferase of the invention can be expressed in any suitable expression host. For example the lipid acyltransferase of the invention may be expressed in *Bacillus subtilis* and may be purified by ultrafiltration and/or by precipitation in ethanol and/or centrifugation, and may be subsequently spray dried using starch  
10 (maltodextrin) as carrier for the enzyme. The spray dried enzyme may be standardised to specified PLU activity by adding further carrier in powder form. The techniques involved are well established and routine in the art.

Alternatively, lipid acyltransferase for use in accordance with the present invention,  
15 for example the heterologously produced lipid acyl-transferase of the invention, once purified, may be stabilised in a suitable liquid formulation, such as those based on glycerol. Other methods of making stabilised enzyme formulations are described in EP 0 770 037 and EP 0 702 712.

20 The acyl transferase in powder form can also be used in combination with other enzymes as listed herein, for the production of enzyme compositions with defined activity according to the product specification.

Typically the dosage of the food enzyme formulation is between 10g and 1000g per  
25 1000kg of foodstuff, preferably 50-200g per 1000kg of foodstuff, preferably, 75-125gm per 1000kg of foodstuff.

Preferably the enzyme according to the present invention is present in an inactive form or in a denatured form in the foodstuff.

30

In one embodiment, the enzyme according to the present invention is preferably not immobilised, in particular is not immobilised on a solid support.



In an alternative embodiment, the enzyme may be immobilised.

Immobilised lipid acyl transferase can be prepared using immobilisation techniques  
5 known in the art. There are numerous methods of preparing immobilised enzymes,  
which will be apparent to a person skilled in the art (for example the techniques  
referred to in EP 0 746 608; or Balcao VM, Paiva AL, Malcata FX., Enzyme Microb  
Technol. 1996 May 1;18(6):392-416; or Reetz MT, Jaeger KE. Chem Phys Lipids.  
1998 Jun;93(1-2):3-14; or Bornscheuer UT, Bessler C, Srinivas R, Krishna SH. Trends  
Biotechnol. 2002 Oct; 20(10):433-7.

In one embodiment, the foodstuff of the invention may contain food ingredients, which  
have been prepared using immobilised lipid acyltransferase, but do not contain the  
15 lipid acyltransferase in the food ingredient or foodstuff. For example the foodstuff  
may contain one or more of the following: an emulsifier, more than one emulsifier,  
one or more flavouring agents, one or more textural enhancers and/or one or more  
sterol esters, such as phytosterol esters or phytostanol esters.

20 The enzyme according to the present invention may be used with one or more  
conventional emulsifiers, including for example monoglycerides, diacetyl tartaric acid  
esters of mono- and diglycerides of fatty acids, and lecithins e.g. obtained from soya.

The enzyme according to the present invention may be used with one or more other  
25 suitable food grade enzymes. Thus, it is within the scope of the present invention that,  
in addition to the enzyme of the invention, at least one further enzyme is added to the  
foodstuff. Such further enzymes include starch degrading enzymes such as endo- or  
exoamylases, pullulanases, debranching enzymes, hemicellulases including xylanases,  
cellulases, oxidoreductases, e.g. peroxidases, phenol oxidases, glucose oxidase,  
30 pyranose oxidase, sulfhydryl oxidase, or a carbohydrate oxidase such as one which  
oxidises maltose, for example hexose oxidase (HOX), lipases, phospholipases,  
glycolipases, galactolipases and proteases.



In one embodiment the enzyme may be Dairy HOX™, which acts as an oxygen scavenger to prolong shelf life of cheese while providing browning control in pizza ovens. Therefore in one aspect the present invention relates to the use of an enzyme  
5 capable of reducing the maillard reaction in a foodstuff (see WO02/39828 ),

such as a dairy product, for example cheese, wherein the enzyme is preferably a maltose oxidising enzyme such as carbohydrate oxidase, glucose oxidase and/or hexose oxidase, in the process of preparing a food material and/or foodstuff according to the present invention.

10

In one preferred embodiment the lipid acyltransferase is used in combination with a lipase having one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26, triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). Suitably, lipase enzymes are  
15 well known within the art and include by way of example the following lipases: LIOPAN® F and/or LECITASE® ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD™ 22L from Biocatalysts, LIPOMAX™ from Genecor), LIPOLASE® (Novozymes A/S, Denmark), the lipases taught in WO03/97835, EP 0 977 869 or EP 1 193 314. This combination of a lipid  
20 acyl transferase as defined herein and a lipase may be particularly preferred in dough or baked products or in fine food products such as cakes and confectionary.

In some embodiments, it may also be beneficial to combine the use of lipid acyltransferase with a lipase such as rennet paste prepared from calf, lamb, kid  
25 stomachs, or Palatase A750L (Novo), Palatase M200L (Novo), Palatase M1000 (Novo), or Piccantase A (DSM), also Piccantase from animal sources from DSM (K, KL, L & C) or Lipomod 187, Lipomod 338 (Biocatalysts). These lipases are used conventionally in the production of cheese to produce cheese flavours. These lipases may also be used to produce an enzymatically-modified foodstuff, for example dairy  
30 product (e.g. cheese), particularly where said dairy product consists of, is produced from or comprises butterfat. A combination of the lipid acyltransferase with one or



more of these lipases may have a beneficial effect on flavour in the dairy product (e.g. cheese for instance).

The use of lipases in combination with the enzyme of the invention may be particularly advantageous in instances where some accumulation of free fatty acids maybe desirable, for example in cheese where the free fatty acids can impart a desirable flavour, or in the preparation of fine foods. The person skilled in the art will be able to combine proportions of lipolytic enzymes, for example LIPOPAN<sup>®</sup> F and/or LECITASE<sup>®</sup> ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD<sup>™</sup> 22L from Biocatalysts, LIPOMAX<sup>™</sup> from Genecor), LIPOLASE<sup>®</sup> (Novozymes A/S, Denmark), the lipases taught in WO03/97835, EP 0 977 869 or EP 1 193, 314 and the lipid acyltransferase of the present invention to provide the desired ratio of hydrolytic to transferase activity which results in a preferred technical effect or combination of technical effects in the foodstuff (such as those listed herein under 'Technical Effects').

It may also be beneficial to combine the use of lipid acyltransferase with a phospholipase, such as phospholipase A1, phospholipase A2, phospholipase B, Phospholipase C and/or phospholipase D.

The combined use may be performed sequentially or concurrently, e.g. the lipid acyl transferase treatment may occur prior to or during the further enzyme treatment. Alternatively, the further enzyme treatment may occur prior to or during the lipid acyl transferase treatment.

In the case of sequential enzyme treatments, in some embodiments it may be advantageous to remove the first enzyme used, e.g. by heat deactivation or by use of an immobilised enzyme, prior to treatment with the second (and/or third etc.) enzyme.

Traditionally the cake industry uses cake improvers for the production of cakes and to secure high quality cakes in terms of taste, structure, eating quality and appearance. These cake improvers are normally based on emulsifiers spray dried on a carrier like



starch and malto dextrin. Some cake improvers are also in a gel form based on emulsifiers, sugars and water. These cake improvers are very important for the cake industry in order to produce cake of high quality. Cake improvers however contain emulsifiers and other “non-natural” ingredients with an E-number. Because of demand  
5 for the consumers to reduce the numbers of E-numbers, the cake industry has asked for alternative ways to produce cakes of high quality without using emulsifiers.

An alternative way to produce cake is to use an enzyme, i.e. the lipid acyltransferase defined herein or an enzyme composition according to the present invention.

10

The lipid acyltransferase as defined herein and/or the food enzyme composition of the present invention may be used in the preparations of a fine food, such as a cake. In such instances, the following constituents may be formed in the fine food:

- i) sugar esters and lysolecithin (from the carbohydrate in the cake recipe and the  
15 lecithin in egg which also form part of the cake recipe); and/or
- ii) acylated peptides and lysolecithin (by transferring a fatty acid from lecithin to a protein or peptide during formation of protein-fatty acid condensates, which are known to be highly efficient emulsifiers (Herstellung und Anwendungsmöglichkeiten von Eiweiss-Fettsäurekondensaten. Andreas  
20 Sander, Eberhard Eilers, Andrea Heilemann, Edith von Kreis. Fett/lipid 99 (1997) Nr. 4, 115-120).

It is considered that in the production of some fine foods, particularly high fat fine foods, such as cakes, it may be desirable to have some accumulation of fatty acids.

25 Therefore the combination of the use of lipolytic enzymes and the lipid acyl transferase as defined herein may be particularly beneficial for production of high fat fine foods. Alternatively, additional free fatty acids or fatty acid soap (E470a) may be selected and used in combination with the lipid acyl transferase.

30 The foodstuff according to the present invention may suitably comprise one or more of the following additives:



soy protein material; carotenoids, flavenoids, antioxidant and phytochemical (especially anthocyanonide, carotenoid, bioflavinoid, glutathione, catechin, isoflavone, lycopene, ginsenoside, pycnogenol, alkaloid, pygeum phytosterol, sulphoraphane, resveretol, grape seed extract or food containing stanol esters), vitamin (especially  
5 vitamin C, vitamin A, vitamin B3, vitamin D, vitamin E, thiamine, riboflavin, niacin, pyridoxine, cyanocobalamin, folic acid, biotin, pantothenic acid or vitamin K), minerals (especially calcium, iodine, magnesium, zinc, iron, selenium, manganese, chromium, copper, cobalt, molybdenum or phosphorus), fatty acid (especially gamma-linoleic acid, ucospentaenoic acid or decosahexaenoic acid), oil (especially borage oil,  
10 high carotenoid canola oil or flax seed oil), amino acid (especially tryptophan, lysine, methionine, phenylalanine, threonine, valine, leucine, isoleucine, alanine, arginine, aspartic acid, cystine, cysteine, glutamic acid, glutamine, glycine, histidine, proline, hydroxyproline, serine, taurine or tyrosine), enzyme (especially bromelain, papain, amylase, cellulase or coenzyme Q), lignin, stanol ester or friendly bacteria (especially  
15 Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus bifidus, Lactobacillus plantarum or Streptococcus faecium), folic acid, and soluble fibre.

#### TECHNICAL EFFECT

20 Surprisingly lipid acyltransferases have significant acyltransferase activity in foodstuffs. This activity has surprising beneficial applications in methods of preparing foodstuffs.

The present invention is predicated upon the surprising finding that the lipid  
25 acyltransferases according to the present invention can perform carbohydrate-esterification via alcohololysis, i.e. acyl transfer from a lipid, in a foodstuff with a significant water content. Prior art suggests that such enzymes if they would function at all in this manner would only function in a solvent environment (i.e. in environments with low or no water content).



The present invention may provide one or more of the following unexpected technical effects in egg products, particularly mayonnaise: an improved heat stability during pasteurisation; improved organoleptic properties, an improved consistency.

- 5 The present invention may provide one or more of the following unexpected technical effects in dough and/or baked products: an improved specific volume of either the dough or the baked products (for example of bread and/or of cake); an improved dough stability; an improved crust score (for example a thinner and/or crispier bread crust), an improved crumb score (for example a more homogenous crumb distribution  
10 and/or a finer crumb structure and/or a softer crumb); an improved appearance (for example a smooth surface without blisters or holes or substantially without blisters or holes); a reduced staling; an enhanced softness; an improved odour; an improved taste.

- The present invention may provide a beneficial effect from formation of highly  
15 surface-active materials in a foodstuff without formation of substantial amount of free fatty acids, which reduce the ability of the foodstuff to oxidize upon storage, because free fatty acids are more prone to oxidation than the corresponding fatty acid esters.

- Suitably, the present invention may provide one or more of the following unexpected  
20 technical effects in a foodstuff: an improved appearance, an improved mouthfeel, an improved stability, in particular an improved thermal stability, an improved taste, an improved softness, an improved resilience, an improved emulsification.

- Suitably, the present invention may provide one or more of the following unexpected  
25 technical effects in dairy products, such as ice cream for example: an improved mouthfeel (preferably a more creamy mouthfeel); an improved taste; an improved meltdown.

- Suitably, the present invention may provide one or more of the following unexpected  
30 technical effects in egg or in egg products: improved stability of emulsion; thermal stability of emulsion; improved flavour; reduced mal-odour; improved thickening properties, improved consistency.



Specific technical effects associated with the use of a lipid acyltransferase as defined herein in the preparation of a foodstuff are listed in the table below:

|    | Foodstuff                    | Effect                                                                                                                                                             |
|----|------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1  | Bread, Muffins and Doughnuts | Strengthens dough and increases mechanical resistance and increases water absorption capacity. Increases volume of bakery products and maintains softness of crumb |
| 2  | Frozen dough                 | Prevents spoiling during refrigeration                                                                                                                             |
| 3  | Sponge cake                  | Makes good cake volume and a uniform soft texture                                                                                                                  |
| 4  | Biscuit, cracker and cookie  | Makes stable emulsions of fat and prevents stickiness to the machine. Prevents blooming of high fat products                                                       |
| 5  | Batter and breading          | Improves texture of fried products.                                                                                                                                |
| 6  | Noodles                      | Prevents dough from sticking to the machine. Increases water content, and decreases cooking loss                                                                   |
| 7  | Instant noodles              | Prevent noodles from adhering to each other                                                                                                                        |
| 8  | Pasta                        | Dough conditioner prevents adhesion on cooking.                                                                                                                    |
| 9  | Custard cream                | Makes starch paste with a smooth and creamy texture, and prevents dehydration.                                                                                     |
| 10 | Coffee whitener              | Prevent oil and water separation                                                                                                                                   |
| 11 | Whipping cream               | Provides stable emulsion                                                                                                                                           |
| 12 | Chocolate                    | Prevents or reduced blooming                                                                                                                                       |
| 13 | Caramel, candy and nougat    | Improves emulsification of molten sugar and oil. Prevents separation of oil.                                                                                       |
| 14 | Processed meat, sausages     | Improves water holding capacity of sausages and pressed ham, and prevents separation of oil phase of pastes and pâté.                                              |

5

Suitably, the present invention may provide one or more of the following unexpected technical effects in cheese: a decrease in the oiling-off effect in cheese; an increase in cheese yield; an improvement in flavour; a reduced mal-odour; a reduced “soapy” taste.

10

Oiling-off is the tendency to form free oil upon storage and melting. Excessive oiling-off is a defect most often related to heated products wherein cheese is used, e. g. pizza and related foods (cf. e.g. Kindstedt J. S; Rippe J. K. 1990, J Dairy Sci. 73: 867873. It



becomes more and more important to control/eliminate this defect, as the consumer concern about dietary fat levels increases. Free oil/fat in a product is perceived as a high fat content, and is generally undesirable. The oiling off effect can not only affect the appearance of the cheese, but in severe cases the oil released by the cheese may spread across the food product, and be absorbed by the food product. This is particularly detrimental to food products which contain a baked components, such as a pizza base, and the effect is not only seen in the undesirable appearance, but also detrimental texture and flavour may also result.

10 In foodstuffs the fat phase is often stabilised by mechanic emulsification, e. g. homogenisation. This technology is generally not applicable in cheese production as homogenisation of the cheese milk has a negative influence on the coagulation properties of the cheese milk and on the yield as well as the taste of the cheese produced therefrom.

15 The use of the enzyme modified foodstuff and/or food material of the present invention (including enzyme modified milk, cream and/or butter fat for example) can be used to produce foodstuffs such as cheese which have a reduced oiling-off effect and/or to improve the homogenization properties of the cheese milk, and/or reduce the negative influence of coagulation properties of homogenised cheese milk when made into cheese, and/or improve the flavour and/or texture of the cheese.

Oiling off effect and cheese yield and fat yield/content can be measured according to the protocols disclosed in WO00/54601.

25 In one embodiment the foodstuff (for example the dairy product, e.g. cheese) prepared in accordance with the present invention may have a higher yield.

Cheese yield increases may occur either when the cheese milk and/or cream is modified directly by enzyme treatment, and/or when the cheese milk is supplemented with the enzyme modified oil or fat, such as enzyme modified butterfat.



A further advantage of the present invention may be the reduction of off-flavours and/or off-tastes, preferably by reducing the amount of free fatty acids in the enzymatically treated foodstuffs (e.g. in the cheese).

5 One advantage of the present invention is that the lipid acyltransferase may be used in a lower dosage to produce the same (or better) effects compared with a phospholipase A2 (PLA2). Thus effectively enzyme may be necessary to achieve the same (or better) results.

10 Another advantage of the present invention is that the lipid acyltransferase for use in the present invention and particularly in cheese manufacture does not necessarily require pre-treatment of the milk and/or cream. In fact the lipid acyltransferase when used in the present invention may be added directly to the cheese vat. This may advantageously simplify the cheese manufacture process for the end user.

15

Another advantage of the present invention is that the lipid acyltransferase may increase the moisture content of the foodstuff, such as for example a cheese (e.g. mozzarella) and/or butterfat, compared to when a phospholipase such as Lecitase™ is used for instance.

20

In one embodiment, the use of the enzyme modified foodstuff and/or food material of the present invention can be used to produce a foodstuff such as cheese that has an increased moisture content compared to when a phospholipase such as Lecitase™ is used for instance. This one embodiment may be particularly advantageous where the  
25 foodstuff and/or food material is a dairy product, for example milk, cream, butterfat, and/or cheese.

Another advantage of the present invention is that sterol esters and/or stanol esters may be produced in foodstuff. This one embodiment this may be particularly advantageous  
30 where the foodstuff and/or food material is a dairy product, for example milk, cream, butterfat, and/or cheese.



Advantageously the present invention may be used to reduce the cholesterol level of a foodstuff, particularly a dairy product, for example cheese.

5 In food production, in particular cheese production, the use of the lipid acyltransferase in accordance with the present invention provides a significant advantage in the ability to recover soluble proteins from dairy products. For example, in cheese production nearly 20% of all milk protein is removed in the whey (i.e. the watery part of the milk that remains after the formation of curds). The whey comprises the soluble milk  
10 proteins, whereas the hydrophobic proteins are maintained in the curd. By use of the lipid acyltransferase in accordance with the present invention it is possible to transfer an acyl group from a lipid (preferably from a glycolipid or a phospholipid), to a protein (in particular to a whey protein such as lactoglobulin) to form a protein fatty acid condensate. Thus, producing a product which is more hydrophobic and which  
15 will stay in the curd rather than being eluted in the whey. In this way, more of the milk protein can be maintained in the final foodstuff, i.e. the final dairy product such as the cheese.

In one aspect, the present invention is based in part on the realisation that yields of  
20 foods – such as cheese - may be improved by the use of a lipid acyl transferase. In addition or alternatively, the flavour, texture, oxidative stability and/or shelf life of the food may be improved. In addition or alternatively, the food may have a reduced cholesterol level or enhanced content of phytosterol/stanol esters.

25 Without wishing to be bound to a particular theory it is considered that the increase in yield may be the result of the transesterification of whey proteins and peptides, resulting in significant increase in the hydrophobicity of the whey proteins and precipitation of the acylated whey proteins in the cheese curd.

30 In biological systems, for example, the deposition of membrane bound proteins and enzymes are achieved by two different mechanisms. The membrane bound proteins either possess a number of membrane-spanning or hydrophobic domains, or they have



alternatively a fatty acid linked to the polypeptide chain. The fatty acids have normally a chain length of 14 or 16 carbon atoms. The fatty acids are covalently linked to the polypeptide chain at 3 different position, the N-terminal amino acid as an amide-bond, a cysteine residue as a thioester linkage, or a serine or threonine amino acid as an ester  
5 linkage. Only one fatty acid per polypeptide molecule is necessary to incorporate the protein into the cell membrane.

When a fatty acid is covalently linked to a non-membrane protein, the physical and functional properties will change drastically. WO97/14713 describes the transformed  
10 soy and gluten proteins into acyl derivatives by treatment with a lipase from *Mucor miehei* (Lipozyme<sup>TM</sup>, Novozymes), and a fatty acid in organic solvent. The lipid acyl transferase according to the present invention may be used in the production of acylated proteins in a low or high water environment.

15 We note that acylated proteins form amphiphilic complexes that can be used for a number of cosmetic products. The acylated protein can form gels, bind water by retaining moisture, have emulsifying properties and is very active in the interphase between water and lipid.

20 Thus, the present invention may in one aspect provide a cosmetic composition comprising a lipid acyl transferase as defined herein.

In addition, the present invention may provide the use of an acyltransferase as defined herein to produce a cosmetic composition.

25

In a further aspect, the present invention provides a method of *in situ* production of a protein ester in a cosmetic composition, wherein the method comprises the step of adding to the cosmetic composition (or components thereof) a lipid acyltransferase as defined herein.

30

Many food proteins are soluble in aqueous solutions and are therefore suitable for *in situ* modification by the lipase acyl transferase. In the cheese production,  $\beta$ -



lactoglobulin is lost to the whey fraction. After acylation with a lipase acyl transferase, or a lipase acyl transferase variant, initial results indicate that b-lactoglobulin may however, be deposited in the casein micelle surface during rennet coagulation.  $\beta$ -lactoglobulin has three potential acylation sites (serine residues) on three surface  
5 loops. Milk contains sufficient amounts of lecithin, a suitable substrate for a lipid acyl transferase enzyme to acylate the  $\beta$ -lactoglobulin. The lysolecithin formed may have an additional emulsifying effect.

The improvements observed with lipid acyltransferase according to the present  
10 invention are in comparison to when lipolytic enzymes without acyltransferase activity, such as triacylglycerol lipases and phospholipases, are used.

## ADVANTAGES

- 15 The generation of an emulsifier and a sterol/stanol ester *in situ* from at least one constituent of the food material, means that the food material will contain at least one less additive material. This is advantageous because of the improvement in the ease of production. For example, no further processing or addition of ingredients or addition of emulsifiers may be required. Moreover, the foodstuff may contain less “additives”.
- 20 The reduction or elimination of “additives” is desirable to consumers and inclusion of additives often must be declared to the consumer in the ingredients listing on the foodstuff. Thus, the present invention is further advantageous.

An advantage of the present invention may be the production *in situ* of an emulsifier in  
25 a foodstuff without a detrimental increase in the free fatty acid content of the foodstuff.

The generation of two emulsifiers and/or a carbohydrate ester *in situ* from at least one constituent of the food material, means that the food material will contain at least one less additive material.

30

In addition, when the lipid acyltransferase acts on a glycolipid it is possible to advantageously produce the emulsifier DGMG *in situ* without a detrimental increase in



the free fatty acid content of the foodstuff. Thus, reducing detrimental effects attributed to an increase in free fatty acids, including but not limited to a reduction in “soapy” taste in cheese, prevention of overdosing in dough and dough baked properties.

5

For some aspects, an advantage of the present invention is the reduction in free cholesterol levels in the foodstuff.

For other aspect, an advantage of the present invention is the increase in stanol and/or sterol esters in the foodstuff. Some sterol/stanol esters may be effective flavourants and/or texturisers. Thus, the present invention may not only results in the *in situ* production of an emulsifier in a foodstuff, but also the *in situ* production of a flavourant and/or a texturiser. Some sterol/stanol esters are known to reduce blood serum cholesterol and/or low density lipoproteins when consumed in a foodstuff. Thus, the present invention may be used to prepare a foodstuff with increased levels of sterol esters and/or stanol esters.

For some aspects, particularly when the enzyme according to the present invention is used in egg based products, an advantage is the removal of unwanted free carbohydrates.

Also advantageously the emulsification properties of the foodstuff are enhanced, leading to improved appearance and/or handling properties and/or structure and/or consistency and/or heat stability without a negative impact on taste.

25

In addition, for some embodiments advantageously the effect of “overdosing” observed when using lipases *per se*, is effectively overcome by the addition of an enzyme in accordance with the present invention. This is due at least in part to the fact that free fatty acids are not produced or only produced to an insignificant degree when using the enzyme according to the present invention.

30



Further and/or alternative advantages are taught in the section entitled “Technical Effects” above.

## ISOLATED

5

In one aspect, preferably the polypeptide or protein for use in the present invention is in an isolated form. The term “isolated” means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

10

## PURIFIED

In one aspect, preferably the polypeptide or protein for use in the present invention is in a purified form. The term “purified” means that the sequence is in a relatively pure state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

15

## CLONING A NUCLEOTIDE SEQUENCE ENCODING A POLYPEPTIDE ACCORDING TO THE PRESENT INVENTION

20

A nucleotide sequence encoding either a polypeptide which has the specific properties as defined herein or a polypeptide which is suitable for modification may be isolated from any cell or organism producing said polypeptide. Various methods are well known within the art for the isolation of nucleotide sequences.

25

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide. If the amino acid sequence of the polypeptide is known, labelled oligonucleotide probes may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be

30



used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, polypeptide-encoding clones could be identified by inserting fragments  
5 of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide, thereby allowing clones expressing the polypeptide to be identified.

10 In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beaucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869, or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA  
15 synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating  
20 fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al* (Science (1988) 239, pp 487-491).

## 25 NUCLEOTIDE SEQUENCES

The present invention also encompasses nucleotide sequences encoding polypeptides having the specific properties as defined herein. The term "nucleotide sequence" as used  
30 herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or antisense strand.



The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence.

5

In a preferred embodiment, the nucleotide sequence *per se* encoding a polypeptide having the specific properties as defined herein does not cover the native nucleotide sequence in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred  
10 embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. Thus, the polypeptide of the present invention can be expressed by a nucleotide sequence in its native organism but  
15 wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Preferably the polypeptide is not a native polypeptide. In this regard, the term "native polypeptide" means an entire polypeptide that is in its native environment and when it has  
20 been expressed by its native nucleotide sequence.

Typically, the nucleotide sequence encoding polypeptides having the specific properties as defined herein is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the  
25 nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

## MOLECULAR EVOLUTION

30

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to



modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

5 Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

10

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR mutagenesis kit from Stratagene\*, or the Diversify PCR random mutagenesis kit from Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis, 15 which can also be combined with the use of mutagenic DNA analogues such as those described in EP 0 866 796. Error prone PCR technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. WO0206457 refers to molecular evolution of lipases.

20 A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence. DNA 25 shuffling and family shuffling technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. Suitable methods for performing 'shuffling' can be found in EP0 752 008, EP1 138 763, EP1 103 606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

30

Thus, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved

\* Trade-mark



functionality of the encoded polypeptide by various means. Using in silico and exo mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974), for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained  
5 may have significant structural analogy to known transferase enzymes, but have very low amino acid sequence homology.

As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other  
10 mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present  
15 invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression  
20 and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

25 As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme.

Suitably, the lipid acyltransferase used in the invention may be a variant, i.e. may contain at least one amino acid substitution, deletion or addition, when compared to a  
30 parental enzyme. Variant enzymes retain at least 1%, 2%, 3%, 5%, 10%, 15%, 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 95%, 97%, 99% homology with the parent



enzyme. Suitable parent enzymes may include any enzyme with esterase or lipase activity. Preferably, the parent enzyme aligns to the pfam00657 consensus sequence.

5 In a preferable embodiment a variant lipid acyltransferase enzyme retains or incorporates at least one or more of the pfam00657 consensus sequence amino acid residues found in the GDSx, GANDY and HPT blocks.

Enzymes, such as lipases with no or low lipid acyltransferase activity in an aqueous environment may be mutated using molecular evolution tools to introduce or enhance  
10 the transferase activity, thereby producing a lipid acyltransferase enzyme with significant transferase activity suitable for use in the compositions and methods of the present invention.

Suitably, the lipid acyltransferase for use in the invention may be a variant with  
15 enhanced enzyme activity on polar lipids, preferably phospholipids and/or glycolipids when compared to the parent enzyme. Preferably, such variants also have low or no activity on lyso polar lipids. The enhanced activity on polar lipids, phospholipids and/or glycolipids may be the result of hydrolysis and/or transferase activity or a combination of both.

20

Variant lipid acyltransferases for use in the invention may have decreased activity on triglycerides, and/or monoglycerides and/or diglycerides compared with the parent enzyme.

25 Suitably the variant enzyme may have no activity on triglycerides and/or monoglycerides and/or diglycerides.

Alternatively, the variant enzyme for use in the invention may have increased activity on triglycerides, and/or may also have increased activity on one or more of the  
30 following, polar lipids, phospholipids, lecithin, phosphatidylcholine, glycolipids, digalactosyl monoglyceride, monogalactosyl monoglyceride.



Variants of lipid acyltransferases are known, and one or more of such variants may be suitable for use in the methods and uses according to the present invention and/or in the enzyme compositions according to the present invention. By way of example only, variants of lipid acyltransferases are described in the following references may be used  
5 in accordance with the present invention: Hilton & Buckley J Biol. Chem. 1991 Jan 15: 266 (2): 997-1000; Robertson *et al* J. Biol. Chem. 1994 Jan 21; 269(3):2146-50; Brumlik *et al* J. Bacteriol 1996 Apr; 178 (7): 2060-4; Peelman *et al* Protein Sci. 1998 Mar; 7(3):587-99.

## 10 AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of polypeptides having the specific properties as defined herein.

15 As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be  
20 made synthetically or it may be prepared by use of recombinant DNA techniques.

Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

25 One suitable method for determining amino acid sequences from isolated polypeptides is as follows:

Purified polypeptide may be freeze-dried and 100 µg of the freeze-dried material may be dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogen  
30 carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl of 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide may be added for



the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135  $\mu$ l of water and 5  $\mu$ g of endoproteinase Lys-C in 5  $\mu$ l of water may be added to the  
5 above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours.

The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18 column (0.46x15cm;10 $\mu$ m; The Separation Group, California, USA) using solvent A:  
10 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

15

## SEQUENCE IDENTITY OR SEQUENCE HOMOLOGY

The present invention also encompasses the use of sequences having a degree of  
20 sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can  
25 be equated with "identity".

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

30

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98%



identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express  
5 homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the  
10 subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express  
15 homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

20 % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

25 Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is  
30 performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and



deletions without penalising unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximise local homology.

However, these more complex methods assign “gap penalties” to each gap that occurs  
5 in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. “Affine gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most  
10 commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for  
15 a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package  
20 (Devereux *et al* 1984 Nuc. Acids Research 12 p387) or the Vector NTI (Invitrogen Corp.). Examples of software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4<sup>th</sup> Ed – Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and Align X for example. Both BLAST and FASTA are available for offline  
25 and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and [tatiana@ncbi.nlm.nih.gov](mailto:tatiana@ncbi.nlm.nih.gov)).

30 Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise



comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. Vector NTI programs and GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if  
 5 supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package or Vector NTI, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment  
 10 feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

Should Gap Penalties be used when determining sequence identity, then preferably the following parameters are used for pairwise alignment:

15

|               |   |
|---------------|---|
| FOR BLAST     |   |
| GAP OPEN      | 0 |
| GAP EXTENSION | 0 |

| FOR CLUSTAL   | DNA  | PROTEIN |          |
|---------------|------|---------|----------|
| WORD SIZE     | 2    | 1       | K triple |
| GAP PENALTY   | 15   | 10      |          |
| GAP EXTENSION | 6.66 | 0.1     |          |

In one embodiment, CLUSTAL may be used with the gap penalty and gap extension  
 20 set as defined above.

Suitably, the degree of identity with regard to a nucleotide sequence is determined over at least 20 contiguous nucleotides, preferably over at least 30 contiguous nucleotides, preferably over at least 40 contiguous nucleotides, preferably over at least



50 contiguous nucleotides, preferably over at least 60 contiguous nucleotides, preferably over at least 100 contiguous nucleotides.

Suitably, the degree of identity with regard to a nucleotide sequence may be  
5 determined over the whole sequence.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

10

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic  
15 nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine,  
20 phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

25

|           |                   |         |
|-----------|-------------------|---------|
| ALIPHATIC | Non-polar         | G A P   |
|           |                   | I L V   |
|           | Polar – uncharged | C S T M |
|           |                   | N Q     |
|           | Polar – charged   | D E     |



|          |  |         |
|----------|--|---------|
|          |  | K R     |
| AROMATIC |  | H F W Y |

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or  $\beta$ -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the  $\alpha$ -carbon substituent group is on the residue's nitrogen atom rather than the  $\alpha$ -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends



of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

5

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

10

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

15

20

25

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed

30



using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

5 The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

10 Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

15 Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40  
20 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in  
25 the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

30

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve



making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating  
5 the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

## 10 HYBRIDISATION

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary  
15 thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR)  
20 technologies.

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the subject sequences discussed herein, or any derivative, fragment or derivative thereof.  
25

The present invention also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences discussed herein.

Hybridisation conditions are based on the melting temperature ( $T_m$ ) of the nucleotide  
30 binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning



Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe); high stringency at about  $5^\circ\text{C}$  to  $10^\circ\text{C}$  below  $T_m$ ; intermediate stringency at about  $10^\circ\text{C}$  to  $20^\circ\text{C}$  below  $T_m$ ; and low stringency at about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridisation can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridisation can be used to identify or detect similar or related polynucleotide sequences.

Preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringency conditions or intermediate stringency conditions to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

More preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$  { $1\times\text{SSC} = 0.15\text{ M NaCl}$ ,  $0.015\text{ M Na-citrate pH } 7.0$ }) to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences discussed herein under conditions of intermediate to maximal stringency.



In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

5

In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

## 10 EXPRESSION OF POLYPEPTIDES

A nucleotide sequence for use in the present invention or for encoding a polypeptide having the specific properties as defined herein can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide  
15 sequence, in polypeptide form, in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two  
20 or more different promoters described above.

The polypeptide produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal  
25 sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

## EXPRESSION VECTOR

30 The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.



Preferably, the expression vector is incorporated in the genome of the organism. The term “incorporated” preferably covers stable incorporation into the genome.

5 The nucleotide sequence of the present invention or coding for a polypeptide having the specific properties as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

10 The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide having the specific properties as defined herein.

15 The choice of vector, e.g. plasmid, cosmid, virus or phage vector, will often depend on the host cell into which it is to be introduced.

The vectors may contain one or more selectable marker genes – such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be accomplished by co-transformation (as  
20 described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

25 Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention or nucleotide sequences encoding polypeptides having the specific properties as defined herein by introducing a nucleotide sequence into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.



The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

## 5 REGULATORY SEQUENCES

In some applications, a nucleotide sequence for use in the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein may be operably linked to a regulatory sequence which is capable of providing  
10 for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

15 The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

20

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

25

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

30

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.



Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

5 Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

## CONSTRUCTS

10 The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence encoding a polypeptide having the specific properties as defined herein for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes  
15 direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

20 The construct may even contain or express a marker which allows for the selection of the genetic construct.

For some applications, preferably the construct comprises at least a nucleotide sequence of the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein operably linked to a promoter.  
25

## HOST CELLS

30 The term "host cell" - in relation to the present invention includes any cell that comprises either a nucleotide sequence encoding a polypeptide having the specific properties as defined herein or an expression vector as described above and which is



used in the recombinant production of a polypeptide having the specific properties as defined herein.

Thus, a further embodiment of the present invention provides host cells transformed or  
5 transfected with a nucleotide sequence of the present invention or a nucleotide sequence that expresses a polypeptide having the specific properties as defined herein. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

10

Examples of suitable bacterial host organisms are gram negative bacterium or gram positive bacteria.

15

Depending on the nature of the nucleotide sequence encoding a polypeptide having the specific properties as defined herein, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these  
20 instances, a different fungal host organism should be selected.

The use of suitable host cells, such as yeast, fungal and plant host cells – may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to  
25 confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain.

30

ORGANISM



The term "organism" in relation to the present invention includes any organism that could comprise a nucleotide sequence according to the present invention or a nucleotide sequence encoding for a polypeptide having the specific properties as defined herein and/or products obtained therefrom.

5

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises a nucleotide sequence coding for a polypeptide having the specific properties as defined herein and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence coding for a polypeptide having the specific properties as defined herein within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

15 The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, a nucleotide sequence coding for a polypeptide having the specific properties as defined herein, constructs as defined herein, vectors as defined herein, plasmids as defined herein, cells as defined herein, or the products thereof. For example the transgenic organism can also comprise a nucleotide sequence coding for a polypeptide having the specific properties as defined herein under the control of a heterologous promoter.

20  
25

#### TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism.

30 Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.



Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as  
5 by removal of introns.

In another embodiment the transgenic organism can be a yeast.

Filamentous fungi cells may be transformed using various methods known in the art –  
10 such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. A review of the general techniques used for  
15 transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

20 General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

#### TRANSFORMED FUNGUS

25 A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera *Thermomyces*, *Acremonium*, *Aspergillus*, *Penicillium*, *Mucor*, *Neurospora*, *Trichoderma* and the like.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which  
30 states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N.*



*crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

5

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

10 A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.( Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

15 Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) Trends Biotechnol 2002 May;20(5):200-6, Archer & Peberdy Crit Rev Biotechnol (1997) 17(4):273-306.

#### TRANSFORMED YEAST

20 In another embodiment, the transgenic organism can be a yeast.

A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

25

In this regard, yeast – such as the species *Saccharomyces cerevisi* or *Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

30 A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a



vehicle for the expression of heterologous genes", *Yeasts*, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

For the transformation of yeast, several transformation protocols have been developed.

5 For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* 75, 1929); Beggs, J D (1978, *Nature*, London, 275, 104); and Ito, H *et al* (1983, *J Bacteriology* 153, 163-168).

10 The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as, but not limited to, yeast species selected from *Pichia* spp.,  
15 *Hansenula* spp., *Kluyveromyces*, *Yarrowinia* spp., *Saccharomyces* spp., including *S. cerevisiae*, or *Schizosaccharomyce* spp. including *Schizosaccharomyce pombe*.

A strain of the methylotrophic yeast species *Pichia pastoris* may be used as the host organism.

20

In one embodiment, the host organism may be a *Hansenula* species, such as *H. polymorpha* (as described in WO01/39544).

#### TRANSFORMED PLANTS/PLANT CELLS

25

A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27), or in WO01/16308. The transgenic plant may produce enhanced levels of  
30 phytosterol esters and phytostanol esters, for example.



Therefore the present invention also relates to a method for the production of a transgenic plant with enhanced levels of phytosterol esters and phytostanol esters, comprising the steps of transforming a plant cell with a lipid acyltransferase as defined herein (in particular with an expression vector or construct comprising a lipid acyltransferase as defined herein), and growing a plant from the transformed plant cell.

## SECRETION

Often, it is desirable for the polypeptide to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the  $\alpha$ -factor gene (yeasts e.g. *Saccharomyces*, *Kluyveromyces* and *Hansenula*) or the  $\alpha$ -amylase gene (*Bacillus*).

## DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia\* Biotech (Piscataway, NJ), Promega\* (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

\* Trade-mark



Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels  
5 include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241.

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

10

## FUSION PROTEINS

A polypeptide having the specific properties as defined herein may be produced as a fusion protein, for example to aid in extraction and purification thereof. Examples of  
15 fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

20

Gene fusion expression systems in *E. coli* have been reviewed in Curr. Opin. Biotechnol. (1995) 6(5):501-6.

In another embodiment of the invention, the amino acid sequence of a polypeptide  
25 having the specific properties as defined herein may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

30

The invention will now be described, by way of example only, with reference to the following Figures and Examples.



Figure 1 shows a pfam00657 consensus sequence from database version 6 (SEQ ID No. 1);

5 Figure 2 shows an amino acid sequence (SEQ ID No. 2) obtained from the organism *Aeromonas hydrophila* (P10480; GI:121051);

Figure 3 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism *Aeromonas salmonicida* (AAG098404; GI:9964017);

10

Figure 4 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NP\_631558);

15 Figure 5 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number: CAC42140);

Figure 6 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number P41734);

20 Figure 7 shows an alignment of selected sequences to pfam00657 consensus sequence;

Figure 8 shows a pairwise alignment of SEQ ID No. 3 with SEQ ID No. 2 showing 93% amino acid sequence identity. The signal sequence is underlined. + denotes differences. The GDSX motif containing the active site serine 16, and the active sites  
25 aspartic acid 116 and histidine 291 are highlighted (see shaded regions). Numbers after the amino acid is minus the signal sequence;

Figure 9 shows a nucleotide sequence (SEQ ID No. 7) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas hydrophila*;

30



Figure 10 shows a nucleotide sequence (SEQ ID No. 8) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas salmonicida*;

5 Figure 11 shows a nucleotide sequence (SEQ ID No. 9) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NC\_003888.1:8327480..8328367);

10 Figure 12 shows a nucleotide sequence (SEQ ID No. 10) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number AL939131.1:265480..266367);

15 Figure 13 shows a nucleotide sequence (SEQ ID No. 11) encoding a lipid acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);

Figure 14 shows an amino acid sequence (SEQ ID No. 12) obtained from the organism  
20 *Ralstonia* (Genbank accession number: AL646052);

Figure 15 shows a nucleotide sequence (SEQ ID No. 13) encoding a lipid acyl transferase according to the present invention obtained from the organism *Ralstonia*;

25 Figure 16 shows SEQ ID No. 20. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [*Streptomyces coelicolor* A3(2)];

Figure 17 shows a nucleotide sequence shown as SEQ ID No. 21 encoding NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein  
30 [*Streptomyces coelicolor* A3(2)];



Figure 18 shows an amino acid shown as SEQ ID No.22. Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

- 5 Figure 19 shows a nucleotide sequence shown as SEQ ID No. 23 encoding Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

- 10 Figure 20 shows an amino acid sequence (SEQ ID No.24) Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

- 15 Figure 21 shows a nucleotide sequence shown as SEQ ID No. 25 encoding Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

- 20 Figure 22 shows an amino acid sequence (SEQ ID No.26) Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

- Figure 23 shows an nucleotide sequence shown as SEQ ID No. 27 encoding Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

- 25 Figure 24 shows an amino acid sequence (SEQ ID No.28) Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

- 30 Figure 25 shows a nucleotide sequence shown as SEQ ID No. 29, encoding Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];



Figure 26 shows an amino acid sequence (SEQ ID No.30) Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [*Streptomyces rimosus*];

Figure 27 shows a nucleotide sequence shown as SEQ ID No. 31 encoding Srim1  
5 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [*Streptomyces rimosus*];

Figure 28 shows an amino acid sequence (SEQ ID No.32)A lipid acyl transferase from *Aeromonas hydrophila* (ATCC #7965);

10

Figure 29 shows a nucleotide sequence (SEQ ID No. 33) encoding a lipid acyltransferase from *Aeromonas hydrophila* (ATCC #7965);

15

Figure 30 shows an amino acid sequence (SEQ ID No.34) of a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 31 shows a nucleotide sequence (SEQ ID No 35) encoding a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

20

Figure 32 shows that homologues of the *Aeromonas* genes can be identified using the basic local alignment search tool service at the National Center for Biotechnology Information, NIH, MD, USA and the completed genome databases. The GDSX motif was used in the database search and a number of sequences/genes potentially encoding enzymes with lipolytic activity were identified. Genes were identified from the genus *Streptomyces*, *Xanthomonas* and *Ralstonia*. As an example below, the *Ralstonia solanacearum* was aligned to the *Aeromonas salmonicida* (satA) gene. Pairwise alignment showed 23% identity. The active site serine is present at the amino terminus and the catalytic residues histidine and aspartic acid can be identified;

25

Figure 33 shows the Pfam00657.11 [family 00657, database version 11] consensus  
30 sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and



Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The – symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue  
5 without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 16, 18, 20, 22, 24, 26, 28 and 30.

Figure 34 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to  
10 the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The – symbol indicates a position where the hidden Markov model of the Pfam consensus expected  
15 to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 2, 16, 18, 20, 26, 28 and 30. All these proteins were found to be active against lipid substrates.

Figure 35 shows a expression vector pet12-AsalGCAT= pSM containing the C-  
20 terminal His-tagged *Aeromonas salmonicida* lipid acyltransferase gene;

Figure 36 shows the results of testing cell extracts in a NEFA Kit Assay, which depicts the activity of a recombinant, *A. salmonicida* lipid acyltransferase, towards lecithin. The wells from left to right indicate: a positive control, a negative control (i.e. extracts  
25 from empty plasmid) and samples collected after 0, 1, 2 and 3 hours cultivation after IPTG induction;

Figure 37 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production  
30 of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay. Wells from left to right: positive control; negative control; 20°C; 30°C;



Figure 38 shows crude cell extracts from BL21(DE3)pLysS expressing active lipid acyltransferase incubated with the substrate lecithin and reaction mixture was analyzed using thin layer chromatography showing the presence of degradation products. Lanes:

- 5 1. No enzyme; 2. + A.sal -10ul 37°C; 3. + A. sal -20ul 37°C; 4. + A.sal -10ul 24°C; 5. + A. sal -20u 24°C;

Figure 39 shows partial purification of the *Aeromonas salmonicida* Acyl Transferase showing the phospholipase activity associated with purified His-tag protein. SE =  
10 Sonicated extracts, His = Purified with Ni-NTA spin-kit from Qiagen\*;

Figure 40 shows the expression vector pet12-A.h. GCAT=pSMa containing the C-terminal His-tagged *Aeromonas hydrophila* Glycerolipid Acyl Transferase (GCAT) gene was used to transform *E.coli* strain BL21(DE3)pLysS;

15

Figure 41 shows the activity of the crude extracts (5. & 10ul) containing the recombinant *Aeromonas hydrophila* GCAT enzyme was tested towards lecithin using Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland), showing the presence of active enzyme towards the phospholipid, lecithin;

20

Figure 42 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay;

25

Figure 43 shows the partial purification of the *Aeromonas hydrophila* & *A. salmonicida* Acyl Transferases showing the phospholipase activity associated with purified His-tag protein. SE = Sonicated extracts,  
His = Purified with Ni-NTA spin-kit from Qiagen);

30

Figure 44 shows the expression of the *Aeromonas* genes in *Bacillus subtilis* 163 showing the production of secreted enzyme with activity towards both lecithin and



DGDG. pUB-AH= construct containing the *A. hydrophila* gene and pUB-AS, construct with the *A. salmonicida* gene, Culture filtrate was incubated with the substrates for 60 minutes.

- 5 Figure 45 and Fig 46 show a TLC plate in developing solvent IV (chloroform:methanol:water (65:25:4)); Lane 1: 40 mg sitosterol 30 min; Lane 2: Transferase+ 40 mg sitosterol 30 min; Lane 3: Transferase+ 80 mg sitosterol 30 min; Lane 4: Transferase+ 40 mg sitosterol 120 min; Lane 5: Transferase+ 80 mg sitosterol 120 min; Lane 6: Transferase+ 40 mg sitosterol 300 min; Lane 7: 40 mg  
10 sitosterol 300 min; Lane 8: Cholesterol; Lane 9: Sitosterol;

Figure 47 depicts the reaction between phosphatidylcholine and cholesterol which is catalysed by a lipid acyltransferase;

- 15 Figure 48 shows a TLC analysis of lipids extracted from enzyme treated or untreated egg yolk., 6) 0.31PLU/g Transferase #179, 7) 1.25PLU/g Transferase #178-9., 8) 23.25 PLU/g Phospholipase #3108., 9) Control.

- 20 Figure 49 shows mayonnaise test samples produced by enzyme treated or untreated egg yolk: 5) Transferase #179, 0.31 PLU/g. 6) Transferase #178-9, 1.25 PLU/g, 7) Phospholipase #3108, 23.3 PLU/g 8) Control, water

Figure 50 shows a TLC (in solvent I) of egg yolk lipid treated with a lipid acyl transferase from *A. hydrophila*;

25

Figure 51 shows a TLC (in solvent IV) of egg yolk lipid treated with a lipid acyl transferase from *A. hydrophila*;

- 30 Figure 52 shows a TLC analysis of transferase treated lipid from egg yolk over a time course;



Figure 53 shows the amount of fatty acid and cholesterol ester produced as a function of time when using a lipid acyltransferase (Tranf #178-9) compared with when using a control lipolytic enzyme, *Thermomyces lanuginosus*;

5 Figure 54 shows relative transferase activity as % of transferase and hydrolytic activity in enzymatic reactions in egg yolk with high water content, #1991 (phospholipase A2) and #2427 (phospholipase A1) are control phospholipases, #178 is a lipid acyltransferase;

10 Figure 55 shows the effect of water content in the assay on the transferase activity of the transferase #178 in transferase reactions in egg yolk with high water content;

Figure 56 shows the transferase activity for a lipid acyltransferase (#178) as a function of reaction time in transferase reactions in egg yolk with high water;

15

Figure 57 and Figure 58 show graphs depicting fatty acid and cholesterol ester as a function of time. The graphs depict results obtained for GLC analysis in the assay for measurement of acyltransferase activity using lecithin and cholesterol in buffer as substrate;

20

Figure 59 shows a TLC in solvent I. Egg yolk treated with lipid acyltransferase #138 from *Aeromonas salmonidica* (lane no. 1 and 2) or with a phospholipase #2938 (LIOPAN<sup>®</sup> F) (lane no. 3) or Untreated egg yolk (lane no. 4);

25 Figure 60 shows a TLC in solvent IV. Egg yolk treated with lipid acyltransferase #138 (lane no. 1 and 2) or with Phospholipase #2938 (lane no. 3). Untreated egg yolk (lane no. 4);

30 Figure 61 shows egg yolk treated with lipid acyltransferase #138 (sample nos. 1 and 2) and with phospholipase #2938 (sample no. 3). Untreated egg yolk (sample no. 4);

Figure 62 shows a food emulsion after 2 hours at 100 °C. 0) Untreated egg yolk



1) Egg yolk treated with lipid acyl transferase #138 for 210 minutes. 3) Egg yolk treated with the control phospholipase #2938 for 210 minutes;

Figure 63 shows TLC plates showing the screening of transferase activity on plant sterol and glycerol. PC = phosphatidylcholine, LPC = lysophosphatidylcholine; PE = phosphatidylethanolamine; monogl = monoglyceride;

Figure 64 shows a TLC plate in solvent I, Samples 1 to 6 after 24 hours and samples 1 to 4 after 4 hours reaction time. The TLC analysis confirms the formation of sterol ester in samples 1, 2, 5 and 6;

Figure 65 shows a TLC plate in solvent I where the transferase activity of an immobilised acyltransferase from *Aeromonas salmonicida* was tested in an oil mixture – with samples taken at 0.5, 1, 3, 6 and 24 h;

Figures 66 and 67 show TLC plates in solvent I and IV. Lane 1 = lecithin; Lane 2 = control – 10mins; Lane 3 = 0.75 PLU, 10mins; Lane 4 = 0.75 PLU, 60mins; Lane 5 = 0.75 PLU, 220mins; Lane 6 = control, 20 h; Lane 7 = 0.75 PLU, 20h; and Lane 8 = cholesterol ester;

Figures 68 and 69 show TLC plates in solvent IV. Lane 1 = lecithin; Lane 2 = control – 10mins; Lane 3 = 1 PLU, 10mins; Lane 4 = 1 PLU, 60mins; Lane 5 = 1 PLU, 180mins; Lane 6 = 1PLU, 220mins; Lane 7 = 1PLU, 1200min; Lane 8 = control, 1200 min; Lane 9 = glucose ester; Lane 10 = cholesterol; and Lane 11 = glucose;

Figure 70 shows the reaction between DGDG and glucose when catalysed by a lipid acyltransferase;

Figure 71 shows an amino acid sequence (SEQ ID No. 36) of the fusion construct used for mutagenesis of the *Aeromonas hydrophila* lipid acyltransferase gene in Example 17. The underlined amino acids is a xylanase signal peptide;



Figure 72 shows a nucleotide sequence (SEQ ID No. 45) encoding an enzyme from *Aeromonas hydrophila* including a xylanase signal peptide;

Figure 73 shows a TLC plate clearly showing the formation of plant sterol ester and monoglyceride. Lane 1 is after 1 hour reaction time, Lane 2 is after 4 hours reaction time, Lane 3 is after 24 hours reaction time and Lane 4 is a plant sterol; and

Figure 74 shows the amino acid sequence of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp (notably, amino acid 80 is in the mature sequence) (SEQ ID 62);

Figure 75 shows SEQ ID No 63 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

Figure 76 shows SEQ ID No 64 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

Figure 77 shows SEQ ID No. 65. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 78 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Thermobifida* (SEQ ID No. 66);

Figure 79 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Thermobifida* (SEQ ID No. 67);

Figure 80 shows a polypeptide of a lipid acyltransferase enzyme from *Corynebacterium efficiens* GDSx 300 amino acid (SEQ ID No. 68);

Figure 81 shows a polypeptide of a lipid acyltransferase enzyme from *Novosphingobium aromaticivorans* GDSx 284 amino acid (SEQ ID No. 69);



Figure 82 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces coelicolor* GDSx 269 amino acid (SEQ ID No. 70);

Figure 83 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces avermitilis* \ GDSx 269 amino acid (SEQ ID No. 71);

Figure 84 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces* (SEQ ID No. 72);

Figure 85 shows an amino acid sequence (SEQ ID No. 73) obtained from the organism *Aeromonas hydrophila* (P10480; GI:121051) (notably, this is the mature sequence);

Figure 86 shows the amino acid sequence (SEQ ID No. 74) of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) (notably, this is the mature sequence);

Figure 87 shows a nucleotide sequence (SEQ ID No. 75) from *Streptomyces thermosacchari*;

Figure 88 shows an amino acid sequence (SEQ ID No. 76) from *Streptomyces thermosacchari*;

Figure 89 shows an amino acid sequence (SEQ ID No. 77) from *Thermobifida fusca*/GDSx 548 amino acid;

Figure 90 shows a nucleotide sequence (SEQ ID No. 78) from *Thermobifida fusca*;

Figure 91 shows an amino acid sequence (SEQ ID No. 79) from *Thermobifida fusca*/GDSx;

Figure 92 shows an amino acid sequence (SEQ ID No. 80) from *Corynebacterium efficiens*/GDSx 300 amino acid;



Figure 93 shows a nucleotide sequence (SEQ ID No. 81) from *Corynebacterium efficiens*;

- 5 Figure 94 shows an amino acid sequence (SEQ ID No. 82) from *S. coelicolor*/ GDSx 268 amino acid;

Figure 95 shows a nucleotide sequence (SEQ ID No. 83) from *S. coelicolor*;

- 10 Figure 96 shows an amino acid sequence (SEQ ID No. 84) from *S. avermitilis*;

Figure 97 shows a nucleotide sequence (SEQ ID No. 85) from *S. avermitilis*;

- 15 Figure 98 shows an amino acid sequence (SEQ ID No. 86) from *Thermobifida fusca*/GDSx;

Figure 99 shows a nucleotide sequence (SEQ ID No. 87) from *Thermobifida fusca*/GDSx;

- 20 Figure 100 shows a nucleotide sequence from *Aeromonas salmonicida* (SEQ ID No. 88) including the signal sequence (preLAT - positions 1 to 87);

Figure 101 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Streptomyces* (SEQ ID No. 89);

25

Figure 102 shows shows the amino acid sequence of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp (notably, amino acid 80 is in the mature sequence) – shown herein as SEQ ID No. 62 - after undergoing post-translational modification (SEQ ID No. 90);

30

Figure 103 shows an alignment of the L131 and homologues from *S. avermitilis* and *T. fusca* illustrates that the conservation of the GDSx motif (GDSY in L131 and



*S. avermitilis* and *T. fusca*), the GANDY box, which is either GGND A or GGND L, and the HPT block (considered to be the conserved catalytic histadine). These three conserved blocks are highlighted;

- 5 Figure 104. TLC (running buffer 5) of 10 butterfat samples, mono-diglyceride and St 17 containing cholesterol, oleic acid and cholesterol ester;

Figure 105 TLC(running buffer 1) of 10 butterfat samples, mono-diglyceride and St 8 containing cholesterol;

10

Figure 106 TLC (running buffer 5) of butterfat samples 1(ref) and 2(enzyme). Reference St. 17 containing cholesterol, oleic acid and cholesterol ester;

15

Figure 107 TLC(running buffer 1) of butterfat sample 1(reference), 2(enzyme), mono-diglyceride and St 17 containing cholesterol, fatty acid and cholesterol ester ;

Figure 108 TLC(running buffer 4) of butterfat sample 1(reference), 2(enzyme) and St. 4 containing phosphatidylcholine(PC) and lyso-phosphatidylcholine;

20

Figure. 109 TLC (running buffer 5) of cream sample 3(ref), 4(enzyme) and reference St. 17 containing cholesterol, oleic acid and cholesterol ester;

Figure. 110 TLC(running buffer 1) of cream sample 3(reference), 4(enzyme), mono-diglyceride and St 17 containing cholesterol, fatty acid and cholesterol ester ;

25

Figure. 111 TLC(running buffer 4) of cream sample 3(reference), 4(enzyme) and St. 4 containing phosphatidylcholine(PC) and lyso-phosphatidylcholine;

30

Figure 112 shows a ribbon representation of the 1IVN.PDB crystal structure which has glycerol in the active site. The Figure was made using the Deep View Swiss-PDB viewer;



Figure 113 shows 1IVN.PDB Crystal Structure – Side View using Deep View Swiss-PDB viewer, with glycerol in active site - residues within 10Å of active site glycerol are coloured black;

- 5 Figure 114 shows 1IVN.PDB Crystal Structure – Top View using Deep View Swiss-PDB viewer, with glycerol in active site – residues within 10Å of active site glycerol are coloured black;

Figure 115 shows alignment 1;

10

Figure 116 shows alignment 2;

- 15 Figures 117 and 118 show an alignment of 1IVN to P10480 (P10480 is the database sequence for *A. hydrophila* enzyme), this alignment was obtained from the PFAM database and used in the model building process;

- 20 Figure 119 shows an alignment where P10480 is the database sequence for *Aeromonas hydrophila*. This sequence is used for the model construction and the site selection. Note that the full protein (SEQ ID No. 36) is depicted, the mature protein (equivalent to SEQ ID No. 73) starts at residue 19. A. sal is *Aeromonas salmonicida* (SEQ ID No. 3) GDSX lipase, A. hyd is *Aeromonas hydrophila* (SEQ ID No. 73) GDSX lipase. The consensus sequence contains a \* at the position of a difference between the listed sequences;

- 25 Figure 120 shows a diagram which illustrates the addition of enzyme to each vat., Han PL is Lecitase, Dan PL is KLM3 a lipid acyltransferase according to the present invention;

- 30 Figure 121 shows a TLC (solvent 6) of lipid extracted from cream and a standard mixture (ST16) of phospholipids; Phosphatidylcholine(PC); Lyso-phosphatidylcholine (LPC); Phosphatidylinositol (PI); Phosphatidylethanolamine (PE); 5.13% Phosphatidic acid (PA); and Spingholipid (SG);



Figure 122 shows a TLC (solvent 1) of lipid extracted from cream and a standard mixture of free fatty acids (FFA), cholesterol (CHL) and cholesterol ester (CHL-ester);

5 Figure 123 shows the ANOVA evaluation of cholesterol in enzyme treated cream (30%) analyzed by TLC (Table 43), A = control, B = Lecitase and C = KLM3';

Figure 124 shows the ANOVA evaluation of Fatty acids in enzyme treated cream (30%) analyzed by TLC (Table 43), A = control, B = Lecitase and C = KLM3';

10

Figure 125 shows ANOVA evaluation of cholesterol analyzed by GLC (Table 44) A = control, B = Lecitase and C = KLM3';

15

Figure 126 shows the ANOVA evaluation of cholesterol ester analyzed by GLC (Table 44) A = control, B = Lecitase and C = KLM3';

20

Figure 127 shows the ANOVA evaluation of Sum FFA (palmitic acid, C:16:0 + oleic acid, C18:1 + Linoleic acid, C18:2 + stearic acid, C 18.0) analyzed by GLC (Table 44) A = control, B = Lecitase and C = KLM3';

25

Figure 128 shows a TLC (solvent 6) of lipid extracted from cheese and a standard mixture of free fatty acids (FFA), cholesterol (CHL) and cholesterol ester (CHL-ester);

Figure 129 shows a TLC (solvent 6) of lipid extracted from cheese and a standard mixture of phospholipids: Phosphatidylcholine (PC), Lyso-phosphatidylcholine (LPC), Phosphatidylinisitol (PI), Phosphatidylethanolamine (PE) and Phosphatidic acid (PA).;

30

Figure 130 shows the ANOVA evaluation of cholesterol in cheese analyzed by GLC (Table 45) A = control, B = Lecitase and C = KLM3';

Figure 131 shows the ANOVA evaluation of cholesterol ester in cheese analyzed by GLC (Table 45) A = control, B = Lecitase and C = KLM3';



Figure 132 shows the ANOVA evaluation of Oleic acid (C18:1) + linoleic acid (C18:2) in cheese analyzed by GLC (Table 45) A = control, B = Lecitase and C = KLM3';

5

Figure 133 shows the ANOVA evaluation of Palmetic acid (C16:0), Stearic acid (C18:0), Oleic acid (C18:1) + linoleic acid (C18:2) in cheese analyzed by GLC (Table 45) A = control, B = Lecitase and C = KLM3';

10 Figure 134 shows a diagram depicting force as an outcome of mass, acceleration and deflection properties of target material;

Figure 135 shows the photos of the control samples DAN011 (left) and the cheese produced with KLM3 DAN013 (right). 5 minutes standing after heating step;

15

Figure 136 shows Pizza baked with cheese DAN011 (left), DAN012 (centre) and DAN013 (right);

Figure 137 shows a gene construct used in Example 32;

20

Figure 138 shows a codon optimised gene construct (no. 052907) used in Example 32; and

25 Figure 139 shows the sequence of the XhoI insert containing the LAT-KLM3' precursor gene, the -35 and -10 boxes are underlined; and

Figure 140 shows BML780-KLM3'CAP50 (comprising SEQ ID No. 90 – upper colony) and BML780 (the empty host strain – lower colony) after 48h growth at 37°C on 1% tributyrin agar.

30



## EXAMPLES

Except where stated TLC analysis was performed as described in Example 6 and GLC analysis was performed as described in Example 11.

5

**EXAMPLE 1: The cloning, sequencing and heterologous expression of a transferase from *Aeromonas salmonicida* subsp. *Salmonicida***

**10 Strains used:**

*Aeromonas salmonicida* subsp. *Salmonicida* (ATCC 14174) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNase A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen)<sup>\*</sup> was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as  
20 host for transformation with the expression vector pet12-AsalGCAT=pSM. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

**25 Construction of expression vector pet12-AsalGCAT- pSM:**

For all DNA amplifications of the transferase genes from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x *pfu* buffer, 1ul each primer (50pmol/ul), 200 uM dNTP in a total reaction  
30 volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30.

\* Trade-mark



seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. salmonicida* was carried in 2  
5 separate PCR reactions. PCR reaction 1 was performed using primer pairs,  
as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ  
ID No. 36]) and asls950new (5' GTG ATG GTG GGC GAG GAA CTC GTA CTG3'  
[SEQ ID No. 37]). A second PCR reaction was performed to incorporate a C-terminal  
Histidine tag using the PCR product from the first reaction and the primers:  
10 as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ  
ID No. 38]) and AHLS1001(5'TTGGATCC GAATTCAT CAATG GTG ATG GTG  
ATG GTG GGC3' [SEQ ID No. 39]). The PCR product from the second reaction was  
purified and digested with restriction enzymes NdeI and BamHI. 2 ug of pET 12a  
vector DNA was also digested with restriction enzymes NdeI and BamHI and treated  
15 with phosphatase. The restriction enzyme-treated pet12a and PCR product from  
reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche,  
Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells.  
Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

20 The T7 promoter primer (5'TAATACGACTCACTATAG3' [SEQ ID No. 40]) and the  
T7 terminator primer (5'CTAGTTATTGCTCAGCGG3' [SEQ ID No. 41]) were used  
to verify the sequences and the orientation of the cloned transferase genes in pET12a  
vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators  
Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter  
25 and terminator primers.

The construct shown in Figure 35 was used to transform competent bacterial host strain  
BL21(DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and  
used for expression analysis.



**Expression of the recombinant *Aeromonas salmonicida* lipid acyltransferase**

Quantification of enzyme activity towards lecithin was determined on cell extracts  
5 using Non-Esterified Fatty Acid (NEFA) kit (Roche,\* Switzerland).

In Figure 36, BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=  
pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at  
37°C until OD<sub>600</sub> = 0.6 to 1.0 is reached. The cultures are then induced using IPTG  
10 (0.4mM) and incubation was continued for the next 3 hours. Samples were taken at 0  
hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the  
NEFA kit and lecithin as substrate.

**Growth Optimisation for the production of more active enzymes**

BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM was  
15 grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different  
growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production  
of active lipid acyltransferase enzyme was when cultures are grown at 30°C as shown  
in Figure 37.

**20 Partial purification of recombinant *Aeromonas salmonicida* transferase**

Strain BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM  
was grown at 37°C & crude cell extracts were prepared by sonication. The  
recombinant enzyme was further purified from the sonicated crude cell extracts using  
the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit &  
25 Lecithin as substrate. Crude cell extracts from BL21(DE3)pLysS expressing active  
transferase incubated with the substrate lecithin and reaction mixture was analysed  
using thin layer chromatography showing the presence of degradation products (see  
Figure 38).

\* Trade-mark



Partial Purification of recombinant *Aeromonas salmonicidae* transferase. Strain BL21(DE3)pLysS harbouring the expression vector pet12-AsalGCAT=pSM was grown at 37°C and crude cell extracts were prepared by sonication. The recombinant enzyme were further purified from the sonicated crude cell extract using the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit and lecithin as substrate was tested (see Figure 39).

**EXAMPLE 2 Cloning and Expression of *Aeromonas hydrophila* transferase in *E.***

10 **coli**

*Aeromonas hydrophila* (ATCC # 7965) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNase A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector pet12a-A.h.GCAT=pSMa. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

25 **Construction of expression vector pet12a-A.h.GCAT- pSMa:**

For all DNA amplifications of the transferase gene from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x *pfu* buffer, 1ul each primer (50pmol/ul), 200 uMdNTP in a total reaction volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.



The PCR amplification of the transferase gene from *A. hydrophila* (ATCC # 7965) was carried out in 2 separate PCR reactions.

5 PCR reaction 1 was performed using primer pairs, AHUS1 (5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3', SEQ ID No. 42) and ahls950 (5'ATGGTGATGGTGGGCGAGGAAGTCTGACTG3', SEQ ID No. 43).

10 A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primer pairs:

AHUS1(5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3' SEQ ID No. 44, ) and

15 AHLS1001(5'TTGGATCCGAATTCATCAATGGTGATGGTGGGCG3' SEQ ID No. 45).

The PCR product from the second reaction was purified and digested with restriction enzymes NdeI and BamHI. 2 ug of pET 12a vector DNA was also digested with  
20 restriction enzymes NdeI and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

25

The T7 promoter primer (5'TAATACGACTCACTATAG3') and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3') were used to verify the sequences and the orientation of the cloned GCAT genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng  
30 plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.



The construct shown in Figure 40 was used to transform competent bacterial host strain BL21 (DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

#### 5 **Expression of the *Aeromonas hydrophila* transferase in BL21(DE3)pLysS**

The *E. coli* strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD<sub>600</sub> = 0.6 to 1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples  
10 where taken at 0hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate (Figure 41).

#### **Growth Optimisation for the production of more active enzymes**

BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was  
15 grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active GCAT enzyme was when cultures are grown at 30°C as shown in Figure 42.

#### **Partial purification of recombinant *A. hydrophila* transferase (GCAT)**

Strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT=pSMa  
20 was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity assay using the NEFA kit & Lecithin as substrate. (Figure 43).

#### 25 **EXAMPLE 3: Expression of *Aeromonas* transferases in *Bacillus subtilis* 163**

##### **Plasmid Construction**

Two different *Bacillus subtilis* expression vectors (pUB 110 & pBE5) were used for the heterologous expression of the *Aeromonas* genes in *Bacillus subtilis*. The pUB110 vector contains the alpha amylase promoter while the pBE vector has the P32 promoter



as the regulatory region for the expression of the fused *Aeromonas* genes. In pUB110, the first amino acid of the mature GCAT genes of *Aeromonas* were fused in frame with the last amino acid of the xylanase signal peptide sequence from *Bacillus subtilis* via the restriction site Nhe1, creating an additional 2 amino acids in front of the mature proteins. pBE5 contains the cgtase signal sequence fusion at the Nco1 site for secretion of the recombinant proteins into the culture filtrate.

PCR reactions were carried out to obtain the *Aeromonas* genes fuse in frame to the signal sequences of the pUB 110 and the pBE5 vectors. PCRs were performed using the following primer pairs for *A. hydrophila* gene:

PCR reaction 1: usAHncol (5'ATGCCATGGCCGACAGCCGTCCCGCC3', SEQ ID No. 46) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 47)

PCR reaction 2: US-AhnheI (5'TTGCTAGCGCCGACAGCCGTCCCGCC3', SEQ ID No. 48.) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3, SEQ ID No. 49)

PCRs were performed using the following primer pairs for *A. salmonicida* gene:

PCR reaction 3: US-Asncol (5'TTGCCATGGCCGACACTCGCCCCGCC3', SEQ ID No. 50) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 51)

PCR reaction 4: US-ASnheI (5'TTGCTAGCGCCGACACTCGCCCCGCC3', SEQ ID No. 52) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 53)

All the PCR products were cloned into PCR blunt II (TOPO vector) and sequenced with reverse & forward sequencing primers.

Clones from PCR reactions 1 & 3 were cut with Nco1 & BamHI and used as inserts for ligation to the pBE5 vector cut with Nco1/BamHI/phosphatase. Clones from PCR



reactions 2 & 4 were cut with Nhe1 & Bam H1 and used as inserts for ligation to the pUB vector that was cut with Nhe1/BamH1/phosphatase.

**5 Expression of the *Aeromonas* transferase genes in *Bacillus subtilis* and characterization of the enzyme activity.**

The acyl transferases from the two *Aeromonas* species have been successfully expressed in *E. coli* (results above). The *Bacillus* pUB110 & pBE5 gene fusion constructs were used to transform *Bacillus subtilis* and transformants were selected by  
10 plating on kanamycin plates. The kanamycin resistant transformants isolated and grown in 2xYT are capable of heterologous expression of the *Aeromonas* genes in *Bacillus*. The culture filtrates have digalactosyldiacylglycerol (DGDG) galactolipase activity, in addition to having both acyl transferase and phospholipase activities. The activity towards digalactosyldiacylglycerol (DGDG) was measured after 60 minutes of  
15 incubation of culture supernatant with the substrate, DGDG from wheat flour (obtainable from Sigma) as well as the activity towards lecithin as shown in Figure 44. *Bacillus* produced the enzyme after overnight (20-24 hours) to 48 hours of cultivation in the culture medium as a secreted protein. In some instances, the expression of the *Aeromonas* genes has been shown to interfere with cell viability and growth in  
20 *Bacillus* & *E. coli*, it is therefore necessary to carefully select expression strains and optimise the growth conditions to ensure expression. For example, several *Bacillus* host strains (B.s 163, DB104 and OS 21) were transformed with the expression vectors for growth comparison. B.s163 is transformable with the 2 *Aeromonas* genes and is capable of expressing active protein. DB104 is transformable with all the constructs  
25 but is only able to express *A. salmonicida* transferase.



**EXAMPLE 4: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *E.coli***

***E.coli* Fermentations:**

**Microorganisms**

- 5 Two strains of *Eschericia coli*, one containing an *Aeromonas hydrophila* (Example 2) lipid acyltransferase and two containing *Aeromonas salmonicida* lipid acyltransferases, (Example 1) were used in this study.

The *E. coli* strain containing the *A. hydrophila* gene was named DIDK0124 , and the  
10 *E. coli* strain containing the *A. salmonicida* gene was named DIDK0125. The fermentation with DIDK0124 was named HYDRO0303 and the fermentation with DIDK0125 was named SAL0302. The purified protein from HYDRO025 was named REF#138. The purified protein from HYDRO0303 was named REF#135.

15 **Growth media and culture conditions**

**LB-agar**

The LB agar plates used for maintaining the strains contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The agar plates were incubated at 30°C.

20

**LB shake flask**

The LB medium (50 mL pr shake flask) used for production of inoculum material for the bioreactor cultivations contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The shake flasks were inoculated  
25 from the LB agar plates, and incubated at 30°C and 200 rpm.

**Bioreactor cultivation**

The bioreactor cultivations were carried out in 6 L in-house built bioreactors filled with 4 L medium containing: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 8 g/L



KH<sub>2</sub>PO<sub>4</sub>, 0.9 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 40 g/L glucose monohydrate, 0.4 mL/ ADD APT® Foamstop Sin 260 (ADD APT Chemicals AG, Helmond, The Netherlands), 10 mg/L (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.7 mg/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 3 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 3 mg/L MnSO<sub>4</sub>·H<sub>2</sub>O, 10 mg/L EDTA, 0.1 mg/L NiSO<sub>4</sub>·6H<sub>2</sub>O, 0.1 mg/L CoCl<sub>2</sub>, 0.1 mg/L  
5 H<sub>3</sub>BO<sub>4</sub>, 0.1 mg/L KI, 0.1 mg/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1 g/L ampicillin and 35 mg/L chloramphenicol.

The bioreactors were inoculated with an amount of LB culture ensuring end of growth after approximately 20 hours of cultivation (calculated from the maximum specific  
10 growth rate of 0.6 h<sup>-1</sup>, the OD<sub>600</sub> of the LB shake flask and the final OD<sub>600</sub> in the bioreactor of approximately 20).

SAL0302 was inoculated with 10 mL of LB culture, and HYDRO0303 was inoculated with 4 mL of LB culture.

15

The bioreactors were operated at the following conditions: temperature 30°C, stirring 800-1000 rpm (depending on experiment), aeration 5 L/min, pH 6.9, pH control 8.75% (w/v) NH<sub>3</sub>-water and 2 M H<sub>2</sub>SO<sub>4</sub>. Induction was achieved by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM, when 0.4 moles (HYDRO0303)  
20 and 0.7 moles CO<sub>2</sub> was produced respectively.

### Harvest

The following procedure was used for harvest and homogenisation of the biomass:

- 1) The fermentation broth from the fermentations was centrifuged at 5000 × g and  
25 4°C for 10 minutes, and the supernatant was discharged. The biomass was stored at -20°C until use. The biomass was thawed and resuspended in 500 mL of 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 500 mM NaCl, 10 mM Imidazole and Complete (EDTA-free) protease inhibitor (Roche, Germany).
- 2) The suspended biomass was homogenized at 2 kbar and 4°C in a cell disrupter  
30 from Constant Systems Ltd (Warwick, UK).
- 3) The cell debris was removed by centrifugation at 10.000 × g and 4°C for 30 minutes followed by collection of the supernatant.



- 4) The supernatant was clarified further by centrifugation at 13.700× g and 4°C for 60 minutes, followed by collection of the supernatant.
- 5) The supernatant was filtered through 0.2 µm Vacu Cap filters (Pall Life Sciences, UK) and the filtrate was collected for immediate chromatographic purification.

#### Chromatographic purification of the Transferases

A column (2.5 x 10 cm) was packed with 50 ml of Chelating Sepharose\* ff. gel and charged with Ni-sulphate (according to the method described by manufacturer, Amersham Biosciences). The column was equilibrated with 200 ml of 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 500 mM NaCl, 10 mM Imidazole. 400 ml of crude was applied to the column at a flow rate of 5 ml/min. The column was then washed with 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 500 mM NaCl, 10 mM Imidazole until the UV<sub>280</sub> reached the base line. The GCAT was then eluted with 40 ml of 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 500 mM NaCl and 500 mM Imidazole.

#### EXAMPLE 5: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *Bacillus subtilis*.

##### 20 Fermentations

BAC0318-19, BAC0323-24

##### Microorganism

The microorganisms used in this study originate from transformation of a *Bacillus subtilis* host strain, #163 with a plasmid containing the gene encoding the *Aeromonas salmonicida* transferase inserted in the vector pUB110OIS. The expression of the gene is controlled by an alpha-amylase promoter, and the secretion of the transferase is mediated by the *B. subtilis* xylanase signal sequence (Example 3). The strains were named DIDK0138 (fermentation BAC0318-19) and DIDK0153 (fermentation BAC0323-24).

30

\* Trade-mark



**Growth media and culture conditions****Pre culture medium**

A shake flask (500 mL total volume, with baffles) was added 100 mL of a medium containing:

|   |                                 |        |
|---|---------------------------------|--------|
| 5 | NaCl                            | 5 g/L  |
|   | K <sub>2</sub> HPO <sub>4</sub> | 10 g/L |
|   | Soy flour                       | 20 g/L |
|   | Yeast extract, BioSpringer 106  | 20 g/L |
|   | Antifoam, SIN260                | 5 mL/L |

10

pH was adjusted to 7.0 before autoclaving

After autoclaving 6 mL 50% (w/w) Nutriose were added pr flask. Kanamycin was added at a concentration of 50 mg/L after autoclaving.

15

**Inoculation**

A pre culture shake flask was inoculated with frozen culture directly from a 25% (w/v) glycerol stock. The shake flask was incubated at 33°C and 175 rpm for approximately 16 hours, whereupon 50 mL was used to inoculate the fermentor.

20

*Fermentations*

The fermentations were carried out in 6 L in house built fermentors.

The batch medium (3 L) contained:

|    |                                                                 |          |
|----|-----------------------------------------------------------------|----------|
|    | Corn steep liquor (50% dw)                                      | 40 g/L   |
| 25 | Yeast extract BioSpringer 153 (50% dw)                          | 10 g/L   |
|    | NaCl                                                            | 5 g/L    |
|    | CaCl <sub>2</sub> , 2H <sub>2</sub> O                           | 0.25 g/L |
|    | Mn(NO <sub>3</sub> ) <sub>2</sub> , H <sub>2</sub> O            | 0.2 g/L  |
|    | Antifoam SIN260                                                 | 1 mL/L   |
| 30 | Kanamycin (filter sterilised to the fermentor after autoclaving | 50 mg/L  |

The feed contained:



160

Glucose monohydrate 540 g/kg

MgSO<sub>4</sub>, 7H<sub>2</sub>O 4.8 g/kg

Antofoam SIN260 4 mL/kg

5

Yeast extract, BioSpringer 153 (50% dw) 150 g/kg  
(autoclaved separately)

10 The feed in fermentation BAC0318 and BAC0323 was started based on the accumulated CO<sub>2</sub>, according to the equations below:

Feed – flow[g/h] = 0, AcCO<sub>2</sub> < 0.15

Feed – flow[g/h] = 2.85 + t · 1.54, AcCO<sub>2</sub> ≥ 0.15 and t < 12

Feed – flow[g/h] = 21.3, t > 12

t: time (hours) from the point when the accumulated CO<sub>2</sub> (AcCO<sub>2</sub>) reached 0.15 moles.

15 The feed in fermentation BAC0319 and BAC0324 was started based on the accumulated CO<sub>2</sub>, according to the equations below:

Feed – flow[g/h] = 0, AcCO<sub>2</sub> < 0.15

Feed – flow[g/h] = 2.0 + t · 1.08, AcCO<sub>2</sub> ≥ 0.15 and t < 12

Feed – flow[g/h] = 15, t > 12

t: time (hours) from the point when the accumulated CO<sub>2</sub> (AcCO<sub>2</sub>) reached 0.15 moles.

20

The pH was controlled at 7.0 by adding 12.5% (w/v) NH<sub>3</sub>-water or 2M phosphoric acid.

The aeration was 3 L/min corresponding to 1 vvm.

The temperature was 33°C.

25 The fermentor was equipped with two 8 cm Ø Rushton impellers placed with a distance of 10 cm.



**Harvest**

The biomass was removed by centrifugation at 16,000× g for 10 minutes at room temperature. The supernatant was filter sterilized, and the filtrate was used for purification and application tests.

**EXAMPLE 6. Application tests in egg yolk.**

- 10 In the following experiments the isolated transferase from *Aeromonas salmonicida* expressed in *E-coli* was tested in egg yolk alone and in egg yolk where a plant sterol had been added.

**Material**

- 15 Transferase from *Aeromonas salmonicida* REF#138  
Egg yolk: from fresh egg (hens eggs)  
Plant sterol:  $\beta$ -sitosterol, Sigma S 5753

- 20 TLC plates: Silica plates Merck nr. 1.05715.0001

**TLC analysis.**

TLC-plate was activated in a heat cupboard (110°C) for ½ h.

- 25 100 ml developing solvent was poured into a chromatography chamber with lid. The walls of the chamber were covered with filter paper (Whatman 2) in order to saturate the chamber with the solvent vapor.

- 30 The TLC-plate was placed in a frame and the sample was applied onto the TLC plate 2 cm from the bottom. The TLC plate was then placed in the TLC chamber with the developing solvent. When the developing solvent reached 14 cm from the bottom of



162

the plate. The TLC plate was taken out and dried in fume board, and then placed in the heat cupboard at 110 °C for 10 minutes.

5 The TLC-plate was then immersed in the developing reagent, and dried in the heat cupboard at 110 °C for 15 minutes

Developing solvent:

Nr. IV: Chloroform : Methanol : H<sub>2</sub>O ( 65:25:4 )

Nr. I : P-ether : MTBE : Acetic acid (60:40:1)

10

Developing Buffer (Vanadate-buffer):

32 g Na<sub>2</sub>CO<sub>3</sub> ad 300 ml H<sub>2</sub>O (1M)

18.2 g vanadate pentoxide (V<sub>2</sub>O<sub>5</sub>) is added and dissolved during gentle heating.

The solution is cooled to ambient.

15 Carefully 460 ml 2.5 M H<sub>2</sub>SO<sub>4</sub>. (460 ml H<sub>2</sub>O +61 ml H<sub>2</sub>SO<sub>4</sub>) is added  
Water is added to 1000 ml.

Phospholipase activity.

20 Substrate:

0.6% L- $\alpha$  Phosphatidylcholine 95% Plant ( Avanti #441601) + 0.4% Triton-X 100( Sigma X-100) + 5 mM CaCl<sub>2</sub> is dissolved in 0.05M HEPES buffer pH 7.

Procedure.

25 400  $\mu$ l substrate was added to an 1.5 ml Eppendorf tube and placed in a Eppendorf thermomixer at 30°C for 5 minutes.

To the time T= 0 50 $\mu$ l enzyme solution was added. Also a blank with water instead of enzyme was analysed.

30

The sample was mixed at 1000 rpm on Eppendorf Termomixer at 30°C for 10 minutes.



163

To the time T=10 min. The Eppendorf tube was placed in another termomixer at 99°C for 10 minutes to stops the reaction.

Free fatty acid in the samples were analyzed by using the NEFA kit from WAKO  
5 GmbH.

Enzyme activity PLU-7 pH 7 was calculated as micromole fatty acid produced per minute under assay conditions.

#### 10 Lipid extraction.

1 g egg yolk and 7.5 ml Chloroform:Methanol 2:1 was mixed on a Whirley and centrifuged at 750 x g for 10 minutes.

3ml of the chloroform phase was isolated and used for further lipid analysis.

15

#### Results:

The transferase (REF#138), from *Aeromonas salmonicida* expressed in *E-coli* was analysed for phospholipase activity as described above, and was  
also tested in egg yolk with and without  $\beta$ -sitosterol. The sample was stirred with a  
20 magnetic stirrer during the reaction. The experimental design is shown in Table 1

Table 1

| Test | Reaction time<br>at 37°C | Egg yolk | Sitosterol | Transferase #138 |
|------|--------------------------|----------|------------|------------------|
| Nr   | Minutes                  | gram     | mg         | Units            |
| 1    | 30                       | 1        | 40         |                  |
| 2    | 30                       | 1        | 40         | 0,75 PLU         |
| 3    | 30                       | 1        | 80         | 0,75 PLU         |
| 4    | 120                      | 1        | 40         | 0,75 PLU         |
| 5    | 120                      | 1        | 80         | 0,75 PLU         |
| 6    | 300                      | 1        | 40         | 0,75 PLU         |



164

|   |     |   |    |  |
|---|-----|---|----|--|
| 8 | 300 | 1 | 40 |  |
|---|-----|---|----|--|

The reaction was stopped by adding 7.5 ml Chloroform:Methanol (2:1) and mixed on a Whirley mixer for 30 seconds. The chloroform phase was isolated by centrifugation and 2 µl of the chloroform phase was transferred to a pre-activated silica TLC plate and eluted with developing solvent nr. I, and another TLC-plate in developing solvent IV.

The results from the TLC analysis are shown in Figures 45 and 46.

10

Transferase reaction with a transferase from *Aeromonas salmonicida* in egg yolk where plant sterol was added has shown that the enzyme transfers fatty acid from lecithin in the egg yolk to the cholesterol during formation of cholesteroles. The TLC chromatogram also indicated that part of the sterol added to egg yolk was transferred to sterol ester.

The amount of sterol ester relative to the amount of cholesterol ester formed during the reaction can be analysed by HPLC or GLC.

It is known that plant sterol esters reduce the absorption of cholesterol in the intestine. It is also indicated in the literature that cholesteroles are absorbed less than free cholesterol in the intestine. When a transferase and plant sterol is added to egg yolk a product with causes reduced cholesterol absorption is obtained, and at the same time lysolecithin is produced which improves the emulsification properties of the egg yolk. A further advantage of adding transferase and plant sterol to egg yolk is that plant sterol ester is ingested together with the natural available cholesterol, which is expected to have the highest effect on the reduction of cholesterol absorption.

30



**EXAMPLE 7: Modification of egg yolk by lipid acyl transferase from *Aeromonas salmonicida*.**

In accordance with the present invention it has now been shown that it is possible to  
5 produce lysolecithin from egg yolk without substantial free fatty acid formation by use  
of a transferase.

The lecithin content of egg yolk is an important emulsifier for the production of  
mayonnaise with the limitation that the mayonnaise is not heat stable. It has therefore  
10 been known for several years to use a phospholipase from pancreas to modify lecithin  
in egg yolk to lysolecithin, which is a more efficient emulsifier. The use of enzyme  
modified egg yolk in mayonnaise production contributes to better heat stability of the  
mayonnaise during pasteurisation. A limitation of using pancreas phospholipase in  
egg yolk is that the amount of free fatty acid also increases, which contributes to  
15 reduced oxidative stability because free fatty acids are more prone to oxidation than  
the corresponding ester. Free fatty acid may also contribute to a soapy off taste.

The transferase from *Aeromonas salmonicida* was successfully expressed in *B. subtilis*  
and fermented in lab scale as described in Example 5, purified by liquid  
20 chromatography and used to modify egg yolk lipids. The enzyme modified egg yolk  
was used to produce heat stable mayonnaise.

The transferase from *A. salmonicida* can be used to produce lysolecithin and  
cholesterolester in egg yolk without production of significant amounts of free fatty  
25 acids. That is to say without increasing or substantially increasing the free fatty acids  
in the foodstuff.

The enzyme modified egg yolk produced by transferase showed improved  
emulsification properties and can be used for heat stable mayonnaise.

30

This enzyme was highly functional in modification of egg yolk by catalysing the lipid  
transfer reaction between lecithin and cholesterol Figure 47.



This study further investigated the use of transferase for modification of egg yolk and the use of modified egg yolk in the production of heat stable mayonnaise.

- 5 This example describes the fermentation, isolation, and application of the transferase in egg yolks as well as the application of the enzyme modified egg yolk in mayonnaise. The example is divided into two parts:

**A. Application of transferase in egg yolk**

- 10 **B. Testing of enzyme modified egg yolk in mayonnaise**

**EXPERIMENTAL**

**A. Application**

- 15 ***Enzyme and substrate***

Transferase #178-9 from *A. salmonicida*, purification 2554-100 C73, 15 PLU-7/ml.

Transferase #179 from *A. salmonicida*, 18.5 PLU-7/ml.

Phospholipase A1 LECITASE<sup>TM</sup> Ultra (Novozymes A/S, Denmark)

Egg yolk: Liquid egg yolk with 8% salt, SANOVA FOODS, DK

20

TLC analysis was performed as described previously (see above Example 6).

Phospholipase activity: See previous examples.

- 25 ***Lipid extraction***

1 g egg yolk and 7.5 ml Chloroform:Methanol 2:1 was mixed on a Whirley for 30 sec. and centrifuged at 750 x g for 10 minutes.

4 ml of the chloroform phase was isolated and used for further lipid analysis.

- 30 **Oxidation stability test**



Oxidation stability of mayonnaise was measured in an ML OXIPRESS equipment where the sample is oxidative stressed by means of heat under pressure in an oxygen atmosphere.

After a certain time, called the induction period (IP), the oxidation of the sample causes a certain consumption of oxygen, which is registered as pressure change of a pressure transducer. Higher induction period indicates better oxidation stability.

Procedure.

5-gram mayonnaise is placed in a glass container and the glass container is closed with the pressure transducer. The container is filled with oxygen to 5 bars. The valve is opened to empty the container. This procedure is repeated twice and the sample with 5 bar oxygen atmosphere is placed at 80 °C. The oxygen pressure as a function of time is measured and the induction period (IP) calculated in hours.

## 15 **Results**

Purified transferase from *Aeromonas salmonicida* sample no. #179 and #178-9 were used to treat egg yolk as outlined in Table 2. The initial test has shown that GCAT transferase should be added with much lower phospholipase (PLU) activity, than a commercial Phospholipase. This is explained by the fact that GCAT is a transferase and therefore has much lower hydrolytic activity than a normal phospholipase.

**Table 2**

|    |                           |                              |                       |                           |       |          |
|----|---------------------------|------------------------------|-----------------------|---------------------------|-------|----------|
|    | Sanofe egg<br>yolk 8%salt | 2344-44 C89<br>18,5 PLU-7/ml | Transferase<br>#178-9 | # 3108,<br>Lecitase Ultra | Water |          |
| nr | Egg yolk                  | Transferase<br>#179          | 18.5 PLU-7/ml         | 1500 PLU-<br>7/ml<br>7/ml |       |          |
|    | gram                      | gram                         | gram                  | ml                        | gram  | PLU-7/ml |
| 6  | 120                       | 2.00                         |                       |                           | 8.00  | 0.31     |
| 7  | 120                       |                              | 10                    |                           | 0     | 1.25     |



|   |     |  |  |      |      |       |
|---|-----|--|--|------|------|-------|
| 8 | 120 |  |  | 1.86 | 8.14 | 23.25 |
| 9 | 120 |  |  |      | 10   | 0     |

The enzymatic reactions were conducted by scaling the egg yolk and the enzyme in a beaker. The samples were placed in a heating cabinet at 37 °C during slow agitation. After 1, 2 and 4 hours reaction time a sample was taken out for TLC analysis. After 4  
5 hours reaction time the product was stored at 5 °C and used for mayonnaise experiments.

The TLC analyses of lipids extracted from enzyme treated egg yolk is shown in Figure 48.

10

The TLC analysis in Figure 48 shows a clear hydrolytic effect of Phospholipase #3108 on triglyceride during formation of free fatty acids, as well as some mono- and diglyceride. Phospholipase #3108 seem to have no effect on cholesterol. Both transferase samples clearly contribute to the formation of cholesterol ester concomitant  
15 with the reduction of the cholesterol content.

#### **D. Enzyme modified egg yolk in Mayonnaise**

In order to investigate the effect of the modification of the egg yolk samples mentioned in Table 2, application trials were performed on mayonnaise with a fat  
20 content of 50%. A mayonnaise containing untreated egg yolk was also produced.

The aim of the investigation was to determine the impact of enzymatically modified egg yolks' emulsification properties and the impact on heat stability. All mayonnaise  
25 samples contained the same oil level and were emulsified with only egg yolk.

The mayonnaise samples were all produced using a Koruma mixer (Disho V60/10) and heated during processing to 95°C for 5 minutes.



Samples of the mayonnaises (Figure 49) produced by enzyme treated egg yolk were nice and homogenous with no oil separation. The control sample separated in an oil and a water phase.

- 5 The particle size of oil droplet in the mayonnaise samples with enzyme treated egg yolk was measured on a Malvern Mastersizer. The sample was mixed with 0.1%SDS in 0.1 M phosphate buffer pH 7 prior to measurement. Reading was mean size of all particles as shown in Table 3.

10 **Table 3.**

| Experiment | Enzyme                            | Mean particle size, $\mu\text{m}$ |
|------------|-----------------------------------|-----------------------------------|
| 6          | Transferase #179, 0.31 PLU-7/g    | 12.9                              |
| 7          | Transferase #178-9, 1.25 PLU-7/g  | 7.2                               |
| 8          | #3108, Lecitase Ultra, 23 PLU-7/g | 5.2                               |

- The results from the particle size measurement clearly show the effect of increased dosage of transferase from *A. salmonicida*. With the high dosage of transferase the particle size is close to the mayonnaise produced by Lecitase Ultra. It should however be kept in mind that Lecitase Ultra produces a lot of fatty acids, which might contribute to a finer particle distribution.
- 15

- The oil droplet size of the mayonnaise prepared with the enzyme is significantly smaller than the oil droplet size of the mayonnaise prepared without the enzyme (i.e. the control mayonnaise).
- 20

#### Oxidation stability

The oxidation stability of the mayonnaise samples 7 and 8 were analyzed on a ML OXIPRES with results shown in Table 4.



**Table 4**

| Sample | Induction period          | Induction period          |
|--------|---------------------------|---------------------------|
|        | 1. determination<br>hours | 2. determination<br>hours |
| 7      | 37.44                     | 38.08                     |
| 8      | 35.68                     | 35.52                     |

- 5 Measurement of oxidation stability gave a clear significant difference in oxidation stability. The mayonnaise with transferase 179-8 treated egg yolk had a significant better oxidation stability than the mayonnaise with Lecitase Ultra treated egg yolk. This might be explained by the fact that Lecitase Ultra produces more free fatty acids which are more prone to oxidation than the corresponding fatty acid esters.

10

A sample of the egg yolks used for mayonnaise production were extracted with chloroform, and the lipids from the egg yolk were analysed by GLC with results shown in Table 5.

15

**Table 5**

| Experiment | Enzyme                   | Fatty acid | Cholesterol | Cholesterol ester | Triglyceride |
|------------|--------------------------|------------|-------------|-------------------|--------------|
| 6          | Transferase #179         | 0.96       | 0.94        | 0.49              | 23.95        |
| 7          | Transferase #178-9       | 1.84       | 0.60        | 1.06              | 24.54        |
| 8          | #3108, Lecitase<br>Ultra | 14.05      | 1.16        | 0.12              | 2.45         |
| 9          | Control                  | 0.48       | 1.16        | 0.13              | 22.87        |

- The GLC results in Table 5 confirm the results from the TLC analysis that Lecitase  
20 Ultra produces a very high amount of free fatty acids and a large part of the



triglyceride is hydrolysed. On the other hand the transferase produces only modest amount of free fatty acids and no triglycerides are hydrolysed. It is also clearly shown that transferase produce cholesterol ester from cholesterol.

5

The results indicate that the amount of PC in the “enzyme treated” mayonnaise is reduced as compared with the control mayonnaise, whilst the amount of LPC is increase in the enzyme treated mayonnaise as compared with the control mayonnaise. The increase in the amount of LPC may well explain the improved emulsification properties of the enzyme treated mayonnaise as compared with the control mayonnaise. The HPLC and GLC analyses also indicate a lower level of free cholesterol in the enzyme treated mayonnaise as compared with the control mayonnaise, probably due to the cholesterol being used as an acceptor molecule in the transferase reaction resulting in an increase in the amount of cholesterol esters in the enzyme treated mayonnaise as compared with the control mayonnaise. In addition, the results indicate that the amount of free fatty acids do not increases significantly when egg yolk is treated with the transferase. The results further indicate that the amount of free fatty acids produced in the foodstuff treated with the lipid acyltransferase is significantly lower than in the foodstuff treated with the control phospholipase, this is true even if the amount of lysolecithin formed in the foodstuffs is the same.

20

**EXAMPLE 8: Effect of *Aeromonas salmonicida* transferase in cakes.**

25

The effect of GCAT acyl-transferase form *Aeromonas salmonicida* is tested in a cake recipe. The enzyme is tested alone and in combination with other lipolytic enzymes. The enzymes are added to some of the cake ingredients or added together with the other cake ingredients before mixing the cake.

30

Preliminary results show that acyl-transferase combined with a triglyceride-hydrolysing enzyme improves the cake volume and crumb structure compared with a control.



In the following experiments a transferase from *A. salmonicida* and variants are tested alone and in combination with triglyceride hydrolysing enzymes. These enzymes are active on the lipid components in the egg and the shortening as well as on the carbohydrates, protein, glycerol and cholesterol (in egg), which forms part of the cake recipe.

#### Materials and method

##### Enzyme

#179, Acyl-transferase from *Aeromonas salmonicida*

Grindamyl EXEL 16, Lipase from *Thermomyces lanuginisus*

#### 15 Cake recipe:

| Ingredients                      | %     | g   |
|----------------------------------|-------|-----|
| Sugar 35/20                      | 20,37 | 204 |
| Cake flour, Albatros             | 18,11 | 181 |
| Wheat starch                     | 5,21  | 52  |
| Baking powder                    | 0,36  | 4   |
| Pasteurised liquid whole egg     | 22,63 | 226 |
| Shortening Vegao (Aarhus United) | 18,11 | 181 |
| Whey powder                      | 0,68  | 7   |
| Glucose sirup ,75% 42 DE         | 4,53  | 45  |
| Glycerol                         | 1,36  | 14  |
| Salt                             | 0,32  | 3   |
| Rape seed oil                    | 6,34  | 63  |
| Potassium sorbate                | 0,18  | 1,8 |



**Equipment:**

Mixer: Hobart N50 with a spatula

5 Oven: Simon cake oven

**Procedure:**

10

All ingredients must be tempered to room temperature.

15

1. Cream up sugar and shortening for 3 minutes – start at 2<sup>nd</sup> speed and move to 3<sup>rd</sup> speed within 30 sec

2. Add remaining ingredients – start at 1<sup>st</sup> speed and move to 2<sup>nd</sup> speed within 30 sec – mix total 5 min

3. Measure the volume of the batter in 1 dl cup

4. The pound cake tins are sprayed with “Babette” oil spread, and covered with paper

20

5. Scale 2 x 350 g into the pound cake tins

6. Spread out the mass evenly with a spatula

7. Before put in the oven – add a string of oil on top of the cake (lengthwise in the middle – to make the cake break in the middle

8. Bake for 50 min. at 180°C

25

9. After baking take the tins out of the oven, and “drop” it on the table, before taking the cakes out of the tins

10. Take paper off the cakes and turn the right side up

30

11. The cakes are cooled on a grating for 60 min. before weighing and measuring of the volume



**Remarks:**

The enzyme(s) used is/are added at the beginning of mixing or is/are added to some of the cake ingredients before added to the other cake ingredients.

- 5 The enzymes are only active during the mixing or reaction of cake components, and the enzymes are inactivated during baking of the cake.

10 **Results.**

The following experiments are conducted as shown in the following table:

|                                  |    | 1   | 2    | 3    | 4   |
|----------------------------------|----|-----|------|------|-----|
| Whole egg                        | G  | 250 | 250  | 250  | 250 |
| Glucose syrup, 75% DE 42         | G  | 10  | 10   | 10   | 10  |
| #179 acyl-transferase, 26 PLU/ml | MI | 25  |      | 25   |     |
| Grindamyl EXEL 16,               | Mg |     | 37,5 | 37,5 |     |
| Water                            |    |     |      |      | 25  |

- 15 Egg, Glucose syrup and enzyme are reacted for 30 minutes at 37 °C and shortly after the eggs are use to produce cake according to the recipe mentioned above.

- Preliminary results show that a combination of acyltransferase and a triglyceride hydrolysing lipase from *Thermomyces lanoginosus* improves the cake volume, and also the crumb structure, eating quality and appearance is improved compared with a water control. Preliminary results indicate in cake it may be preferably to use a combination of lipid acyltransferase and a lipase.
- 20



**EXAMPLE 9: The purpose of these experiments was to test a transferase from *A. hydrophila* expressed in *E. coli*.**

The transferase reaction of *A. hydrophila* #135 (0.5 NEFA-PLU/ml) was tested in egg  
5 yolk . The experimental set-up is shown in Table 6.

Table 6

|    | Reaction time | Egg yolk | #135 conc.      |
|----|---------------|----------|-----------------|
| Nr | Minutes       | Gram     | Units, PLU-NEFA |
| 1  | 30            | 1        | 0,000           |
| 2  | 30            | 2        | 0,100           |
| 3  | 60            | 2        | 0,100           |
| 4  | 150           | 2        | 0,100           |
| 5  | 240           | 2        | 0,100           |
| 6  | 1560          | 2        | 0,100           |
| 7  | 1560          | 1        | 0,000           |

The egg yolk was heated to 37°C and the enzyme added. After reaction time 7 ml  
10 CHCl<sub>3</sub>:Methanol 2:1 was added and mixed on a Whirley for 30 sec.

The sample was centrifuged 800 x g for 10 minutes and the lower solvent phase  
isolated. 2 µl of this sample was applied onto a TLC Silica plate and eluted in elution  
solvent IV. The results from the TLC analysis is shown in Figures 50 and 51.

15

The methods and materials mentioned in this Example are those detailed in Examples  
above.

Samples from this experiment was also analysed by GLC as TMS derivatives. The  
20 results from the GLC analysis are shown in Table 7.



**Table 7.** GLC analysis of lipid from egg yolk

| No. | Reaction | Transferase<br>#135 conc. |                 |             |                   |
|-----|----------|---------------------------|-----------------|-------------|-------------------|
|     | time     | Units/g egg yolk          | Free fatty acid | Cholesterol | Cholesterol ester |
|     | min      |                           | %               | %           | %                 |
| 7   | control  | 0                         | 0,25            | 2,88        | 0,34              |
| 3   | 60       | 0,025                     | 0,25            | 2,68        | 0,56              |
| 4   | 150      | 0,025                     | 0,29            | 1,85        | 1,72              |
| 5   | 240      | 0,025                     | 0,53            | 1,42        | 3,54              |
| 6   | 1560     | 0,025                     | 0,95            | 0,3         | 4,43              |

From the GLC analysis of free fatty acid, cholesterol and cholesterol ester it is possible  
 5 to calculate the molar concentration of each component and calculate % transferase  
 activity as shown in Table 7.

#### Calculation of % transferase activity

From the results the increase in free fatty acid, sterol esters are calculated

10  $\Delta \% \text{ fatty acid} = \% \text{ Fatty acid(enzyme)} - \% \text{ fatty acid(control)}$

$\Delta \% \text{ sterol ester} = \% \text{ sterol/sterol ester(enzyme)} - \% \text{ sterol/sterol ester(control)}$

The transferase activity is calculated as % of the total enzymatic activity:

15  $\% \text{ transferase activity} =$

$$\frac{(\Delta \% \text{ sterol ester}/(\text{Mv sterol ester}) \times 100}{\Delta \% \text{ sterol ester}/(\text{Mv sterol ester}) + \Delta \% \text{ fatty acid}/(\text{Mv fatty acid})}$$

$\Delta \% \text{ sterol ester}/(\text{Mv sterol ester}) + \Delta \% \text{ fatty acid}/(\text{Mv fatty acid})$

20 where:

Mv sterol ester = average molecular weight of the sterol esters

Mv fatty acid = average molecular weight of the fatty acids



Table 8 Transferase activity in egg yolk of *A. hydrophila* #135

| No. | Reaction | Transferase<br>#135 conc. |                    |             |                       |                         |
|-----|----------|---------------------------|--------------------|-------------|-----------------------|-------------------------|
|     | Time     | Units/g egg yolk          | Free fatty<br>acid | Cholesterol | Cholesterol-<br>ester | Transferase<br>activity |
|     | min      |                           | mM                 | mM          | mM                    | %                       |
| 7   | Control  | 0                         | 8,9                | 74,5        | 5,3                   | -                       |
| 3   | 60       | 0,05                      | 8,9                | 69,3        | 8,7                   | 100                     |
| 4   | 150      | 0,05                      | 10,4               | 47,8        | 26,5                  | 93                      |
| 5   | 240      | 0,05                      | 18,9               | 36,7        | 54,6                  | 77                      |
| 6   | 1560     | 0,05                      | 33,9               | 7,8         | 68,4                  | 48                      |

5

Both TLC and GLC analysis confirm that initially the transferase reaction of *A. hydrophila* #135 is the dominating reaction. After 150 minutes reaction time some hydrolytic activity occurs. After 1560 minutes the transferase reaction and the hydrolytic reaction has almost reached the same level. The results also indicate that as long as the acceptor molecule cholesterol is available the transferase reaction is the dominating reaction. When the concentration of cholesterol decreases the hydrolytic activity becomes more dominant.

10

**EXAMPLE 10: Assay for measurement of transferase activity using egg yolk as substrate – hereinafter referred to as the “Egg Yolk Assay”**

15

A lipid acyltransferase was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. The purpose of this work is to develop an analytical method, which is able to measure both transferase and hydrolytic activity of enzymes and from these analyses it is possible to define both transferase and hydrolytic activity of enzymes using a substrate which contain lecithin and cholesterol.

20



In this work egg yolk was used as substrate for the enzyme assay because egg yolk contain both lecithin and cholesterol and it is known that transferases and phospholipases works very well in this substrate.

- 5 The drawback by using egg yolk is that this substrate is a complex mixture of water, lipids, and proteins. Lipid components include glycerides, 66.2%; phospholipids, 29.6%; and cholesterol, 4.2%. The phospholipids consist of 73% lecithin, 15% cephalin , and 12% other phospholipids. Of the fatty acids, 33% are saturated and 67% unsaturated, including 42% oleic acid and 7% linoleic acid (ref. Kirk-Othmer  
10 Encyclopedia of Chemical Technology, John Wiley & Sons, Inc.)

Some variations in the egg yolk composition might be expected. In the literature (Biochimica et Biophysica Acta, 1124 (1992) 205-222) it is however mentioned that  
15 “The mature egg yolk of the domestic hen possesses remarkably constant lipid and lipoprotein composition despite much variation in dietary and environmental conditions”, and further it is quoted “As a result the egg yolk continues to provide a food product of nearly constant composition, which serves to maintain its chemical and physical-chemical properties for reliable utilization in the baking, cosmetic and pharmaceutical industries”

20

This reference indicates that egg yolk composition is very constant and it was therefore decided to use hens egg yolk as substrate for the Egg Yolk Assay.

Quantification of lipid reaction products from enzymatic treatment of egg yolk was  
25 made by extraction of lipids from the substrate followed by GLC analysis of the lipid components.

## Procedure

### 30 Materials.

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde.

HEPES buffer Sigma cat. no. H 3375



Chloroform, Analytical grade

Enzymes.

Purified lipid acyltransferase from *A. salmonicida* #178-9

- 5 *Thermomyces lanuginosus* lipase. GRINDAMYL EXEL 16 , item nr. 147060  
(Control)

Enzyme assay with egg yolk substrate.

- 10 5 gram liquid egg yolk was scaled in a 20ml Wheaton glass and heated to 35 °C  
0.25ml enzyme solution was added and a stopwatch is started.  
At regular intervals 0.5g samples were transferred to a 10ml Dram glass.  
20 µl 4M HCl was added in order to stop the enzyme reaction and acidify the fatty  
acid soap.
- 15 3 ml Chloroform was added. And the sample was mixed on a Whirley mixer for 30  
sec.  
The sample was centrifuged at 3000 g for 10 min and 0.8 ml of the chloroform phase  
was transferred to a tarred Dram glass. Chloroform was evaporated at 60 °C under a  
steam of nitrogen. The dram glass was scaled again.
- 20 The isolated lipids were analysed by GLC and TLC.

TLC analysis – as described herein.

GLC analysis – as described herein.

25

## Results

For the Egg Yolk Assay using egg yolk as substrate the experiment shown in Table 9  
was conducted.

30



**Table 9**

|                                   |      | <b>1</b> | <b>2</b> | <b>3</b> |
|-----------------------------------|------|----------|----------|----------|
| Egg yolk, liquid.                 | gram | 5        | 5        | 5        |
| Transferase# 178-9, 32 PLU-7/ml*  | ml   |          |          | 0.25     |
| T.lanuginosus lipase, 200 LIPU/ml | ml   |          | 0.25     |          |
| Water                             | ml   | 0.25     |          |          |

0.5g samples were taken out after 15, 30, 60 120 and 1080 minutes, and the lipid isolated by solvent extraction. The lipids were analysed by TLC using solvent I and IV  
5 respectively. Picture of the TLC plate is shown in Figure 52.

The TLC analysis clearly indicates the activity of transferase #178-9 from *A. salmonicida* (sample 3). This can be seen from the decrease in the phospholipids PC and PE. The results also indicate that the amount of lysolecithin LPC is not as high as  
10 expected. This might indicate hydrolytic activity on lysolecithin or it might also be caused by insufficient extraction because lysolecithin is very polar and therefore could be partly distributed in the water phase.

The lipids isolated by solvent extraction was also analysed by GLC in order to  
15 quantify the amount of free fatty acid, cholesterol and cholesterol ester. The GLC results are shown in Table 10.



Table 10. GLC analysis of lipid from enzyme treated egg yolk. Results are in % based on lipid content.

|                   |                       |   | 15      | 30      | 60      | 120     | 1080    |
|-------------------|-----------------------|---|---------|---------|---------|---------|---------|
|                   |                       |   | Minutes | Minutes | Minutes | Minutes | Minutes |
| Free fatty acids  | Control               | 1 | 0.328   | 0.304   | 0.332   | 0.333   | 0.369   |
|                   | T. lanuginosus        | 2 | 0.391   | 0.376   | 0.459   | 0.627   | 22.909  |
|                   | A. salmonicida #178-9 | 3 | 1.007   | 1.668   | 4.013   | 6.761   | 15.098  |
|                   |                       |   |         |         |         |         |         |
| Cholesterol       | Control               | 1 | 3.075   | 2.968   | 3.103   | 3.056   | 3.099   |
|                   | T. lanuginosus        | 2 | 3.130   | 3.032   | 3.045   | 3.026   | 3.225   |
|                   | A. salmonicida #178-9 | 3 | 2.835   | 1.912   | 0.356   | 0.220   | 0.206   |
|                   |                       |   |         |         |         |         |         |
| Cholesterol ester | Control               | 1 | 0.416   | 0.397   | 0.422   | 0.408   | 0.437   |
|                   | T. lanuginosus        | 2 | 0.436   | 0.400   | 0.425   | 0.419   | 0.416   |
|                   | A. salmonicida #178-9 | 3 | 1.414   | 2.988   | 6.107   | 6.694   | 5.760   |
|                   |                       |   |         |         |         |         |         |
| Triglyceride      | Control               | 1 | 76.153  | 73.505  | 75.565  | 79.344  | 77.382  |
|                   | T. lanuginosus        | 2 | 74.099  | 74.413  | 77.079  | 74.284  | 21.781  |
|                   | A. salmonicida #178-9 | 3 | 73.781  | 73.342  | 77.857  | 82.040  | 72.117  |

- 5 From the results it was observed that almost all the cholesterol was esterified after 60 minutes in sample 3. It was concluded that for the first 30 minutes there was surplus substrate for the reaction. The results from samples taken out after 15 and 30 minutes were therefore used to calculate the activities of the enzymes.
- 10 Based on the information in table 10 and the fact that egg yolk contain 27% lipid the amount of micromole fatty acid and cholesterol ester produced per ml enzyme was calculated with results shown in Table 11



182

The results in Table 11 were obtained by the following calculations of the results from fatty acids in sample no.3 (*A. salmonicida*, 15 min.)

Lipid in 5 g egg yolk =  $5 \times 0.27 = 1.35$  gram

5 1.35 gram lipid contain 1.007% fatty acids =  $1.35 \times 1.007 / 100 = 0.01359$  gram

Average molecular weight of fatty acids is 272

$0.01359 \text{ gram} = 0.01359 \times 1000000 / 272 \text{ } \mu\text{mol} = 49.9798 \text{ } \mu\text{mol}$

0.25 ml enzyme is added

$\mu\text{mol Fatty acid/ml enzyme} = 49.9798 / 0.25 = 199.9$

10

**Table 11**

| Micromole/ml enzyme |                      |       |        |        |
|---------------------|----------------------|-------|--------|--------|
|                     |                      | 0 min | 15 min | 30 min |
| Free fatty acid     | Control              |       | 65.01  | 60.37  |
|                     | <i>T. lanuginosa</i> |       | 77.61  | 74.71  |
|                     | Transferase #178-9   |       | 199.86 | 331.06 |
|                     |                      |       |        |        |
| Cholesterol ester   | Control              |       | 35.09  | 33.50  |
|                     | <i>T. lanuginosa</i> |       | 36.77  | 33.73  |
|                     | Transf. #178-9       |       | 119.29 | 252.15 |

From the results in Table 11 it is possible to calculate the change in amount of fatty acid and cholesterol ester caused by the enzyme relative to control as shown in Table

15 12.



**Table 12.**

| $\Delta$ Micromole/ml enzyme |                | 0 min | 15 min  | 30 min  |
|------------------------------|----------------|-------|---------|---------|
| Free fatty acid              | T. lanuginosus | 0     | 12.593  | 14.340  |
|                              | Transf. #178-9 | 0     | 134.843 | 270.691 |
|                              |                |       |         |         |
| Cholesterol ester            | T. lanuginosus | 0     | 1.677   | 0.235   |
|                              | Transf. #178-9 | 0     | 84.196  | 218.652 |

The amount of fatty acid or cholesterol ester produced as a function of time is shown in Figure 53.

5

From the slope of the curve the hydrolytic activity (FFA formation) and the lipid acyltransferase activity (cholesterol ester formation) as a function of time was calculated. The relative transferase activity (% acyltransferase activity) and the relative hydrolytic activity were then calculated as shown in Table 13. The relative transferase activity was determined using the protocol for the determination of % acyltransferase activity as described hereinbefore. For example, calculation of relative activity for #178-9: Total activity is FFA activity + transferase activity = 9,023+7,2884= 16,311  $\mu\text{mol}/\text{min}/\text{ml}$ , Relative transferase activity=  $7,2884 \times 100 / 16,311 = 44,7$ , Relative hydrolytic activity=  $9,023 \times 100 / 16,311 = 55,3$

15

**Table 13.**

|                       |                             |        |                                      |
|-----------------------|-----------------------------|--------|--------------------------------------|
| T. lanuginosus        | FFA activity                | 0.4780 | $\mu\text{mol}/\text{min}/\text{ml}$ |
| A. salmonicida #178-9 | FFA activity                | 9.0230 | $\mu\text{mol}/\text{min}/\text{ml}$ |
|                       |                             |        |                                      |
| T. lanuginosus        | Cholesterol ester. Activity | 0.0078 | $\mu\text{mol}/\text{min}/\text{ml}$ |
| A. salmonicida #178-9 | Cholesterol ester. Activity | 7.2884 | $\mu\text{mol}/\text{min}/\text{ml}$ |
|                       |                             |        |                                      |
| T. lanuginosus        | Relative transferase        | 1.6    |                                      |



|                       |                              |      |  |
|-----------------------|------------------------------|------|--|
|                       | activity                     |      |  |
| A. salmonicida #178-9 |                              | 44.7 |  |
|                       |                              |      |  |
| T. lanuginosus        | Relative hydrolytic activity | 98.4 |  |
| A. salmonicida #178-9 |                              | 55.3 |  |

The results in Table 13 confirmed that the transferase enzyme from *A. salmonicida* has a significant transferase activity, but the results also confirmed that this enzyme has a significant hydrolytic activity.

5

The lipase from *T. lanuginosus* mainly has hydrolytic activity, and the relative transferase activity 1.6 was not a proof of any transferase activity but was explained by the uncertainty of the analysis.

## 10 Conclusion.

Egg yolk was used as substrate for the measurement of transferase and hydrolase activity of lipid acyltransferase from *Aeromonas salmonicida* and a lipase from *Thermomyces lanuginosus*. Under assay conditions there was initially a linear relation between cholesterol ester and free fatty acid formation and time for the lipid acyltransferase enzyme. Based on this linear relationship it was possible to calculate the hydrolytic activity (FFA formation) and the transferase activity (cholesterol ester formation). The relative hydrolytic and transferase activity was also calculated. The lipid acyltransferase (in this case a GCAT) from *Aeromonas salmonicida* showed almost equal hydrolytic and transferase activity under these assay conditions.

15

Lipase from *Thermomyces lanuginosus* showed very low hydrolytic activity and the transferase activity was not significant.

20

## **EXAMPLE 11: Transferase Assay in High Water Egg Yolk.**

25

### **Introduction**



A lipid acyltransferase in accordance with the present invention was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. Initial experiments have shown that this enzyme is very efficient in transferring fatty acid from lecithin to cholesterol using egg yolk as a substrate.

In the following experiments the transferase reaction was studied in further detail using egg yolk as a substrate with special focus on the water concentration in the substrate.

## 10 Procedure

### Materials.

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde.

HEPES buffer Sigma cat. no. H 3375

15 Chloroform, Analytical grade

Squalane, analytical grade

### Enzymes.

#178-9 Lipid acyl transferase in accordance with present invention from *A. salmonicida*

#2427 Phospholipase A1 from *Fusarium oxysporum*. LIPOPAN<sup>®</sup> F from Novozymes, DK (comparative lipolytic enzyme)

#1991 Phospholipase A2 from Pancreas, LIPOMOD 22L from Biocatalysts, UK (comparative lipolytic enzyme)

25

### Enzyme assay with egg yolk substrate.

5 gram liquid egg yolk substrate was scaled in a 20ml Wheaton glass and heated to 35 °C

Water and enzyme solution was added and a stopwatch is started.

At regular intervals 0.5g samples was transferred to a 10ml Dram glass.



20 µl 4M HCl was added in order to stop the enzyme reaction and acidify the fatty acid soap.

3 ml Chloroform was added. And the sample was mixed on a Whirley mixer for 30 sec.

- 5 The sample was centrifuged at 3000 g for 10 min and 0.8 ml of the chloroform phase was transferred to a tarred Dram glass. Chloroform was evaporated at 60 °C under a steam of nitrogen. The dram glass is scaled again.

The isolated lipids are analysed by GLC

## 10 GLC analysis

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm ID x 0.1 µ film thickness 5% phenyl-methyl-silicone (CP Sil 8 CB from Chrompack).

- 15 Carrier gas: Helium.

Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0µl

Detector FID: 395°C

Oven program:

|                           | 1  | 2   | 3   |
|---------------------------|----|-----|-----|
| Oven temperature, °C.     | 90 | 280 | 350 |
| Isothermal, time, min.    | 1  | 0   | 10  |
| Temperature rate, °C/min. | 15 | 4   |     |

Oven temperature, °C.

90 280 350

- 20 Isothermal, time, min.

1 0 10

Temperature rate, °C/min.

15 4

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml. 300µl sample solution was transferred to a crimp vial, 300 µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) was added and reacted for 20 minutes at 60°C.

- 25 Calculation: Response factors for mono-di-triglycerides and free fatty acid were determined from Standard 2 (mono-di-triglyceride), for Cholesterol, Cholesteryl palmitate and Cholesteryl stearate the response factors were determined from pure reference material (weighing for pure material 10mg).
- 30

## Results



Egg yolk containing 2% squalane was used as substrate for the reactions. Squalane was added as an internal standard for the GLC analysis, in order to quantify the lipid components in egg yolk.

- 5 The experiment was set up as shown in Table 14.

**Table 14.**

|                                              |    | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> |
|----------------------------------------------|----|----------|----------|----------|----------|----------|----------|----------|----------|
| Substrate, egg yolk with 2% squalane         | g  | 5        | 5        | 5        | 5        | 5        | 5        | 2.5      | 2.5      |
| Transferase # 178-9 , 14 PLU-7/ml            | ml |          | 0.25     |          |          | 0.25     |          | 0.13     |          |
| LIOPAN <sup>®</sup> Fsolution , 200 PLU-7/ml | ml |          |          | 0.25     |          |          |          |          | 0.13     |
| #1991 Phospholipase A2, 6300 PLU/ml          | ml |          |          |          | 0.25     |          | 0.25     |          |          |
|                                              |    |          |          |          |          |          |          |          |          |
| Water                                        | ml | 0.25     |          |          |          | 3.8      | 3.8      | 8.75     | 8.75     |

- 10 Samples were taken out after 30, 60 and 120 minutes and analysed according the method described above (0.5 ml (exp 1-4) 0.86 ml (exp. 5-6) and 2.2 ml(exp.7-8) samples were taken).

- 15 The results from the GLC analysis are shown in Table 15. The GLC results were expressed in percent of the substrate (egg yolk). The table also indicate the reaction time and the total amount of water in the reaction mixture.

**Table 15.**

| Enzyme  | Reaction time | Water %     | GLC          | GLC           | GLC                 |
|---------|---------------|-------------|--------------|---------------|---------------------|
|         | minutes       | in reaction | % Fatty acid | % cholesterol | % cholesterol ester |
| Control | 120           | 54          | 0,247        | 0,863         | 0,083               |
| # 178   | 30            | 54          | 0,422        | 0,669         | 0,445               |



188

|       |     |    |       |       |       |
|-------|-----|----|-------|-------|-------|
| # 178 | 60  | 54 | 0,515 | 0,549 | 0,672 |
| # 178 | 120 | 54 | 0,711 | 0,364 | 1,029 |
| #2427 | 30  | 54 | 2,366 | 0,848 | 0,090 |
| #2427 | 60  | 54 | 3,175 | 0,837 | 0,088 |
| #2427 | 120 | 54 | 3,926 | 0,833 | 0,082 |
| #1991 | 30  | 54 | 1,606 | 0,911 | 0,083 |
| #1991 | 60  | 54 | 1,701 | 0,838 | 0,080 |
| #1991 | 120 | 54 | 1,781 | 0,763 | 0,053 |
| # 178 | 30  | 73 | 0,377 | 0,764 | 0,495 |
| # 178 | 60  | 73 | 0,488 | 0,665 | 0,719 |
| # 178 | 120 | 73 | 0,626 | 0,426 | 0,931 |
| #2427 | 30  | 73 | 2,471 | 0,853 | 0,092 |
| #2427 | 60  | 73 | 3,284 | 0,858 | 0,087 |
| #2427 | 120 | 73 | 4,176 | 0,837 | 0,081 |
| # 178 | 30  | 89 | 0,344 | 0,720 | 0,308 |
| # 178 | 60  | 89 | 0,443 | 0,725 | 0,446 |
| # 178 | 120 | 89 | 0,610 | 0,597 | 0,607 |
| #2427 | 30  | 89 | 0,510 | 0,167 | 0,010 |
| #2427 | 60  | 89 | 0,602 | 0,133 | 0,010 |
| #2427 | 120 | 89 | 0,867 | 0,147 | 0,009 |

Based on the analyses of fatty acid, cholesterol and cholesterol ester it was possible to calculate the amount of free fatty acid, and cholesterol ester produced as a function of reaction time and water content. Based on these results it was then possible to calculate the total enzymatic activity as the sum of the fatty acid formation and the cholesterol ester formation. The relative hydrolytic activity and the relative transferase activity (i.e. % acyltransferase activity) were then calculated with the results shown in Table 16.

10

The results in Table 16. were also analysed statistically using a Statgraphic



Multifactor ANOVA . The statistical results in Figure 54 confirm that Phospholipase A1, #2427 and phospholipase A2, #1991 have no transferase activity whereas the transferase #178-9 showed almost 50% transferase activity under these assay conditions.

5

The effect of water content in the assay on the transferase activity of the transferase #178 was also analysed statistically as shown in Figure 55. These results indicate that in the range from 54 to 89% water in the assay there was no strong effect of the water content on the relative transferase activity.

10

The impact of reaction time on transferase activity for transferase #178 was evaluated with results shown in Table 16 and Figure 56. The results in Figure 56 indicate that the relative transferase activity decreases as a function of reaction time. This might be explained by the fact that most of the acceptor molecule cholesterol is consumed and therefore the relative hydrolytic activity increases. The negative values for transferase reaction for #2427 only indicate no transferase activity within the variation for the analytical method.

15

**Table 16.**

| Enzyme | Reaction time minutes | Water % in reaction mixture | Fatty acid Produced | Cholesterol Consumed | Cholesterol ester produced | Hydrolytic activity % | Transferase activity % |
|--------|-----------------------|-----------------------------|---------------------|----------------------|----------------------------|-----------------------|------------------------|
| # 178  | 30                    | 54                          | 0,175               | 0,194                | 0,362                      | 53                    | 47                     |
| # 178  | 60                    | 54                          | 0,268               | 0,314                | 0,589                      | 52                    | 48                     |
| # 178  | 120                   | 54                          | 0,464               | 0,499                | 0,946                      | 53                    | 47                     |
| #2427  | 30                    | 54                          | 2,119               | 0,015                | 0,007                      | 100                   | 0                      |
| #2427  | 120                   | 54                          | 2,928               | 0,026                | 0,005                      | 100                   | 0                      |
| #2427  | 60                    | 54                          | 3,679               | 0,030                | -0,001                     | 100                   | 0                      |
| #1991  | 30                    | 54                          | 1,359               | -0,048               | 0,000                      | 100                   | 0                      |
| #1991  | 60                    | 54                          | 1,454               | 0,025                | -0,003                     | 100                   | 0                      |



190

|       |     |    |       |       |        |     |     |
|-------|-----|----|-------|-------|--------|-----|-----|
| #1991 | 120 | 54 | 1,534 | 0,100 | -0,030 | 101 | -1  |
| # 178 | 30  | 73 | 0,130 | 0,099 | 0,412  | 42  | 58  |
| # 178 | 60  | 73 | 0,241 | 0,198 | 0,636  | 47  | 53  |
| # 178 | 120 | 73 | 0,379 | 0,437 | 0,848  | 51  | 49  |
| #2427 | 30  | 73 | 2,224 | 0,010 | 0,009  | 100 | 0   |
| #2427 | 60  | 73 | 3,037 | 0,005 | 0,004  | 100 | 0   |
| #2427 | 120 | 73 | 3,929 | 0,026 | -0,002 | 100 | 0   |
| # 178 | 30  | 89 | 0,097 | 0,143 | 0,225  | 50  | 50  |
| # 178 | 60  | 89 | 0,196 | 0,138 | 0,363  | 56  | 44  |
| # 178 | 120 | 89 | 0,363 | 0,266 | 0,524  | 62  | 38  |
| #2427 | 30  | 89 | 0,263 | 0,696 | -0,073 | 113 | -13 |
| #2427 | 60  | 89 | 0,355 | 0,730 | -0,073 | 110 | -10 |
| #2427 | 120 | 89 | 0,620 | 0,716 | -0,074 | 105 | -5  |

5

### Conclusion.

The lipid acyltransferase from *Aeromonas salmonicida* was tested in egg yolk as substrate and with different levels of water content. This enzyme was compared with control lipolytic enzymes, namely Phospholipase A1 from *Fusarium oxysporum* and a Phospholipase A2 from pancreas.

The results have proved that only the transferase catalysed the transferase reaction between lecithin and cholesterol during formation of cholesterol ester. The results showed that in the range from 54% to 89 % water in the substrate the relative transferase activity was almost the same for transferase from *Aeromonas salmonicida*.



**EXAMPLE 12 : The "Transferase Assay in Buffered Substrate" for measurement of acyltransferase activity (e.g. for use in a foodstuff using lecithin and cholesterol).**

- 5 The lipid acyltransferase was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. This enzyme is very efficient in transferring fatty acid from lecithin to cholesterol during formation of cholesterol esters. It has also been shown that the enzyme has some hydrolytic activity, which is observed by the formation of free fatty acid. Traditional phospholipases (EC3.1.1.4 and EC3.1.1.32) have the ability to  
10 hydrolyse lecithin during formation of free fatty acids and lysolecithin, and no transferase reactions has been reported for these enzymes.

We detail herein an assay that is able to measure both transferase and hydrolytic activity of enzymes and thus to identify lipid acyltransferases in accordance with the  
15 present invention, the assay uses a substrate which contains lecithin and cholesterol. In this work a substrate based on phosphatidylcholine and cholesterol dispersed in a buffer was used. Quantification of reaction products was made by extraction of lipids from the substrate followed by GLC analysis of the lipid components.

20

#### Procedure

#### Materials

L-alpha-Phosphatidylcholine 95% (Plant) Avanti no. 441601

25 Cholesterol: Sigma cat. C 8503

Cholesteryl Palmitate, Sigma C 6072

Cholesteryl Stearate, Sigma C 3549

HEPES buffer Sigma cat. No. H 3375

Chloroform, Analytical grade.

30

#### Enzymes

Purified GCAT from *A. salmonicida* #178-9



TLC analysis was carried out as described in Example 6.

GLC analysis was carried out as described in Example 11.

5

Results: Transferase assay based on phosphatidylcholine and cholesterol as substrate.

In the following the transferase activity of the transferase was tested in a substrate based on phosphatidylcholine and cholesterol according to the following procedure.

10

450 mg phosphatidylcholine (>95% PC Avanti item no. 441601) and 50 mg cholesterol was dissolved in chloroform and evaporated to dryness under vacuum. 300 mg cholesterol/phosphatidylcholine mixture was transferred to a Wheaton glass and 15 ml 50mM HEPES buffer pH 7 was added. The lipid was dispersed in the buffer during agitation.

15

The substrate was heated to 35 °C during mixing with a magnetic stirrer and 0.25 ml enzyme solution was added. This is a very high water environment of approximately 95% water.

20

Samples of 2 ml were taken out after 0, 5, 10, 15, 25, 40 and 60 minutes reaction time. Immediately 25 µl 4M HCl was added to acidify the free fatty acid and stop the enzyme reaction. 3.00 ml chloroform was added, and the sample was shaken vigorously on a Whirley for 30 seconds. The sample was centrifuged and 2 ml of the chloroform phase was isolated and filtered through 0.45-µm filters into a 10 ml tared Dram glass. The chloroform was evaporated under a stream of nitrogen at 60°C, and the samples were scaled again. The extracted lipid was analysed by GLC.

25

The results from the GLC analysis are shown in Table 17. The results are expressed in % calculated on extracted lipid. The amount of fatty acid and cholesterol ester formed as a function of time is illustrated in. Figure 57 It can be concluded from Figure 57 that the enzyme reaction is not linear as a function of time, because an initially strong

30



both hydrolytic and transferase activity is observed. After approximately 10 minutes and until approximately 60 minutes the reaction shows an almost linear response of fatty acid and cholesterol ester formation as a function of time. It was therefore decided to look at the enzymatic reaction in this time interval.

5

**Table 17**

| Minutes              | 0      | 5     | 10    | 15    | 25    | 40    | 60    |
|----------------------|--------|-------|-------|-------|-------|-------|-------|
|                      |        |       |       |       |       |       |       |
| Cholesterol, %       | 10.064 | 8.943 | 8.577 | 8.656 | 8.102 | 7.856 | 7.809 |
| Cholesterol ester, % | 0.000  | 1.571 | 2.030 | 2.058 | 2.282 | 2.659 | 3.081 |
| FFA total, %         | 0.260  | 1.197 | 1.239 | 1.466 | 2.445 | 2.943 | 3.940 |

From the knowledge about the amount of lipid in the reaction mixture and the amount of enzyme added it was possible to calculate the formation of fatty acid and cholesterol ester expressed in  $\mu\text{mol/ml}$  enzyme (Table 18 and Figure 58 )

10

**Table 18**

| Minutes           | 10                 | 15                 | 25                 | 40                 | 60                 |
|-------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                   | $\mu\text{mol/ml}$ | $\mu\text{mol/ml}$ | $\mu\text{mol/ml}$ | $\mu\text{mol/ml}$ | $\mu\text{mol/ml}$ |
| FFA total         | 58.1               | 68.7               | 114.6              | 138.0              | 184.7              |
| Cholesterol ester | 88.8               | 90.0               | 99.3               | 115.6              | 133.8              |

From the results in Table 18 and the slope of the curves in Figure 58 it was possible to calculate the amount of fatty acid and cholesterol ester as a function of time expressed in  $\mu\text{mol/min}$  per ml enzyme.

15

The calculation of the hydrolytic activity and the transferase activity is shown in Table 19. The relative transferase activity was determined using the protocol for the determination of % acyltransferase activity as described hereinbefore.

20



**Table 19**

|                                         |      |                        |
|-----------------------------------------|------|------------------------|
| Hydrolytic activity (fatty acid)        | 2.52 | μmol/min per ml enzyme |
| Transferase activity(cholesterol ester) | 0.94 | μmol/min per ml enzyme |
| Total activity                          | 3.45 | μmol/min per ml enzyme |
|                                         |      |                        |
| Relative Transferase activity           | 27.1 | %                      |
| Relative hydrolytic activity            | 72.9 | %                      |

## 5 Screening of other enzymes for transferase activity.

The method mentioned above was used to screen different lipolytic enzymes for transferase and hydrolytic activity. The enzymes were tested as shown in Table 20

10 **Table 20**

|                                              |    | 1    | 2    | 3    | 4    | 5    |
|----------------------------------------------|----|------|------|------|------|------|
| Substrate                                    | ml | 15   | 15   | 15   | 15   | 15   |
| #178-9Transferase A. salmonicida 32 PLU-7/ml | ml | 0.25 |      |      |      |      |
| 5% #3016, LIOPAN® F (F. oxysporum)           | ml |      | 0.25 |      |      |      |
| 5%, Thermomyces lanuginosus                  | ml |      |      | 0.25 |      |      |
| 5% Candida rugosa #2983                      | ml |      |      |      | 0.25 |      |
| 5% Candida cylindracea #3076                 | ml |      |      |      |      | 0.25 |

The substrate containing 300mg phosphatidylcholine/cholesterol dispersed in 50 mM HEPES buffer pH 7.0 was heated to 35 °C with agitation. Enzyme solution was added and the sample was kept at 35 °C with agitation. Samples were taken out with regular interval and extracted with Chloroform. The isolated lipids were analysed by GLC with results shown in Table 21.



**Table 21**

| Sample   |                                           |       |       |       |      |       |       |       |
|----------|-------------------------------------------|-------|-------|-------|------|-------|-------|-------|
| <b>1</b> | <b>Transferase 178-9</b>                  |       |       |       |      |       |       |       |
|          | Minutes                                   | 0     | 5     | 10    | 15   | 25    | 40    | 60    |
|          | FFA                                       | 1.216 | 2.516 | 2.983 | 2.62 | 2.894 | 3.448 | 3.911 |
|          | Cholesterol                               | 7.547 | 6.438 | 6.365 | 6.15 | 6.136 | 5.936 | 5.662 |
|          | Chl. Ester                                | 0     | 1.835 | 2.177 | 2.44 | 2.58  | 2.851 | 3.331 |
|          |                                           |       |       |       |      |       |       |       |
| <b>2</b> | <b>Fusarium oxysporum<br/>(LIOPAN® F)</b> | 0     | 5     | 10    | 15   | 25    | 40    | 60    |
|          | FFA                                       | 1.216 | 1.345 | 1.796 | 1.95 | 2.487 | 2.424 | 2.977 |
|          | Cholesterol                               | 7.547 | 7.309 | 7.366 | 7.33 | 7.429 | 7.341 | 7.326 |
|          | Chl. Ester                                | 0     | 0.26  | 0.386 | 0.35 | 0.267 | 0.36  | 0.394 |
|          |                                           |       |       |       |      |       |       |       |
| <b>3</b> | <b>Thermomyces lanuginosus</b>            | 0     | 5     | 10    | 15   | 25    | 40    | 60    |
|          | FFA                                       | 1.216 | 0.853 | 0.875 | 1    | 0.896 | 1.105 | 1.009 |
|          | Cholesterol                               | 7.547 | 7.384 | 7.639 | 7.63 | 7.675 | 7.603 | 7.529 |
|          | Chl. Ester                                | 0     | 0     | 0     | 0    | 0     | 0     | 0     |
|          |                                           |       |       |       |      |       |       |       |
| <b>4</b> | <b>Candida rugosa (#2938)</b>             | 0     | 5     | 10    | 15   | 25    | 40    | 60    |
|          | FFA                                       | 1.216 | 0.982 | 0.987 | 1.02 | 1.135 | 1.131 | 1.15  |
|          | Cholesterol                               | 7.547 | 7.438 | 7.656 | 7.66 | 7.638 | 7.575 | 7.585 |
|          | Chl. Ester                                | 0     | 0     | 0     | 0    | 0     | 0     | 0     |
|          |                                           |       |       |       |      |       |       |       |
| <b>5</b> | <b>Candida cylandracea<br/>(#3076)</b>    | 0     | 5     | 10    | 15   | 25    | 40    | 60    |
|          | FFA                                       | 1.216 | 1.032 | 1.097 | 1.07 | 1.203 | 1.131 | 1.43  |
|          | Cholesterol                               | 7.547 | 7.502 | 7.425 | 7.65 | 7.619 | 7.502 | 7.411 |
|          | Chl. Ester                                | 0     | 0     | 0     | 0    | 0     | 0     | 0     |



From the GLC analysis it was observed that only the lipid acyltransferase (178-9) produced significant amount of cholesterol ester and fatty acids. Phospholipase from *Fusarium oxysporum* also gave a steady increase in free fatty acid but only an initial small amount formation of cholesterol ester was formed but no increase in cholesterol ester as a function of time was observed.

Based on the knowledge about the amount of lipid substrate and the GLC analyses it was possible to calculate the relative transferase activity and the relative hydrolytic activity based on the results from 10 to 60 minutes reaction time. The results from Transferase 178-9 and *Fusarium oxysporum* lipase are shown in Table 21. The other enzymes tested showed no activity.

**Table 21**

|                                                   | Transferase 178-9 | <i>Fusarium oxysporum</i> |
|---------------------------------------------------|-------------------|---------------------------|
| Hydrolytic activity, micromole/min per ml enzyme  | 1.03              | 0.96                      |
| Transferase activity, micromole/min per ml enzyme | 0.40              | 0.01                      |
| Total activity, micromole/min per ml enzyme       | 1.43              | 0.98                      |
|                                                   |                   |                           |
| Relative hydrolytic activity                      | 71.8              | 98.7                      |
| Relative transferase activity                     | 28.2              | 1.3                       |

The result shown in Table 21 confirm a significant transferase activity from the lipid acyltransferase (sample 178-9). It is also observed that the relative transferase activity is in good agreement with the experiment mentioned in Table 19

A very low transferase activity from *Fusarium oxysporum* phospholipase is however observed. This transferase level is so low that it falls within the uncertainty of the



analysis. As expected *Fusarium oxysporum* phospholipase has a significant hydrolytic activity.

#### Conclusion.

5

Instead of egg yolk (shown in Example 11) an artificial substrate based on purified phosphatidylcholine and cholesterol was used as a substrate to measure the activity of transferase from *Aeromonas salmonicida*. Between 10 minutes and 60 minutes reaction time the assay gave an almost linear formation of free fatty acids and  
10 cholesterol ester as a function of time. Based on the activity between 10 and 60 minutes reaction time the hydrolytic activity and the transferase activity was calculated.

The concentration of substrates in this assay was relatively lower than in egg yolk, and  
15 the amount of water in the assay was relatively higher.

Based on the results from the assay of the lipid acyltransferase (in this instance a GCAT) from *Aeromonas salmonicida* in a artificial substrate of phosphatidylcholine/cholesterol in buffer it is concluded that this enzyme has very  
20 good transferase activity also in a system with a very high water content.

Both assays based on egg yolk (see Example 11) and phosphatidylcholine/cholesterol in buffer (Example 12), can be used to measure the transferase and hydrolytic activity of enzymes. The egg yolk is preferred from the point of view that the hydrolytic and  
25 the transferase activity is linear as a function of time, but the phosphatidylcholine/cholesterol in buffer is only linear within a certain time limit.

#### **EXAMPLE 13: Food Emulsions**

30

The effect of enzyme modified liquid egg yolk was tested in a standard Food emulsion recipe with 60 % oil.



Standard methods and materials are as per those detailed in the Examples above.

The egg yolk was treated with a lipid acyl transferase from *Aeromonas salmonicida*(  
5 #138) or phospholipase, namely a commercially available enzyme LipopanF®  
(Novozymes A/S, Denmark) (#2938) as shown in Table 22.

**Table 22.** Enzyme treatment of egg yolk.

|                             |         | 1   | 2   | 3   | 4   |
|-----------------------------|---------|-----|-----|-----|-----|
| Egg Yolk, Sanofi product no |         |     |     |     |     |
| 1123P2                      | Gram    | 10  | 10  | 10  | 10  |
| #138, 10 PLU/ml             | ml      | 1   | 1   |     |     |
| #2938, 200 PLU/ml           | ml      |     |     | 1   |     |
| Water                       | ml      |     |     |     | 1   |
| Reaction time               | Minutes | 210 | 360 | 210 | 210 |

10

TLC analysis of the egg yolk lipids from enzyme treated egg yolk (Table 9) is shown  
in Figures 59 and 60.

In this experiment the dosage of #2938 was increased by a factor of 10 and this gave a  
15 very clear activity on egg yolk. The amount of free fatty acid increased significantly  
and lecithin (PC) was hydrolysed to lysolecithin (LPC). The transferase #138 gave a  
clear transferase reaction because free cholesterol was converted to cholesterol ester  
and part of the lecithin was converted to lysolecithin.

20 Another interesting aspect of the enzyme modification was the consistency of the  
product. The sample treated with Phospholipase #2938 became very solid, whereas  
the samples treated with the lipid acyltransferase #138 kept the same liquid  
consistency as the control sample (see Figure 61).

25 These modified egg yolks were tested in a Food Emulsion recipe shown in Table 23.



**Table 23.** Mayonnaise with enzyme modified egg yolk.

|                                    | 0   | 1a   | 2a   | 3a   | 4a   |
|------------------------------------|-----|------|------|------|------|
|                                    | %   | %    | %    | %    | %    |
| Rapsolie                           | 60  | 60   | 60   | 60   | 60   |
| Egg yolk, Sanofa product no.1123P2 | 2,8 |      |      |      |      |
| Enz. Modified egg yolk no. 1       |     | 2,8  |      |      |      |
| Enz. Modified egg yolk no. 2       |     |      | 2,8  |      |      |
| Enz. Modified egg yolk no. 3       |     |      |      | 2,8  |      |
| Control (untreated) egg yolk no. 4 |     |      |      |      | 2,8  |
| Water                              | 39  | 36,2 | 36,2 | 36,2 | 36,2 |
| Vinegar, 10% acetic acid           | 1   | 1    | 1    | 1    | 1    |

Modified egg yolks 1 and 2 were treated with the lipid acyl transferase; and modified  
5 egg yolk 3 was treated with the commercially available phospholipase.

The food emulsion was produced as an oil in water emulsion according to the  
following procedure: Egg yolk and water was scaled in a beaker. The oil was scaled  
separately.

10

A Turrax mixer(20000 rpm) was immersed in the water phase. Oil was pumped to  
the water phase at a constant speed over 2 minutes. The mixing continued for further 1  
minute. The vinegar was then added and mixed for 5 seconds.

15 The stability of the emulsion was tested in a heating cabinet at 100 °C. After 2 hours at  
100 °C the emulsion was evaluated (see Figure 62).

The emulsion stability of untreated egg yolk was quite good in this experiment.  
Treatment of egg yolk with the lipid acyltransferase #138 however improved the  
20 stability because the amount of water separation was reduced. Egg yolk treated with



phospholipase #2938 gave a very unstable emulsion with almost complete separation of the oil-and the water phase at 100 °C.

It is considered that in some applications the use of the compositions and methods of the invention can provide enhanced thermal stability of emulsions, such as oil in water salad dressings and the like. This is particularly important in food emulsions which are pasturised to ensure long shelf life and/or are heated prior to serving, e.g. in pre-prepared meals for re-heating prior to serving (e.g. microwave meals). Although not wishing to be bound by any particular theory, it is considered that in some applications the accumulation of free fatty acid may be detrimental to the thermal stability of such emulsions. It should be recognised that the enhanced thermal stability of the food emulsions produced using the methods of the invention, may not be found, or even desirable, in all food applications. It will be apparent to the person skilled in the art in which applications such characteristics are desirable, and the stability of the emulsions can be easily determined using a simple heat tests, equivalent to, for example pasturisation and or microwave reheating. The inventors have discovered that in a preferable embodiment the food emulsions obtained using the enzymes of the invention have enhanced thermal stability.

**EXAMPLE 14: Transferase reaction in plant sterol enriched egg yolk:**

Transferase from *Aeromonas salmonicida* was able to catalyse to formation of lysolecithin, monoglyceride and plant sterol esters in egg yolk enriched with plant sterol and glycerol. The same enzyme was also tested in a low water system containing palm oil, lecithin, plant sterol and glycerol. By TLC and GLC analyses it was shown that monoglyceride, and plant sterol esters were produced under these reaction conditions.

**Introduction:**

The transferase from *Aeromonas salmonicida* was tested for transferase activity in almost water free system of lecithin, fat, plant sterol and glycerol.



**Materials:**

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde

GCAAT transferase purification 178-9 , 32 PLU-7/ml(Journal 2254-100)

Soya lecithin. Yolkin from Aarhus United, Denmark.

5 Palm oil 43, from Aarhus United, Denmark.

L- $\alpha$  Phosphatidylcholine 95% Plant (Avanti #441601)

Sitosterol, Sigma no S5753

Plant Sterol: Generol N122 from Cognis, Germany

Glycerol Item no.085915

10

**Results**

Initial screening of transferase activity on plant sterol and glycerol was conducted in egg yolk as shown in Table 24.

15

**Table 24**

|                     |       | 1   | 2   | 3    | 4    |
|---------------------|-------|-----|-----|------|------|
| Egg yolk            | Gram  | 1   | 1   | 1    | 1    |
| Glycerol            | Gram  | 0.1 | 0.1 |      |      |
| Sitosterol:olie 3:7 | Gram  |     |     | 0.13 | 0.13 |
| Transferase #178-9  | Units | 1   |     | 1    |      |
| Water               |       |     | *   |      | *    |

\*Water corresponding to the amount of water in the enzyme solution= 83 $\mu$ l

The ingredients were mixed and heated to 37 °C and kept at this temperature during agitation with a magnetic stirrer.

20 0.1 gram samples were taken out after 3 and 23 hours and analysed by TLC.

The results from the TLC analysis is shown in Figure 63.

The result in Figure 63 indicated that both cholesterol and plant sterols were esterified by the transferase reaction, concomitant with the formation of lysolecithin (sample 3



and 4), because almost all free sterol and cholesterol was converted to the corresponding ester in sample 3.

The results also indicated that the sample with only glycerol and egg yolk produced monoglyceride. The amount of monoglyceride needs to be confirmed by GLC analysis. When sterol was added together with glycerol (sample 3) the amount of monoglyceride was very low and not detectable by TLC. This indicated that as long as there were surplus of sterol or cholesterol the transferase reaction using glycerol was modest.

10

In another experiment the transferase enzyme 178-9 was added to a mixture soybean lecithin, glycerol and plant sterol, in order to study the catalytic activity of the enzyme in this reaction mixture.

15 The composition of the reaction mixtures in these experiments are shown in Table 25

**Table 25**

|                                |      | 1     | 2     | 3     | 4     | 5     | 6     |
|--------------------------------|------|-------|-------|-------|-------|-------|-------|
| Soya lecithin                  | gram | 1.875 | 2.25  | 1.875 | 2.5   | 3.5   | 3.5   |
| Plantesterol; Generol N 122    | gram | 0.225 | 0.225 | 0     | 0     | 0.225 | 0.5   |
| Palm oil 43                    | gram | 2.675 | 2.25  | 2.8   | 2.125 | 1.062 | 0.831 |
| Glycerol                       | gram | 0.225 | 0.275 | 0.325 | 0.375 | 0.248 | 0.238 |
|                                |      |       |       |       |       |       |       |
| Transferase #178 -9, 32 PLU/ml | ml   | 0.2   | 0.2   | 0.2   | 0.2   | 0.2   | 0.2   |

The experiment was conducted by mixing the lipid components during agitation at 46 °C. The enzyme was added and samples were taken out after 4 and 24 hours.

20

The samples were analysed by TLC as shown in Figure 64.



Sample from experiment 2, 4 and 5 after 24 hours reaction time were also analysed by GLC with results shown in Table 26

**Table 26.**

|             |   | 2    | 4    | 5    |
|-------------|---|------|------|------|
| Glycerol    | % | 3.16 | 5.71 | 4.17 |
| Fatty acids | % | 4.23 | 5.36 | 6.67 |
| Mono        | % | 2.24 | 3.87 | 3.92 |
| Sterol      | % | 2.13 |      | 2.62 |
| Sterolester | % | 2.89 |      | 2.14 |

5

The results confirmed that transferase 178-9 was able to catalyse to formation plant sterol esters and monoglyceride from a reaction mixture containing soybean lecithin, glycerol and plant sterol. Such reaction mixture could be of interest for use in margarine production where monoglyceride is wanted for their emulsification properties and plant sterol esters for their cholesterol lowering effect.

#### Conclusion

CGAT transferase from *Aeromonas salmonicida* was able to catalyse the formation of plant sterol esters and monoglyceride in egg yolk where plant sterol and glycerol was added. The same enzyme also catalysed the formation of plant sterol esters and monoglyceride in a mixture of palm oil, lecithin, plant sterol and glycerol. This enzyme therefore is of interest for use in margarine and other oil containing food products where monoglyceride and lysolecithin are needed for improved emulsification and the plant sterol ester for their cholesterol lowering effects.

20



**EXAMPLE 15 : Immobilisation of a lipid acyltransferase from *Aeromonas salmonicida* and the use in the synthesis of Sterol esters.**

5 A lipid acyltransferase (in this instance a GCAT) from *A. salmonicida* was immobilised on Celite by acetone precipitation. 10 ml enzyme solution in 20 mM TEA buffer pH 7 was agitated slowly with 0,1 gram Celite 535 535 (from Fluka) for 2 hours at room temperature.

50ml cool acetone was added during continued agitation.

10 The precipitate was isolated by centrifugation 5000 g for 1 minute.

The precipitate was washed 2 times with 20 ml cold acetone.

The Celite was tried at ambient temperature for about 1 hour

The immobilised transferase was tested in a oil mixture containing 13 %

15 Phosphatidylcholin and 7 % plant sterol.(Table 27)

**Table 27**

|                                      | %    |
|--------------------------------------|------|
| Avanti lecithin                      | 12.0 |
| Plant sterol, Generol 122N           | 6.6  |
| Palm 43                              | 71.4 |
| Glycerol                             | 5.0  |
| Immobilised Transferase #178, 45 U/g | 2.0  |
| Water                                | 3.0  |

20 Lecithin, plant sterol and soybean oil was heated to 46 °C and the plant sterol was dissolved. The immobilised transferase was added.

The transferase reaction continued at 46 °C during gentle agitation with a magnetic stirrer. Samples were taken out for analyses after ½, 1 3 6 and 24 hours and analysed by TLC. The reaction was stopped after 24 hours reaction time and the immobilised enzyme was filtered off.



The samples were analysed by TLC as shown in Figure 65.

The TLC analysis clearly shows the effect of immobilised transferase from *A. salmonicida* in the transformation of cholesterol into cholesterol ester. It is also  
5 observed that small amount of monoglyceride is formed. The enzyme has also been shown to have a high activity in environments with high water content (6- 89 % )water environments, the use of the transferase, and other transferases for use in the invention can therefore also be used in immobilised enzyme applications with a significant water content. This allows the replacement of the solvents used by the current immobilised  
10 lipases in the bioconversion of lipids using transferases.

15 **EXAMPLE 16 The Aeromonas hydrophila transferase can transfer from a phospholipid to a sterol to form a sterol ester, and/or a sugar molecule to form a sugar ester.**

20 A lipid acyltransferase from *Aeromonas hydrophila* expressed in *E. coli* (Hydro 0303 HVP ), labelled #139 was purified on a Chelating Sepharose FF, HR 2.5/10column and analysed for Phospholipase activity. The transferase activity was evaluated in egg yolk for enzyme activity and functionality in egg yolk. The enzyme was also tested in  
egg yolk containing glucose.

25

Phospholipase activity.

Transferase #139 isolated from a Chelating Sepharose FF, HR 2.5/10column was assayed by NEFA-PLU(pH7) The activity was 1,15 Units NEFA-PLU/ml.

30

Egg yolk



In an initial application test transferase #139 was tested in egg yolk according to the following procedure.

1-gram fresh egg yolk was scaled in a 10 ml flask with screw lid. The enzyme preparation was added and mixed on a Vortex mixer. The sample was placed at 37 °C  
5 and agitated with a magnetic stirrer.

The reaction was stopped by adding 7.5 ml Chloroform:Methanol (2:1) and mixed on a Whirley mixer for 30 seconds. The chloroform phase was isolated by centrifugation and 2 µl of the chloroform phase was transferred to a pre-activated silica TLC plate and eluted with running buffer nr. I and another TLC-plate in running buffer IV,  
10

The experimental set up is shown in table 28

15 **Table 28**

| Test | Reaction time | Egg yolk | Transferase #139 |
|------|---------------|----------|------------------|
| no.  | min.          | gram     | units            |
| 1    | 10            | 1        |                  |
| 2    | 10            | 1        | 0.75 NEFA-PLU    |
| 3    | 60            | 1        | 0.75 NEFA-PLU    |
| 4    | 300           | 1        | 0.75 NEFA-PLU    |
| 5    | 1200          | 1        |                  |
| 6    | 1200          | 1        | 0.75 NEFA-PLU    |

TLC analysis are shown in Figure 66 and Figure 67. The TLC analysis clearly demonstrates the transferase reaction of transferase #139. The cholesterol is converted  
20 to cholesterol ester and the amount of lecithin is reduced. The results however also indicate that lysolecithin are only accumulated in very small amount because transferase #139 also is active on lysolecithin. This observation is supported by the formation of free fatty acids(FFA).



### Egg yolk and glucose

It was earlier shown that a transferase from *Aeromonas salmonicida* (#138) was able to use glucose as acceptor molecule in a transferase reaction. It has also been tested if transferase #139 can use glucose as acceptor molecule. The experimental set up is  
5 seen in Table 29.

**Table 29**

| Test | Reaction time | Egg yolk | Glucose, 70% | Transferase #139 |
|------|---------------|----------|--------------|------------------|
| no.  | Minutes       | gram     | mg           | units            |
| 1    | 10            | 1        | 500          |                  |
| 2    | 10            | 1        | 500          | 1 NEFA-PLU       |
| 3    | 60            | 1        | 500          | 1 NEFA-PLU       |
| 4    | 180           | 1        | 500          | 1 NEFA-PLU       |
| 5    | 300           | 1        | 500          | 1 NEFA-PLU       |
| 6    | 1200          | 1        | 500          | 1 NEFA-PLU       |
| 7    | 1200          | 1        | 500          |                  |

10 The reaction products were analysed by TLC (Figures 68 and Figure 69).

The TLC analysis indicates formation of glucose ester after 220 min. reaction time (Figure 69 lane 6) but after 1200 min reaction time no glucose ester is seen.

It must therefore be concluded that transferase #139 has both transferase and  
15 hydrolytic activity. This is also supported by the fact that the amount of free fatty acids steadily increases as a function of reaction time.

### Resume:

Transferase from *Aeromonas hydrophila* was tested in egg yolk. The results confirm that this enzyme catalyses the formation of cholesterol ester concomitant with the  
20 formation of lysolecithin. After extended reaction time when most of the cholesterol is consumed free fatty acid are also formed. It can therefore be concluded that the enzyme has primary transferase activity but also hydrolytic activity was observed when only water was available as donor molecule.



In an experiment with egg yolk and glucose it has been observed that transferase from *Aeromonas hydrophila* is able to catalyse the formation of glucose ester *in situ* in a high water food environment (Figure 70).

5

**EXAMPLE 17: Variants of a lipid acyltransferase from *Aeromonas hydrophila* (Ahyd2) (SEQ ID No. 36 (see Figure 71))**

10 Mutations were introduced using the QuikChange® Multi-Site Directed Mutagenesis kit from Stratagene, La Jolla, CA 92037, USA following the instructions provided by Stratagene.

Variants at Tyr256 showed an increased activity towards phospholipids.

15 Variants at Tyr256 and Tyr260 showed an increased activity towards galactolipids.

Variants at Tyr265 show an increased transferase activity with galactolipids as the acyl donor.

20 The numbers indicate positions on the following sequence: An enzyme from *Aeromonas hydrophila* the amino acid sequence of which is shown as SEQ ID No. 36 in Figure 71 (the underlined amino acids show a xylanase signal peptide). The nucleotide sequence is as shown as SEQ ID No 54 in FIGURE 72.

25 **EXAMPLE 18: Use of Acyl-transferase reaction for the production of plant sterol ester and monoglyceride for margarine production.**

30 An acyltransferase from *Aeromonas salmonicida* expressed in *Bacillus subtilis* was tested in a palm oil mixture containing plant lecithin, plant sterol and glycerol. The acyl-transferase showed the ability to utilise both plant sterol and glycerol as acceptor molecules during production of plant sterol ester and monoglyceride. The reaction mixture was used to produce table margarine of good quality based on the



monoglyceride in the reaction mixture and at the same time the margarine was enriched with plant sterol ester, which has been shown to have a cholesterol lowering effect.

- 5 The aim of this work was to study to possibility to produce monoglyceride and plant sterol ester by enzymatic reaction of lecithin, plant sterol and glycerol dissolved in vegetable fat.

Initial experiments has shown that it was possible to use acyl-transferase from  
10 *Aeromonas salmonicida* to produce monoglyceride and plant sterol ester from lecithin, glycerol and plant sterol.

In this experiment such reaction mixture was used to produce table margarine.

15 Materials:

Lipid acyltransferase from *Aeromonas salmonicida*, # 196 C101, 18.6 PLU/g (Journal 2254-104)

Palm Oil 43, from Aarhus United, DK

L- $\alpha$  Phosphatidylcholine 95% Plant (Avanti #441601)

20 Plant Sterol: Generol N122 from Cognis, Germany

Glycerol Item no.085915

Distilled Monoglyceride, Dimodan HP from Danisco.

Margarine production.

25

1. Blend the water phase ingredients. (If required, pasteurise the water phase by heating to approx. 80°C). Adjust pH 5.5.

2. Melt the fat phase, and temper to approx. 40-45°C..

30

3. Heat the emulsifier with some of the oil in a ratio of  
1 part emulsifier to 5 parts oil to a temperature (75-80°), which is 5-10°C higher



than the melting point of the emulsifier. When this blend is fully melted and well stirred, add it to the remaining heated oil, stirring continuously.

4. Add the flavouring.

5

5. Add the water phase to the fat phase, stirring continuously.

6. Cool in a tube chiller (normal capacity, normal cooling) to an outlet temperature of 8-10°C.

10

### Results

Acyltransferase from *A. salmonicida* was tested in an palm oil mixture as shown in Table 30. Lecithin, plant sterol, glycerol and palm oil was heated to 60°C during agitation in order to solubilize plant sterol and lecithin.

15

Table 30

| Substrate:                 | %    |
|----------------------------|------|
| Avanti lecithin            | 12   |
| Plant sterol, Generol 122N | 6.6  |
| Palm oil, melting point 43 | 76.4 |
| Glycerol                   | 5    |

The substrate was cooled to 48 °C and acyl-transferase #196 was added in the amount shown in Table 31. The reaction mixture was kept at 48 °C for 24 hours during slow agitation.

20

Table 31

| Substrate                    | gram |
|------------------------------|------|
| Transferase # 196 C101, 18.6 | 15   |



PLU/g

Samples from the reaction mixture were taken out after 1, 4 and 24 hours reaction time, and analysed by TLC in solvent I (Figure 73). The TLC results clearly show the formation of plant sterol ester and monoglyceride. In Figure 73, the first lane is after 1 hour reaction time, Lane 2 is 4 hours reaction time, Lane 3 is 24 hours reaction time and Lane 4 is a plant sterol.

The reaction was stopped after 24 hours reaction time and residues of undissolved plant sterol was removed, and the clear solution was used to produce margarine.

Margarine.

The reaction mixture containing monoglyceride and plant sterol ester was used to produce table margarine according to the recipe shown in Table 32.

Table 32

| Jour. No 3734      | 1     | 2     |
|--------------------|-------|-------|
| <b>Water phase</b> |       |       |
| Water phase        | 16    | 16    |
| Salt               | 0.5   | 0.5   |
| Skim milk powder   | 1     | 1     |
| Potassium sorbate  | 0.1   | 0.1   |
| EDTA               | 0.015 | 0.015 |
| PH                 | 5.5   | 5.5   |
|                    |       |       |
| Water phase total  | 16.6  | 16.6  |
|                    |       |       |
| <b>Fat phase</b>   |       |       |



|                  |      |      |
|------------------|------|------|
| Palm 43          | 25   | 25   |
| Rapeseed Oil     | 75   | 75   |
|                  |      |      |
|                  |      |      |
| Fat phase total  | 83.2 | 78.4 |
| Dimodan HP       | 0.2  |      |
| Reaction mixture |      | 5    |

The margarine produced from the reaction mixture was evaluated of good quality with good spreadability, and good mouth feel and without any off flavour. The margarine was compared to be on quality level with the reference margarine produced by using distilled monoglyceride Dimodan HP.

The only difference observed was that the margarine jour. 3734 no 2 with the reaction mixture was slightly more firm, which was explained by the fact that this recipe contained more Palm 43 than the reference margarine.

#### **EXAMPLE 19: Use of a lipid acyltransferase during bread production.**

One of the limitations of using lipases in bread making is that free fatty acid is formed during the lipase reaction. It is well known that formation of too much free fatty acid will have a negative impact on the baking performance of flour, because the gluten gets too stiff and a bucky (i.e. less elastic) dough is formed which can not expand during fermentation and baking.

Formation of free fatty acid should also be avoided from the point of oxidative stability, because free fatty acids are more prone to lipid oxidation than the corresponding triglyceride.

In the present invention the problems with free fatty acid formation when adding a lipolytic enzyme to a dough has been overcome by using a lipid acyltransferase which,



instead of producing free fatty acids, transfers one or more fatty acids from the lipid acyl donor to a non water acceptor molecule present in the dough, such as a carbohydrate, a protein or peptide, or if used in bread with milk fat, a sterol, alternatively or in combination other acceptors listed above may be added to a dough,  
5 for example phytosterols or phytosteranols. Preferably, the acceptor molecule in a dough may be one or more of glucose, sucrose or maltose and/or other carbohydrates normally available in a dough.

10 In the following experiments acyl transferase is tested in mini scale baking experiments. The formation of reaction products, and the lipid components in fully proved dough is extracted by water saturated butanol and analysed by HPLC and GLC analysis.

#### Materials and methods

15

##### Enzymes:

Acyl Transferase, 550 PLU-7/ml

Lipopan<sup>TM</sup> F BG, a commercial lipase from Novozymes. 12000 LIPU/g or Grindamyl

Exel 16. 12000 LIPU/g

20 Lecithin powder, 95% phospholipid (available from Danisco A/S Denmark)

Digalactosyldiglyceride from whole wheat flour (from Sigma D4651)

Flour : Sølvmeel nr. 2001084 (Danish wheat flour, obtained from Havnemøllerne, Odense, Denmark)

25

##### Mini baking test.

Flour , 50 gram, Dry yeast 10 gram, glucose 0.8 gram, salt 0.8 gram, 70 ppm ascorbic acid and, water 400 Brabender units was kneaded in a 50 g Brabender mixing bowl for 5 min at 30 °C.

30 Resting time was 10 min. at 34 °C . The dough was scaled 15 gram per dough. Then moulded on a special device where the dough is rolled between a wooden plate and a



plexiglas frame. The doughs were proofed in tins for 45 min. at 34 °C, and baked in a Voss household oven 8 min. 225 °C.

After baking the breads are cooled to ambient temperature and after 20 min. the breads are scaled and the volume is determined by rape seed displacement method. The  
5 breads are also cut and crumb and crust evaluated.

#### Results and conclusion:

Preliminary results indicate that the lipid acyltransferase clearly demonstrates a  
10 positive effect on both bread volume and bread appearance. In particular, preliminary results indicate that the use of the lipid acyltransferase results in increased specific bread volume as compared with that obtained with the control (no enzyme) and that obtained with the use of a commercially available lipolytic enzyme, namely Grindamyl Exel 16 or LipopanF<sup>TM</sup>.

15

#### **EXAMPLE 20: Standard ice cream with dairy fat**

The function of emulsifiers used in ice cream is to bring about controlled fat  
crystallisation and mild destabilization due to protein desorption during ageing of the  
20 ice cream. This change improves the ice cream quality. Mono-diglycerides are normally used for the production of ice cream, but is also known to use polar emulsifiers like polysorbate and sugar esters in ice cream production in combination with mono-diglyceride to facilitate controlled fat destabilization and produce ice cream with very good creamy and smooth eating texture.

25

Emulsifiers used for ice cream are normally added the ice cream mix as a powder. Recently it has however been shown that mono-diglyceride can be produced by enzymatic reaction of the fat in the ice cream recipe using lipases. The problem by using lipases is however that lipases also catalyse the formation of free fatty acids,  
30 when water is available in the reaction mixture.



It has however surprisingly been shown that lipid acyl-transferase overcomes the limitation by lipase because acyl-transferase is able to transfer fatty acid from lecithin and other lipids to acceptor molecules like sterol, cholesterol, glucose, glycerol and proteins/peptides without formation of significant amount of free fatty acids.

One of the main ingredients in ice cream is dairy cream containing 38 % milk fat. Dairy cream also contains smaller amount of lecithin, which is a donor molecule for acyl-transferase. (*Complex milk lipids* account for about 1 % of the total milk fat and are mainly composed of phospholipids". Ref. Ullmann's Encyclopedia of Industrial Chemistry Copyright © 2003 by Wiley-VCH Verlag GmbH & Co. KGaA.). Dairy cream also contains small amount of cholesterol, which is an acceptor molecule for acyl-transferase.

From the constituents of ice cream it is thus possible to produce both monoglyceride and polar emulsifiers like lyso-lecithin and sugar ester, which are known for the beneficial effects in ice cream production.

A further beneficial effect from the reaction of acyl-transferase in dairy cream is the formation of cholesterol ester, which might slow down the absorption of cholesterol in the intestine.

#### Ice cream Recipe

|                              | With emulsifier | With enzyme |
|------------------------------|-----------------|-------------|
| Dairy cream, 38%             | 23,65           | 23,65       |
| Skimmed milk                 | 53,30           | 53,30       |
| Skimmed milk powder          | 4,90            | 11,30       |
| Sugar                        | 12,00           | 12,00       |
| Glucose sirup, DE 42, 75% TS | 4,25            | 4,25        |



216

|                                   |     |     |
|-----------------------------------|-----|-----|
| Glycerol                          | 1,0 | 1,0 |
| Stabilizer blend                  | 0,2 | 0,2 |
| Cremodan SE 30                    | 0,6 |     |
| Lipid acyl transferase, 500 PLU/g |     | 0,1 |
| Grindsted Flavouring 2976         | 0,1 | 0,1 |
| Colour                            | +   | +   |

Ice Cream production proces.

1. Heat dairy cream, glucose syrup and glycerol to approx. 40 °C Add the lipid acyl transferase and let the mixture react for 30 minutes. A sample is taken out for analysis
- 5 2. Heat all the other liquid ingredients to approx. 40 °
3. Add the other dry ingredients. (stabiliser blend is mixed with sugar before addition)
4. When the dry ingredients are dissolved add the dairy cream-glucose mixture.
5. Pasteurise at 80 -85 °C/20-40 seconds
6. Homogenise at 80 °C (190 bar for recipe 1 and 175 bar for recipe 2)
- 10 7. Cool to ageing temperature , 4 °C
8. Freeze in continuous freezer to desired overrun (100% recommended)
9. Harden in tunnel at -40 °C
10. Store below -25 °C

15 Results:

Uses of Acyl-transferase in the production of ice cream contribute to the production of ice cream with very good taste and excellent creamy mouth feel comparable the ice cream produced by using a commercial emulsifier Cremodan SE 30. The melt down of  
20 the ice cream produced by the lipid acyl transferase is also improved.



**Example 21: Acyl transferase in Cheese.**

5 Cheese is the fresh or matured solid or semisolid product obtained by coagulating milk, skimmed milk, partly skimmed milk, cream, whey cream, or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and partially draining the whey that results from such coagulation.

10 The cheese yield depends primarily on the fat and protein contents of the milk. The salt (particularly calcium salts) and protein concentrations, as well as the acidity, are very important for coagulation. (ref. Ullmann's Encyclopedia of Industrial Chemistry Copyright © 2003 by Wiley-VCH Verlag GmbH & Co).

15 Such effort has been made in order to optimise and increase the cheese yield by optimisation of the cheese making procedure (USP 4,959,229) or by using improved clotting method (USP 4,581,240), which increase the amount of whey protein in the curd.

20 In the present invention the amount of whey protein in the curd is increased by enzymatic modification of the whey protein by treatment of the milk during cheese making with a lipid acyl transferase.

25 When a fatty acid is covalently linked to a non-membrane protein like  $\beta$ -lactoglobulin, the physical and functional properties will change drastically.

For cheese production of the present invention acyl transferase is added to the milk before or at the same time as rennet is added to the milk.

30 During casein precipitation acyl transferase is able to use lecithin and other lipids in the milk as donor and peptides or protein as acceptor molecule during formation of acylated protein or acylated peptides.



The change in hydrophobic properties of milk protein contributes to increased protein precipitation in the curd during cheese production.

5 Since the increase in cheese yield obtained by the present invention originates from increased retention in the cheese coagulum of proteins that are normally lost in the whey, a suitable method, directly related to the mechanism of the invention, is based on determination of the amount of protein that ends up in the whey. Less protein in the whey necessarily means more protein in the curd, and higher cheese yield.

10

The test for the amount of protein in the whey can be performed in the following way. Skim or whole milk is warmed to a temperature suitable for rennet coagulation, typically 30-35°C in a 100 ml beaker. Optionally 1% of a bulk lactic acid bacteria starter is added, and standard rennet is added in an amount corresponding to e.g. 0.03-  
15 0.05%. When the milk has turned into a coagulum solid enough to allow it to be cut into cubes with a side length of about 0.5 cm, such cutting is performed with a sharp knife. Syneresis is thereby initiated, and after 30 min holding period, that allows the curd to settle, a whey sample is withdrawn, and centrifuged in a laboratory centrifuge for 10 min. This sample is analyzed for protein content, using e.g. the Kjeldahl  
20 method. Alternatively, and/or as a supplement, the sample may be analyzed with methods that allow the type and quantity of the individual protein components to be established.

#### **EXAMPLE 22 "Assay in Low Water Environment"**

25

Transferase reactions of lipolytic enzymes in low water environment .

#### **Procedure**

30

#### **Materials.**

Cholesterol Sigma cat. C 8503

L-alpha-Phosphatidylcholine 95% (Plant) Avanti #441601



Soybean oil, Aarhus United, DK.

Chloroform, Analytical grade

Enzymes.

5 #179, GCAT from *A. salmonicida*

#2427, Phospholipase A1 from *Fusarium oxysporum*. LIPOPAN<sup>®</sup> F from Novozymes, Denmark

#1991, Phospholipase A2 from Pancreas, LIPOMOD 22L from Biocatalysts, UK

#2373, *Candida Antarctica* lipase, Novozyme 525 L from Novozymes Denmark.

10

#### Enzyme assay

13.1 % Lecithin and 6.6% cholesterol was dissolved in soybean oil by heating to 60 °C during agitation

The substrate was scaled in a 20ml Wheaton glass and heated to 46 °C

15 Water and enzyme solution was added and a stopwatch is started.

At regular intervals 50 mg samples were transferred to a 10ml Dram glass and frozen.

The isolated lipids were analysed by GLC

#### GLC analysis

20

GLC analysis was carried out as described in Example 11

#### Results

The experiment was set up as shown in Table 33

25 The substrate based on soybean oil containing 13.1 % lecithin and 6.6% cholesterol was heated to 46°C. The enzyme solution was added and a stopwatch started.

After 30, 60 and 120 minutes reaction time samples were taken out for GLC analysis.

30



220

Table 33

|                                      |      | 1   | 2   | 3   | 4   | 5   |
|--------------------------------------|------|-----|-----|-----|-----|-----|
| Substrate                            | gram | 5   | 5   | 5   | 5   | 5   |
| Transferase #179-C72, 56 PLU-7/ml    | ml   |     | 0.3 |     |     |     |
| #2427, 200 PLU-7/ml                  | ml   |     |     | 0.3 |     |     |
| Pancreas PLA 2 #1991 6300 PLU/ml     | ml   |     |     |     | 0.3 |     |
| Novozyme 525 L, #2373, 200 LIPU/mlml |      |     |     |     |     | 0.3 |
| Water                                | ml   | 0.3 |     |     |     |     |
| % water                              |      | 6   | 6   | 6   | 6   | 6   |

The results from the GLC analysis is shown in Table 34 . The results are expressed in percent based total sample composition. Based on the GLC results it was possible to calculate the amount of fatty acid and cholesterol ester produced by enzymatic reaction relative to the control sample without enzyme added. Under these experimental conditions the total enzymatic activity was estimated as the hydrolytic activity measured as free fatty acid formation and the transferase activity estimated as cholesterol ester formation. From these results and the information about molecular weight of fatty acid and cholesterol ester it was possible to calculate to relative molar hydrolytic activity and the relative molar transferase activity as shown in Table 35.

Table 34

| Enzyme  | Reaction        |                 |                  |                        |
|---------|-----------------|-----------------|------------------|------------------------|
|         | time<br>minutes | Fatty acid<br>% | cholesterol<br>% | Cholesterol ester<br>% |
| Control | 120             | 0.533           | 7.094            | 0.000                  |
| #179    | 30              | 0.770           | 5.761            | 2.229                  |



221

|       |     |       |       |       |
|-------|-----|-------|-------|-------|
| #179  | 60  | 0.852 | 5.369 | 2.883 |
| #179  | 120 | 0.876 | 4.900 | 3.667 |
| #2427 | 30  | 3.269 | 7.094 | 0.000 |
| #2427 | 60  | 3.420 | 7.094 | 0.000 |
| #2427 | 120 | 3.710 | 7.094 | 0.000 |
| #1991 | 30  | 2.871 | 7.094 | 0.000 |
| #1991 | 60  | 3.578 | 7.094 | 0.000 |
| #1991 | 120 | 3.928 | 7.094 | 0.000 |
| #2373 | 30  | 1.418 | 7.094 | 0.000 |
| #2373 | 60  | 1.421 | 7.094 | 0.000 |
| #2373 | 120 | 1.915 | 7.094 | 0.000 |

Table 35

| Enzyme | Reaction |            | CholesterolCholesterol ester Hydrolytic Transferase |                   |          |          |
|--------|----------|------------|-----------------------------------------------------|-------------------|----------|----------|
|        | time     | Fatty acid | Cholesterol                                         | Cholesterol ester | activity | activity |
|        | minutes  | produced   | Used                                                | produced          | %        | %        |
| #179   | 30       | 0.238      | 1.334                                               | 2.229             | 20       | 80       |
| #179   | 60       | 0.319      | 1.725                                               | 2.883             | 21       | 79       |
| #179   | 120      | 0.343      | 2.195                                               | 3.667             | 18       | 82       |
| #2427  | 30       | 2.737      | 0.000                                               | 0.000             | 100      | 0        |
| #2427  | 60       | 2.887      | 0.000                                               | 0.000             | 100      | 0        |
| #2427  | 120      | 3.177      | 0.000                                               | 0.000             | 100      | 0        |
| #1991  | 30       | 2.338      | 0.000                                               | 0.000             | 100      | 0        |
| #1991  | 60       | 3.046      | 0.000                                               | 0.000             | 100      | 0        |
| #1991  | 120      | 3.395      | 0.000                                               | 0.000             | 100      | 0        |
| #2373  | 30       | 0.885      | 0.000                                               | 0.000             | 100      | 0        |
| #2373  | 60       | 0.888      | 0.000                                               | 0.000             | 100      | 0        |
| #2373  | 120      | 1.383      | 0.000                                               | 0.000             | 100      | 0        |



### Conclusion

In these experiments it was observed that all the tested enzymes showed hydrolytic activity because the amount of fatty acid increased. However the only enzyme which showed transferase activity was GCAT from *A. salmonicida*. It is therefore concluded that in an oily system with lecithin and cholesterol containing 6% water phospholipase A1 from *Fusarium oxysporum*, phospholipase A2 from *pancreas* and a lipase from *Candida antarctica* only showed hydrolytic activity.

### Example 23 - Treatment of butterfat

10

Lipid acyl transferase derived from *Aeromonas salmonicida* (SEQ ID No. 90, N80D variant) was expressed in *Bacillus licheniformis* (hereinafter referred to as KLM3) (see below)..

15

The lipid acyl transferase was tested in butterfat with the aim to investigate the transfer reaction when 0,5% glycerol and 1% phospholipid was added to the butterfat.

20

The reaction products were analysed by TLC and the results clearly showed the formation of monoglyceride which confirm that lipid acyl transferase utilizes glycerol as acceptor molecule.

### Experimental

#### Enzymes:

Lipid acyl transferase (LAT) expressed in *B. licheniformis*: 2005876 (5500 TIPU/ml)

25

Lipomod 699L, pancreatic phospholipase from Biocatalysts. 10000 U/ml

Butterfat: Anhydrous Butterfat A0019659 lot 0130547 from Croman Belgium.

Glycerol:

Lecithin: Phosphatidylcholine 95% Plant (Avanti #441601),

30



## HPTLC

Applicator: LINOMAT 5, CAMAG applicator.

HPTLC plate: 10 x 10 cm (Merck no. 1.05633)

- 5 The plate was activated before use by drying in an oven at 160°C for 20-30 minutes.

Application: 1,0µl of a 15,0% solution of reacted butterfat dissolved in Chloroform:Methanol (2:1) was applied to the HPTLC plate using LINOMAT 5 applicator.

- 10 Running-buffer:1: P-ether:MTBE:Acetic acid (60:40:1)

Application/Elution time: 14 minutes.

Running-buffer:5: P-ether:MTBE:Acetic acid (70:30:1)

Application/Elution time: 12 minutes.

15

Running-buffer:4: Chloroform:Methanol:water (75:25:4)

Application/Elution time: 20 minutes.

Developing fluid: 6% Cupriacetate in 16% H<sub>3</sub>PO<sub>4</sub>

20

After elution the plate was dried in an oven at 160°C for 5 minutes, cooled and immersed in the developing fluid and then dried additional in 5 minutes at 160°C. The plate was evaluated visually and scanned (Camag TLC scanner).

## 25 Results

Samples of butterfat, glycerol, lecithin and enzyme were scaled in a 20 ml Wheaton glass

As outlined in table 36

30

**Table 36.**



|                            |    | 1  | 2  | 3   | 4  | 5   | 6   | 7   | 8   | 9   | 10  |
|----------------------------|----|----|----|-----|----|-----|-----|-----|-----|-----|-----|
| Croman Anhydrous Butterfat | g  | 10 | 10 | 10  | 10 | 10  | 9,9 | 9,9 | 9,9 | 9,9 | 9,9 |
| lecithin,                  | g  |    |    |     |    |     | 0,1 | 0,1 | 0,1 | 0,1 | 0,1 |
| LAT, 500                   | mg |    | 20 | 100 |    |     |     | 20  | 100 |     |     |
| Lipopmod 699L 1000         | mg |    |    |     | 20 | 100 |     |     |     | 20  | 100 |
| Glycero                    | mg | 50 | 30 |     | 30 |     | 50  | 30  |     | 30  |     |
| Units/g                    |    | 0  | 1  | 5   | 2  | 10  | 1   | 1   | 5   | 2   | 10  |

5

\* LAT 2005876 (5000 TIPU/ml) dissolved in glycerol:enzyme 9:1

\*\* Lipomod 699L (#3332) dissolved in glycerol:enzyme 9:1

10 The samples were placed in a heating block at 50 °C for 4 hours and then a sample was taken out for analysis and dissolved in chloroform : methanol 2:1.

The samples were analyzed by TLC in running buffer 5, 1 and 4 as shown in Figure. 104.

15 The TLC plate shown in Figure. 105 was scanned by a Camag Densiometric scanner and based on the amount of monoglyceride in the reference sample of monoglyceride the amount of monoglyceride in the butterfat is calculated as shown in table 37



Table 37. Monoglyceride in the butterfat samples calculated by densitometric

| Sample<br>Jour. 2390-67 | % Monoglyceride |
|-------------------------|-----------------|
| 1                       | 0,005           |
| 2                       | 0,005           |
| 3                       | 0,009           |
| 4                       | 0,005           |
| 5                       | 0,005           |
| 6                       | 0,004           |
| 7                       | <b>0,423</b>    |
| 8                       | <b>0,449</b>    |
| 9                       | 0,004           |
| 10                      | 0,004           |

measurement of TLC plate.

### Conclusion

- 5 The TLC results from enzymatic treatment of butter oil containing glycerol/phospholipids with lipid acyltransferase conform the ability of the enzyme to convert cholesterol into

cholesterolester and glycerol to monoglyceride using phospholipid as acyl donor.

- 10 In the experiment conducted it was shown that all phospholipids both phosphatidylcholine (PC) and lyso-phosphatidylcholine(LPC) can be completely converted to glycerophosphocholine.

The experiments also indicated that the pancreatic phospholipase is less active in low water environment and had no significant acyltransferase activity.

- 15 The enzyme modified butterfat (samples 7 & 8 of Table 37) is added to skimmed milk to a final concentration of 3.6wt% fat to produce a milk for use in the preparation of cheese.

### Example 24 – treatment of butterfat and cream

20

The lipid acyl transferase was tested in butterfat and cream (38% fat) with the aim to investigate the transfer reaction when 0,5% glycerol and 1% phospholipid was added to the butterfat.



The reaction products was analysed by TLC and the results from butterfat clearly showed the formation of monoglyceride and lysophospholipid. The results from experiment with cream also confirmed the formation of monoglyceride although at lower level, possibly due to a competitive hydrolytic reaction causing the formation of free fatty acids. In the experiments with cream little increase in lysophospholipid was observed, but this might be explained by too high enzyme dosage.

### Experimental

Enzymes:

10 Lipid acyl transferase (as per Example 23)

Butterfat: Anhydrous Butterfat A0019659 lot 0130547 from Croman Belgium.

Cream: 38% fat from ARLA, DK

Glycerol:

15 Lecithin: Phosphatidylcholine 95% Plant (Avanti #441601),

### HPTLC

Applicator: LINOMAT 5, CAMAG applicator.

20 HPTLC plate: 10 x 10 cm (Merck no. 1.05633)

The plate was activated before use by drying in an oven at 160°C for 20-30 minutes.

Application: 1,0µl of a 15,0% solution of reacted butterfat dissolved in Chloroform:Methanol (2:1) was applied to the HPTLC plate using LINOMAT 5 applicator.

25

Running-buffer: 1: P-ether:MTBE:Acetic acid (60:40:1)

Application/Elution time: 14 minutes.

Running-buffer: 5: P-ether:MTBE:Acetic acid (70:30:1)

30 Application/Elution time: 12 minutes.



Running-buffer:4: Chloroform:Methanol:water (75:25:4)

Application/Elution time: 20 minutes.

## 5 Developing fluid: 6% Cupriacetate in 16% H<sub>3</sub>PO<sub>4</sub>

After elution the plate was dried in an oven at 160°C for 5 minutes, cooled and immersed in the developing fluid and then dried additional in 5 minutes at 160°C. The plate was evaluated visually and scanned (Camag TLC scanner).

## Results

Samples of butterfat, glycerol, lecithin and enzyme were scaled in a 20 ml Wheaton glass

As outlined in table 38

Table 38.

|                                                    | 1   | 2   |
|----------------------------------------------------|-----|-----|
| Croman, Anhydrous Butterfat A0019659 lot 0130547 g | 10  | 10  |
| Cream, 38% g                                       |     |     |
| Lecithin, Avanti g                                 | 0,1 | 0,1 |
| LAT, 500 TIPU/ml* mg                               |     | 50  |
| Glycerol mg                                        | 50  |     |
| Units/g                                            | 0   | 2,5 |

\*LAT (5000 TIPU/ml) dissolved n glycerol:enzyme 9:1

The samples were placed in a heating block at 45°C and samples were taken out after 10, 30, 60, and 120 minutes and dissolved in chloroform : methanol 2:1.

The samples were analyzed by TLC in running buffer 5, 1 and 4 as shown in Figure. 106, 107 and 108.



### Conclusion. Butterfat experiment.

The TLC results from enzymatic treatment of butter oil containing glycerol/phospholipids with lipid acyltransferase confirm the ability of this enzyme to  
 5 convert cholesterol into cholesterol ester and glycerol to monoglyceride using phospholipid as acyl donor.

In the experiment conducted it was shown that phospholipid(PC) was converted to lyso-phosphatidylcholine(LPC). By extended reaction time lyso-phospholipid (LPC)  
 10 was further converted to glycerophosphocholine. It is therefore possible to optimize enzyme dosage and reaction time in order to identify the optimum level of monoglyceride and lysophospholipid production for any particular application.

The enzyme modified butterfat is added to skimmed milk to a final concentration of  
 15 3.6wt% fat to produce a milk for use in the preparation of cheese. Initial experiments indicate that the enzyme modified butter fat may be more easily incorporated into the skimmed milk when compared to non modified butter fat.

### Results with cream

20 Samples of cream, glycerol, lecithin and enzyme were scaled in a 20 ml Wheaton glass  
 As outlined in table 39

Table 39.

|                                                    |    | 3   | 4   |
|----------------------------------------------------|----|-----|-----|
| Croman, Anhydrous Butterfat A0019659 lot 0130547 g |    |     |     |
| Cream, 38%                                         | g  | 10  | 10  |
| lecithin, Avanti                                   | g  | 0,1 | 0,1 |
| LAT, 500 TIPU/ml*                                  | mg |     | 50  |
| Glycerol                                           | mg | 50  |     |

\*LAT (5000 TIPU/ml) dissolved in glycerol:enzyme 9:1



The samples were placed in a heating block at 45°C and samples were taken out after 10, 30, 60, and 120 minutes and dissolved in chloroform : methanol 2:1.

- 5 The samples were analyzed by TLC in running buffer 5, 1 and 4 as shown in figures. 109, 110 and 111.

Conclusion. Cream experiment.

10 The TLC results from treatment of cream containing phospholipid and glycerol with an enzyme lipid acyl transferase clearly confirm the transfer reaction of acyl groups from phospholipid (PC) to cholesterol during formation of cholesterol ester.

The transferase reaction of acyl groups to glycerol was also observed. There was also a noticeable hydrolytic activity. Further optimization of to produce optimum level of monoglyceride via modulation of enzyme dosage, glycerol dosage and reaction time is  
15 therefore is therefore possible.

### EXAMPLE 25 – Production of Mozzarella

#### Enzymes

- 20 EDS 188: Lipid acyltransferase in accordance with the present invention, (herein referred to KLM3) expressed in *B. lichiniiformis*: 2005876 (1460 TIPU/ml) (SEQ ID No. 90, N80D variant).

Lecitase, pancreas phospholipase, Sigma P0861, 10,000 unit/ml.

#### 25 Day 1.

1. Milk was separated at 55 °C into skim (~ 0.075% w/w fat) and cream (30%, w/w) fat A “skim” (0.83%, w/w) fat was prepared by blending the skim milk and cream (see Figure 120)
- 30 2. 0.4g CaCl<sub>2</sub> (50%, w/v) per kg of cream (30% fat) was added and the cream was divided into 3 equal lots- namely for control (Vat 1), Lecitase (Vat 2) and KLM3' (Vat 3).



3. 0,2%(w/w of fat content), Lecitase to Vat 2 equivalent to 0.06% (w/w of 30 % fat cream) or equivalent to 0.6 g per kg of 30% fat cream was added.
4. KLM3' 25 TIPU/kg cream was added to Vat 3.
5. In the control (Vat 1), no enzyme solution (or water) was added.
- 5 6. All cream treatments (incl. control) were incubated at 50 °C for 30 min.
7. Immediately thereafter, the correct weight of each cream to the correct of cold (10 °C) "skim milk (0.83% w/w) fat was added to get the correct fat content (3.5%, w/w) in the mixtures, which are the standardized milks.
8. These were pasteurised at 72 °C for 26 seconds.
- 10 9. Cooled to 5 C and held overnight.

## Day 2.

10. The milk was heated to 41°C and kept it for 30 minutes (This was done so as to  
15 reverse the cold storage ageing effects on the milk).
11. The milk was cooled to 34.4°C
12. Starter culture was added (Choozit Ta 61 100DCU, Choozit LH100 50 DCU in  
DAN 011, Dan 012, DAN 013; and Choozit Ta 61 100 DCU, Choozit LH100  
23.3 DCU in DAN 021, DAN 022, DAN 023). DAN 021, DAN 022 and DAN  
20 023 were dosed with a reduced amount of Choozit LH100 to reflect the addition  
rates of *Helveticus* culture normally used in industrial mozzarella production.  
The cultures were added directly to the cheese milk and left for 45 min. with  
agitation
13. The rennet was added ((145 ml Marzyme10 (140 imcu/ml) diluted to 1 litre  
25 with water),
14. The rennet was mixed in for 2 min.  
A sample of the rennet milk was taken and placed in a rheometer to measure the  
change in the elastic modulus,  $G'$ , as a function of time.
15. The gel (curd) in the vat was cut when the firmness ( $G'$ ) reached 40 Pa as  
30 determined on a controlled stress rheometer.
16. The gel was cut using a wire grid – (speed 2 - 15 seconds, stand 1 min, cut  
speed 1 -15 seconds, stand 1 min, Cut speed 1 -10 seconds) and the curd whey



mix was allowed to sit quiescently (heal). This healing step is incorporated in industrial cheesemaking to minimize fat losses to the whey.

17. The curd whey mixture was stirred (at 10 min from beginning of cut period) for 5 min, so as to get curd/whey mix in motion.

5 18. The curd/whey mix was heated to 41.1°C in 30 min.

19. Stirring continued until curd pH (as measured on whey squeezed from the curd) reached 5.9.

20. The curd whey mixture was drained into finishing vat, and the whey removed by gravity flow.

10 21. The curd was trenched to sides of vat, leading to 2 curd trenches.

22. The curd trenches were cut into slabs.

23. The curd slabs were turned every 15-20 min and held in the finishing vat until the pH (as measured by inserting pH probe into sample of curd) reached 5.25.

24. The curd was then milled into chips (~ 0.75 cm x ~ 0.75 cm x ~ 7 cm long).

15 25. Covered with cold water (17°C) for 15 mins.

26. The water drained for 10 mins.

27. The curd was weighed and salt added to the curd at a level of 0.2% (w/w) of cheese milk weight (0.9kg to cheese curd from 450 kg of milk). The curd was left to absorb applied salt for 20 min

20 28. The curd was placed into a plasticization kneading/stretching unit (via the shredding unit built into the equipment).

29. The curd was kneaded/stretched while it is heated to 63°C by circulating water at 80C.

30. The curd was placed in 7°C water for 30 min

25 31. The curd was then placed in 7°C brine (23% NaCl) for 90 min.

32. The curd was remove from the brine and left to drain for 10 mins.

33. The brined curd was weighed

34. Vacuum packed, and placed at 4°C.



## Results

Table 40 – Cheese yield and fat content of whey

| Code   | Wt Milk<br>kg | wt curd in<br>moulds<br>kg | unmoulded<br>curd<br>kg | Curd ex<br>brine<br>kg | Total wt of<br>salted cheese<br>kg | Cheese yield<br>kg/100<br>milk<br>kg | Fat in whey<br>%, w/w |
|--------|---------------|----------------------------|-------------------------|------------------------|------------------------------------|--------------------------------------|-----------------------|
| DAN011 | 454,1         | 26,62                      | 18,36                   | 26,78                  | 45,25                              | 9,96                                 | 0,48                  |
| DAN012 | 454,6         | 26,62                      | 21,66                   | 26,69                  | 48,41                              | 10,65                                | 0,41*                 |
| DAN013 | 454,2         | 26,6                       | 23,56                   | 26,83                  | 50,59                              | 11,14*                               | 0,34*                 |
| DAN021 | 454,4         | 26,55                      | 18,63                   | 26,7                   | 45,44                              | 10,00                                | 0,51                  |
| DAN022 | 454,1         | 26,5                       | 20,69                   | 26,69                  | 47,53                              | 10,47                                | 0,41*                 |
| DAN023 | 454,3         | 26,4                       | 21,61                   | 26,52                  | 48,23                              | 10,62*                               | 0,35*                 |

DAN011 and DAN021 = control, DAN 012 and DAN022 = Lecitase, DAN013 and  
 5 DAN023 = KLM3 [\* means statistically significant compared with the control]

**EXAMPLE 26: PIZZA MADE WITH ENZYME MODIFIED CHEESE**

10 The cheese prepared according to Example 25 is used in the preparation of pizza.

## Pizza base

500gms strong white flour

15 12gms fresh yeast dissolved in 200-250ml water water containing 1 teaspoon of  
 dissolved sugar, and allowed to stand at 20°C for 10 minutes.

1 egg

1-2 tablespoons olive oil to taste.

Salt to taste

20

The above are mixed and subsequently kneaded by hand for 5 minutes to produce a  
 dough. The dough is left, covered by a damp cloth, to rise until at least doubled in  
 volume. The dough is then rolled until approximately 5mm-1cm thin depending on  
 taste.

25



A tomato sauce is prepared by briefly frying finely chopped onion and garlic in a pan with olive oil and adding chopped tomatoes. The sauce is reduced to a desirable consistency. When cool, the sauce is added to the rolled out pizza dough.

- 5 The cheese prepared in Example 25 is added, vegetable, meat and seafood toppings may also be added. The pizza is baked at 200°C on a stone base in a fan assisted oven.

The pizza made with the cheese comprising the edible oil/fat of the invention appears to have noticeably less surface oil and the baked pizza base appear to be less saturated  
10 with the oil, especially around the edges, and on the surface of the sauce and toppings (see Figure 136). This makes the pizza more appetizing to handle and to eat.

The pizza has an improved over appearance with less visible oiling off.

15 **EXAMPLE 27 – Lipid analysis**

Cream and cheese from the production of mozzarella as detailed in Example 25 were analysed as follows:

20 **Lipid analysis**

Cream and cheese from the production of Mozzarella cheese as detailed in Example 25 were extracted with organic solvents and the isolated lipids were analysed by HPTLC and GLC. In the cheese experiment the cream used to produce the cheese was treated  
25 with a pancreatic phospholipase (Lecitase) or a lipid acyltransferase according to the present invention (KLM3). A control experiment without any enzyme treatment was also conducted. All three experiments were made in duplicate over two days.

Lipid analysis of isolated lipids from enzyme treated cream as well as the cheese  
30 produced from the creams showed that both Lecitase and KLM3 were active on the phospholipids in the products, and the main phospholipids, phosphatidylcholine(PC) and phosphadidylethanolamine(PE) were almost completely degraded.



In the Lecitase treated sample the degradation of PC and PE was followed by concomitant formation of free fatty acids, mainly oleic acid and linoleic acid. In the experiment with KLM3 the formation of free fatty acids were significantly lower than  
5 the degradation of phospholipids because this enzyme carried out a transfer reaction of fatty acids from phospholipids to cholesterol which resulted in the formation of cholesterol esters. In the cheese samples treated with KLM3 only 40% cholesterol was left compared with control and Lecitase treated cheeses. In the cheese treated with KLM3 small amounts of saturated free fatty acids were formed, because of unspecific  
10 activity on the saturated fatty acids in the sn-1 position of the phospholipids.

The enzyme treatment was made in a 30% cream which after enzymation was added to skim milk and adjusted to 3.5% fat for cheese production.

15 In this report the analyses of lipid components in the cream used for the cheese production as well as the cheese were analysed.

## Materials and Methods

### Enzymes:

20 EDS 188: Lipid acyltransferase in accordance with the present invention, (hereinafter referred to KLM3) expressed in *B. lichiniformis*: 2005876 (1460 TIPU/ml), (SEQ ID No. 90, N80D variant).

Lecitase, pancreas phospholipase, Sigma P0861, 10,000 unit/ml.

25 TLC standards:

ST16: 0,5% solution of phospholipids containing 14.76% Phosphatidylcholine(PC), 0.49% Lyso-phosphatidylcholine(LPC), 10.13% Phosphatidylinositol(PI), 12.74% Phosphatidylethanolamine(PE) and 5.13% Phosphatidic acid(PA).

30

ST17: 0.1% solution of cholesterol, 0,1% cholesterolsteareate and 0,1% oleic acid.



**Enzymation of cream used for Mozzarella cheese production**

Was carried out as disclosed in Example 25.

**5 HPTLC**

Applicator: CAMAG applicator AST4.

HPTLC plate: 20 x 10 cm (Merck<sup>\*</sup> no. 1.05641)

The plate was activated before use by drying in an oven at 160°C for 20-30 minutes.

- 10 Application: 3,0µl of extracted lipids dissolved in Chloroform:Methanol (2:1) was applied to the HPTLC plate using AST4 applicator. 0.1, 0.3, 0.5, 0.8, 1.5µl of a standard solution of standard components with known concentration are also applied to the HPTLC plate.

- 15 Running-buffer:1: P-ether:MTBE:Acetic acid (50:50:1)

Application/Elution time: 12 minutes.

Running-buffer:6: Methyl-acetate: Chloroform:Methanol:Isopropanol:0,25% KCl solution in water. (25:25:25:10:9)

- 20 Application/Elution time: 20 minutes.

Developing fluid: 6% Cupriacetate in 16% H<sub>3</sub>PO<sub>4</sub>

- 25 After elution the plate was dried in an oven at 160°C for 10 minutes, cooled and immersed in the developing fluid and then dried additional in 5 minutes at 160°C. The plate was evaluated visually and scanned (Camag TLC scanner).

- 30 After drying the TLC spots are quantified by scanning the plate in a TLC Scanner 3 from Camag. Based on the density of the standard component a calibration curve is constructed, and used for quantification of the components in the sample.

\* Trade-mark



GLC analysis

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT  
fused silica column 12.5 m x 0.25 mm ID x 0.1  $\mu$  film thickness 5% phenyl-methyl-  
5 silicone (CP Sil 8 CB from Chrompack).

Carrier gas: Helium.

Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0 $\mu$ l

Detector FID: 395°C

|                                       |                           |    |     |     |
|---------------------------------------|---------------------------|----|-----|-----|
| Oven program (used since 30.10.2003): |                           | 1  | 2   | 3   |
| 10                                    | Oven temperature, °C.     | 90 | 280 | 350 |
|                                       | Isothermal, time, min.    | 1  | 0   | 10  |
|                                       | Temperature rate, °C/min. | 15 | 4   |     |

Sample preparation: Lipid extracted from cheese or cream samples was dissolved in  
15 0,5 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml.  
300 $\mu$ l sample solution is transferred to a crimp vial, 300  $\mu$ l MSTFA (N-Methyl-N-  
trimethylsilyl-trifluoroacetamide) is added and reacted for 20 minutes at 60°C.

Calculation: Response factors for Free Fatty Acid (FFA), Cholesterol, Cholesteryl  
palmitate and Cholesteryl stearate were determined from pure reference material.

20

Extraction Cream.

Cream samples in Eppendorph tubes were heated at 99°C for 10 min. in order to  
inactivate the enzyme, and cooled to ambient temperature. 1 ml cream was transferred  
25 to a 10 ml dram glass with screw lid. 3 Ml Chloroform:Methanol 2:1 was added and  
mixe on a Whirley. The sample was extracted for 30 min on a Rotamix. The sample  
was centrifuged for 10 min. at 1700 g. The lower organics phase was isolated and used  
for TLC and GLC analysis.

30 Extraction Cheese



0.5 g cheese was scaled in a 12 ml centrifuge with screw lid. 2 ml 99% Ethanol was added and the sample was homogenized with a Ultra Turrax Mixer for 30 sec at 20000 rpm. The mixer was rinsed with 1.5 ml Ethanol. 5 ml Chloroform was added and mixed on a whirley. The sample was extracted for 30 min on a Rotamix 25 rpm. The sample was centrifuged for 10 min. at 1700 g.

The lower organics phase was isolated and used for TLC and GLC analysis

### Samples

Table 41 - Cream samples taken out after 30 min enzymation.

| Test No. | Enzyme   | Dosage, ppm | Day |
|----------|----------|-------------|-----|
| DAN011   | Control  | 0           | 1   |
| DAN012   | Lecitase | 600         | 1   |
| DAN013   | KLM3     | 17.1        | 1   |
| DAN021   | Control  | 0           | 2   |
| DAN022   | Lecitase | 600         | 2   |
| DAN023   | KLM3     | 17.1        | 2   |

Table 42 - Labeliing of Mozzarella Cheese samples

| Test No. | Enzyme   | Day |
|----------|----------|-----|
| DAN011   | Control  | 1   |
| DAN012   | Lecitase | 1   |
| DAN013   | KLM3     | 1   |
| DAN021   | Control  | 2   |
| DAN022   | Lecitase | 2   |
| DAN023   | KLM3     | 2   |

### Results



Cream lipid analysis.

Samples of cream used for the production of cheese were extracted with Chloroform methanol according to the procedure mentioned under Materials and Methods and  
5 analysed by HPTLC.

The results from TLC analysis of the cream samples are shown in Figures 121 and 122.

10 Figure 121 shows the TLC (solvent 6) of lipid extracted from cream and a standard mixture (ST16) of phospholipids; Phosphatidylcholine(PC); Lyso-phosphatidylcholine (LPC); Phosphatidylinisitol (PI); Phosphatidylethanolamine (PE); 5.13% Phosphatidic acid(PA); and Spingholipid (SG)

15 Figure 122 shows a. TLC (solvent 1) of lipid extracted from cream and a standard mixture of free fatty acids (FFA), cholesterol (CHL) and cholesterol ester (CHL-ester).

The density of the bands from the TLC chromatogram were determined, and based on the standard mixture of phospholipids the amount of PC and PE were calculated from  
20 the TLC chromatogram in Figure 121 and based on the standard mixture of cholesterol and fatty acids the amount of free fatty acids and cholesterol in the samples were calculated from the TLC chromatogram. The results are shown in table 43.

Table 43. Analysis of Phosphatidylcholine(PC), phosphatidylethanolamine (PE) ,  
25 cholesterol (CHL) and free fatty acids (FFA) based on TLC chromatograms Figures 121 and 122

| Enzyme   | Day | ppm<br>PC | Ppm<br>PE | ppm<br>CHL | ppm<br>FFA |
|----------|-----|-----------|-----------|------------|------------|
| Control  | 1   | 149       | 278       | 713        | 201        |
| Lecitase | 1   | 23        | 17        | 638        | 396        |
| KLM3     | 1   | 11        | 24        | 328        | 274        |



|          |   |     |     |     |     |
|----------|---|-----|-----|-----|-----|
| Control  | 2 | 117 | 214 | 638 | 166 |
| Lecitase | 2 | 39  | 29  | 629 | 345 |
| KLM3     | 2 | 15  | 28  | 311 | 201 |

The results in table 43 were evaluated statistically by ANOVA using Statgraphic Plus for Windows 3.1. The statistical evaluation for cholesterol and free fatty acid are illustrated graphically in Figures 123 and 124.

5

TLC analysis of cream treated with Lecitase and KLM3 has shown a strong effect of phospholipases in the cream (Figure 121) and it is seen that the two main phospholipid components PC and PE are almost completely hydrolyzed (table 43).

10 In Figure 122 it is shown that KLM3 has a strong impact on the cholesterol compared to Lecitase. It is also observed that the amount of fatty acids produced in sample treated with Lecitase are clearly higher than the samples treated with KLM3 and control.

15 A statistical evaluation of the amount of fatty acids (Figure 124) shows that KLM3 produces a small but not significant amount of free fatty acids compared with control. The amount of fatty acids in the sample treated with Lecitase is however significantly higher. This is explained by the fact that Lecitase hydrolyses phospholipids resulting in the formation of free fatty acids. KLM3 also degrades the phospholipids (Table 43) but  
20 results in the fatty acids from the phospholipids being transferred to cholesterol, thus resulting in the formation of cholesterol ester. This is also confirmed by the fact that the amount of cholesterol is significantly lower in the sample treated with KLM3 whereas control and Lecitase treated samples are on the same level (see Figure 123).

25 On a molar ratio it can be calculated that the amount of degraded PC and PE is 0,6 mmol/kg for both Lecitase and KLM3 and the amount of fatty acids produced is 0,65 mmol/kg in Lecitase treated cream and 0,2mmol/kg for the KLM3 treated cream, which confirms the observations that Lecitase hydrolyzes phospholipids, but KLM3 catalyses a transfer reaction.



The lipids extracted from cream after 30 minutes enzymation were also analyzed by GLC in order to quantify specific fatty acids, cholesterol and cholesterol ester.

5 The results from GLC analysis are shown in table 44

Table 44. GLC analysis of palmitic acid (FFA-16), oleic acid (C18:1), linoleic acid (C18:2), stearic acid (C:18:0), Sum FFA (C16:0, C18:0, C18:1 and C18:2), cholesterol and cholesterol ester.

| Enzyme   | Day | FFA-16<br>ppm | FFA-18:1<br>and C:18:2<br>ppm | FFA-C18:0<br>ppm | Sum FFA<br>ppm | Cholesterol<br>ppm | Cholesterol<br>ester<br>Ppm |
|----------|-----|---------------|-------------------------------|------------------|----------------|--------------------|-----------------------------|
| Control  | 1   | 119           | 154                           | 54               | 327            | 551                | 0                           |
| Lecitase | 1   | 133           | 316                           | 60               | 508            | 546                | 0                           |
| KLM3     | 1   | 125           | 177                           | 51               | 353            | 216                | 286                         |
| Control  | 2   | 111           | 152                           | 54               | 317            | 520                | 0                           |
| Lecitase | 2   | 130           | 314                           | 62               | 507            | 547                | 0                           |
| KLM3     | 2   | 130           | 195                           | 63               | 388            | 238                | 335                         |

10

The results in table 44 are evaluated statistically by ANOVA using Statgraphic Plus for Windows 3.1. The statistical evaluation for cholesterol, cholesterol ester and Sum free fatty acid (FFA) are illustrated in Figures 125 to 127.

15 The GLC analysis confirms what already was observed by TLC analysis, that KLM3 significantly reduces the amount of cholesterol (see Figure 126) compared with control and Lecitase treated cream. The cholesterol in the KLM3 treated cream is converted to cholesterol ester (see Figure 121), whereas cream treated with Lecitase and control contain no cholesterol ester. The formation of cholesterol ester also has an impact on  
 20 the level of free fatty acid (see Figure 127) where Lecitase produces a significant amount of free fatty acids by hydrolysis of phospholipids, and KLM3 only produces a small and not significant amount of free fatty acids. It is also observed that it is mainly the unsaturated fatty acid which increases during enzymation, because Lecitase is a sn-



2 specific phospholipase and KLM3 is sn-2 specific with regard to transferase reaction. In naturally occurring phospholipids the sn-2 position contains mainly unsaturated fatty acids.

5 Cheese lipids analysis

Samples of cheese produced from enzyme modified cream were extracted with chloroform ethanol according to the procedure mentioned above and analyzed by HPTLC and GLC.

10

Each sample was analyzed in duplicate.

The results from the HPTLC analysis are shown in Figures 128 and 129.

15 The TLC chromatogram shown in Figure 129 indicates that both Lecitase and KLM3 has completely hydrolyzed the phospholipids phosphatidylcholine and phosphatidylethanolamine. The chromatogram in Figure 128 illustrates that cheese treated with KLM3 has a reduced content of cholesterol compared with control and Lecitase treated cheese. It is also observed the amount of free fatty acids in cheese  
20 treated with KLM3 is lower than cheese treated with Lecitase although both enzymes completely hydrolysis phospholipids PC and PE.

GLC analysis of lipids from Mozzarella cheese.

25 The lipids extracted from cheese were also analyzed by GLC in order to quantify specific fatty acids, cholesterol and cholesterol ester. Each cheese was extracted and analyzed in duplicate.

The results from the GLC analysis is shown in Table 45. The fatty acid analysis is  
30 split up in the amount of palmetic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18.2) and stearic acid (C:18:0).



Table 45: GLC analysis of lipids from Mozzarella cheese.

| Enzyme   | Day | FFA-16 | FFA-18:1 and<br>18:2 | FFA-18:0 | Sum FFA | Cholesterol | Cholesterol<br>ester |
|----------|-----|--------|----------------------|----------|---------|-------------|----------------------|
| Control  | 1   | 291    | 291                  | 158      | 740     | 689         | 0                    |
| Control  | 1   | 304    | 275                  | 156      | 735     | 758         | 0                    |
| Lecitase | 1   | 345    | 566                  | 195      | 1105    | 688         | 0                    |
| Lecitase | 1   | 336    | 546                  | 180      | 1062    | 690         | 0                    |
| KLM3     | 1   | 374    | 453                  | 202      | 1030    | 296         | 440                  |
| KLM3     | 1   | 399    | 481                  | 228      | 1109    | 304         | 492                  |
| Control  | 2   | 285    | 259                  | 160      | 703     | 726         | 0                    |
| Control  | 2   | 302    | 261                  | 167      | 730     | 702         | 0                    |
| Lecitase | 2   | 354    | 584                  | 202      | 1140    | 728         | 0                    |
| Lecitase | 2   | 357    | 591                  | 202      | 1150    | 744         | 0                    |
| KLM3     | 2   | 377    | 458                  | 221      | 1056    | 302         | 419                  |
| KLM3     | 2   | 388    | 485                  | 227      | 1099    | 315         | 487                  |

The results in table 45 showing the GLC analysis of lipids in Mozzarella cheese were evaluated statistically by ANOVA using Statgraphic Plus for Windows 3.1. The statistical evaluation for cholesterol, cholesterol ester, Oleic acid + linoleic acid and Sum FFA are illustrated in Figures 130 to 133.

GLC analysis of lipids in Mozzarella has confirmed the effect of KLM3 on cholesterol (see Figure 130) and the formation of cholesterol ester (see Figure 131). Cheese produced with KLM3 contains only 40% cholesterol compared with the control cheese. Lecitase did not show any affect on the cholesterol level and no cholesterol ester was formed in control and Lecitase treated cheese.

Because of the transfer reaction it is also seen that the amount of free fatty acids in the cheeses produced with KLM3 is lower than in cheese produced with Lecitase. This is clearly seen for the unsaturated fatty acids oleic acid and linoleic acids (see Figure 132), which are lower in the trials with KLM3 compared with Lecitase. However the



differences are less pronounced for Palmitic acid and Stearic acid (see Table 45). It is known that pancreas phospholipase - Lecitase is very specific for the sn-2 position of the phospholipids and thus primary produces unsaturated fatty acids. Some unspecific hydrolytic activity of KLM3 is known, which can explain the formation of saturated fatty acids from sn-1 position of phospholipids in milk fat.

In this experiment it is seen that almost all phospholipids are degraded after 30 minutes enzymation of the cream. However the enzyme reaction continues during the standardization of the cheese milk until the cheese milk was pasteurized. The ongoing enzyme reaction after enzymation of cream, until the cheese milk is pasteurized explains the formation saturated fatty acids C16:0 and C18:0 in the experiment with KLM3. This is also confirmed by the fact that no saturated fatty acids are formed in cream after 30 minutes enzymation with KLM3, but is only seen in the cheese. The formation of saturated fatty acids in the experiment with KLM3 can be reduced or prevented by reducing the incubation time of the cream.

### Conclusion

Enzymation of cream for use in Mozzarella cheese production has shown that KLM3 and Lecitase were very active on phospholipids in milk fat. An almost complete conversion of the phospholipids phosphatidylcholine and phosphatidylethanolamine were observed.

The activity of Lecitase on phospholipids contributed to an increase in free fatty acids. The fatty acids produced were mainly the unsaturated fatty acids oleic acid and linoleic acid, because Lecitase is sn-2 specific and the unsaturated fatty acids are most abundant in the sn-2 position of the phospholipids.

KLM3 however produced less free fatty acids because this enzyme transfers fatty acids from phospholipids to cholesterol during formation of cholesterol ester.



Lipid analysis of lipid extracted from the final product Mozzarella cheese showed almost the same lipid profiles as observed for the cream used to produce the Mozzarella cheese.

#### 5 **Example 28: Moisture analysis**

Cheese from six experiments with the use of enzyme in pilot scale Mozzarella cheese production (see Example 25) were analyzed for moisture content by standard method IDF 4A, 1982 and the fat content was determined by the standard method IDF 5B,  
10 1986 from International Dairy Federation.

Results:

Table 46 – Analysis of moisture and fat content.

| Cheese |          | %Moisture | %Fat  |
|--------|----------|-----------|-------|
| DAN011 | Control  | 48,75     | 23,26 |
| DAN012 | Lecitase | 50,95     | 23,02 |
| DAN013 | KLM3     | 52,03     | 22,70 |
| DAN021 | Control  | 48,67     | 24,69 |
| DAN022 | Lecitase | 49,60     | 24,25 |
| DAN023 | KLM3     | 51,66     | 23,67 |

15

The moisture content of the cheese was influenced by the enzyme treatment; the KLM3 acyl transferase significantly increased the moisture content of the cheese, both when compared to the lecitase as well as the control. This partly explains the  
20 increased yield obtained by the enzyme treatment. The percentage of fat in the cheese thus decreases slightly due to the total increase in yield.

25



**Example 29: Oiling off analysis**

Cheese from experiments with the use of enzyme in pilot scale Mozzarella cheese production (see Example 25) were analyzed for oiling off by a diffusion test. After  
5 production the cheeses matured for 8 days at 6°C.

**Oiling Off Diameter Test:**

Cheese samples (2 g) were ground up and pressed into a 2 cm wide ring using a weight  
10 of 16g dropped from a 5 cm height, applied three times in order to make a compact mass. This is a key point for measuring the oiling off, unless the amount of force used to create the sample is known (along with the resistance of the material being compacted) it will be unclear as to the density of the final mass which has a direct effect on oiling off during heating (see Figure 134).

15

The samples were placed on Whatman number 4 filter papers and heated together in a drying oven at 90.0°C for 5 minutes.

20

Measurements of oiling off as determined by the diameter of translucent zones seen on the filter papers were measured after 10 minutes.

**Results:**

Table 47 – oiling off

| Cheese | Mean  | SD   | Av. Area<br>/mm <sup>2</sup> | % of<br>control<br>area |
|--------|-------|------|------------------------------|-------------------------|
| DAN011 | 32.33 | 1.25 | 821.09                       |                         |
| DAN013 | 25.00 | 2.16 | 490.87                       | 59.78                   |

25

DAN011 was a no enzyme control. DAN013 was treated with KLM3.



Figure 135 shows the photos of the control samples DAN011 (left) and the cheese produced with KLM3 DAN013 (right). 5 minutes standing after heating step.

## 5 Conclusions:

As can be seen from the results, after 10 minutes the day the KLM3 cheese did indeed register significantly less oiling off than the control.

## 10 Example 30 - Melting test:

Cheese from experiments with the use of enzyme in pilot scale Mozzarella cheese production (see Example 25) were analyzed for melting ability by the tube method described by Olsen (Olsen, NF. & WV. Price, Journal of Dairy Science 1958, Vol. 41: 999-1000). The cheese flow is measured as percentage change from the starting point before heating the tube (Olsen 1958).

Results:

20 Table 48 – cheese flow results.

| Cheese |          | Cheese flow (%) |
|--------|----------|-----------------|
| DAN011 | Control  | 211             |
| DAN012 | Lecitase | 217             |
| DAN013 | KLM3     | 221             |
| DAN021 | Control  | 168             |
| DAN022 | Lecitase | 200             |
| DAN023 | KLM3     | 200             |

No statistically significant difference was observed in the melting test for the cheese, thus neither Lecitase nor the acyl transferase KLM3 changed the melting properties of the cheese.



247.

Melting properties was also determined by baking a pizza, to determine visual changes of the mozzarella cheese as compared to the control without enzyme. The cheese showed less oiling off on the pizza and normal melting properties.

5

### **Example 31 - Expression of a lipid acyltransferase in *Bacillus licheniformis***

A nucleotide sequence (SEQ ID No. 100) encoding a lipid acyltransferase (SEQ. ID No. 90, hereinafter KLM3) was expressed in *Bacillus licheniformis* as a fusion protein with the signal peptide of *B. licheniformis* [alpha]-amylase (LAT) (see FIGS. 137 and 138). For optimal expression in *Bacillus*, a codon optimized gene construct (no. 052907) was ordered at Geneart (Geneart AG, Regensburg, Germany).

Construct no. 052907 contains an incomplete LAT promoter (only the -10 sequence) in front of the LAT-KLM3' precursor gene and the LAT transcription (Tlat) downstream of the LAT-KLM3' precursor gene (see FIGS 137 and 139). To create a *XhoI* fragment that contains the LAT-KLM3' precursor gene flanked by the complete LAT promoter at the 5' end and the LAT terminator at the 3' end, a PCR (polymerase chain reaction) amplification was performed with the primers Plat5XhoI\_FW and EBS2XhoI\_RV and gene construct 052907 as template.

Plat5XhoI\_FW:

ccccgctcgaggcttttcttttgaagaaaatatagggaatggtactgttaaaaattc  
ggaatattatacaatatcatatgtttcacattgaaagggg

EBS2XhoI\_RV: tggaatctcgaggttttatcctttacctgtctcc

PCR was performed on a thermocycler with Phusion High Fidelity DNA polymerase (Finnzymes OY, Espoo, Finland) according to the instructions of the manufacturer (annealing temperature of 55[deg.] C.).

30



The resulting PCR fragment was digested with restriction enzyme *Xho*I and ligated with T4 DNA ligase into *Xho*I digested pICatH according to the instructions of the supplier (Invitrogen, Carlsbad, Calif. USA).

- 5 The ligation mixture was transformed into *B. subtilis* strain SC6.1 as described in U.S. Patent Application US20020182734 (International Publication WO 02/14490). The sequence of the *Xho*I insert containing the LAT-KLM3' precursor gene was confirmed by DNA sequencing (BaseClear, Leiden, The Netherlands) and one of the correct plasmid clones was designated pICatH-KLM3'(ori1) (Figure 137). pICatH-  
10 KLM3'(ori1) was transformed into *B. licheniformis* strain BML780 (a derivative of BRA7 and BML612, see WO2005111203) at the permissive temperature (37[deg.] C.).

- One neomycin resistant (neoR) and chloramphenicol resistant (CmR) transformant was  
15 selected and designated BML780(pICatH-KLM3'(ori1)). The plasmid in BML780(pICatH-KLM3'(ori1)) was integrated into the catH region on the *B. licheniformis* genome by growing the strain at a non-permissive temperature (50[deg.] C) in medium with 5 [mu]g/ml chloramphenicol. One CmR resistant clone was selected and designated BML780-pICatH-KLM3'(ori1). BML780-pICatH-  
20 KLM3'(ori1) was grown again at the permissive temperature for several generations without antibiotics to loop-out vector sequences and then one neomycin sensitive (neoS), CmR clone was selected. In this clone, vector sequences of pICatH on the chromosome are excised (including the neomycin resistance gene) and only the catH - LATKLM3' cassette is left. Next, the catH - LATKLM3' cassette on the chromosome  
25 was amplified by growing the strain in/on media with increasing concentrations of chloramphenicol. After various rounds of amplification, one clone (resistant against 50 [mu]g/ml chloramphenicol) was selected and designated BML780-KLM3'CAP50. To verify KLM3' expression, BML780-KLM3'CAP50 and BML780 (the empty host strain) were grown for 48h at 37 [deg.] C on a Heart Infusion (Bacto) agar plate with  
30 1% tributyrin. A clearing zone, indicative for lipid acyltransferase activity, was clearly visible around the colony of BML780-KLM3'CAP50 but not around the host strain BML780 (see Figure 140). This result shows that a substantial amount of KLM3' is



# **DEMANDES OU BREVETS VOLUMINEUX**

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS  
COMPREND PLUS D'UN TOME.**

**CECI EST LE TOME \_\_1\_\_ DE \_\_2\_\_**

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

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# **JUMBO APPLICATIONS / PATENTS**

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE  
THAN ONE VOLUME.**

**THIS IS VOLUME \_\_1\_\_ OF \_\_2\_\_**

NOTE: For additional volumes please contact the Canadian Patent Office.



## CLAIMS

1. A lipid acyltransferase wherein the lipid acyltransferase comprises the amino acid sequence shown as SEQ ID No. 90, or an amino acid sequence which has 95% or more sequence identity with SEQ ID No. 90.
2. The lipid acyltransferase according to claim 1 wherein the lipid acyltransferase is an enzyme which possesses acyltransferase activity and which comprises the amino acid sequence motif GDSX, wherein X is any one of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.
3. The lipid acyltransferase according to claim 1 or claim 2 wherein the lipid acyltransferase is obtainable from the genera *Aeromonas*.
4. The lipid acyltransferase according to any one of claims 1-3 wherein the lipid acyltransferase has one of the following characteristics: (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.
5. The lipid acyltransferase according to any one of claims 1-3 wherein the lipid acyltransferase has two of the following characteristics: (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.
6. The lipid acyltransferase according to any one of claims 1-3 wherein the lipid acyltransferase has all of the following characteristics: (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%;



(b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; and (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.

7. The lipid acyltransferase according to any one of claims 1-6 wherein the lipid acyltransferase comprises an amino acid sequence which has 98% or more sequence identity with SEQ ID No. 90.
8. The lipid acyltransferase according to any one of claims 1-7 wherein the lipid acyltransferase comprises the amino acid sequence shown as SEQ ID No. 90.
9. The lipid acyltransferase according to any one of claims 1-8 wherein the lipid acyltransferase consists of the amino acid sequence shown as SEQ ID No. 90.
10. The lipid acyltransferase enzyme according to any one of claims 1-9 wherein the lipid acyltransferase is immobilised.
11. A food or feed enzyme composition which contains said lipid acyltransferase according to any one of claims 1-10 and a carrier.



Figure 1

## SEQ ID No. 1

```

1  ivafGD$1Td geayygdsdg ggwgagladr Ltallrlrar prgvdvfnrg isGrtsdGr1
61  ivDalvallF laqslglpnL pPYLsgdflr GANFAsagAt Ilptsgpfli QvqFkdfksq
121 vlelrqalgl lqellrllpv ldakspdlvt imiGtN$lit saffgpkste sdrnsvvpef
181 kdnlrqlikr Lrsnngarii vlitlviInl gplGC1Plkl alalassknv dasgclerln
241 eavadfneal relaiskled qlrkdglpDv kgadvpyvDl ysifqdldgi qnpsayvyGF
301 ettkaCCGyG gryNynrvCG naglcnvtaK aCnpssylls flfwDgfHps ekGykavAea
361 1

```

Figure 2

## SEQ ID No. 2

```

1  mkkwfvcllg lvaltvqaad srpafsrivm fgdsIsdtgk myskmrgylp ssppyyegrif
61  sngpvwleql tnefpgltia neaeggptav aynkiswnpk yqvinnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakeill fnlpldgqnp
181 sarsqkvvea ashvsayhnq lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdqr
241 nacyggsyvw kpfasrsast dsqIsafnpq erlaiagnpl laqavaspma arsastlnce
301 gkmfwdqvhP ttvvhaalse paatfiesqy eflah

```

Figure 3

## SEQ ID No. 3

```

1  mkkwfvcllg lialtvqaad trpafsrivm fgdsIsdtgk myskmrgylp ssppyyegrif
61  sngpvwleql tkqfpgltia neaeggatav aynkiswnpk yqvynnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakqill fnlpldgqnp
181 sarsqkvvea vshvsayhnk lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdve
241 npcydggyvw kpfatrsvst drqlsafspq erlaiagnpl laqavaspma rrsasplnce
301 gkmfwdqvhP ttvvhaalse raatfietqy eflahg

```

Figure 4

## SEQ ID No. 4

```

1  mpkpallrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
61  anllclrsta nyphviadtT garltdvtcg aaqtadfta qypgvapqld algtgtdlvt
121 ltiggnndst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahIndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnaIg errmaehtmd vlglD

```



Figure 5

## SEQ ID No. 5

```
1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
61 anllclrsta nypvhiadt garltdvtcg aaqtadfta qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsaggks pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpplr aigahldav rraaetgat
241 yvdifsgvsg hdaceapgtr wiepllfghs lvpvhpnlq errmaehtmd vlgld
```

Figure 6

## SEQ ID No. 6

```
1 mdyekfllfg dsitefafnt rpiedgkdqy algaalvney trkmdilqrg fkgytsrwal
61 kilpeilkhe snivmatifl gandacsagp qsvplpefid nirqmvslmk syhirpiiig
121 pglvdrekwe kekseeialg yfrtnenfai ysdalaklan eekvpfvaln kafqqeggda
181 wqqlitdglh fsgkgykifh dellkvietf ypqyhpknmq yklkdwravl ddgsnims
```



Figure 7

Alignment of pfam00657.6 consensus sequence with P10480

```

*->ivafGDSlTdg.....eayygsdgggwgagladrL
iv+fGDSl+d+++ ++ ++ ++++++ +++s+g w ++l + +
P10480 28 IVMFGDSSLSDTgkmyskmrgylpssppYYEGRFSNGFPVWLEQLTNEF 74

tall..rlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqlglpn
+ l + ++++++ +n+ +
P10480 75 PGLTIANEAEGGPTAVAYNKISWNP----- 100

LpPYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqalg
++ ++
P10480 101 -----YQVINN 106

llqellrllpvldakspdlvtimiGtNDlitsaffgpkstesdrnsvpe
l++e+ ++l +++ k+ dlv++++G+ND+ ++ ++ +++++
P10480 107 LDYEVTTQFLQKDSFKPDDLVLWVGANDY-----LAYGWNTQDAKR 148

fkdnlrqlikrLrsnngariivlitlviilngplGClPlklalalasskn
++d +++++r+ nga+ +++++nl+ lG+ P+
P10480 149 VRDAISDAANRMV-LNGAK-----EILLFNLPDLGQNPS----- 181

vdasgcclerlneavadfnealrelaiskledqlrkdglpdkgadvpyvD
++++ +e + ++a++n++l +la +ql+++g++++++d +++++
P10480 182 ARSQKVVEAASHVSAYHNQLLLNLA-----RQLAPTGMVKLFEIDKQFAE 226

lysifqldldgignpsayv.y...GFe.ttkaCCGyGgr.yNyn.rv.CG
+ +q+++ + + +a+++++ +++ ++++++ +N+++r+ ++
P10480 227 MLRDPQNFGLSDQRNACYgGsyvwKPFaSRsASTDSQLSaFNPQeRLaIA 276

nag.l.c.nvtakaC.npssyll.sflfwDgfHpskGykavAeal<-*
+++ l + +++++a++ +s+ +++++fwD++Hp+ ++a+ e
P10480 277 GNPLLaQaVASPMARrSASTLNCEGKMFWQVHPTTVVHAALSEPA 322

```

Alignment of pfam00657.6 consensus sequence with AAG09804

```

*->ivafGDSlTdg.....eayygsdgggwgagladrL
iv+fGDSl+d+++ ++ ++ ++++++ +++s+g w ++l + +
AAG09804 28 IVMFGDSSLSDTgkmyskmrgylpssppYYEGRFSNGFPVWLEQLTKQF 74

tallrlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqlglpnLp
+g+++ n + +G+t
AAG09804 75 -----PGLTIANEAEGGAT----- 88

PYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqa....
++++ + +++++
AAG09804 89 -----AVAYNKISWNPkyq 102

..lgllqellrllpvldakspdlvtimiGtNDlitsaffgpkstesdrnv
++l++e+ ++l +++ k+ dlv++++G+ND+ ++ ++ ++
AAG09804 103 vYNNLDYEVTTQFLQKDSFKPDDLVLWVGANDY-----LAYGWNTQ 144

svpefkdnlrqlikrLrsnngariivlitlviilngplGClPlklalala
+++++d +++++r+ nga+ +++++nl+ lG+ P+
AAG09804 145 DAKRVRDAISDAANRMV-LNGAK-----QILLFNLPDLGQNPS----- 181

ssknvdasgcclerlneavadfnealrelaiskledqlrkdglpdkgadv
++++ +e + ++a++n++l +la +ql+++g++++++d
AAG09804 182 ----ARSQKVVEAVSHVSAYHNKLLNLA-----RQLAPTGMVKLFEIDK 222

pyvDlysifqldldgignpsayv.y...GFe.ttkaCCGyGgr.yNyn.r
+++++ +q+++ + ++ ++++++ +++ t++ +++ +++ + +r
AAG09804 223 QFAEMLRDPQNFGLSDVENPCYdGgyvwKPFaTRSVSTRQLSaFSPQeR 272

v.CGnag.l.c.nvtakaC.npssyll.sflfwDgfHpskGykavAeal
+ +++++ l + +++++a++ +s +++++fwD++Hp+ ++a+ e+
AAG09804 273 LaIAGNPILaQaVASPMARrSASPLNCEGKMFWQVHPTTVVHAALSERA 322

<-*
AAG09804 - -

```

Alignment of pfam00657.6 consensus sequence with NP\_631558

```

*->ivafGDSlTdgayygsdgggwgagladrLtallrlrarprgvdvf
+va+GDS ++g +g + +++L + + + ++ +

```



NP\_631558 42 YVALGDSYSAG-----SGVLPVDPANL----LCLRSTANYPHV 75  
nrgisGrtsdGrlivD.a.l.vallFlaqlslglpnLpPYLsgdflrGANF  
+ ++G++ D + + +  
NP\_631558 76 IADTTGAR-----LTDvTcGaAQ----- 93  
AsagAtIlptsgpfliQvqFkdfksqvlrlqalglqellrllpvldak  
+++ ++ + ++ ++  
NP\_631558 94 -----TADFTRAQYPGVAPQLDALGT 114  
spdlvtimiGtNDl.....itsaffgpkstesdrnvsvp  
+ dlvt+ iG+ND ++ + + ++ + ++ + +k ++ + ++  
NP\_631558 115 GTDLVTLTIGGNDNstfinaitacgtagvlSGGKGSPCKDRHGTSFDDEI 164  
efkdn..lrqlikrLrs.nngariivlitlvilnlg.....plG  
e +++ l++++ +r+++ +ar+ +l ++i+++ +++ + + G  
NP\_631558 165 EANTYpaLKEALLGVRARAPHARVAALGYPWITPATadpscflklplAAG 214  
ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk  
P+ l+ ++a n a+r a  
NP\_631558 215 DVPY-----LRAIQAHLNDAVRRAA----- 234  
dglpdvkgadvpyvDlysifqdlldgiqnpsayvyGFettkaCCGyGgryN  
++ + +yvD+ ++  
NP\_631558 235 -----EETGATYVDFSGVSDG----- 250  
ynrvCGnaglcnavtakaC.npssyll.sflfwDgf...HpsekGykavAe  
++aC+ p +++ + lf + + + Hp++ G +++Ae  
NP\_631558 251 -----HDACeAPGTRWIEPLLFGHSLvpvHPNALGERRMAE 286  
al<-\*  
+  
NP\_631558 287 HT 288

Alignment of pfam00657.6 consensus sequence with CAC42140

\*->ivafGDSlTdgeayygdsgggwgagladrltallrlrarprgvdvf  
+va+GDS ++g +g + +++L + + + ++ +  
CAC42140 42 YVALGDSYSAG-----SGVLPVDPANL----LCLRSTANYPHV 75  
nrgisGrtsdGrlivD.a.l.vallFlaqlslglpnLpPYLsgdflrGANF  
+ ++G++ D + + +  
CAC42140 76 IADTTGAR-----LTDvTcGaAQ----- 93  
AsagAtIlptsgpfliQvqFkdfksqvlrlqalglqellrllpvldak  
+++ ++ + ++ ++  
CAC42140 94 -----TADFTRAQYPGVAPQLDALGT 114  
spdlvtimiGtNDl.....itsaffgpkstesdrnvsvp  
+ dlvt+ iG+ND ++ + + ++ + ++ + +k ++ + ++  
CAC42140 115 GTDLVTLTIGGNDNstfinaitacgtagvlSGGKGSPCKDRHGTSFDDEI 164  
efkdn..lrqlikrLrs.nngariivlitlvilnlg.....plG  
e +++ l++++ +r+++ +ar+ +l ++i+++ +++ + + G  
CAC42140 165 EANTYpaLKEALLGVRARAPHARVAALGYPWITPATadpscflklplAAG 214  
ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk  
P+ l+ ++a n a+r a  
CAC42140 215 DVPY-----LRAIQAHLNDAVRRAA----- 234  
dglpdvkgadvpyvDlysifqdlldgiqnpsayvyGFettkaCCGyGgryN  
++ + +yvD+ ++  
CAC42140 235 -----EETGATYVDFSGVSDG----- 250  
ynrvCGnaglcnavtakaC.npssyll.sflfwDgf...HpsekGykavAe  
++aC+ p +++ + lf + + + Hp++ G +++Ae  
CAC42140 251 -----HDACeAPGTRWIEPLLFGHSLvpvHPNALGERRMAE 286  
al<-\*  
+  
CAC42140 287 HT 288

Alignment of pfam00657.6 consensus sequence with P41734

\*->ivafGDSlTdg....eayygdsgggwgagladrltallrlrarprg  
++fGDS+T+ +++ + + d+ ga+l + + +r+  
P41734 6 FLLFGDSITEfaintrPIEDGKDQYALGAALVNEY-----TRK 43  
vdvfnrgisGrtsdGrlivDalvallFlaqlslglpnLpPYLsgdflrGAN  
+d+ rg++G+t



P41734 44 MDILQRGFKGYT----- 55  
FAsagAtIlptsgpfliQvqFkdfksqvlelrqalglqellrllpvlda  
+r+al++l+e+l+ +  
P41734 56 -----SRWALKILPEILKH-----E 70  
kspdlvtimiGtNDlitsaffgpkstesdrnsvpefkdnlrqlikrLrs  
+ + ti++G+ND+ ++ +++ v++pef+dn+rq+++++s  
P41734 71 SNIVMATIFLGANDA-----CSAGPQSVPLPEFIDNIRQMVSLMKS 111  
nngariivlitlvilnlgplGC1Plklalalassknvdasgcclerlneav  
++++ii++++lv ++ ++ k ++ + + r+ne +  
P41734 112 YHIRPIIIGPGLVDREKW-----EKEKSEEIALGYFRTNENF 148  
adfnealrelaiskledqlrkdglpdkvgadvpyvDlysifqldldgiqnp  
a + al +la ++ +vp+v l+++fq+ +g++++  
P41734 149 AIYSDALAKLA-----NEEKVPFVALNKAFQQEGGDAWQ 182  
sayvyGFettkaCCGyGgryNynrvCGnaglcnvtakaCnpssyllsflf  
+ l+  
P41734 183 Q-----LL 185  
wDgfHpsekGykavAeal<-\*  
Dg+H+s kGyk+++++l  
P41734 186 TDGLHFSGKGYKIFHDEL 203



Figure 8

```

A.sal 1  MKKWFVCLLGLIALTVQAADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF 60
          +      +
A.hyd 1  MKKWFVCLLGLVALTVQAADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF 60
          ++      +
A. sal 61 SNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
          ++      +
A. hyd 61 SNGPVWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
A. sal 121 KPDDLVLWVGANDYLAYGWNTAQDAKVRDAISDAANRMVLNGAKQILLFNLPDLGQNP 180
          +
A. hyd 121 KPDDLVLWVGANDYLAYGWNTAQDAKVRDAISDAANRMVLNGAKEILLFNLPDLGQNP 180
A. sal 181 SARSQKVVEAVSHVSAYHNKLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVE 240
          +      +
A.hyd 181 SARSQKVVEAASHVSAYHNQLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQR 240 ++
A. sal 241 NPCYDGGYVWKPFATRSVSTDRQLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE 300
          + ++ +      + + +      +      +
A. hyd 241 NACYGGSYVWKPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMARSASTLNCE 300
A. sal 301 GKMFWDQVHPTTVVHAALSERAAATFIETQYEFLAH 335
          +      +
A. hyd 301 GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH 335

```



Figure 9

```
1  ATGAAAAAAT GGTTCGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA GGCAGCCGAC
61  AGCCGTCCCG CCTTCTCCCG GATCGTGATG TTTGGCGACA GCCTCTCCGA TACCGGCAAG
121 ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCCC CCTACTATGA GGGCCGCTTC
181 TCCAACGGGC CCGTCTGGCT GGAGCAGCTG ACCAACGAGT TCCCGGGCCT GACCATAGCC
241 AACGAGGCGG AAGGCGGACC GACCGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCCTGCAAAA AGACAGCTTC
361 AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGCGCCAACG ACTATCTGGC CTATGGCTGG
421 AACACAGAGC AGGATGCCAA GCGGGTGCGC GACGCCATCA GCGATGCGGC CAACCGCATG
481 GTGCTGAACG GCGCCAAGGA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCC
541 TCGGCCCCGA GCCAGAAGGT GGTGAGGCG GCCAGCCATG TCTCCGCCTA CCACAACCAG
601 CTGCTGCTGA ACCTGGCAGC CCAGCTGGCT CCCACCGGCA TGGTGAAGCT GTTCGAGATC
661 GACAAGCAGT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACCAGAGG
721 AACGCCTGCT ACGGTGGCAG CTATGTATGG AAGCCGTTTG CCTCCGCGAG CGCCAGCACC
781 GACAGCCAGC TCTCCGCCTT CAACCCGCGAG GAGCGCCTCG CCATCGCCGG CAACCCGCTG
841 CTGGCCGAGG CCGTCGCCAG CCCCATGGCT GCGCGCAGCG CCAGCACCTT CAACTGTGAG
901 GGCAAGATGT TCTGGGATCA GGTCCACCCC ACCACTGTCG TGCACGCCGC CCTGAGCGAG
961 CCGCCGCCA CCTTCATCGA GAGCCAGTAC GAGTTCCTCG CCCAC
```



Figure 10

```
1  ATGAAAAAAT GGT TTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA GGCAGCCGAC
61  ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA GCCTCTCCGA TACCGGCAAA
121 ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCGC CCTACTATGA GGGCCGTTTC
181 TCCAACGGAC CCGTCTGGCT GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC
241 AACGAAGCGG AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAG
301 TATCAGGTCT ACAACAACCT GGACTACGAG GTCACCCAGT TCTTGAGAA AGACAGCTTC
361 AAGCCGGACG ATCTGCTGAT CCTCTGGGTC GGTGCCAATG ACTATCTGGC ATATGGCTGG
421 AATACGGAGC AGGATGCCAA GCGAGTTCGC GATGCCATCA GCGATGCGGC CAACCGCATG
481 GTACTGAACG GTGCCAAGCA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG
541 TCAGCCCGCA GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
601 CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT GTTCGAGATC
661 GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACGTCGAG
721 AACCCTGCT ACGACGGCGG CTATGTGTGG AAGCCGTTTG CCACCCGCAG CGTCAGCACC
781 GACCGCCAGC TCTCCGCCTT CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG
841 CTGGCACAGG CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
901 GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC CCTGAGCGAG
961 CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG CCCACGGATG A
```



Figure 11

```
1  ATGCCGAAGC CTGCCCTTCG CCGTGTCATG ACCGCGACAG TCGCCGCCGT CGGCACGCTC
61  GCCCTCGGCC TCACCGACGC CACCGCCAC GCGCGGCCG CCCAGGCCAC TCCGACCCTG
121 GACTACGTCG CCCTCGGCGA CAGCTACAGC GCGGGCTCCG GCGTCCTGCC CGTCGACCCC
181 GCCAACCTGC TCTGTCTGCG CTCGACGGCC AACTACCCC ACGTCATCGC GGACACGACG
241 GCGCGCCGCC TCACGGACGT CACCTGCGGC GCGCGCAGA CCGCCGACTT CACGCGGGCC
301 CAGTACCCGG GCGTCGCACC CCAGTTGGAC GCGCTCGGCA CCGGCACGGA CCTGGTCACG
361 CTCACCATCG GCGGCAACGA CAACAGCACC TTCATCAACG CCATCACGGC CTGCGGCACG
421 GCGGGTGTCC TCAGCGGCGG CAAGGGCAGC CCCTGCAAGG ACAGGCACGG CACCTCCTTC
481 GACGACGAGA TCGAGGCCAA CACGTACCCC GCGCTCAAGG AGGCGCTGCT CGGCGTCCGC
541 GCCAGGGCTC CCCACGCCAG GGTGGCGGCT CTCGGCTACC CGTGGATCAC CCCGGCCACC
601 GCCGACCCGT CCTGCTTCCT GAAGCTCCCC CTCGCCCGG GTGACGTGCC CTACCTGCGG
661 GCCATCCAGG CACACCTCAA CGACGCGGTC CCGCGGGCCG CCGAGGAGAC CGGAGCCACC
721 TACGTGGACT TCTCCGGGGT GTCCGACGGC CACGACGCCT GCGAGGCCCC CGGCACCCGC
781 TGGATCGAAC CGCTGCTCTT CGGGCACAGC CTCGTTCCCG TCCACCCCAA CGCCCTGGGC
841 GAGCGGCGCA TGGCCGAGCA CACGATGGAC GTCCTCGGCC TGGACTGA
```



Figure 12

```
1 TCAGTCCAGG CCGAGGACGT CCATCGTGTG CTCGGCCATG CGCCGCTCGC CCAGGGCGTT
61 GGGGTGGACG GGAACGAGGC TGTGCCCCGAA GAGCAGCGGT TCGATCCAGC GGGTGCCGGG
121 GGCCTCGCAG GCGTCGTGGC CGTCGGACAC CCCGGAGAAG TCCACGTAGG TGGCTCCGGT
181 CTCCTCGGCG GCCCGCCGGA CCGCGTCGTT GAGGTGTGCC TGGATGGCCC GCAGGTAGGG
241 CACGTCACCG GCGGCGAGGG GGAGCTTCAG GAAGCAGGAC GGGTCGGCGG TGGCCGGGGT
301 GATCCACGGG TAGCCGAGAG CCGCCACCCT GGCGTGGGGA GCCCTGGCGC GGACGCCGAG
361 CAGCGCCTCC TTGAGCGCGG GGTACGTGTT GGCCTCGATC TCGTCGTCTGA AGGAGGTGCC
421 GTGCCTGTCC TTGAGGGGCG TGCCCTTGCC GCCGCTGAGG ACACCCGCCG TGCCGCAGGC
481 CGTGATGGCG TTGATGAAGG TGCTGTTGTC GTTGCCGCCG ATGGTGAGCG TGACCAGGTC
541 CGTGCCGGTG CCGAGCGCGT CCAACTGGGG TCGGACGCCC GGGTACTGGG CCCGCGTGAA
601 GTCGGCGGTC TCGCGGGCGC CGCAGGTGAC GTCCGTGAGG CGGGCGCCCG TCGTGTCCGC
661 GATGACGTGG GGGTAGTTGG CCGTCGAGCG CAGACAGAGC AGGTGCGCGG GGTGACGGG
721 CAGGACGCCG GAGCCGGCGC TGTAGCTGTC GCCGAGGGCG ACGTAGTCCA GGGTCGGAGT
781 GGCCTGGGCG GCGCGGGCGT GGGCGGTGGC GTCGGTGAGG CCGAGGGCGA GCGTGCCGAC
841 GCGGCGGACT GTCGCGGTCA TGACACGGCG AAGGGCAGGC TTCGGCAT
```



Figure 13

```
1  ATGGATTACG AGAAGTTTCT GTTATTTGGG GATTCCATTA CTGAATTTGC TTTTAATACT
61  AGGCCCATTG AAGATGGCAA AGATCAGTAT GCTCTTGGAG CCGCATTAGT CAACGAATAT
121  ACGAGAAAAA TGGATATTCT TCAAAGAGGG TTCAAAGGGT ACACTTCTAG ATGGGCGTTG
181  AAAATACTTC CTGAGATTTT AAAGCATGAA TCCAATATTG TCATGGCCAC AATATTTTGT
241  GGTGCCAACG ATGCATGCTC AGCAGGTCCC CAAAGTGTC CCCTCCCCGA ATTTATCGAT
301  AATATTCGTC AAATGGTATC TTTGATGAAG TCTTACCATA TCCGTCCTAT TATAATAGGA
361  CCGGGGCTAG TAGATAGAGA GAAGTGGGAA AAAGAAAAAT CTGAAGAAAT AGCTCTCGGA
421  TACTTCCGTA CCAACGAGAA CTTTGCCATT TATTCCGATG CCTTAGCAAA ACTAGCCAAT
481  GAGGAAAAAG TTCCCTTCGT GGCTTTGAAT AAGGCGTTTC AACAGGAAGG TGGTGATGCT
541  TGGCAACAAC TGCTAACAGA TGGACTGCAC TTTTCCGGAA AAGGTACAA AATTTTTCAT
601  GACGAATTAT TGAAGGTCAT TGAGACATTC TACCCCAAT ATCATCCCA AAACATGCAG
661  TACAACTGA AAGATTGGAG AGATGTGCTA GATGATGGAT CTAACATAAT GTCTTGA
```



Figure 14

(SEQ ID No. 12)

|            |            |            |            |            |            |
|------------|------------|------------|------------|------------|------------|
| 10         | 20         | 30         | 40         | 50         | 60         |
|            |            |            |            |            |            |
| MNLRQWMGAA | TAALALGLAA | CGGGGTDQSG | NPNVAKVQRM | VVFGDSLSDI | GTYPVAQAV  |
| 70         | 80         | 90         | 100        | 110        | 120        |
|            |            |            |            |            |            |
| GGGKFTTNPG | PIWAETVAAQ | LGVTLTPAVM | GYATSVQNCP | KAGCFDYAQG | GSRVTDPNGI |
| 130        | 140        | 150        | 160        | 170        | 180        |
|            |            |            |            |            |            |
| GHNGGAGALT | YPVQQQLANF | YAASNNTFNG | NNDVVFVLAG | SNDIFFWTTA | AATSGSGVTP |
| 190        | 200        | 210        | 220        | 230        | 240        |
|            |            |            |            |            |            |
| AIATAQVQQA | ATDLVGYVKD | MIAKGATQVY | VFNLPDSSLT | PDGVASGTTG | QALLHALVGT |
| 250        | 260        | 270        | 280        | 290        | 300        |
|            |            |            |            |            |            |
| FNTTLQSGLA | GTSARIIDFN | AQLTAAIQNG | ASFGFANTSA | RACDATKINA | LVPSAGGSSL |
| 310        | 320        | 330        | 340        |            |            |
|            |            |            |            |            |            |
| FCSANTLVAS | GADQSYLFAD | GVHPTTAGHR | LIASNVLARL | LADNVAH    |            |



Figure 15

(SEQ ID No. 13)

|            |             |            |            |             |              |      |
|------------|-------------|------------|------------|-------------|--------------|------|
| atgaacctgc | gtcaatggat  | gggcgcgcgc | acggctgccc | ttgccttggg  | cttggccgcg   | 60   |
| tgccggggcg | gtgggaccga  | ccagagcggc | aatcccaatg | tcgccaagggt | gcagcgcgcatg | 120  |
| gtggtgttcg | gcgacagcct  | gagcgatata | ggcacctaca | cccccgtcgc  | gcaggcggtg   | 180  |
| ggcggcggca | agttcaccac  | caaccggggc | ccgatctggg | ccgagaccgt  | ggccgcgcaa   | 240  |
| ctgggcgtga | cgctcacgcc  | ggcggtgatg | ggctacgcca | cctccgtgca  | gaattgcccc   | 300  |
| aaggccggct | gcttcgacta  | tgcgcagggc | ggctcgcgcg | tgaccgatcc  | gaacggcatc   | 360  |
| ggccacaacg | gcggcgcggg  | ggcgtgacc  | taccgggttc | agcagcagct  | cgccaacttc   | 420  |
| tacgcggcca | gcaacaacac  | attcaacggc | aataacgatg | tcgtcttcgt  | gctggccggc   | 480  |
| agcaacgaca | ttttcttctg  | gacctctgcg | gcggccacca | gcggctccgg  | cgtgacgccc   | 540  |
| gccattgcca | cggcccagggt | gcagcaggcc | gcgacggacc | tggtcggcta  | tgtcaaggac   | 600  |
| atgatcgcca | aggggtgcgac | gcaggtctac | gtgttcaacc | tgcccgacag  | cagcctgacg   | 660  |
| ccggacggcg | tggcaagcgg  | cacgaccggc | caggcgctgc | tgacgcgct   | ggtgggcacg   | 720  |
| ttcaacacga | cgctgcaaag  | cgggctggcc | ggcacctcgg | cgcgcacat   | cgacttcaac   | 780  |
| gcacaactga | ccgcggcgat  | ccagaatggc | gcctcgttcg | gcttcgcca   | caccagcgcc   | 840  |
| cgggcctgcg | acgccaccaa  | gatcaatgcc | ctggtgccga | gcgccggcgg  | cagctcgctg   | 900  |
| ttctgctcgg | ccaacacgct  | ggtggcttcc | ggtgcggacc | agagctacct  | gttcgcccgc   | 960  |
| ggcgtgcacc | cgaccacggc  | cggccatcgc | ctgatcgcca | gcaacgtgct  | ggcgcgcctg   | 1020 |
| ctggcggata | acgtcgcgca  | ctga       |            |             |              | 1044 |



Figure 16 (SEQ ID No. 20)

```
1 migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avrgrlldqi
61 vaeqvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaaltaaa gtvltvtgfd
121 trgvpvikh1 rgkiatyngv vraiadrygc pvldlws1rs vqdrrowdad rhlspgght
181 rvalraggal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241 hvtakgtlsp daiktriaav a
```

Figure 17 (SEQ ID No. 21)

```
1 gtgatcgggt cgtacgtggc ggtgggggac agcttcaccg agggcgctcg cgaccccggc
61 cccgacgggg cgttcgtcgg ctggggccgac cggctcgccg tactgctcgc ggaccggcgc
121 cccgagggcg acttcacgta cacgaacctc gccgtgcgcg gcaggctcct cgaccagatc
181 gtggcggaac aggtcccgcg ggtcgtcgga ctccgcgccg acctcgtctc gttcggggcg
241 ggcggcaacg acatcatccg gcccggcacc gatcccgacg aggtcgccga gcggttcgag
301 ctggcggtgg ccgcgctgac cgcgcgggcc ggaaccgtcc tggtagccac cgggttcgac
361 acccgggggg tgcccgctct caagcacctg cgcggcaaga tcgccacgta caacgggcac
421 gtccgcgcca tcgccgaccg ctacggctgc ccggtgctcg acctgtggtc gctgcggagc
481 gtccaggacc gcagggcggt ggacgccgac cggctgcacc tgcgcggga ggggcacacc
541 cgggtggcgc tgccgcgggg gcaggccctg ggcctgcgcg tcccgccga ccctgaccg
601 ccctggccgc ccctgccgcc gcgcggcacg ctccagctcc ggcgcgacga cgtgactgg
661 gcgcgcgagt acctggtgcc gtggatcggg cgcgggctgc ggggcgagtc gtcgggcgac
721 cacgtgacgg ccaaggggac gctgtcgccg gacgccatca agacgcggat cgcgcgggtg
781 gcctga
```



Figure 18  
(SEQ ID No. 22)

```
1  mqtncpytsl vavgdsfteg msdlldgsy rgwadllatr maarspgfry anlavrgkli
61  ggiqvdeqv dv aaamgadvit lvgglnndtlr pkcdmarvrd lltqaverla phceqlvlmr
121 spgrqgppvle rfrprmealf aviddlagrh gavvvdlyga qsladprmw dvrhlhtaeg
181 hrrvaeavwq slghepedpe whapipatpp pgwvtrrtad vrfarqhllp wigriltgrs
241 sgdglpakrp dllpyedpar
```

Figure 19 (SEQ ID No. 23)

```
1  atgcagacga accccgcgta caccagtctc gtcgccgtcg gcgactcctt caccgagggc
61  atgtcggacc tgctgcccga cggctcctac cgtggctggg ccgacctcct cgccaccggg
121 atggcgggcc gctccccggg cttccggtac gccaacctgg cgggtgcggg gaagctgac
181 ggacagatcg tcgacgagca ggtggacgtg gccgcgcgca tgggagccga cgtgatcacg
241 ctggtcggcg ggctcaacga cacgctcgcg cccaagtgcg acatggcccg ggtgcgggac
301 ctgctgacct aggcogtgga acggctcgcc ccgcactgcg agcagctggt gctgatgcgc
361 agtcccggtc gccaggggtc ggtgctggag cgcttcggc cccgatgga ggccctgttc
421 gccgtgatcg acgacctggc cgggcggcac ggcccggtgg tcgtcgacct gtacggggcc
481 cagtcgctgg ccgacctcg gatgtgggac gtggaccggc tgcacctgac cgccgagggc
541 caccgccggg tcgcggaggg ggtgtggcag tcgctcggcc acgagccga ggacccgag
601 tggcacgcgc cgatccggc gacgcggcc cgggggtggg tgacgcgcag gaccgcggac
661 gtccggttcg cccggcagca cctgctgccc tggataggcc gcaggctgac cgggcgctcg
721 tccggggacg gcctgcccgc caagcggccg gacctgctgc cctacgagga cccgcacgg
781 tga
```



Figure 20 (SEQ ID No. 24)

```
1 mtrgrdggag apptkhrall aaivtlivai saaiyagasa ddgsrdhalq aggrlprgda
61 apastgawvg awatapaaae pgtettglag rsvrnvvhts vggtagariti snlyggsplt
121 vthasialaa gpdtaaaiaa tmrrltfggs arviipaggq vmsdtarlai pyganvlvtt
181 yspipsgpyt yhpqarqtsy ladgdrtadv tavayttptp ywryltaldv lsheadgtvv
241 afgdsitdga rsqsdanhrw tdvlaarlhe aagdgrdtpr ysvvnegisg nrlltsrpgr
301 padnpsglr fqrdivlertn vkavvvvlgv ndvlinspela drdailtglr tlvdraharg
361 lrvvgatitp fggyggytea retmrqevne eirsgrvfdt vvdfdkalrd pydprmrds
421 ydsghlhpq dkgymrgav idlaalkgaa pvka
```

Figure 21 (SEQ ID No. 25)

```
1 atgacccggg gtcgtgacgg ggggtgcgggg gcgcccccca ccaagcaccg tgccctgctc
61 gcggcgatcg tcacctgat agtggcgatc tccgcggcca tatacgccgg agcgcccgcg
121 gacgacggca gcagggacca cgcgctgcag gccggaggcc gtctcccacg aggagacgcc
181 gcccccggt ccaccgggtg cttgggtgggc gcctgggcca ccgcaccggc cgcggccgag
241 ccgggcaccg agacgaccgg cctggcgggc cgtccgtgc gcaacgtcgt gcacacctcg
301 gtcggcgcca ccggcgcgcg gatcacctc tccaacctgt acgggcagtc gccgctgacc
361 gtcacacacg cctcgatcgc cctggccgcc gggcccgaca ccgcgccgc gatcgccgac
421 accatgcgcc ggctcacctt cggcgcgagc gcccggtga tcatcccggc gggcgggccg
481 gtgatgagcg acaccgcccg cctcgccatc ccctacgggg cgaacgtcct ggtcaccacg
541 tactcccca tcccgtccgg gccggtgacc taccatccgc agggccggca gaccagctac
601 ctggccgacg gcgaccgcac ggcgagcgtc accgccgtcg cgtacaccac cccacgccc
661 tactggcgct acctgaccgc cctcgacgtg ctgagccacg agggcgacgg caccgctctg
721 gcgttcggcg actccatcac cgacggcgcc cgtcgcaga gcgacgcaa ccaccgctg
781 accgacgtcc tcgcgcacg cctgcacgag gcggcgggcg acggccggga caccgcccgc
841 tacagcgtcg tcaacgaggg catcagcggc aaccggctcc tgaccagcag gccggggcg
901 ccggccgaca acccgagcgg actgagccgg ttccagcggg acgtgctgga acgcaccaac
961 gtcaaggccg tcgtcgtcgt cctcgcgctc aacgacgtcc tgaacagccc ggaactcgcc
1021 gaccgcgacg ccatactgac cggcctgccc accctcgtcg accggcgcca cggccgggga
1081 ctgcgggctg tcggcgccac gatcacgccg ttcgggcggt acggcggcta caccgagggc
1141 cgcgagacga tgcggcagga ggtcaacgag gagatccgt ccggccgggt cttcgacacg
1201 gtcgtcgact tcgacaaggc cctgcgcgac ccgtacgacc cgcgccggat gcgctccgac
1261 tacgacagcg gcgaccacct gcaccccgcc gacaaggggt acgcgcgcac gggcgcggtc
1321 atcgacctgg ccgcgtgaa gggcgcgggc ccggtcaagg cgtag
```



Figure 22 (SEQ ID No. 26)

```
1 mtsmsrarva rriaagaayg gggiglagaa avglvvaevq larrrvvgvgt ptrvpnaqgl
61 yggtlptagd pplrlmmlgd staagqgvhr agqtpgalla sglaavaerp vrlgsvaqpg
121 acsddldrqv alvlaepdrv pdicvimvga ndvthrmpt rsvrhlssav rrlrtagaev
181 vvgtcpdlgt iervrqplrw larrasrqla aaqtigaveq ggtrvslgdl lgpefaqnpr
241 elfgpdnyhp saegyataam avlpsvcaal glwpadeehp dalrregflp varaaaaaas
301 eagtevaaaam ptgprgpwal lkrrrrrrvs eaepsspsgv
```

Figure 23 (SEQ ID No. 27)

```
1 atgacgagca tgtcgagggc gagggtggcg cggcggatcg cggccggcgc ggcgtacggc
61 ggcggcgcca tcggcctggc gggagcggcg gcggtcggtc tggtagtggc cgaggtgcag
121 ctggccagac gcaggggtggg ggtgggcacg ccgacccggg tgccgaacgc gcagggactg
181 tacggcgcca ccctgcccac ggccggcgac ccgccgctgc ggctgatgat gctgggcgac
241 tccacggccg ccgggcaggg cgtgcaccgg gccgggcaga cggccggcgc gctgctggcg
301 tccgggctcg cggcgggtggc ggagcggccg gtgcggtcg ggctcgctgc ccagccgggg
361 gcgtgctcg acgacctgga ccggcaggtg gcgctgggtc tcgccagacc ggaccgggtg
421 cccgacatct gcgtgatcat ggtcggcgcc aacgacgtca cccaccgat gccggcgacc
481 cgctcggtgc ggcacctgtc ctggcggtta cggcggtgc gcacggccgg tcgggaggtg
541 gtggtcggca cctgtccgga cctgggcacg atcgagcggg tcggcgagcc gctgcgctgg
601 ctggcccggc gggcctcacg gcagctcgcg gcggcacaga ccatcggcgc cgtcgagcag
661 ggcgggcgca cgggtgcgt gggcgacctg ctgggtccgg agttcgcgca gaaccgcgg
721 gagctcttcg gccccgacaa ctaccacccc tccgccgagg ggtacgccac ggccgcgatg
781 ggggtactgc cctcggtgtg cgcgcgctc ggcctgtggc cggccgacga ggagcaccg
841 gacgcgctgc gccgcgaggg ctctctgcg gtggcgcgcg cggcggcgga ggcggcgctc
901 gaggcgggta cggaggtcgc cgcgcctatg cctacggggc ctggggggcc ctgggcgctg
961 ctgaagcgcc ggagacggcg tcgggtgtcg gaggcggaac cgtccagccc gtccggcggtt
1021 tga
```



Figure 24 (SEQ ID No. 28)

```
1 mgrgtdqrtr ygrrrarval aaltaavlgv gvagcdsvgg dspapsgsps krtrtapawd
61 tspasvaavg dsitrgfdac avlsdcpevs watgssakvd slavrllgka daaehswnya
121 vtgarmadlt aqvtraagre pelvavmaga ndacrsttsa mtpvadfrac feeamatlrk
181 klpkaqvys sipdlkrlws qgrtnplgkq vwklglcp sm lgdadslds atlrrntvrd
241 rvadynevlr evcakdrrcr sddgavhefr fgtdqlshwd wfhpsvdgqa rlaeiayrav
301 taknp
```

Figure 25 (SEQ ID No. 29)

```
1 atgggtcgag ggacggacca gcgacgcgg tacggccgtc gccgggcgcg tgctgcgctc
61 gccgccctga ccgcccgcgt cctgggcggtg ggcgtggcgg gctgcgactc cgtgggcggc
121 gactcaccgg ctccttccgg cagcccgtcg aagcggacga ggacggcgcc cgctgggac
181 accagcccg cgtccgtcgc cgcgtgggc gactccatca cgcgcggctt cgacgcctgt
241 gcggtgctgt cggactgccc ggaggtgtcg tgggcgaccg gcagcagcgc gaaggtcgac
301 tcgctggccg tacggctgct ggggaaggcg gacgcggccg agcacagctg gaactacgcg
361 gtcaccgggg cccgatggc ggacctgacc gctcaggtga cgcgggcggc gcagcgcgag
421 ccggagctgg tggcgggtgat ggccggggcg aacgacgcgt gccggtccac gacctggcg
481 atgacgccgg tggcggactt ccgggcgcag ttcgaggagg cgatggccac cctgcgcaag
541 aagctcccca aggcgcaggt gtacgtgtcg agcatcccg acctcaagcg gctctggtcc
601 cagggcgcga ccaaccgcgt gggcaagcag gtgtggaagc tcggcctgtg cccgtcgatg
661 ctgggcgacg cggactccct ggactcggcg gcgacctgc ggcgcaacac ggtgcgcgac
721 cgggtggcg actacaacga ggtgctgcgg gaggtctcg cgaaggaccg gcggtgccgc
781 agcgacgacg gcgcggtgca cgagttccgg ttcggcacgg accagttgag cactggggac
841 tggttccacc cgagtgtgga cggccaggcc cggctggcg agatcgcta ccgcgcggtc
901 accgcgaaga atccctga
```



Figure 26 (SEQ ID No. 30)

```
1 mrlsrraata sallltpala lfgasaavsa priqatdyva lgdsyssgvv agsydsssgs
61 ckrstksypa lwaashtgtr fnftacsgar tgdvlakqlt pvnsgtdlvs itiggndagf
121 adtmittcnlq gesaclaria karayiqgtl paqldqvyda idsrapaaqv vvlgyprfyk
181 lggscavglis eksraaina addinavtak raadhgfafg dvnttfaghe lcsgapwlhs
241 vtlpvensyh ptangqskgy lpvlinsat
```

Figure 27 (SEQ ID No. 31)

```
1 ttcatcacaa cgatgtcaca acaccggcca tccgggtcat ccctgatcgt gggaaatgggt
61 gacaagcctt cccgtgacga aagggtcctg ctacatcaga aatgacagaa atcctgctca
121 gggaggttcc atgagactgt cccgacgcgc ggccacggcg tccgcgctcc tcctcacccc
181 ggcgctcgcg ctcttcggcg cgagcgcgcg cgtgtccgcg ccgcgaatcc aggccaccga
241 ctacgtggcc ctccggcgact cctactcctc gggggtcggc gcgggcagct acgacagcag
301 cagtggctcc tgtaagcgca gcaccaagtc ctaccgggcc ctgtgggccc cctcgcacac
361 cggtagcgcg ttcaacttca ccgcctgttc gggcgcccgc acaggagacg tgetggccaa
421 gcagctgacc ccggtcaact ccggcacoga cctggtcagc attaccatcg gcggcaacga
481 cgcgggcttc gcgacacca tgaccacctg caacctccag ggcgagagcg cgtgcctggc
541 gcggatcgcc aaggcgcgcg cctacatcca gcagacgctg cccgcccagc tggaccaggt
601 ctacgacgcc atcgacagcc gggccccgcg agcccaggtc gtcgtcctgg gctacccgcg
661 cttctacaag ctgggcgga gctgcgcccgt cgggtctctcg gagaagtccc gcgcggccat
721 caacgccgcc gccgacgaca tcaacgccgt caccgccaag cgcgccgccg accacggctt
781 cgccttcggg gacgtcaaca cgacctcgc cgggacagag ctgtgctccg gcgccccctg
841 gctgcacagc gtcacccttc ccgtggagaa ctcctaccac cccacggcca acggacagtc
901 caagggtac ctgcccgtcc tgaactccgc cacctgatct cgcggctact ccgcccctga
961 cgaagtcccg cccccggcg gggcttcgcc gtaggtgcgc gtaccgccgt cgcgcgtcgc
1021 gccggtggcc ccgccgtacg tgccgccgcc cccggacgcg gtcggttc
```



Figure 28 (SEQ ID No. 32)

```
1  MKKWFVCLLG LVALTVQAAD SRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101 YQVINNLDE VTQFLQKDSF KPDDLVLWV GANDYLAYGW NTEQDAKRVR
151 DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNQ
201 LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251 KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMR RRSASPLNCE
301 GKMFWQVHP TTVVHAALSE RAATFIANQY EFLAH*
```



Figure 29 (SEQ ID No. 33)

```
1  ATGAAAAAAT GGTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA
   TACTTTTTTA CCAAACACAC AAATAACCCT AACCAGCGCG ACTGTCAAGT

51  GGCAGCCGAC AGTCGCCCCG CCTTTTCCCG GATCGTGATG TTCGGCGACA
   CCGTCGGCTG TCAGCGGGGC GGAAAAGGGC CTAGCACTAC AAGCCGCTGT

101 GCCTCTCCGA TACCGGCAA AATGTACAGCA AGATGCGCGG TTACCTCCCC
   CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

151 TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
   AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA

201 GGAGCAGCTG ACCAAACAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
   CCTCGTCGAC TGGTTTGTCA AGGGCCCAGA CTGGTAGCGG TTGCTTCGCC

251 AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
   TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
   ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

351 AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
   TCTGTCAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

401 ACTATCTGGC CTATGGCTGG AACACGGAGC AGGATGCCAA GCGGGTTCGC
   TGATAGACCG GATACCGACC TTGTGCCTCG TCCTACGGTT CGCCCAAGCG

451 GATGCCATCA GCGATGCGGC CAACCGCATG GACTGAACG GTGCCAAGCA
   CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

501 GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCTCGCA
   CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGAGCGT

551 GTCAGAAGGT GGTGAGGCG GTCAGCCATG TCTCCGCTA TCACAACCAG
   CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTGGTTC

601 CTGCTGCTGA ACCTGGCAGC CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
   GACGACGACT TGGACCGTGC GGTGACCGG GGTGGCCGT ACCATTTCGA

651 GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
   CAAGCTCTAG CTGTTCGTTA AACGGCTCTA CGACGCACTA GCGTCTTGA

701 TCGGCCTGAG CGACGTCGAG AACCCTGCT ACGACGGCGG CTATGTGTGG
   AGCCGGACTC GCTGCAGTC TTGGGGACGA TGCTGCCGCC GATACACACC

751 AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCTT
   TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTGC AGAGGCGGAA

801 CAGTCCGCAG GAACGCCTCG CCATCGCCCG CAACCCGCTG CTGGCACAGG
   GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGCGGAC GACCGTGTCC

851 CCGTTGCCAG TCCTATGGCC CGCCGCGAGC CCAGCCCCCT CAACTGTGAG
   GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTGGGGGA GTTGACACTC

901 GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCTG TGCACGCAGC
   CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCCTCG

951 CCTGAGCGAG CGCGCCGCCA CCTTCATCGC GAACAGTAC GAGTTCCTCG
   GGACTCGCTC GCGCGGCGGT GGAAGTAGCG CTTGGTCATG CTCAAGGAGC

1001 CCCAC TGA
      GGGTG ACT
```



Figure 30 (SEQ ID No. 34)

```
1  MKKWFVCLLG LIALTVQAAD TRPAFSRIVM FGDSLSDTGM MYSKMRGYLP
51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNP
101 YQVINNL DYE VTQFLQKDSF KPDDLVLWV GANDYLAYGW NTEQDAKRVR
151 DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNK
201 LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251 KPFATRSVST DRQLSAFSPQ ERLAIAAGNPL LAQAVASPMA RRSASPLNCE
301 GKMFWQVHP TTVVHAALSE RAATFIETQY EFLAHG*
```



Figure 31 (SEQ ID No. 35)

```
1  ATGAAAAAAT GGTTTGTGTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA
   TACTTTTTTA CCAAACAAAC AAATAACCCC AACTAGCGCG ACTGTCAAGT
51  GGCAGCCGAC ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA
   CCGTCGGCTG TGAGCGGGGC GGAAGAGGGC CTAGCACTAC AAGCCGCTGT
101 GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATGCGCGG TTACCTCCCC
   CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG
151 TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
   AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA
201 GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
   CCTCGTCGAC TGTTTCGTCA AGGGCCAGA CTGGTAGCGG TTGCTTCGCC
251 AAGCCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
   TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC
301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
   ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT
351 AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
   TCTGTCAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC
401 ACTATCTGGC ATATGGCTGG AATACGGAGC AGGATGCCAA GCGAGTTCGC
   TGATAGACCG TATACCGACC TTATGCCTCG TCCTACGGTT CGCTCAAGCG
451 GATGCCATCA GCGATGCGGC CAACCGCATG GTACTGAACG GTGCCAAGCA
   CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT
501 GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCCCGCA
   CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGGGCGT
551 GTCAGAAGGT GGTGAGGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
   CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTGTGTC
601 CTGCTGCTGA ACCTGGCAGC CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
   GACGACGACT TGGACCGTGC GGTGACCGG GGGTGGCCGT ACCATTTCTGA
651 GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
   CAAGCTCTAG CTGTTGCTTA AACGGCTCTA CGACGCACTA GCGTCTTGA
701 TCGGCCTGAG CGACGTCGAG ACCCCCTGCT ACGACGGCGG CTATGTGTGG
   AGCCGGACTC GCTGCAGTC TTGGGGACGA TGCTGCCGCC GATACACACC
751 AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
   TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTCT AGAGGCGGAA
801 CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
   GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC
851 CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
   GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTGCGGGGA GTTGACACTC
901 GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCTG TGCACGCAGC
   CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCCTCG
951 CCTGAGCGAG CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG
   GGACTCGCTC GCGCGGCGGT GGAAGTAGCT CTGGGTCATG CTCAAGGAGC
1001 CCCACGGATG A
     GGGTGCCTAC T
```



Figure 32

|           |                                                      |     |     |     |     |     |
|-----------|------------------------------------------------------|-----|-----|-----|-----|-----|
|           | 1                                                    | 10  | 20  | 30  | 40  | 50  |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | ADTRPAFSRIYMFQDSLSDTGKMYSKMRGYLPSSPPYYEGRFSN--G      |     |     |     |     |     |
| R.sol     | QSGNPNVAKYQRHYVFGDSLSDIGT-----YTPVAQAYGGGKFTTNP      |     |     |     |     |     |
| Consensus | ...adnraafqRiYnFGDSLSDiGk.....YLPsaqaygeGrFsn..G     |     |     |     |     |     |
|           | 51                                                   | 60  | 70  | 80  | 90  | 100 |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | PYWLEQLTKQFPGLTIANEAREGGATAYAYNKISWNPKYQVINNLDYEY    |     |     |     |     |     |
| R.sol     | PIWAETVAAQL-GVTLTPAYMGYATSYQNCPCAGCFDYAQGGSRYTDPNG   |     |     |     |     |     |
| Consensus | P!WaEq1aaQ1.GlTianaaeGgATaYannkiagnfdYaqqnnrdt#pnq   |     |     |     |     |     |
|           | 101                                                  | 110 | 120 | 130 | 140 | 150 |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | FLQKDSFKPDOLYILWYGANDYLAIG--WNTAQDAKRYRDAISDAANRMY   |     |     |     |     |     |
| R.sol     | IGHNGGAGALTYPYQQQLAWFYAASNNTFNGANDVYFYLAGSNDIFFHTT   |     |     |     |     |     |
| Consensus | igqndgagaddlp!qqqgANDYaRsn..fNg##DakrYraainDaanrnt   |     |     |     |     |     |
|           | 151                                                  | 160 | 170 | 180 | 190 | 200 |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | LNGAKQILLFNLPLDQNPASRSQKYVEAYSHYSAYHNKL-LLNLARQLA    |     |     |     |     |     |
| R.sol     | AAATSGSGVTPAIATAQYQQAATDLYGYVKDHIKAGATQYVYFNLPDSSL   |     |     |     |     |     |
| Consensus | aaaakqiglfnaialaQnqqRas#lVgeakdh!aaganql.11NLarqla   |     |     |     |     |     |
|           | 201                                                  | 210 | 220 | 230 | 240 | 250 |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | PTGHYKLFEDKQFAEHLRDPQNFGLSDYENPCYDGGYVWKPFATRSYST    |     |     |     |     |     |
| R.sol     | TPDGYASGTTGQALLHALYGTFTTLQSGLAGTSARIIDFNAQLTAAIQN    |     |     |     |     |     |
| Consensus | ppdgValgeidqalaeaLrdpqHfgLqdgeagcsargidfnaqaTaa!qn   |     |     |     |     |     |
|           | 251                                                  | 260 | 270 | 280 | 290 | 300 |
|           | -----+-----+-----+-----+-----                        |     |     |     |     |     |
| satA      | DRQLSAFSPQERLAIAG--NPLLAQAYASPM---ARRSASPLNCEGKHFW   |     |     |     |     |     |
| R.sol     | GASFGFANTSARACDATKINALVPSAGGSSLFCSANTLYASGADQSYLFA   |     |     |     |     |     |
| Consensus | daqlgaanpqaRaadAg..NaLlaqAgaSp\$...Arrlaapgad#gk\$Fa |     |     |     |     |     |
|           | 301                                                  | 310 | 320 | 330 |     |     |
|           | -----+-----                                          |     |     |     |     |     |
| satA      | DQYHPTTYVHAALSERAAATFIETQYEFLAH                      |     |     |     |     |     |
| R.sol     | DGYHPTTAGHRLIASNVLARLLA--DNVAH                       |     |     |     |     |     |
| Consensus | DqYHPTTagHaaiaeraaariae..#nLAH                       |     |     |     |     |     |



Figure 33

|       |                                                                      |                                                                       |     |
|-------|----------------------------------------------------------------------|-----------------------------------------------------------------------|-----|
| Pfam  | *->ivafGDSltldggg.....ayygdsgggwgagladrltsla..rlrargrgv              |                                                                       |     |
| Srim1 | 38                                                                   | YVALGDSYSSGVG.....agSYDSSSGSCKRSTKSYPALWAAS..-----HTGTRF              | 81  |
| Scoe1 | 5                                                                    | YVAVGDSFTEG--.....--VGDPGPDGAFVWADRLAVLL..ADRRPEGDFTY                 | 47  |
| Scoe2 | 10                                                                   | LVAVGDSFTEG--.....--MSDLLPDGSYRGWADLLATRM..--AARSPGFY                 | 50  |
| Scoe3 | 239                                                                  | VVAFGDSITDG--.....ARSQSDANHRWTDVLAARLHEAA..GDGRDTPRYSV                | 283 |
| Scoe4 | 75                                                                   | LMMLGDSTAAG--.....-----QGVHRAGQTPGALLASG..LAAVAERPVR                  | 113 |
| Scoe5 | 66                                                                   | VAAVGDSITRGFD.....acAVLSDCPEVSWATGSSAKVDSLAvrLLGKADAAEHS              | 116 |
| Ahyd1 | 28                                                                   | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTNEFPGLTiaNEAEGGPTAVA      | 91  |
| Asall | 28                                                                   | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTKQF-----PGLTI             | 79  |
| Ahyd2 | 40                                                                   | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTKQFPGLTiaNEAEGGATAVA      | 103 |
| ▼     |                                                                      |                                                                       |     |
| Pfam  | fnrgisGrtsdGrlvvDarlvatl1FlaqflGlnlpPYLsgdflrGANFAsagAtIlgtslipflni  |                                                                       |     |
| Srim1 | 82                                                                   | NFTACSGAR-----                                                        | 90  |
| Scoe1 | 48                                                                   | TNLAVRGRL-----                                                        | 56  |
| Scoe2 | 51                                                                   | ANLAVRGKL-----                                                        | 59  |
| Scoe3 | 284                                                                  | VNEGISGNR-----                                                        | 292 |
| Scoe4 | 114                                                                  | GSVAQPGAC-----                                                        | 122 |
| Scoe5 | 117                                                                  | WNYAVTGAR-----                                                        | 125 |
| Ahyd1 | 92                                                                   | YNKISWNPk-----                                                        | 100 |
| Asall | 80                                                                   | ANEAEGGAT-----                                                        | 88  |
| Ahyd2 | 104                                                                  | YNKISWNPk-----                                                        | 112 |
| ▼     |                                                                      |                                                                       |     |
| Pfam  | QvqFkdfkskvlrlrqa.....lgllqellrlvpvldakspdlvtimiGtND1...itvakfgpks   |                                                                       |     |
| Srim1 | 91                                                                   | -----TGDLVLAQLTPVNSGTDLSITIGGNDagfaDTMTTCNLQ                          | 131 |
| Scoe1 | 57                                                                   | -----LDQIVAEQVPRVVG LAPDLVSFAAGGNDI...-----I-----                     | 86  |
| Scoe2 | 60                                                                   | -----IGQIVDEQVDVAAAMGADVITLVGGLNDT...-----                            | 88  |
| Scoe3 | 293                                                                  | -----LLTSRPGRPA.....DNPSGLSRFQRDVLETRNVKAVVVVLGVNDV...-----           | 333 |
| Scoe4 | 123                                                                  | -----SDDLDRQVALVLAEPDRVPDICVIMVGANDV...-----                          | 153 |
| Scoe5 | 126                                                                  | -----MADLTAQVTRAAQREPELVAVMAGANDA...-----CR                           | 155 |
| Ahyd1 | 101                                                                  | -----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA                | 137 |
| Asall | 89                                                                   | -----AVAYNKISWNPkyqvyNNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA         | 137 |
| Ahyd2 | 113                                                                  | -----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA                | 149 |
| ▼     |                                                                      |                                                                       |     |
| Pfam  | .....tksdnrvsvpefrdnrlrklkrLrsangariililtvl1nlp1.....plGCl           |                                                                       |     |
| Srim1 | 132                                                                  | esaclariAKARAYIQOTLPAQLDQVYDAIDSRAPAA-----QVVVLGYP-----               | 176 |
| Scoe1 | 87                                                                   | -----RPGTDPDEVAERFELAVAAALT-AAAGTVLVTTGFDTRGVP-----                   | 125 |
| Scoe2 | 89                                                                   | -----LRPKCDMARVRDLTQAVERLAPHCEQLVLMRSP-----                           | 122 |
| Scoe3 | 334                                                                  | -----LNSPELADRDAITGLRTLVDRAHARGLRVVGATITPFGGYGG-----                  | 376 |
| Scoe4 | 154                                                                  | -----THRMPATRSVRHLSSAVRRLR-TAGAEVVVGTCPDLGTE-----                     | 192 |
| Scoe5 | 156                                                                  | -----STTSAMTPVADFRAQFEEAMATLR-KKLPKAQVYVSSIPDLKRLwsqgrtnplgkQVWKL     | 214 |
| Ahyd1 | 138                                                                  | -----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----EILLFNLP-----                  | 174 |
| Asall | 138                                                                  | -----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----                  | 174 |
| Ahyd2 | 150                                                                  | -----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----                  | 186 |
| ▼     |                                                                      |                                                                       |     |
| Pfam  | pq.klalalassknvdatgclerlneavadynealrelaei.ek.1.q.aqlrkdglpdlkeanvpy  |                                                                       |     |
| Srim1 | 177                                                                  | ---RFYKLGGSCAVGLSEKSRAAINAAADDINAVTAKRA---.---.---ADHGFAF             | 219 |
| Scoe1 | 126                                                                  | ---VLKHLRGKIATYNGHVRAIA---.---.---DRYGCPV                             | 152 |
| Scoe2 | 123                                                                  | ---GRQGPVLERFRPRMEALFAVIDDLA---.---.---GRHGAVV                        | 154 |
| Scoe3 | 377                                                                  | ---YTEARETMROEVNEEIRSGRVFDTIVDFDKALRDPY---.---.---412                 |     |
| Scoe4 | 193                                                                  | ---RVQRPLRWLaRRaSrQlAAAQTIGAVEQGGRTVSL                                | 227 |
| Scoe5 | 215                                                                  | GLcPSMLGDADSLDAATLRRNTVRDRVADYNEVLREVC---.---.---AkDRRCRSDDGAVHEFRFGT | 273 |
| Ahyd1 | 175                                                                  | ---DLGQNPSARSQKVVEAASHVSAYHNQLLLNLA---.---.---RQLAPTGMVKLFEIDKQF      | 224 |
| Asall | 175                                                                  | ---DLGQNPSARSQKVVEAASHVSAYHNQLLLNLA---.---.---RQLAPTGMVKLFEIDKQF      | 224 |
| Ahyd2 | 187                                                                  | ---DLGQNPSARSQKVVEAASHVSAYHNQLLLNLA---.---.---RQLAPTGMVKLFEIDKQF      | 236 |
| ▼     |                                                                      |                                                                       |     |
| Pfam  | VDlysifqldldgiqnpsayv.y....GFeet.kaCCGyGgr.yNyn.rv.CGnag.1.ck.vtakaC |                                                                       |     |
| Srim1 | 220                                                                  | GDVNT-----TFAGHElCSGAPwL.HS.VT----                                    | 242 |
| Scoe1 | 153                                                                  | LDLWSLRSVQDRRA-----                                                   | 166 |
| Scoe2 | 155                                                                  | VDLYGAQSLADPRM-----                                                   | 168 |
| Scoe3 | 413                                                                  | -----                                                                 | 413 |
| Scoe4 | 228                                                                  | GDLLGPEFAQNPREL-----                                                  | 242 |
| Scoe5 | 274                                                                  | DQL-----                                                              | 276 |
| Ahyd1 | 225                                                                  | AEMLRDPQNFGLSQDNACyGsyvwwKPFASrSASTDSQLSaFNPQeRLaIAGNP1LaQAvASPMMA    | 291 |
| Asall | 225                                                                  | AEMLRDPQNFGLSQDNACyGsyvwwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR    | 291 |
| Ahyd2 | 237                                                                  | AEMLRDPQNFGLSQDNACyGsyvwwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR    | 303 |



```
Pfam      .dassyll.atlfdWgf.HpsekGykavAeal<-*
Srim1 243 .-----LPVENSyHPTANGQSKGYLPV 263
Scoe1 167 .-----WDADRL.HLSPEGHTRVALRA 186
Scoe2 169 .-----WDVDRL.HLTAEGHRRVAEAV 188
Scoe3 413 .-DPRRMRsDYDSGDHL.HPGDKGYARMGAVI 441
Scoe4 243 .-----FGPDNY.HPSAEGYATAAMAV 262
Scoe5 277 .-----SHWDWF.HPSVDGQARLAEIA 296
Ahyd1 292 rSASTLNCEGKMFWQV.HPTTVVHAALSEPA 322
Asa11 292 rSASPLNCEGKMFWQV.HPTTVVHAALSERA 322
Ahyd2 304 rSASPLNCEGKMFWQV.HPTTVVHAALSERA 334
```



Figure 34

|       |     |                                                                      |     |
|-------|-----|----------------------------------------------------------------------|-----|
|       |     | ▼                                                                    |     |
| Pfam  |     | *->ivafGDSltddggg.....ayygdsgggwgagladrltsla..rlrargrgv              |     |
| Srim1 | 38  | YVALGDSYSSGVG.....agSYDSSSGSCKRSTKSYPALWAAS..-----HTGTRF             | 81  |
| Scoe1 | 5   | YVAVGDSFTEG--.....--VGDPGPDGAFVWADRLAVLL..ADRRPEGDFTY                | 47  |
| Scoe2 | 10  | LVAVGDSFTEG--.....--MSDLLPDGSYRGWADLLATRM..--AARSPGFY                | 50  |
| Ahyd1 | 28  | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTNEFPGLTiaNEAEGGPTAVA     | 91  |
| Asa11 | 28  | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTKQF-----PGLTI            | 79  |
| Ahyd2 | 40  | IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTKQFPGLTiaNEAEGGATAVA     | 103 |
|       |     |                                                                      |     |
| Pfam  |     | fnrgisGrtsdGrlvvDarlvatllFlaqflGlnlpPYLsgdflrGANFAsagAtIlgtslipflni  |     |
| Srim1 | 82  | NFTACSGAR-----                                                       | 90  |
| Scoe1 | 48  | TNLAVRGRL-----                                                       | 56  |
| Scoe2 | 51  | ANLAVRGKL-----                                                       | 59  |
| Ahyd1 | 92  | YNKISWNP-----                                                        | 100 |
| Asa11 | 80  | ANEAEGGAT-----                                                       | 88  |
| Ahyd2 | 104 | YNKISWNP-----                                                        | 112 |
|       |     |                                                                      |     |
|       |     | ▼                                                                    |     |
| Pfam  |     | QvqFkdfkskvlrlqa.....lgllqellrlvpvldakspdlvtimiGtNDl...itvakfgpks    |     |
| Srim1 | 91  | -----TGDLVLAQLTPVNSGTDLVSTIGCNDAGfaDTMTTCNLQG                        | 131 |
| Scoe1 | 57  | -----LDQIVAEQVPRVVG LAPDLVSFAAGNDI...-----I-----                     | 86  |
| Scoe2 | 60  | -----IGQIVDEQVDVAAAMGADVITLVGGLNDT...-----                           | 88  |
| Ahyd1 | 101 | -----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA               | 137 |
| Asa11 | 89  | -----AVAYNKISWNPkyqvyNNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA        | 137 |
| Ahyd2 | 113 | -----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLWVGANDY...-----LA               | 149 |
|       |     |                                                                      |     |
| Pfam  |     | .....tksdnrvsvpefrdnrlrklkrLrsangariililitvlvlnlplplGCl              |     |
| Srim1 | 132 | esacIarIAKARAYIQOTLPAQLDQVYDAIDSRAPAA-----QVVVLGY-----               | 176 |
| Scoe1 | 87  | -----RPGTDPDEVAERFELAVAALT-AAAGTVLVTTGFDTRGVP-----                   | 125 |
| Scoe2 | 89  | -----LRPKCDMARVRDILLTQAVERLAPHCEQLVLMRSP-----                        | 122 |
| Ahyd1 | 138 | .....YGWNTAQDAKRVRDAISDAANRMV-LNGAK-----EILLFNLP-----                | 174 |
| Asa11 | 138 | .....YGWNTAQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----                | 174 |
| Ahyd2 | 150 | .....YGWNTAQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----                | 186 |
|       |     |                                                                      |     |
| Pfam  |     | pqklalalassknvdatgclerlneavadynealrelaeieklqaqlrkdglpdlkeanvpy       |     |
| Srim1 | 177 | --RFYKLGSCAVGLSEKSRAAINAAADDINAVTAKRA-----ADHGFAF                    | 219 |
| Scoe1 | 126 | -----VLKHLRGKIATYNGHVRAIA-----DRYGCPV                                | 152 |
| Scoe2 | 123 | -----GRQGPVLERFRPRMEALFAVIDDLA-----GRHGAVV                           | 154 |
| Ahyd1 | 175 | -----DLGQNPSARSQKVVEAASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEIDKQF        | 224 |
| Asa11 | 175 | -----DLGQNPSARSQKVVEAASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEIDKQF        | 224 |
| Ahyd2 | 187 | -----DLGQNPSARSQKVVEAASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEIDKQF        | 236 |
|       |     |                                                                      |     |
| Pfam  |     | VDlysifqldldgignpsayv.y...GFeet.kaCCGyGgr.yNyn.rv.CGnag.1.ck.vtakaC  |     |
| Srim1 | 220 | GDVNT-----TFAGHELCSGAPwL.HS.VT-----                                  | 242 |
| Scoe1 | 153 | LDLWSLRSVQDRRA-----                                                  | 166 |
| Scoe2 | 155 | VDLYGAQSLADPRM-----                                                  | 168 |
| Ahyd1 | 225 | AEMLRDPQNFGLSQQRNACYgGsyvwKPFASrSASTDSQLSaFNPQeRLaIAGNPllLaQAvASPMMA | 291 |
| Asa11 | 225 | AEMLRDPQNFGLSQQRNACYgGsyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPllLaQAvASPMAR | 291 |
| Ahyd2 | 237 | AEMLRDPQNFGLSQQRNACYgGsyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPllLaQAvASPMAR | 303 |
|       |     |                                                                      |     |
|       |     | ▼                                                                    |     |
| Pfam  |     | .dassyll.atlfdwDgf.HpsekGykavAeal<-*                                 |     |
| Srim1 | 243 | -----LPVENSyHPTANGQSKGYLPV                                           | 263 |
| Scoe1 | 167 | -----WDADRL.HLSPEGHTRVALRA                                           | 186 |
| Scoe2 | 169 | -----WDVDRL.HLTAEGHRRVAEAV                                           | 188 |
| Ahyd1 | 292 | rSASTLNCeGKMFWQV.HPTTVVHAALSEPA                                      | 322 |
| Asa11 | 292 | rSASPLNCeGKMFWQV.HPTTVVHAALSER                                       | 322 |
| Ahyd2 | 304 | rSASPLNCeGKMFWQV.HPTTVVHAALSER                                       | 334 |



FIG. 35

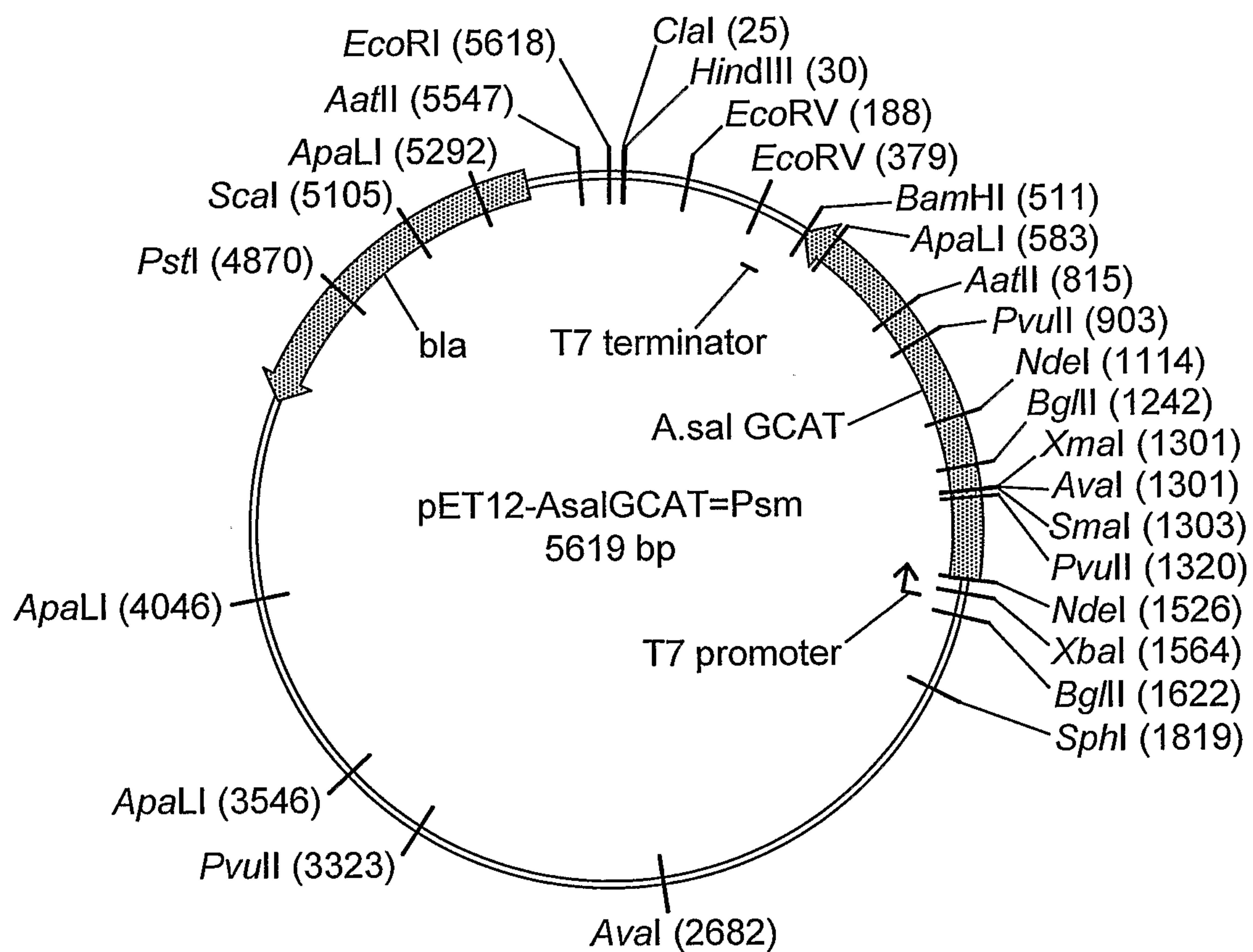




FIG. 36

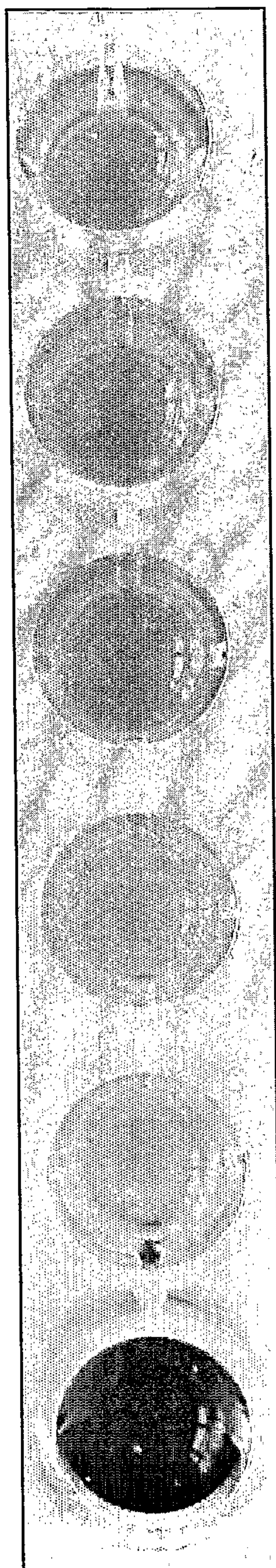




FIG. 37

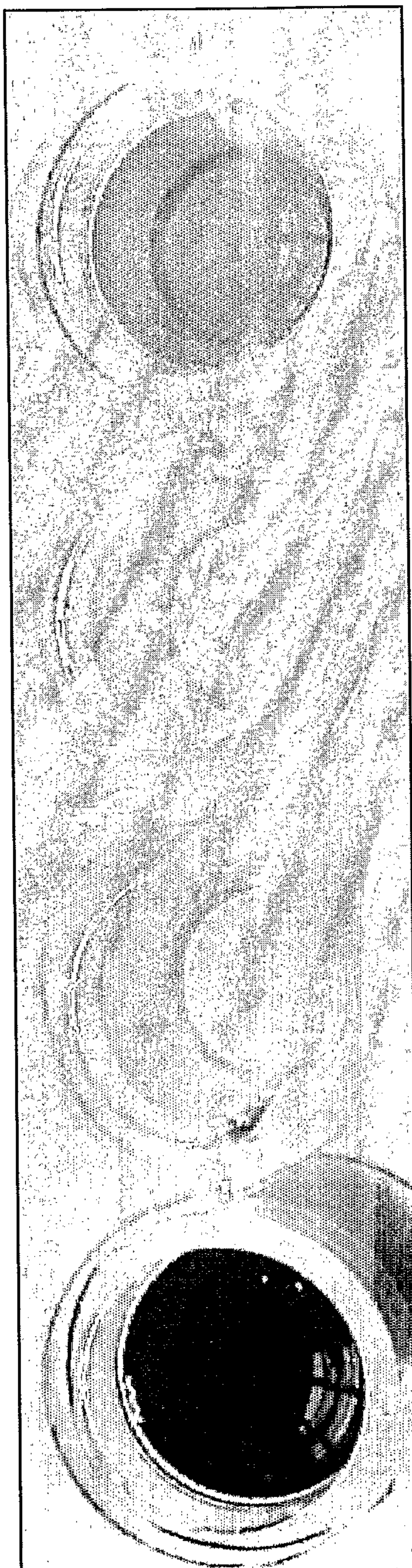




FIG. 38

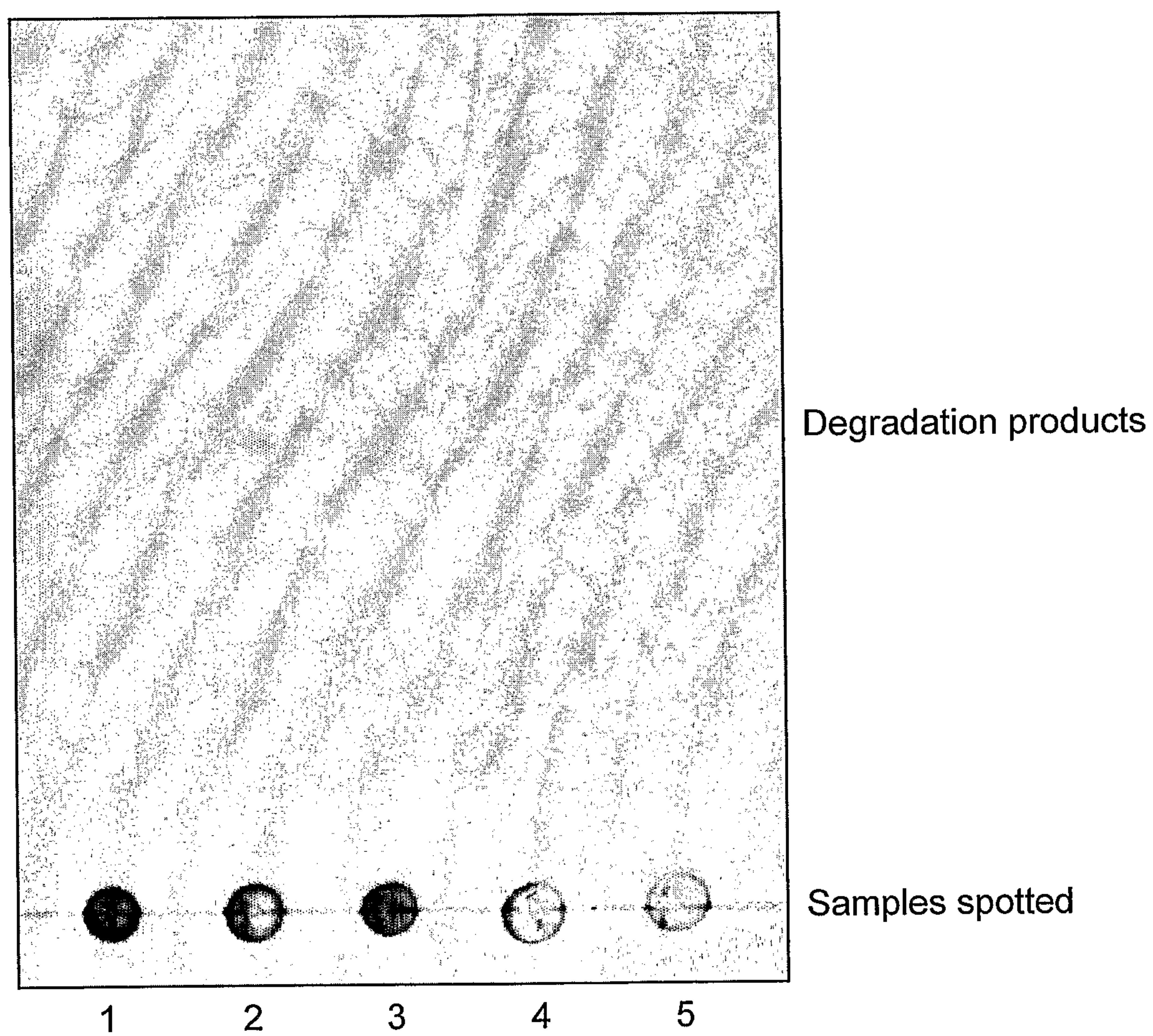




FIG. 39

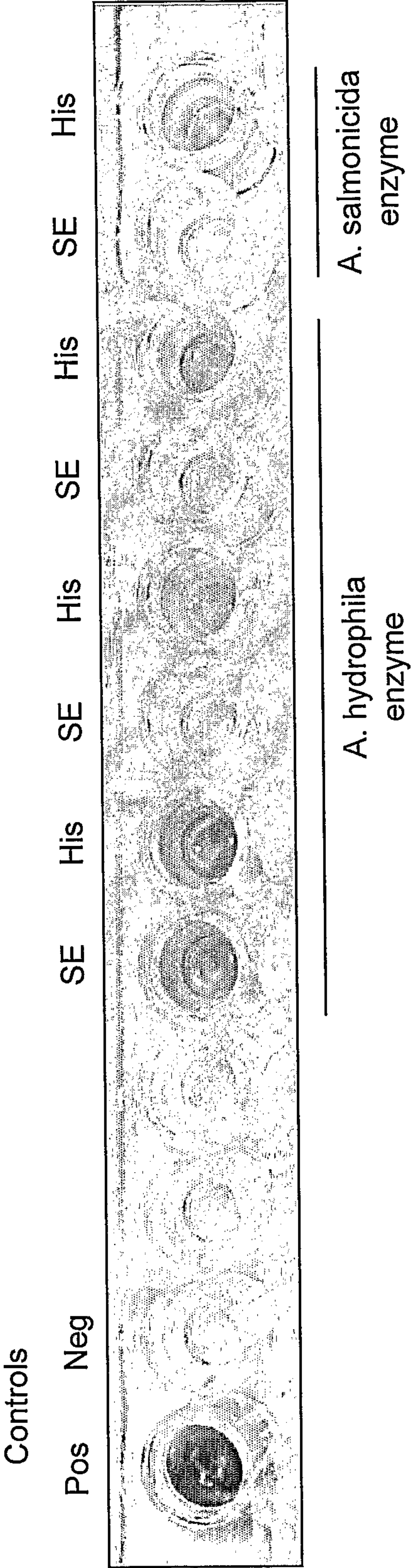
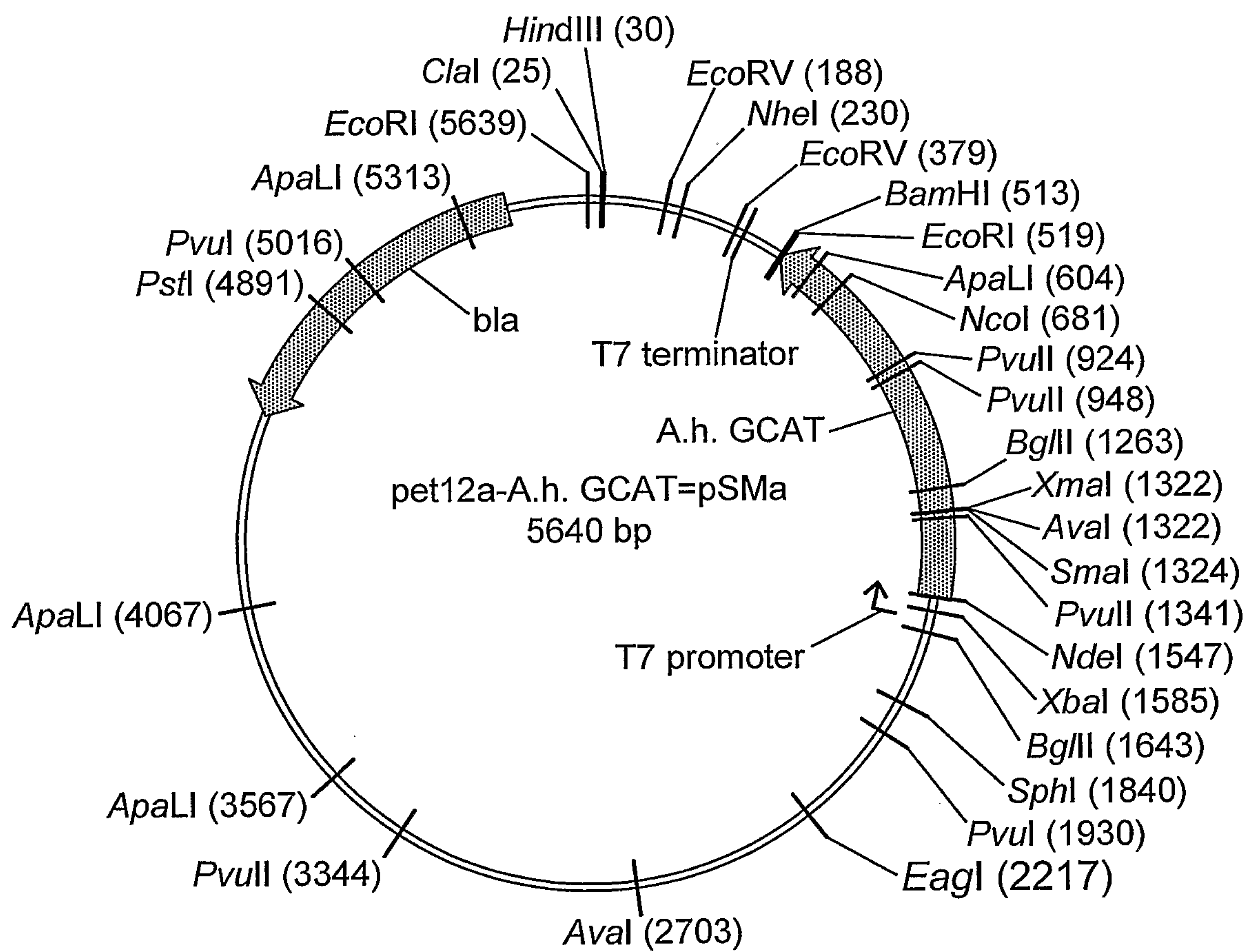




FIG. 40





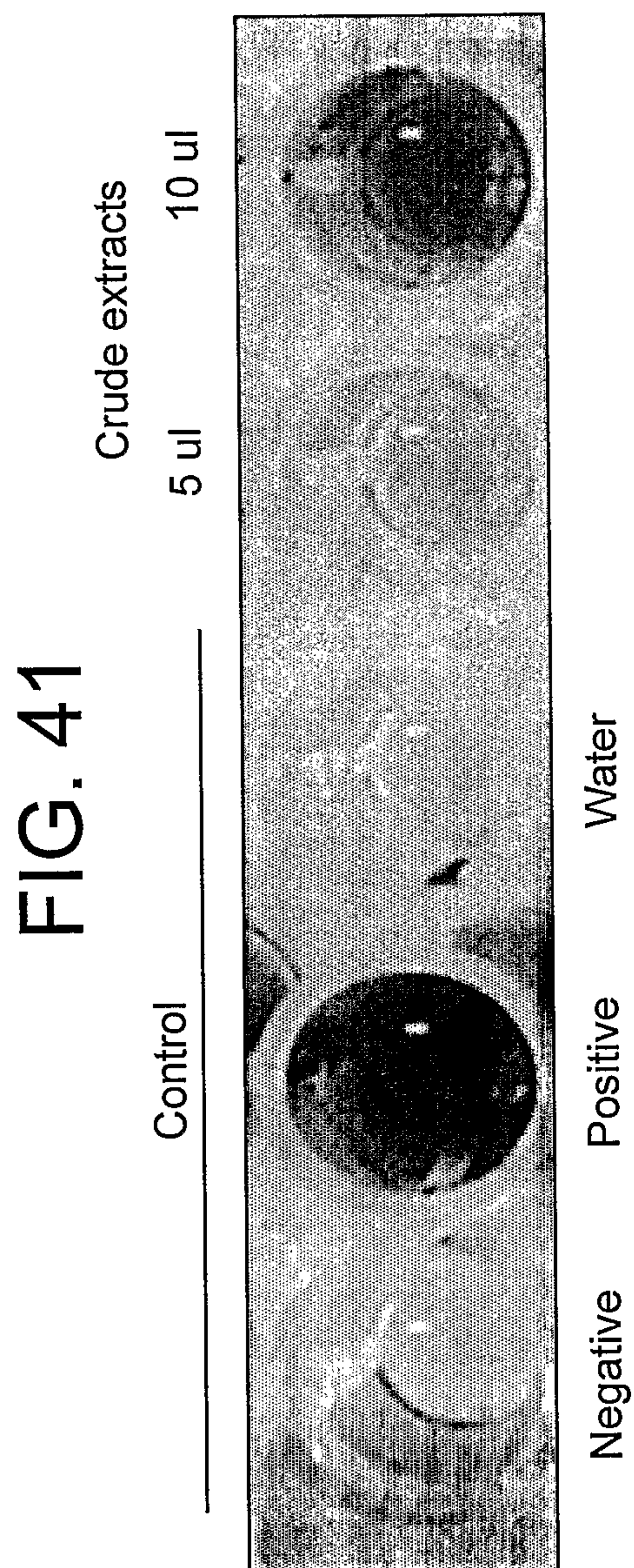




FIG. 42

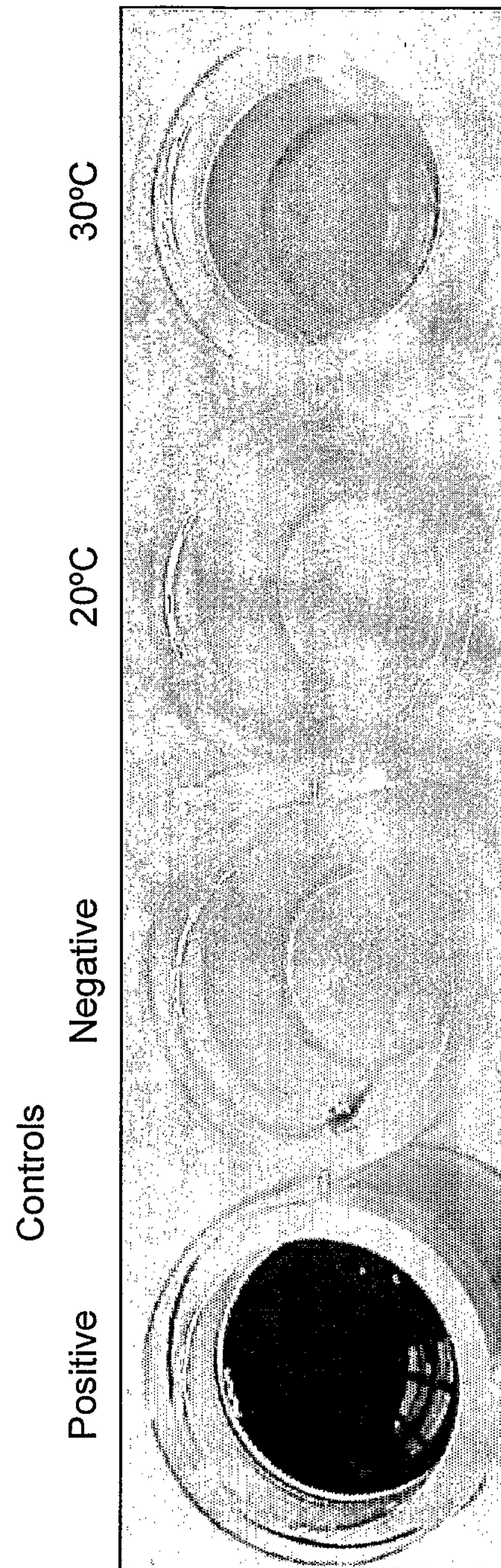




FIG. 43

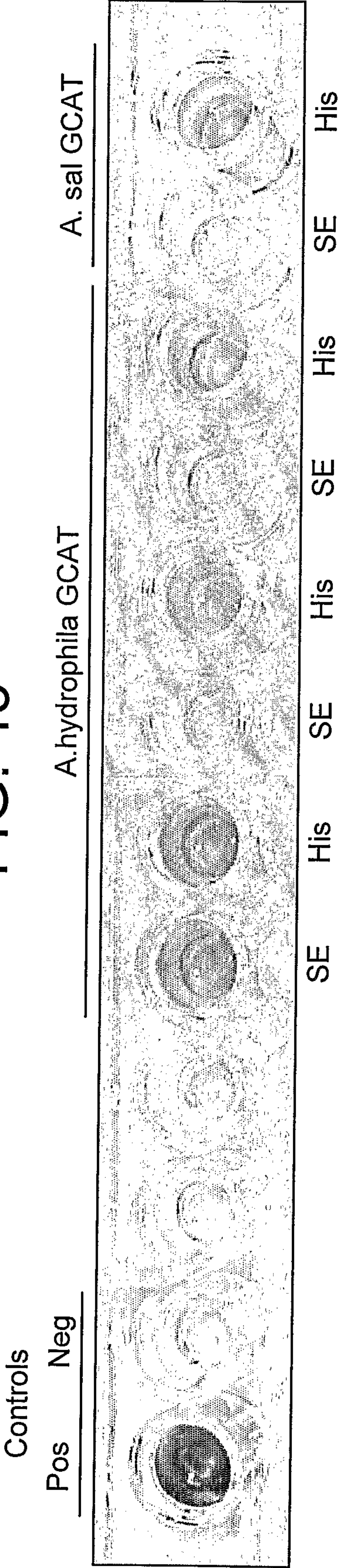
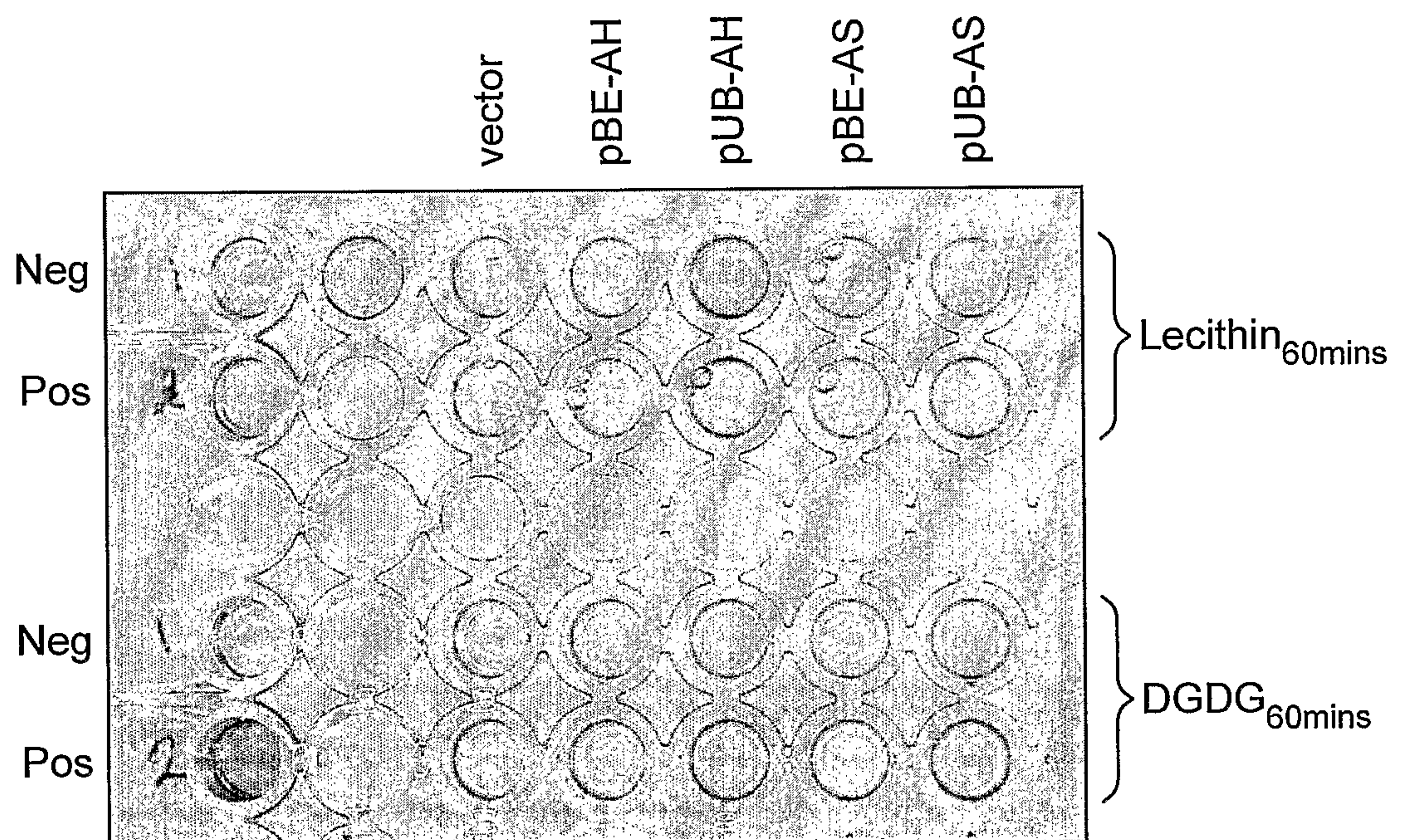




FIG. 44





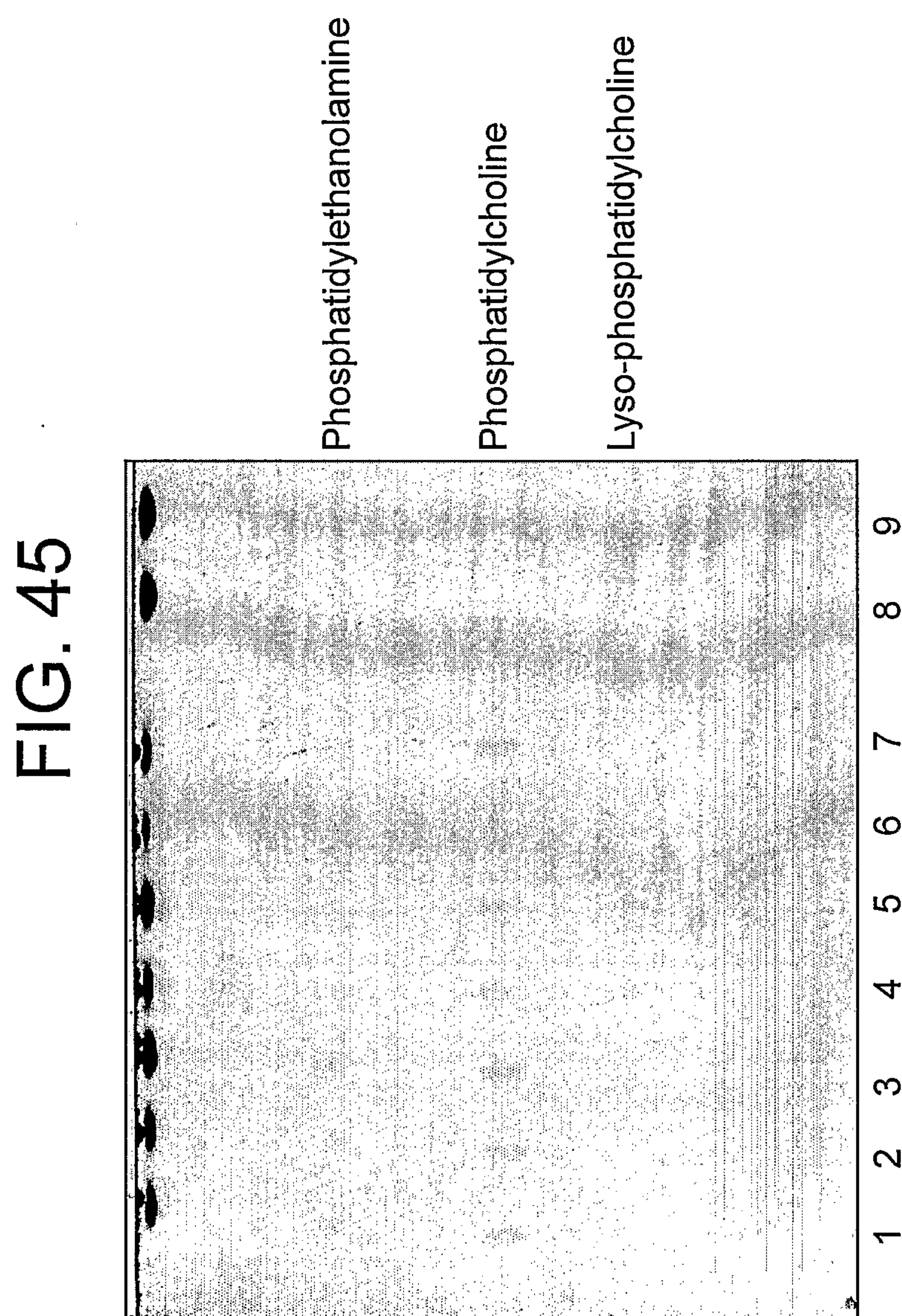




FIG. 46

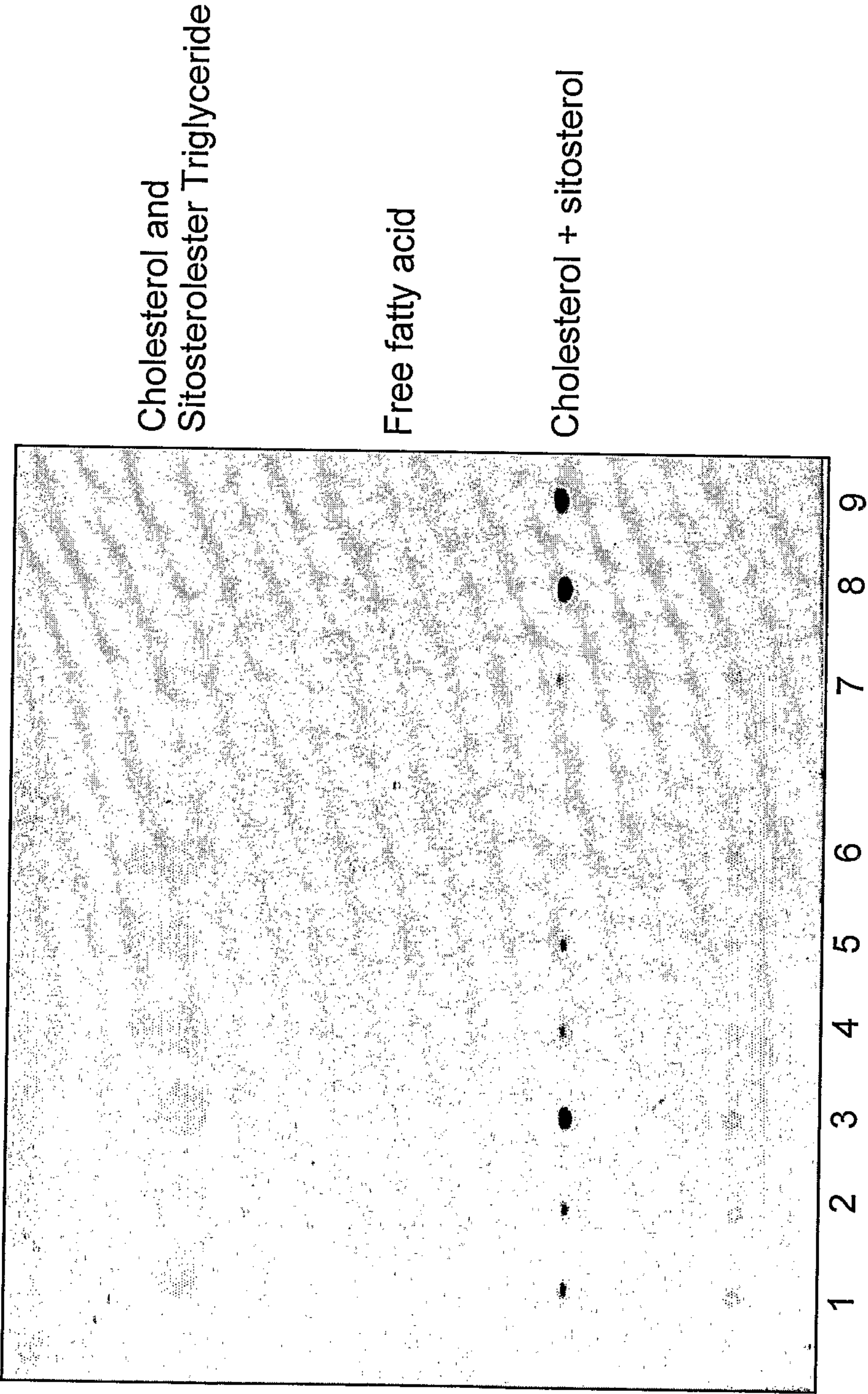




Figure 47

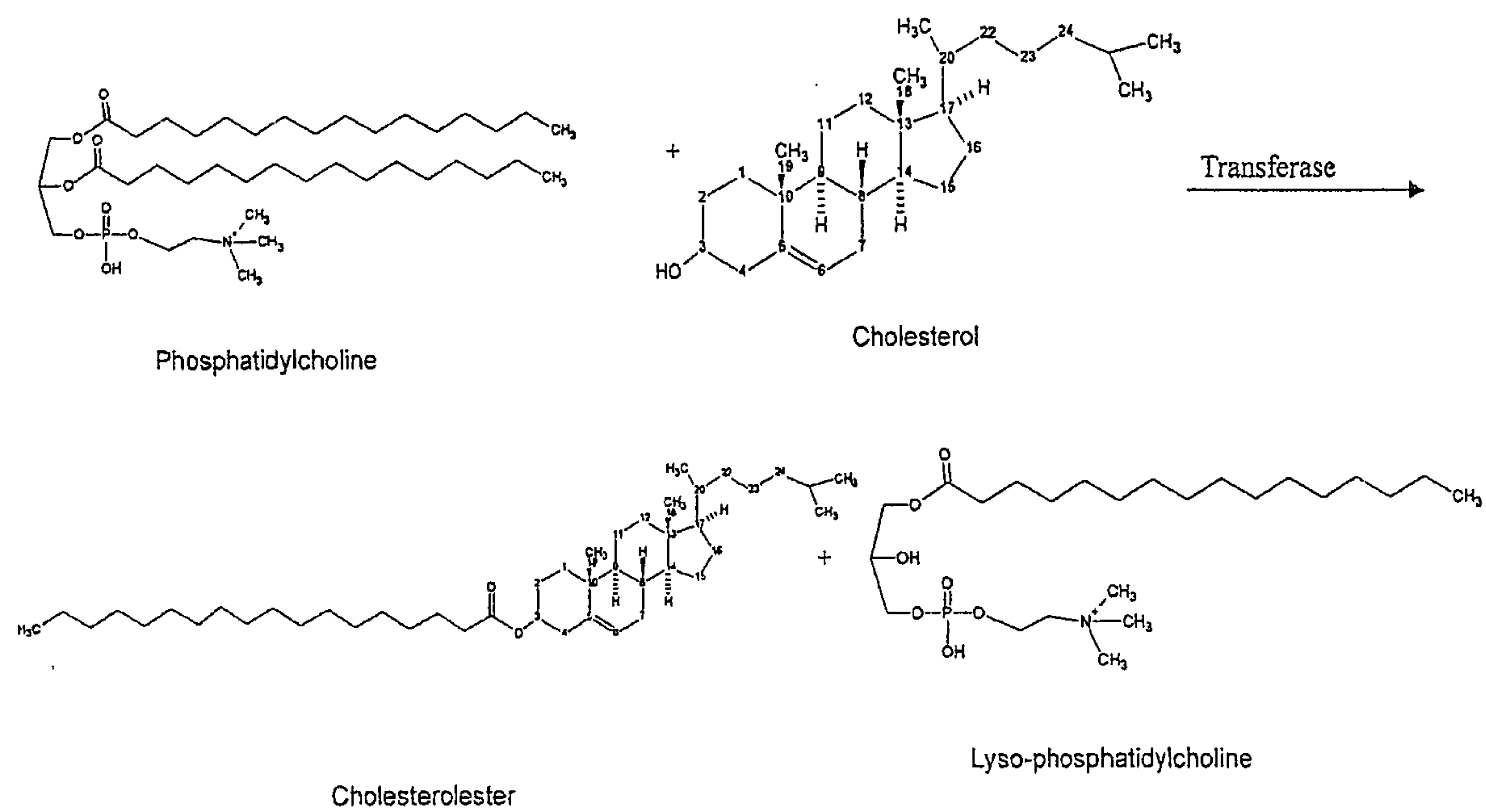




FIG. 48

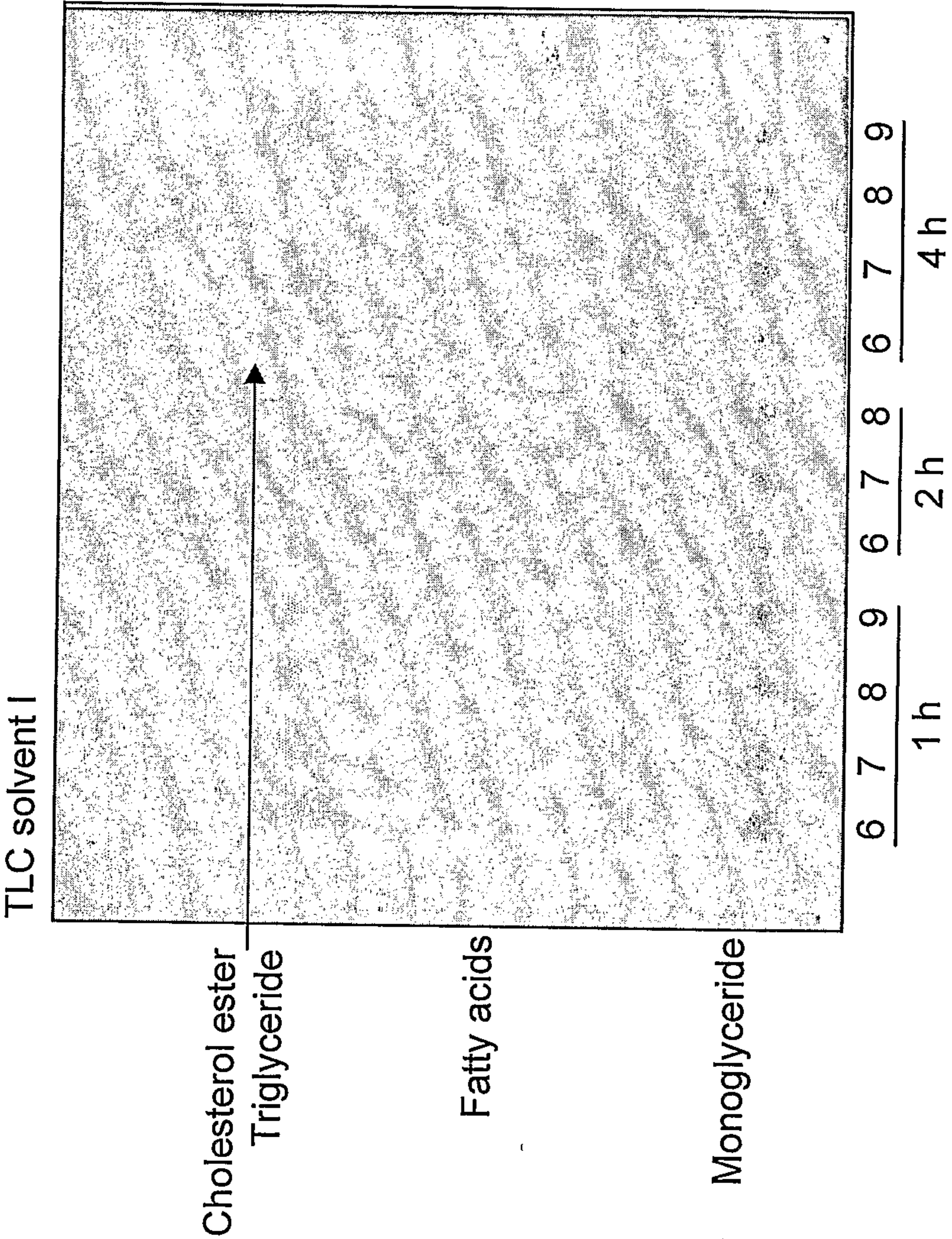
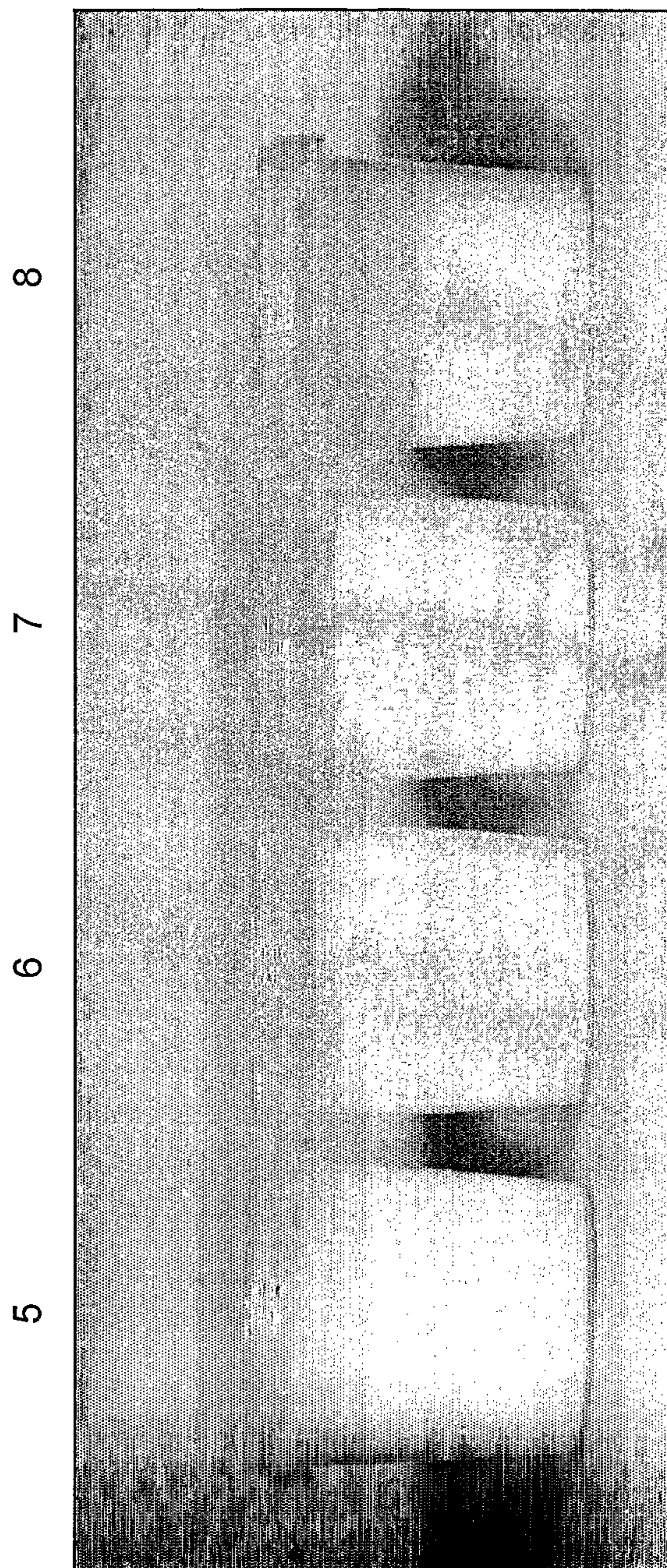




FIG. 49





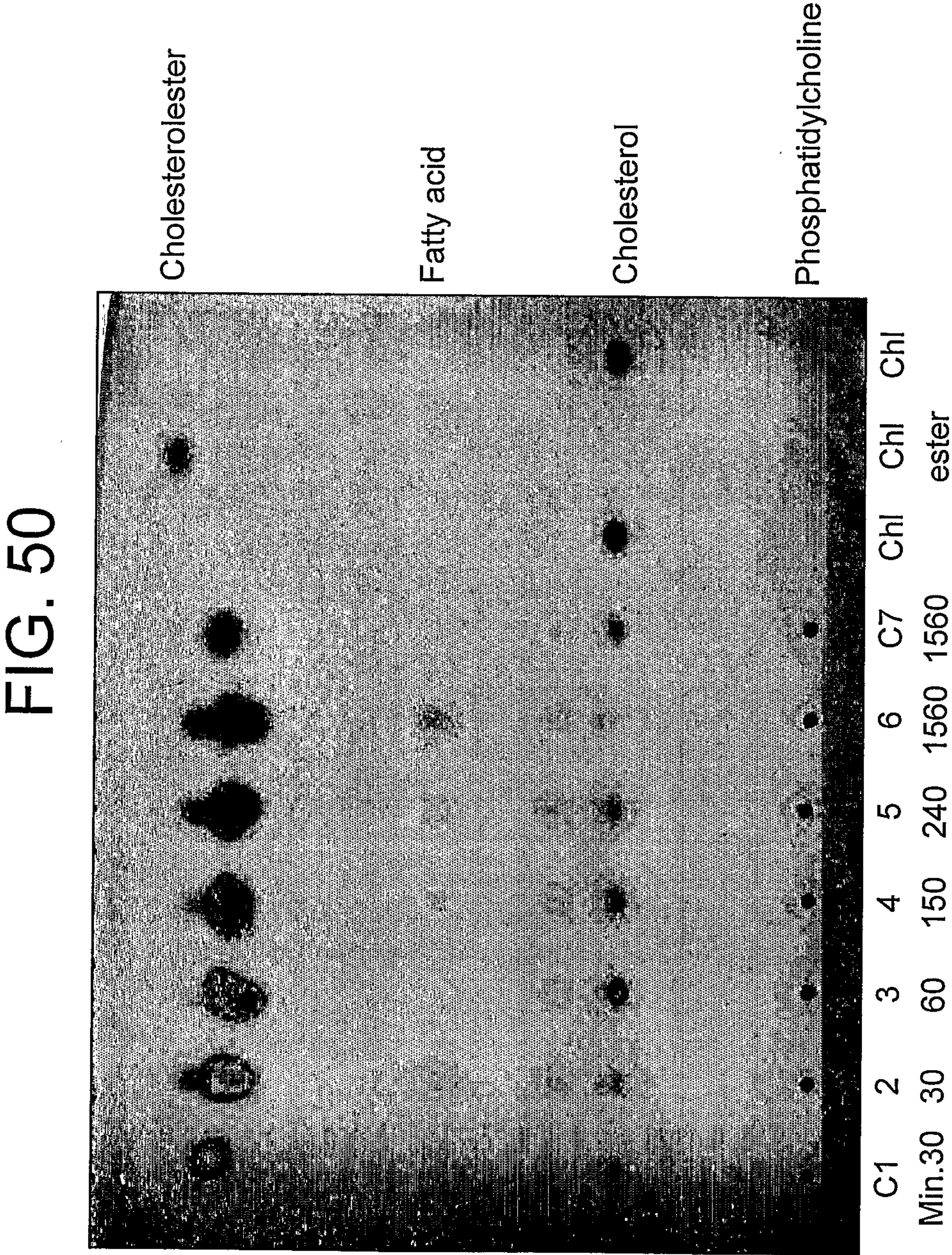




FIG. 51

2254-82-5. TLC of egg yolk lipid treated with transferase #135

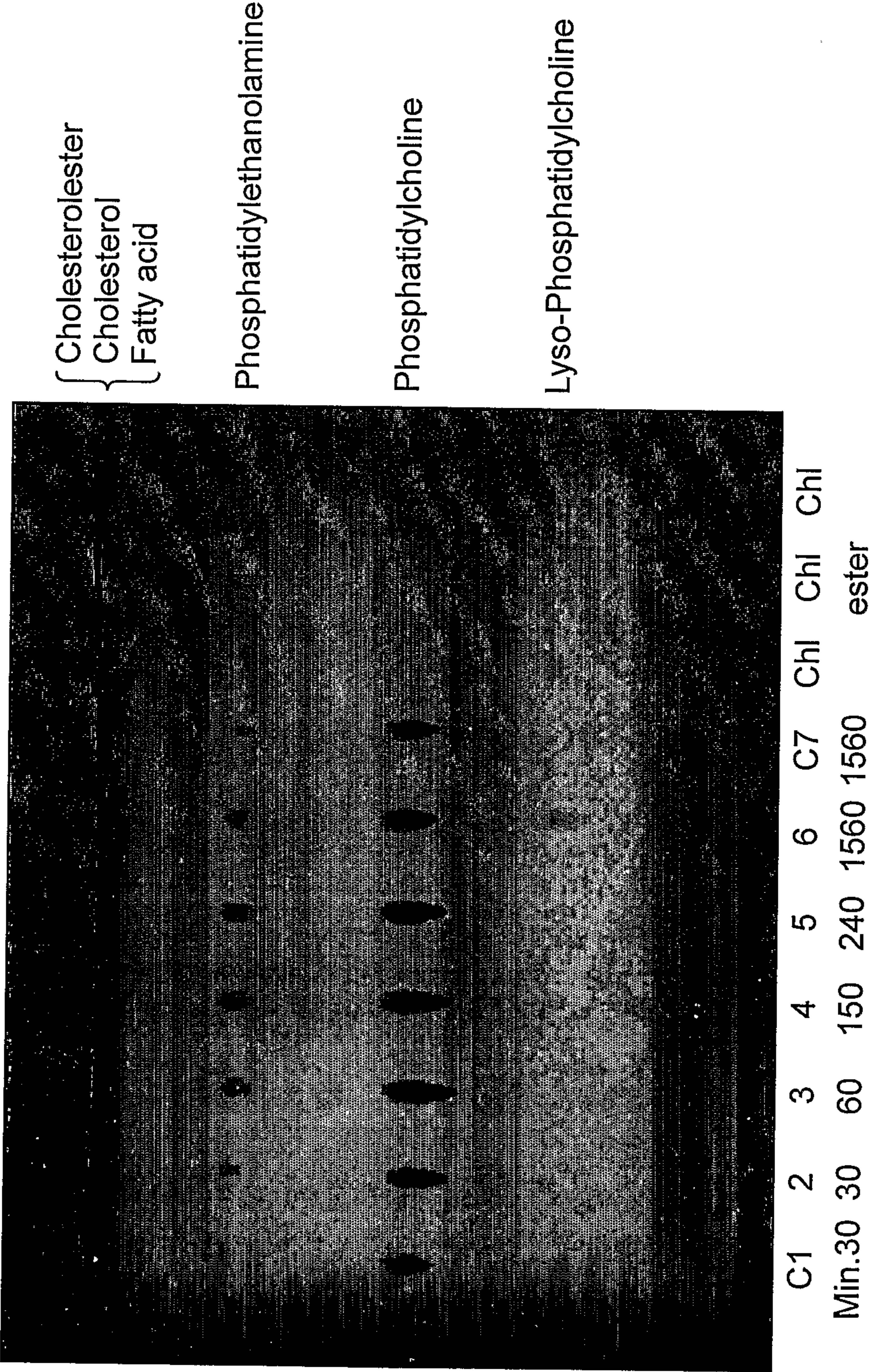




FIG. 52

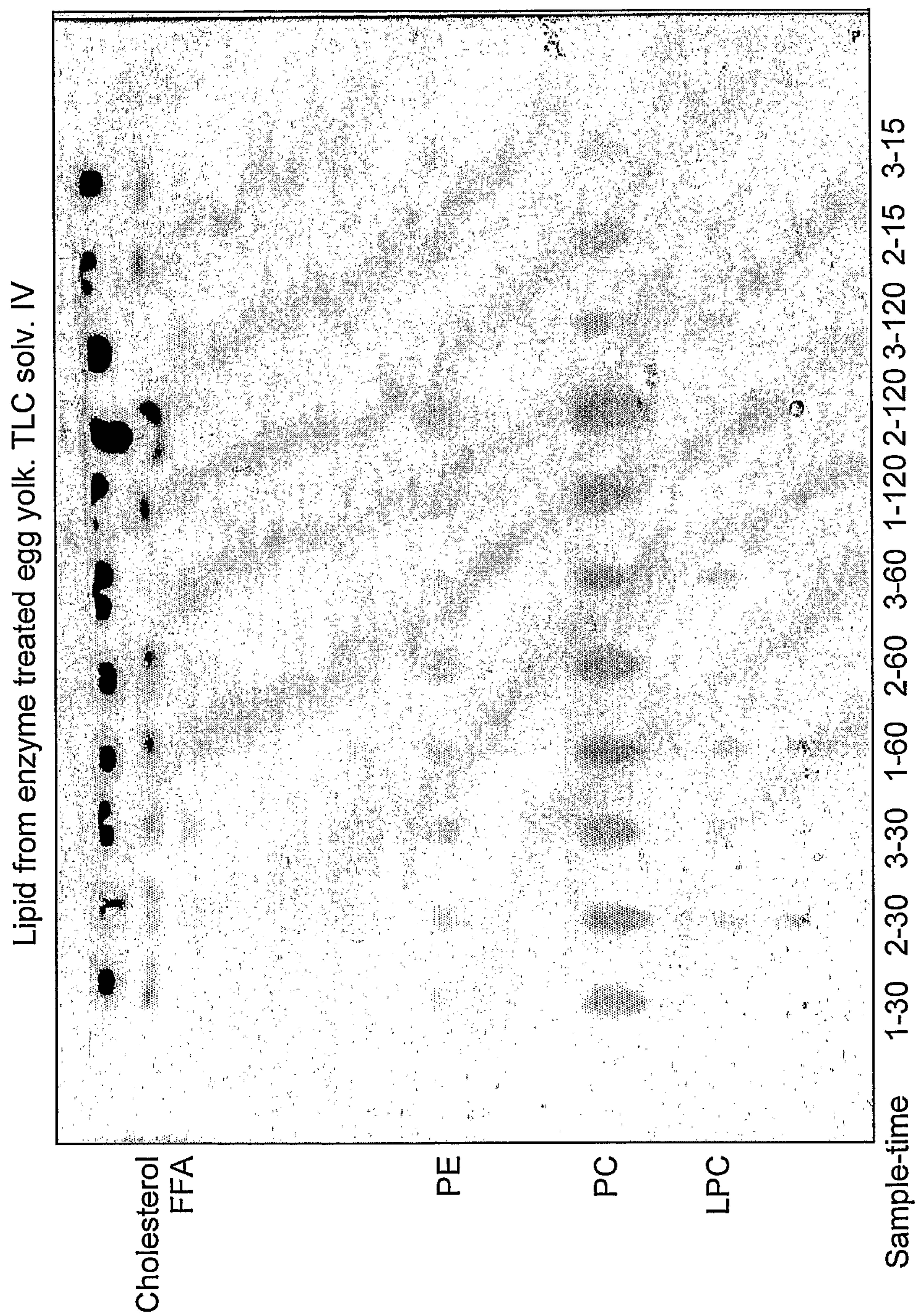




Figure 53

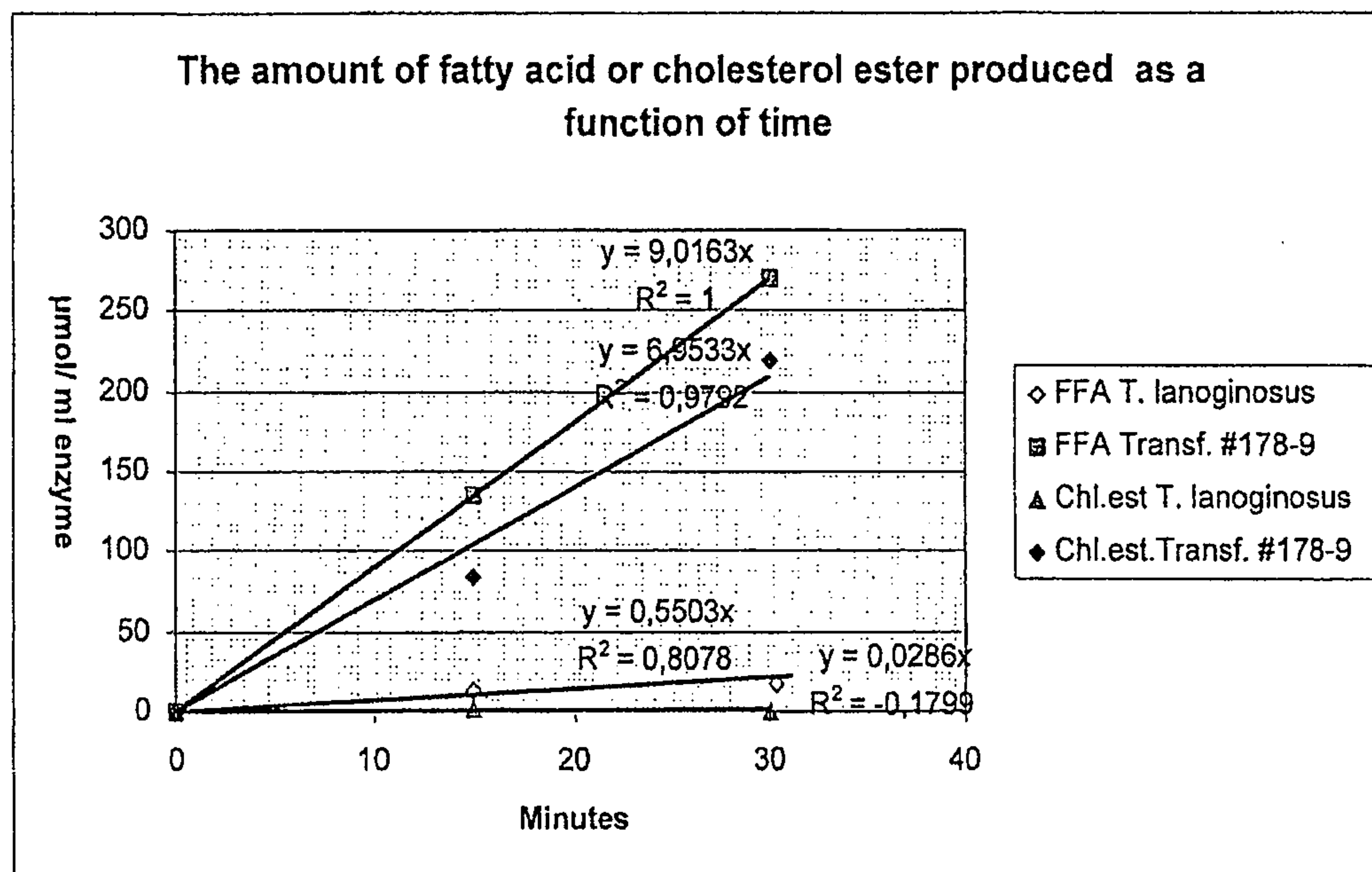




Figure 54

Relative transferase activity as % of transferase and hydrolytic

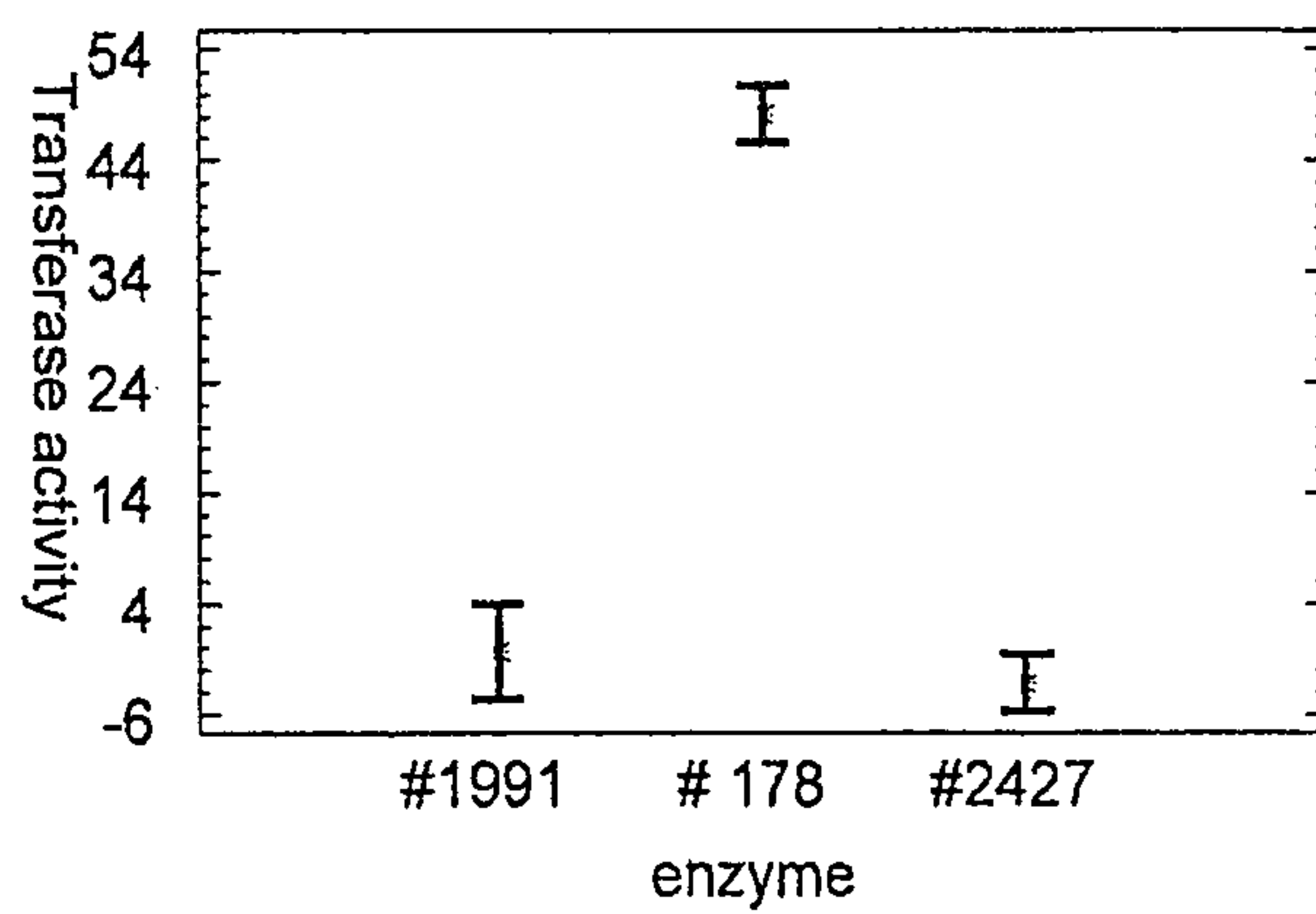




Figure 55

Transferase activity for #178 as a function of % water in the assay.

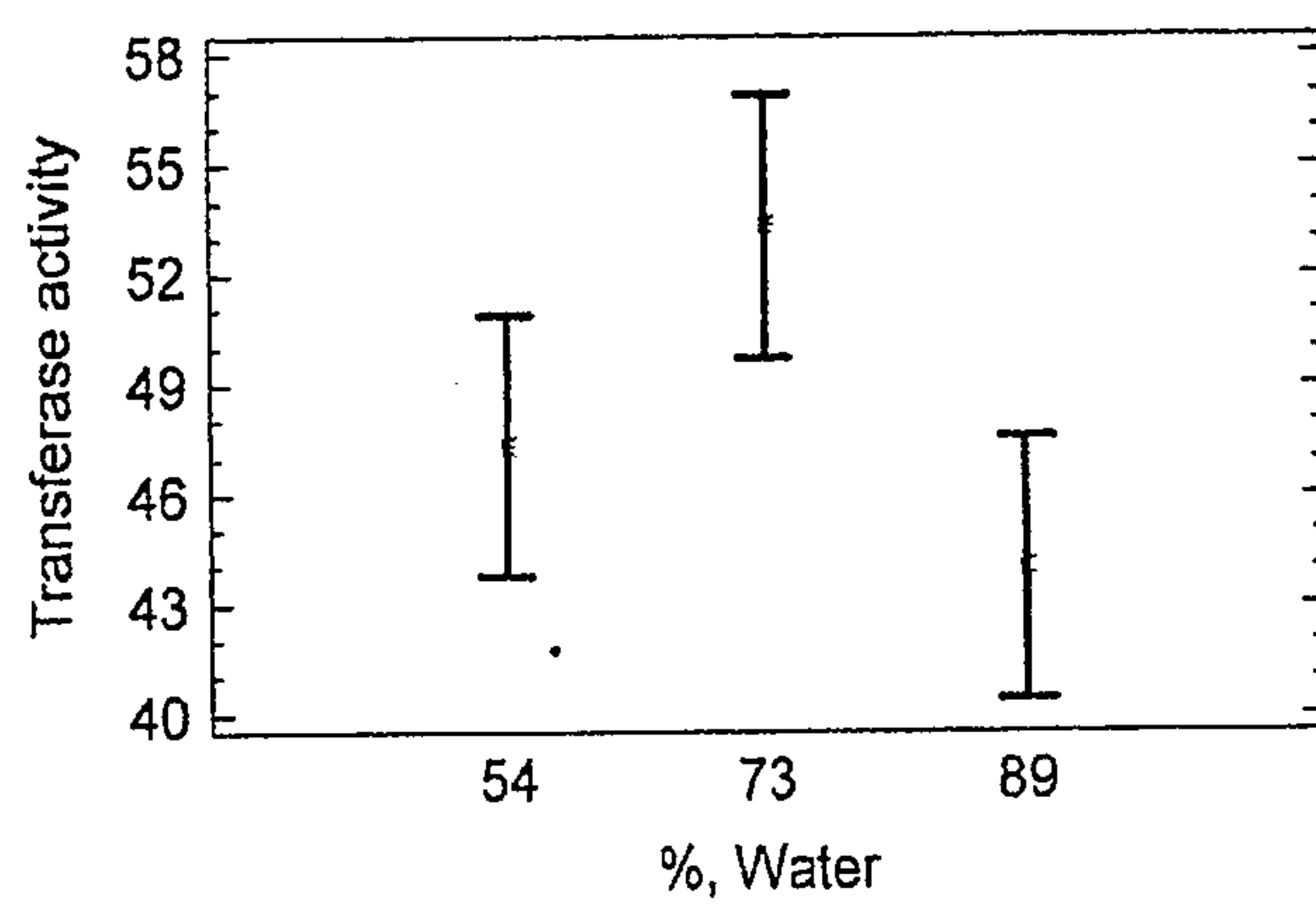




Figure 56

Transferase activity for #178 as a function of reaction time the assay.

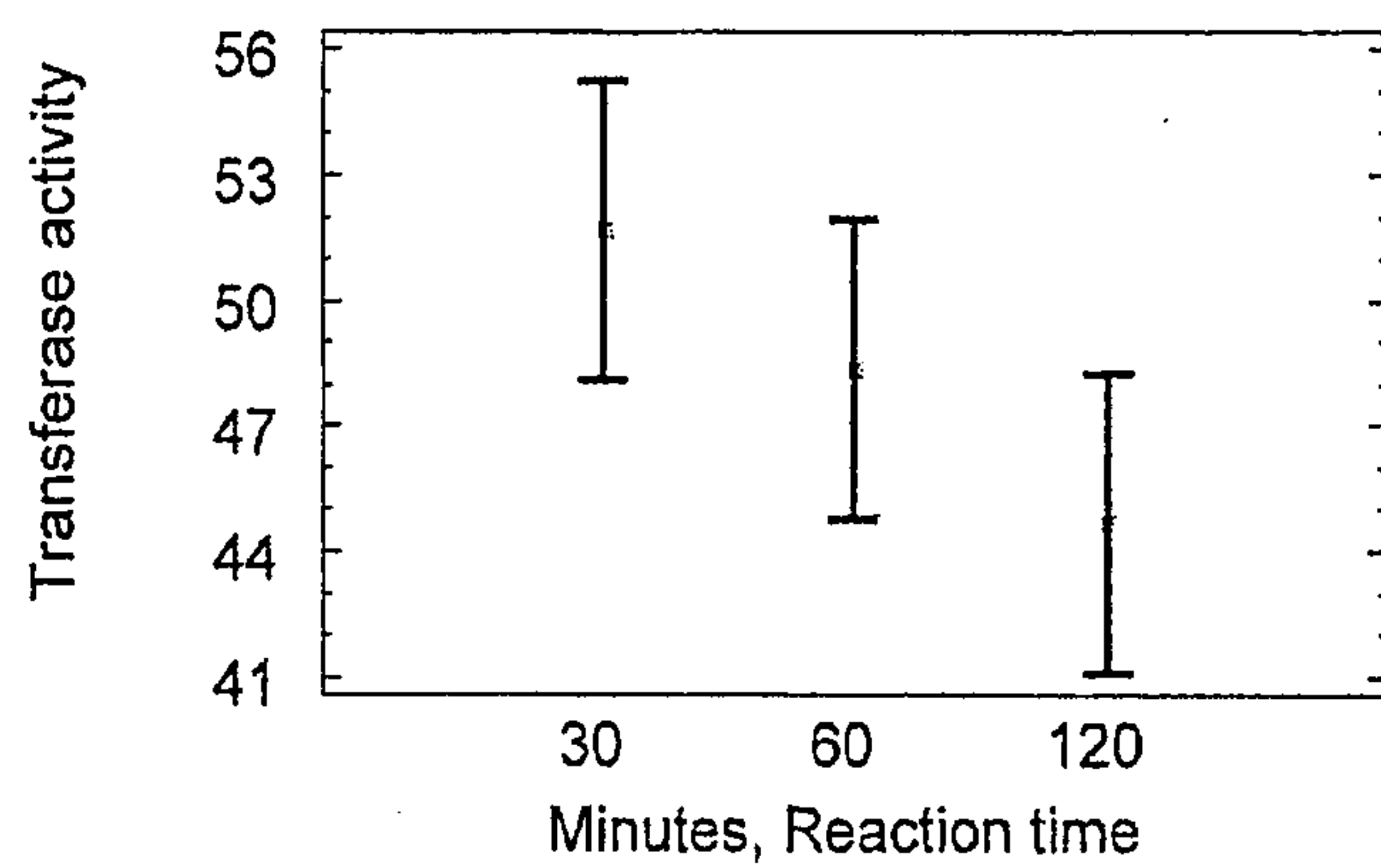




Figure 57

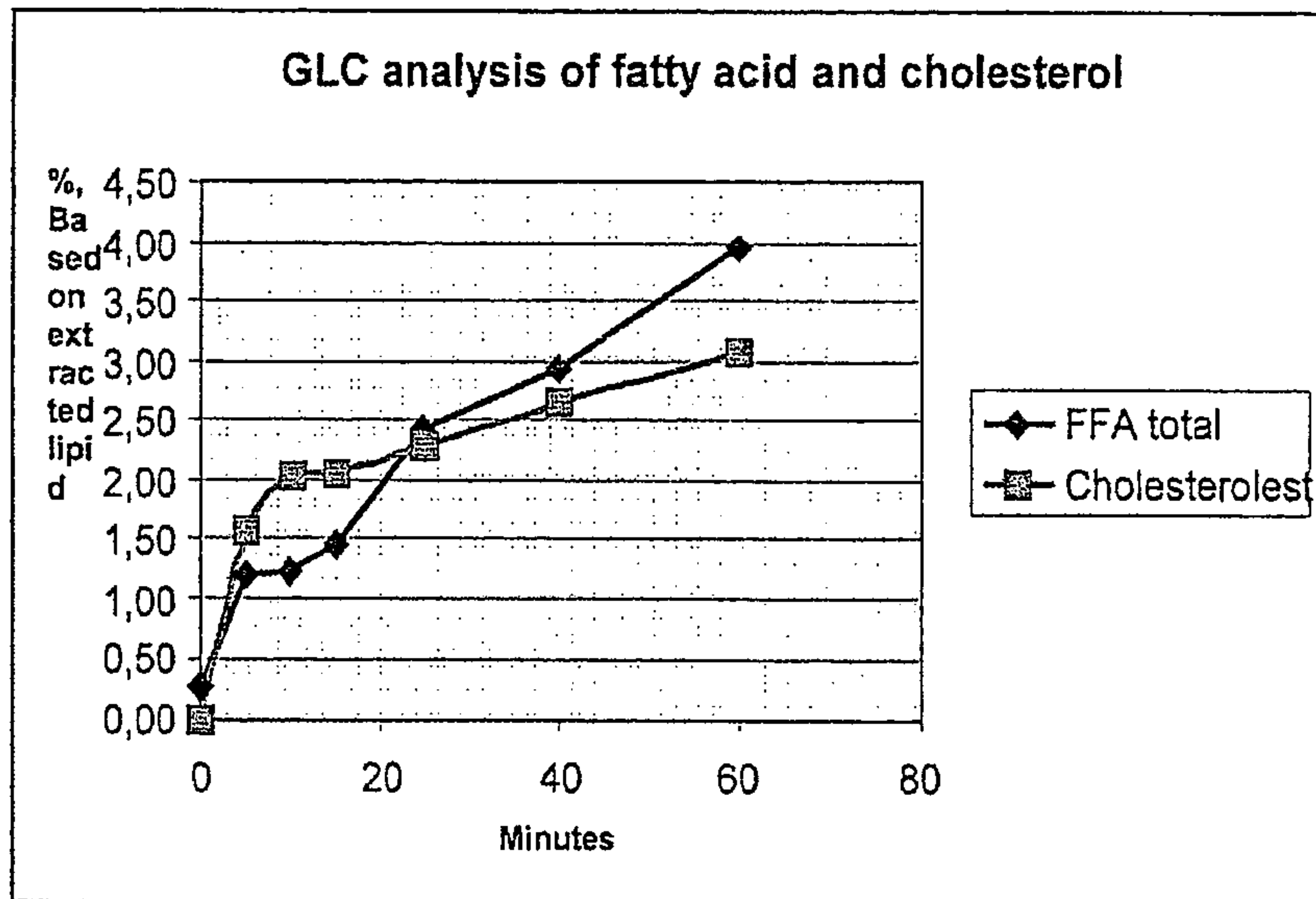




Figure 58

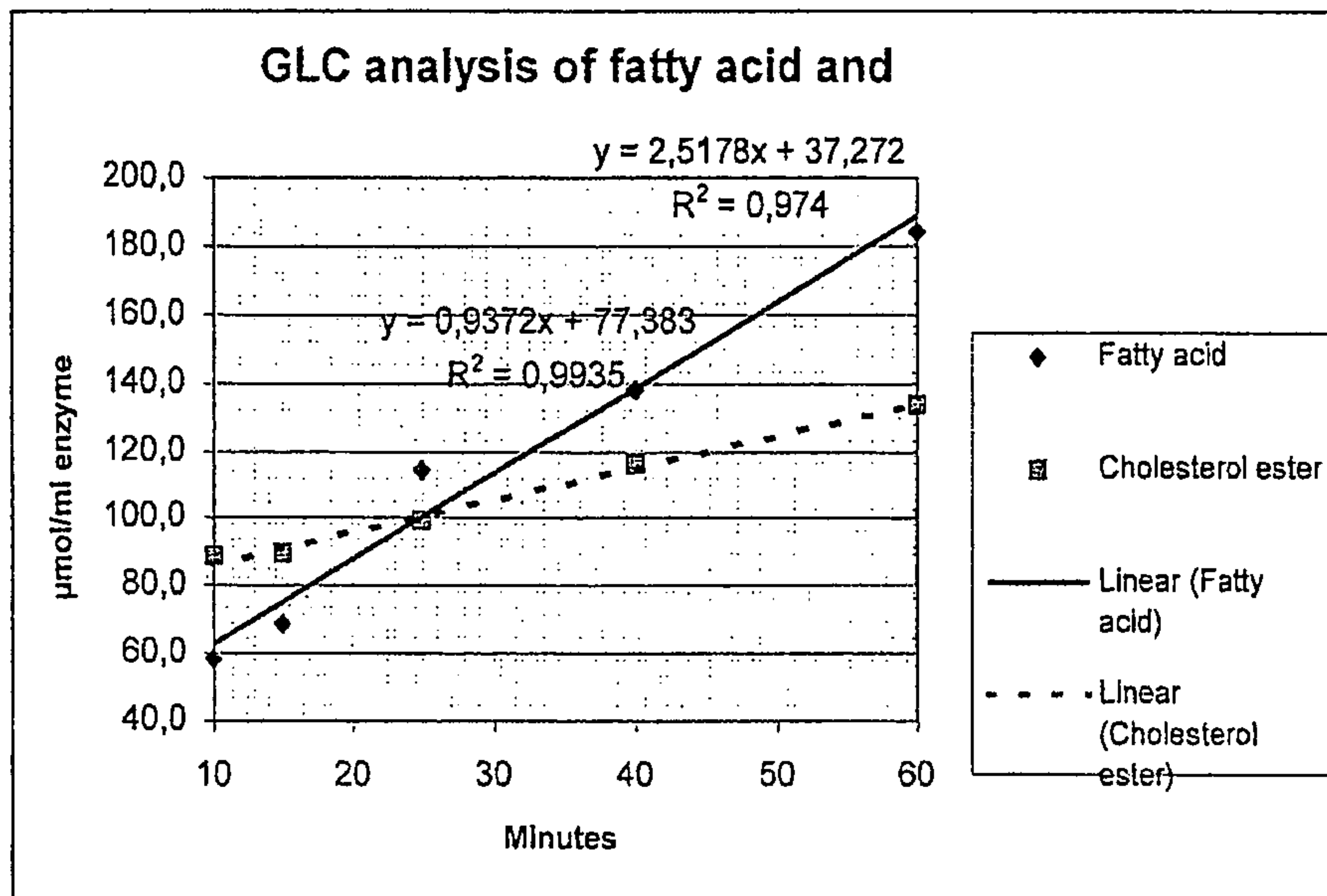




FIG. 59

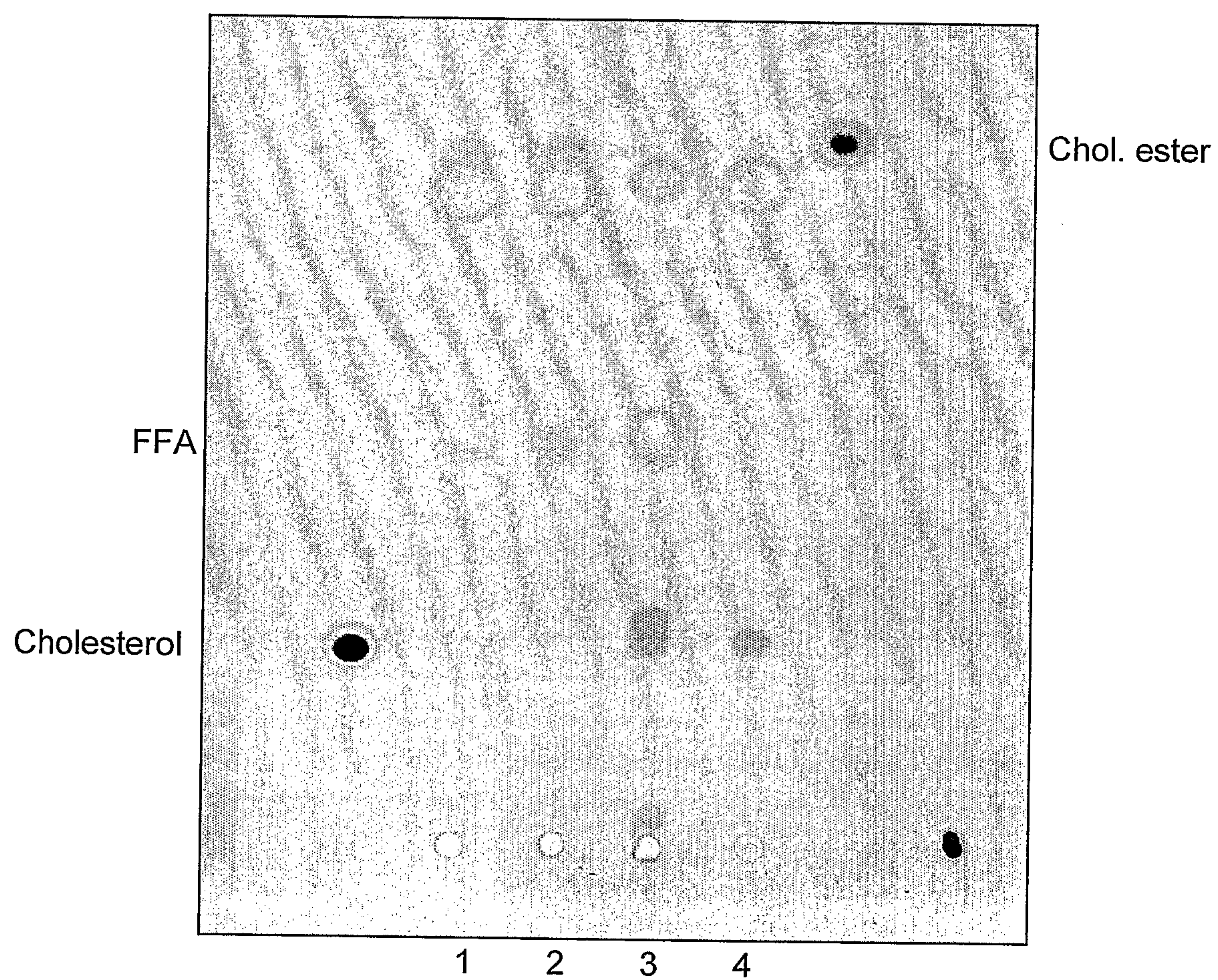




FIG. 60

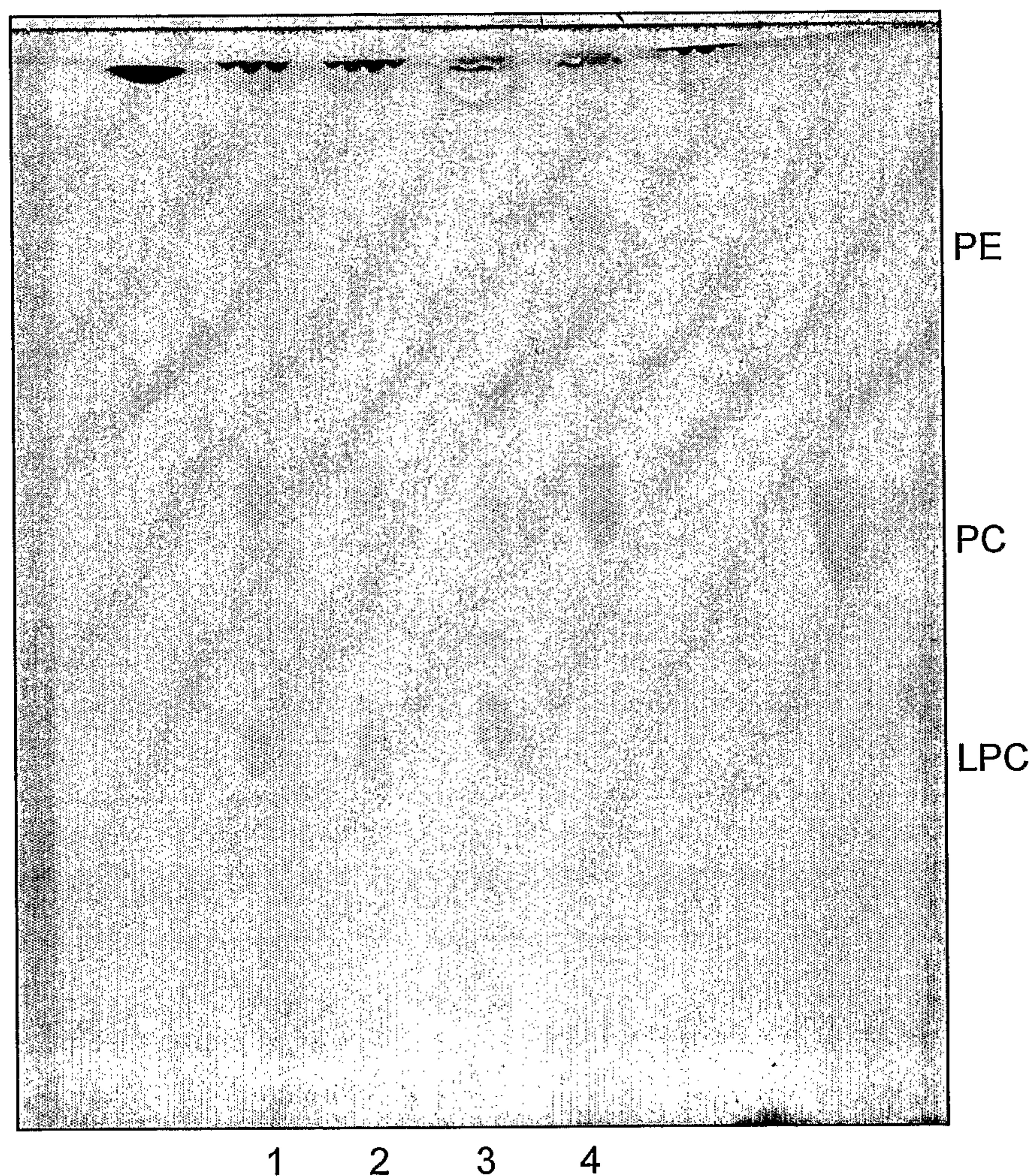




FIG. 61

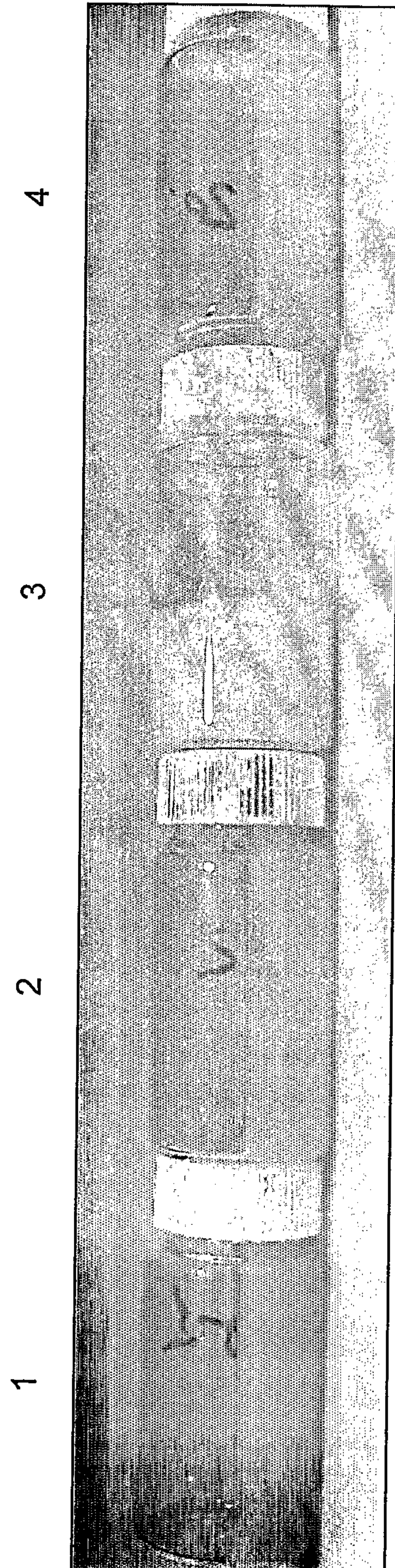




FIG. 62



0

1

3



FIG. 63

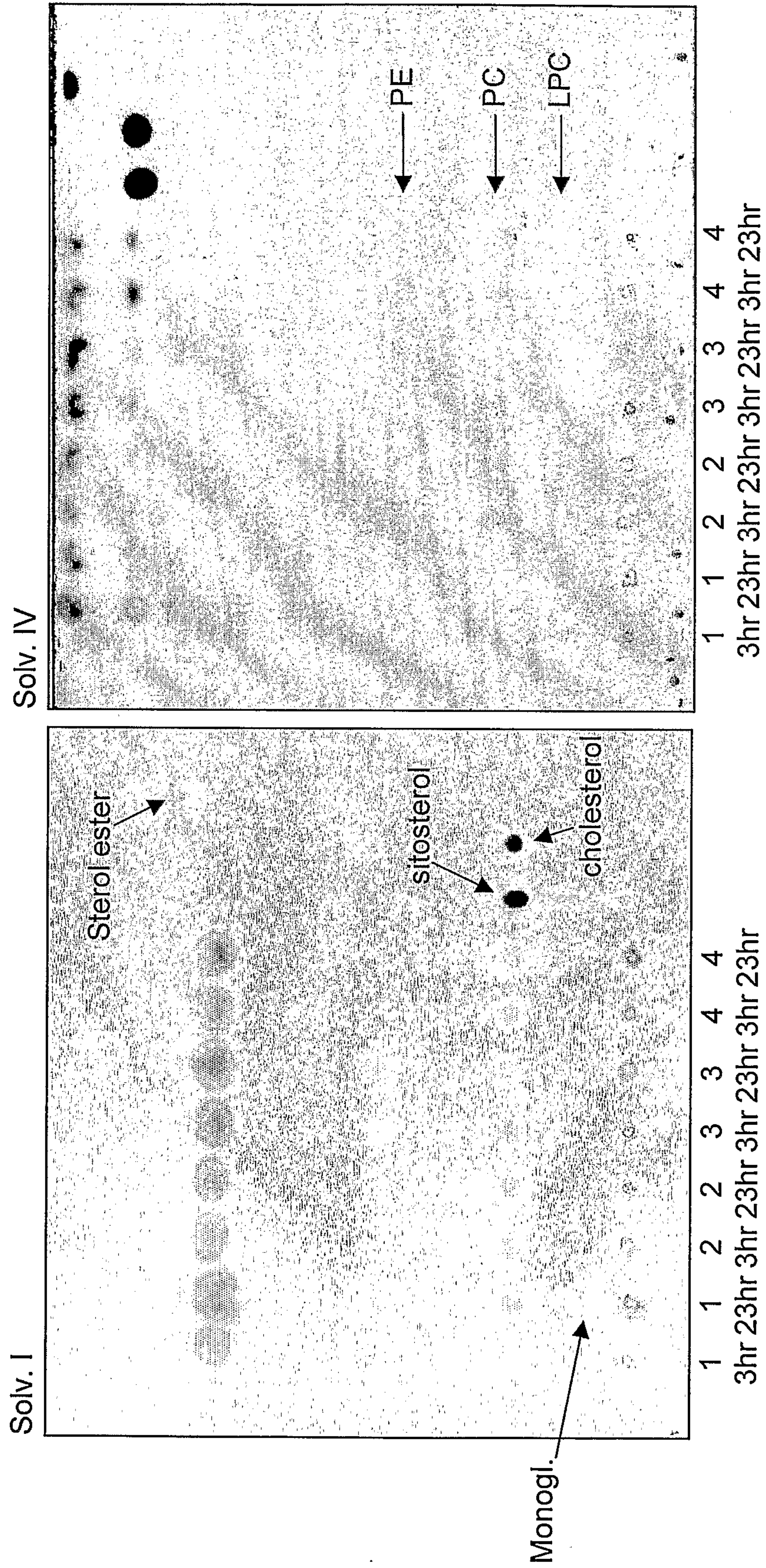




FIG. 64

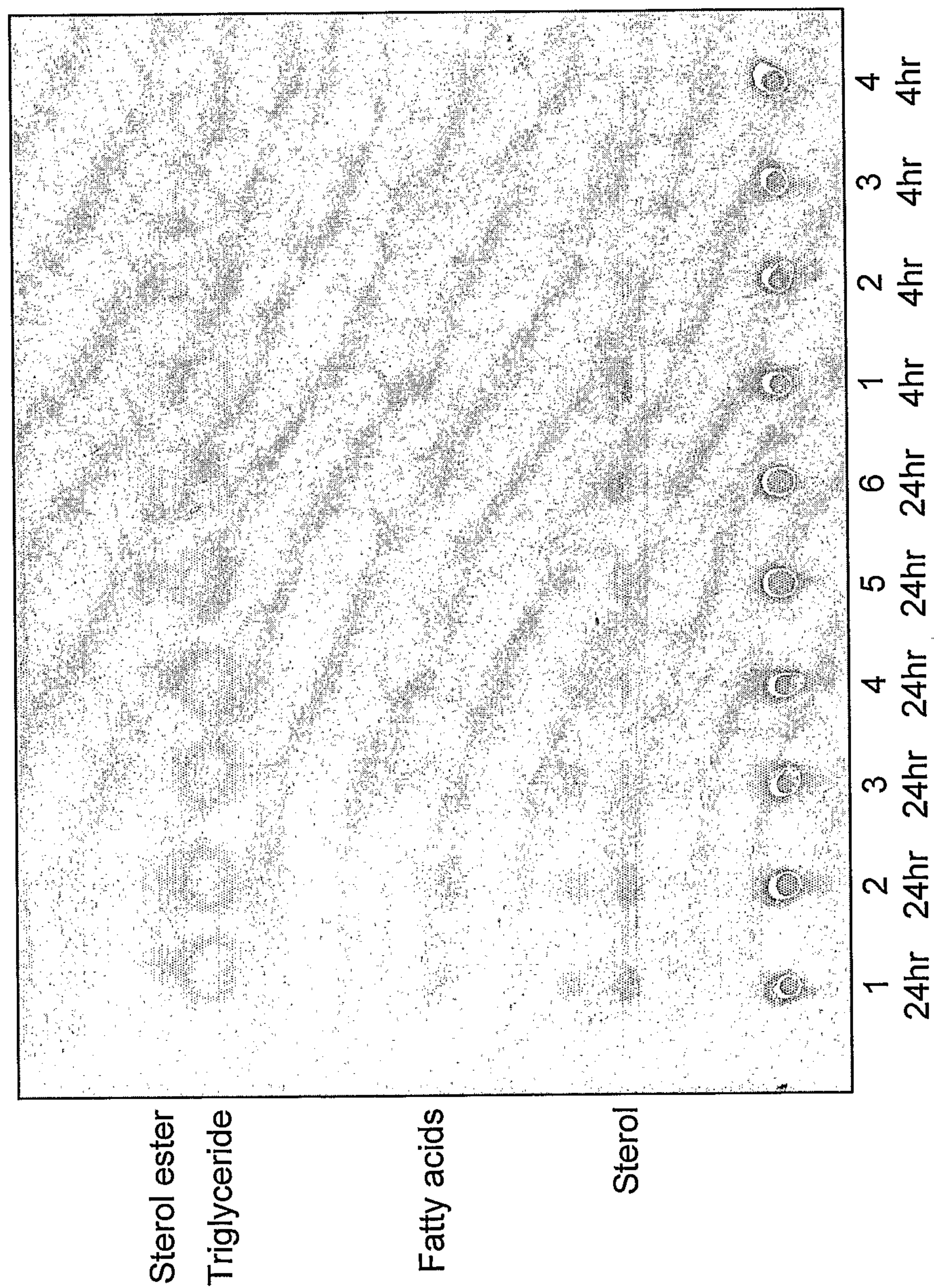




FIG. 65

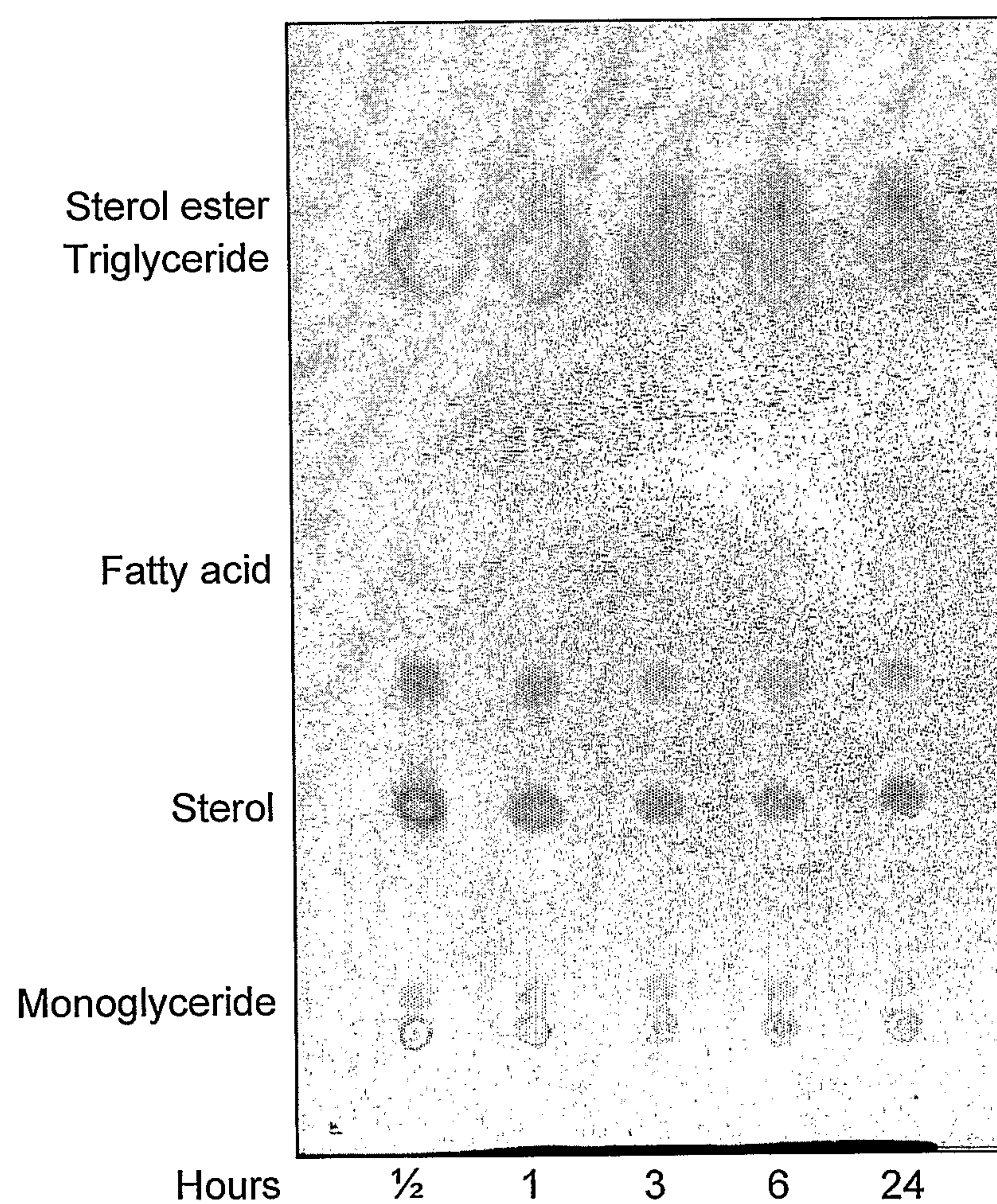




FIG. 66

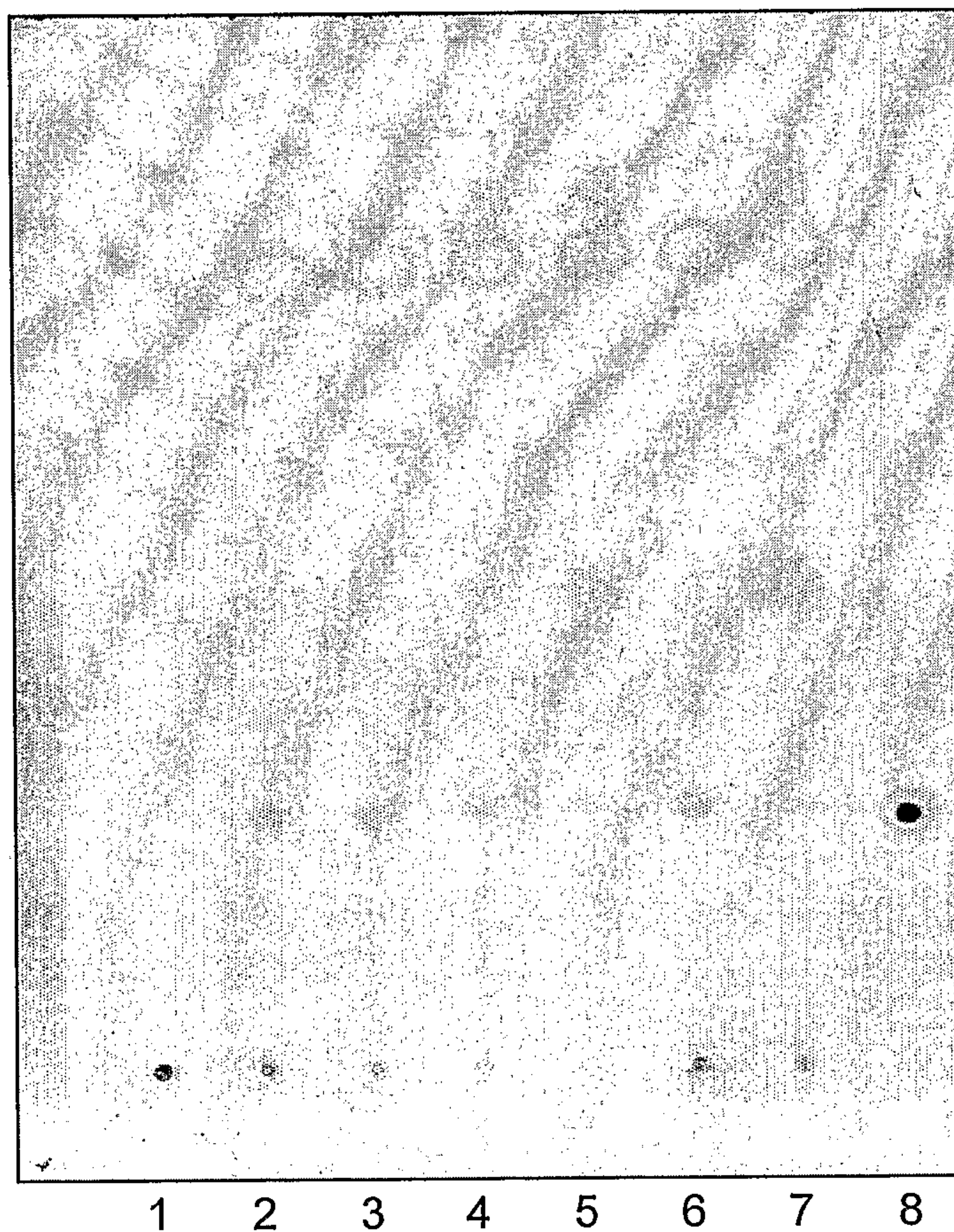




FIG. 67

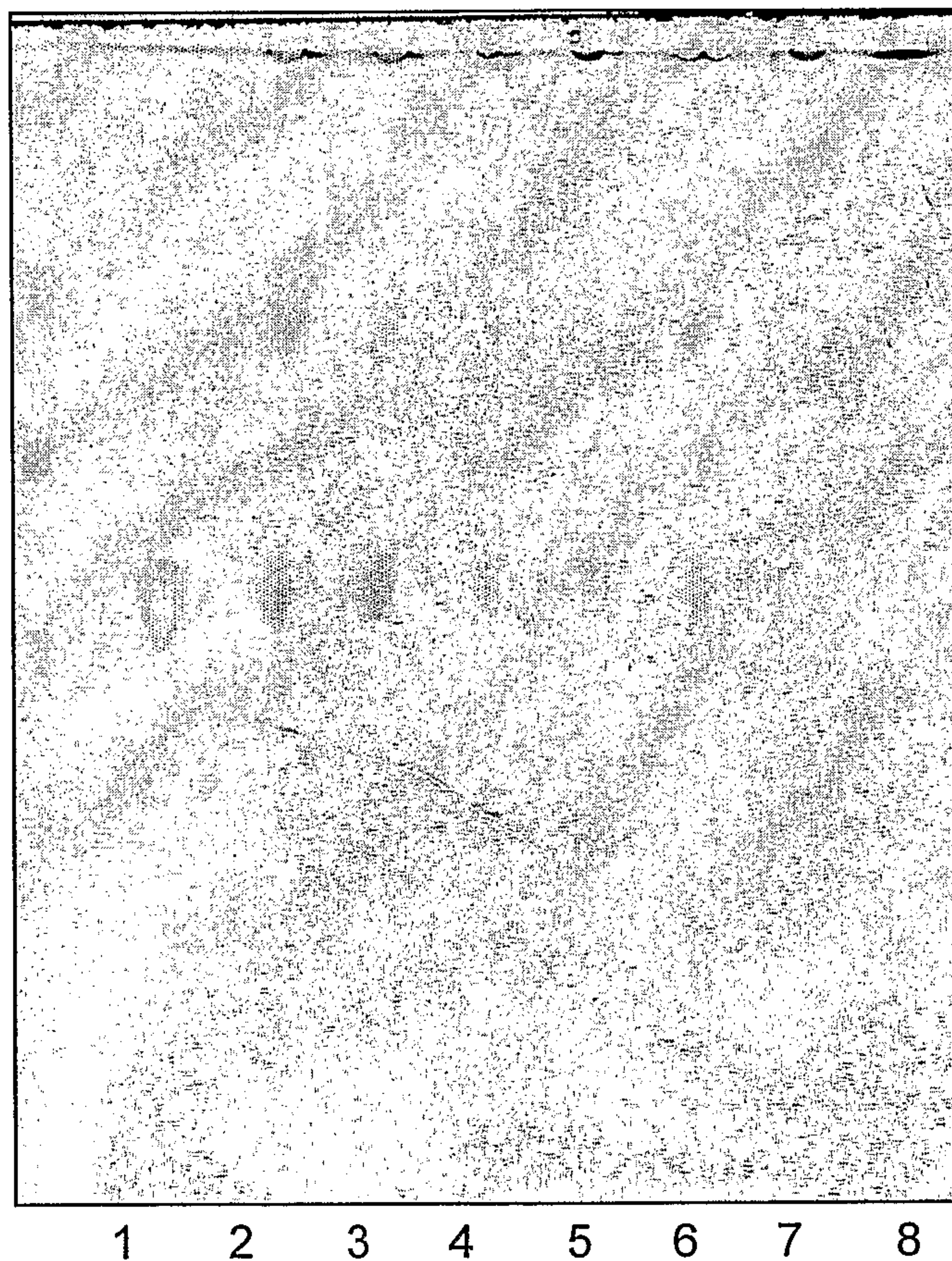




FIG. 68

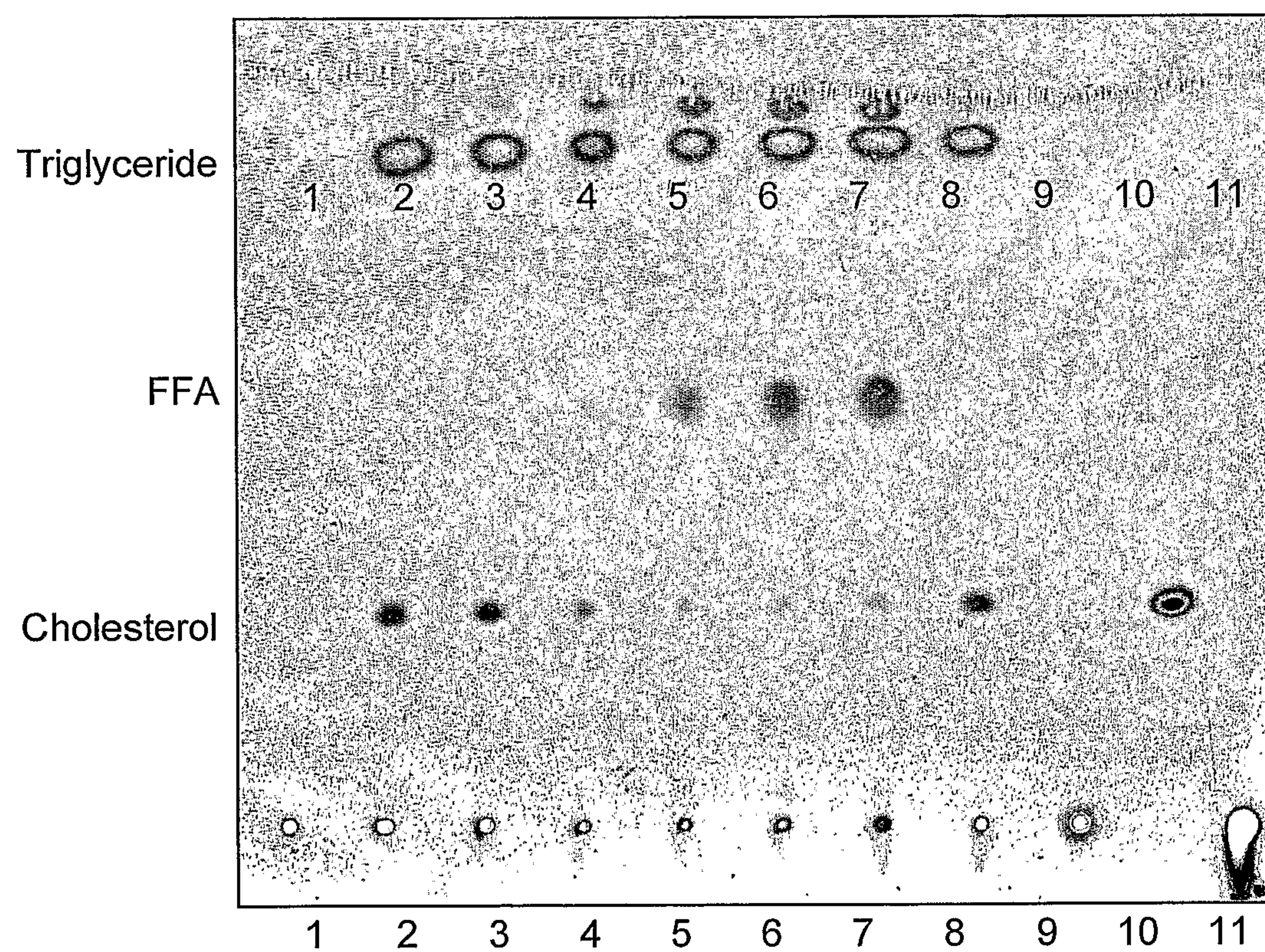




FIG. 69

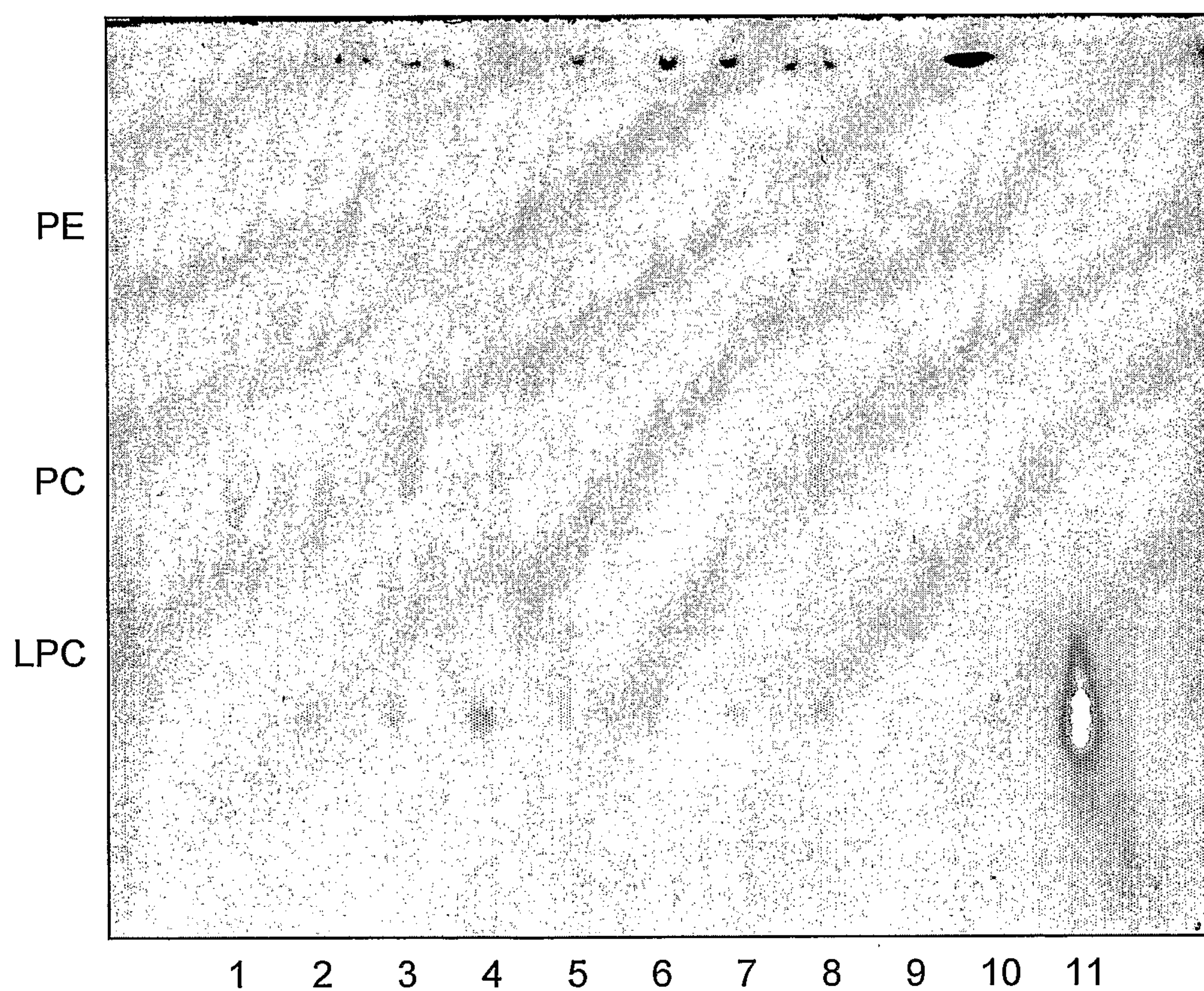




Figure 70

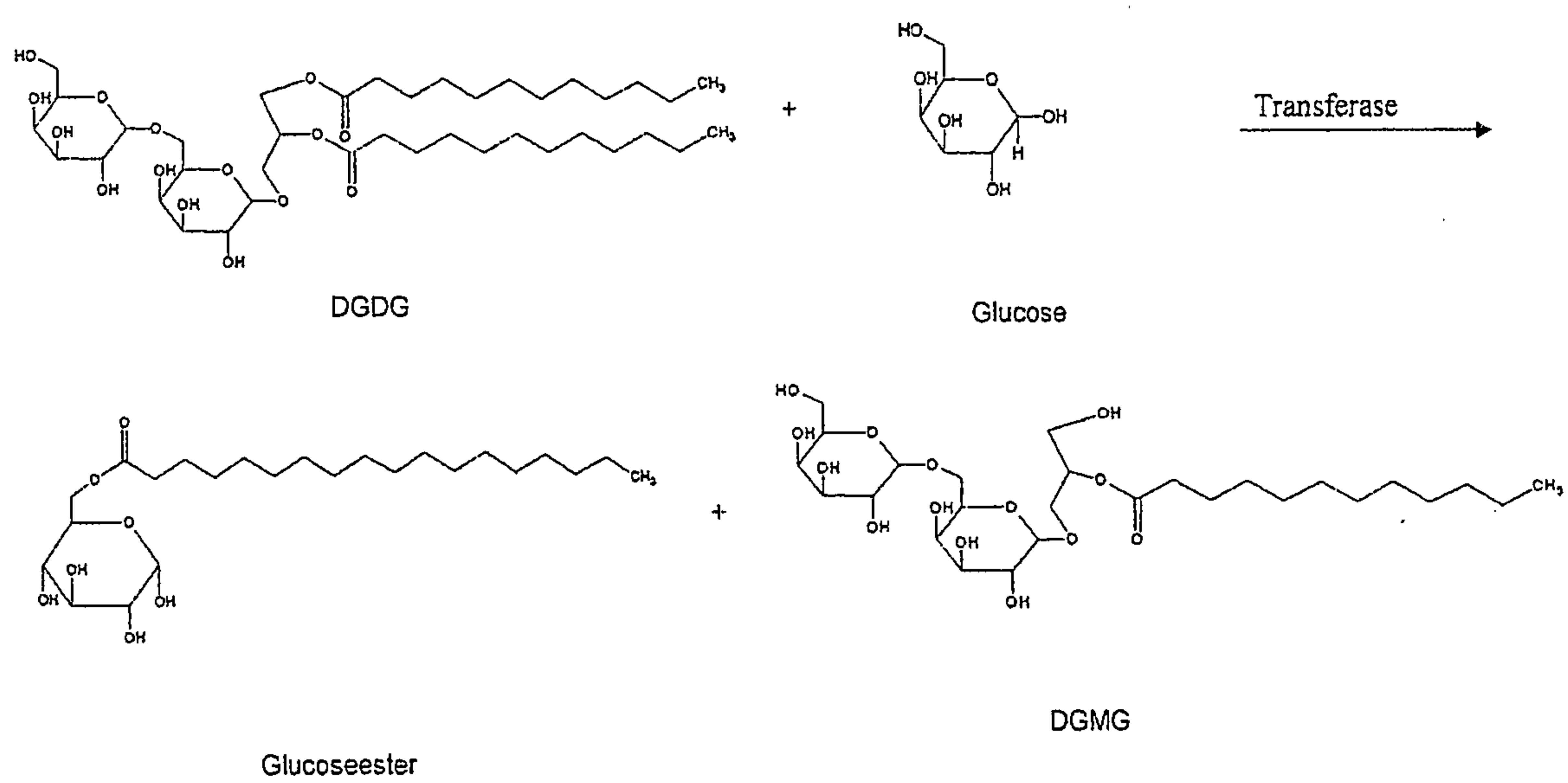




Figure 71

(SEQ ID No. 36)

```
1  MFKFKNFLV GLSAALMSIS LFSATASAAS ADSRPAFSRI VMFGDSLSDT
51  GKMYSKMRGY LPSSPPYYEG RFSNGFPVWLE QLTQFFPGLT IANEAEGGAT
101 AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVL WVGANDYLAY
151 GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLENLPDLGQ NPSARSQKV
201 EAVSHVSAYH NQLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD
251 VENPCYDGGY VWKPFATRSV STDROLSAFS POERLAIAGN PLLAQAVASP
301 MARRSASPLN CEGKMFWDQV HPTTVVHAAL SERAATFIAN QYEFLAR**
```



Figure 72 (SEQ ID No. 54)

1 ATGTTTAAGT TTAAAAAGAA TTTCTTAGTT GGATTATCGG CAGCTTTAAT  
TACAAATTCA AATTTTTCTT AAAGAATCAA CCTAATAGCC GTCGAAATTA

51 GAGTATTAGC TTGTTTTCGG CAACCGCCTC TGCAGCTAGC GCCGACAGCC  
CTCATAATCG AACAAAAGCC GTTGGCGGAG ACGTCGATCG CGGCTGTCGG

101 GTCCCGCCTT TTCCCGGATC GTGATGTTCG GCGACAGCCT CTCCGATACC  
CAGGGCGGAA AAGGGCCTAG CACTACAAGC CGCTGTCGGA GAGGCTATGG

151 GGCAAAATGT ACAGCAAGAT GCGCGGTTAC CTCCCCTCCA GCCCGCCCTA  
CCGTTTACA TGTCGTTCTA CGCGCCAATG GAGGGGAGGT CGGGCGGGAT

201 CTATGAGGGC CGTTTCTCCA ACGGACCCGT CTGGCTGGAG CAGCTGACCA  
GATACTCCCG GCAAAGAGGT TGCCTGGGCA GACCGACCTC GTCGACTGGT

251 AACAGTTCCC GGGTCTGACC ATCGCCAACG AAGCGGAAGG CGGTGCCACT  
TTGTCAAGGG CCCAGACTGG TAGCGGTTGC TTCGCCTTCC GCCACGGTGA

301 GCCGTGGCTT ACAACAAGAT CTCCTGGAAT CCCAAGTATC AGGTCATCAA  
CGGCACCGAA TGTGTTCTA GAGGACCTTA GGTTTCATAG TCCAGTAGTT

351 CAACCTGGAC TACGAGGTCA CCCAGTTCTT GCAGAAAGAC AGCTTCAAGC  
GTTGGACCTG ATGCTCCAGT GGGTCAAGAA CGTCTTTCTG TCGAAGTTCTG

401 CGGACGATCT GGTGATCCTC TGGGTCGGTG CCAATGACTA TCTGGCCTAT  
GCCTGCTAGA CCACTAGGAG ACCCAGCCAC GGTTACTGAT AGACCGGATA

451 GGCTGGAACA CGGAGCAGGA TGCCAAGCGG GTTCGCGATG CCATCAGCGA  
CCGACCTTGT GCCTCGTCTT ACGGTTTCGCC CAAGCGCTAC GGTAGTCGCT

501 TGCGGCCAAC CGCATGGTAC TGAACGGTGC CAAGCAGATA CTGCTGTTCA  
ACGCCGGTTG GCGTACCATG ACTTGCCACG GTTCGTCTAT GACGACAAGT

551 ACCTGCCGGA TCTGGGCCAG AACCCGTCAG CTCGCAGTCA GAAGGTGGTC  
TGGACGGCCT AGACCCGGTC TTGGGCAGTC GAGCGTCAGT CTCCACCAG

601 GAGGCGGTCA GCCATGTCTC CGCCTATCAC AACCAGCTGC TGCTGAACCT  
CTCCGCCAGT CCGTACAGAG GCGGATAGTG TTGGTCGACG ACGACTTGGA

651 GGCACGCCAG CTGGCCCCCA CCGGCATGGT AAAGCTGTTC GAGATCGACA  
CCGTGCGGTC GACCGGGGGT GGCCGTACCA TTTGACAAG CTCTAGCTGT

701 AGCAATTTGC CGAGATGCTG CGTGATCCGC AGAACTTCGG CCTGAGCGAC  
TCGTTAAACG GCTCTACGAC GCACTAGGCG TCTTGAAGCC GGAATCGCTG

751 GTCGAGAACC CCTGCTACGA CGGCGGCTAT GTGTGGAAGC CGTTTGCCAC  
CAGCTCTTGG GGACGATGCT GCCGCCGATA CACACCTTCG GCAAACGGTG

801 CCGCAGCGTC AGCACCGACC GCCAGCTCTC CGCCTTCAGT CCGCAGGAAC  
GGCGTCGCAG TCGTGGCTGG CGGTCGAGAG GCGGAAGTCA GCGTCCTTG

851 GCCTCGCCAT CGCCGGCAAC CCGCTGCTGG CACAGGCCGT TGCCAGTCCT  
CGGAGCGGTA GCGGCCGTTG GGCGACGACC GTGTCCGGCA ACGGTCAGGA

901 ATGGCCCGCC GCAGCGCCAG CCCCCTCAAC TGTGAGGGCA AGATGTTCTG  
TACCGGGCGG CGTCGCGGTC GGGGGAGTTG AACTCCCGT TCTACAAGAC

951 GGATCAGGTA CACCCGACCA CTGTCGTGCA CGCAGCCCTG AGCGAGCGCG  
CCTAGTCCAT GTGGGCTGGT GACAGCACGT GCGTCGGGAC TCGCTCGCGC

1001 CCGCCACCTT CATCGCGAAC CAGTACGAGT TCCTCGCCCA CTGATGA  
GGCGGTGGAA GTAGCGCTTG GTCATGCTCA AGGAGCGGGT GACTACT



FIG. 73

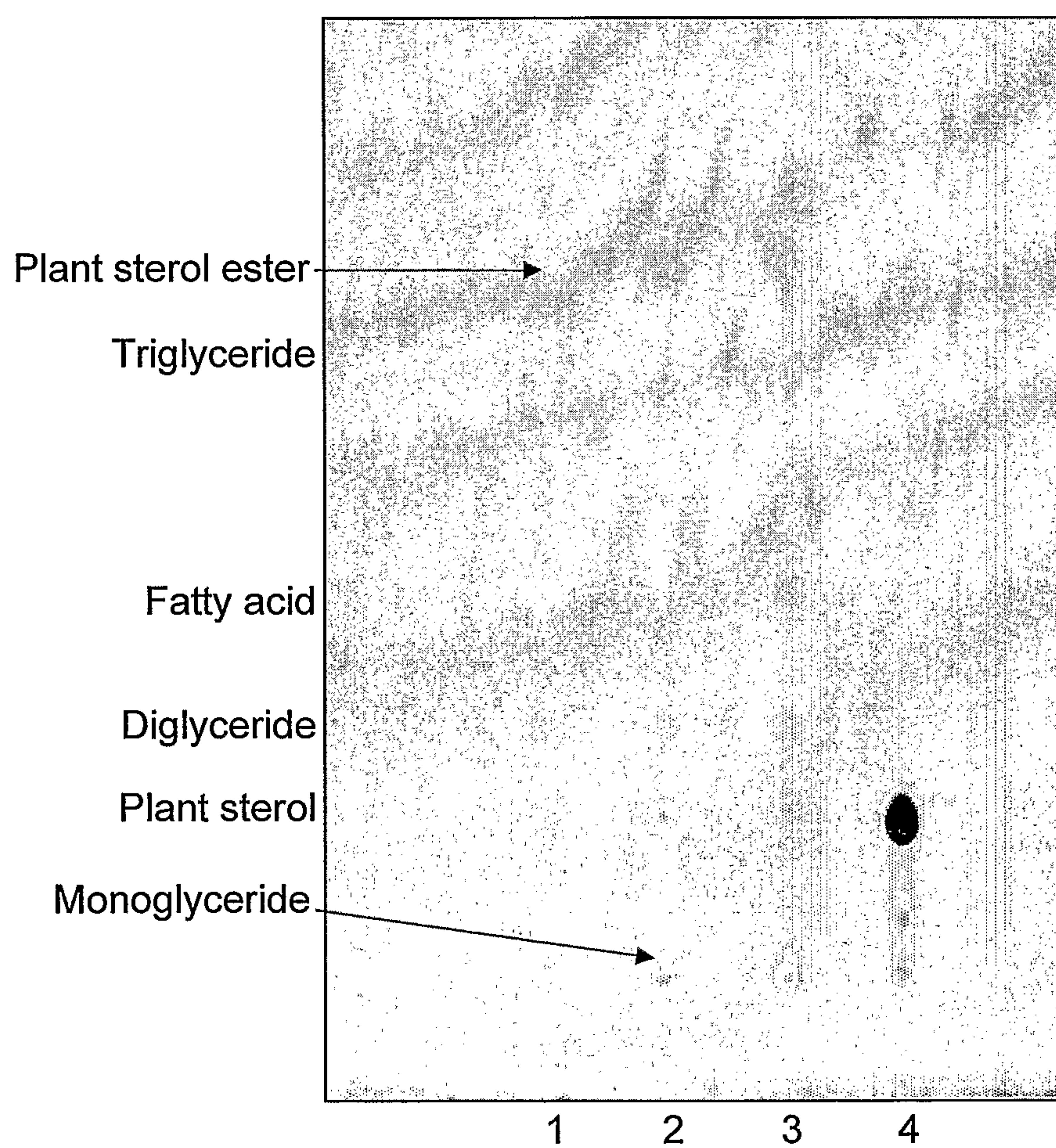




Figure 74

SEQ ID No. 62

```
1  ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTQFPGLT
61  IANEAEggAT AVAYNKISWD PKYQVINNLD YEVTQFLQKD SFKPDDLVL WVGANDYLAY
121 GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV EAVSHVSAYH
181 NKLLNLARQ LAPTMVKLF EIDKQFAEML RDPQNFGLSD VENPCYDGGY VWKPFATRSV
241 STDRQLSAFS PQERLAIAGN PLLAQAVASP MARRSASPLN CEGKMFWDQV HPTTVVHAAL
301 SERAATFIET QYEFLAHG
```

Figure 75

SEQ ID No 63 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

```
MRYFAIAFLL INTISAFVLA PKKPSQDDFY TPPQGYEAQP LGSILKTRNV PNPLTNVFTP VKVQNAWQLL VRSEDTEGPN NAIVTIIQP
FNAKKDKLVS YQTFEDSGKL DCAPSYAIQY GSDISTLTQ GEMYYISALL DQGYVVVTPD YEGPKSTFTV GLQSGRATLN SLRATLKSGN
LTGVSSDAET LLWGYSGGSL ASGWAAAIQK EYAPELSKNL LGAALGGFVT NITATAEAVD SGPFAGIISN ALAGIGNEYF DFKNYLLKKV
SPLLSITYRL GNTHCLLDGG IAYFGKSFFS RIIRYFPDGW DLVNQEPIKT ILQDNGLVYQ PKDLTPQIPL FIYHGTLDAL VPIVNSRKTF
QQWCDWGLKS GEYNEDLTNG HITESIVGAP AALTWIINRF NGQPPVDGCQ HNVRSANLEY PGTPQSIKNY FEALHAILG FDLGPDVKRD
KVTLGGLLKL ERFAF
```

Figure 76

SEQ ID No 64 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

```
MRYFAIAFLL INTISAFVLA PKKPSQDDFY TPPQGYEAQP LGSILKTRNV PNPLTNVFTP VKVQNAWQLL VRSEDTEGPN NAIVTIIQP
FNAKKDKLVS YQTFEDSGKL DCAPSYAIQY GSDISTLTQ GEMYYISALL DQGYVVVTPD YEGPKSTFTV GLQSGRATLN SLRATLKSGN
LTGVSSDAET LLWGYSGGSL ASGWAAAIQK EYAPELSKNL LGAALGGFVT NITATAEAVD SGPFAGIISN ALAGIGNEYF DFKNYLLKKV
SPLLSITYRL GNTHCLLDGG IAYFGKSFFS RIIRYFPDGW DLVNQEPIKT ILQDNGLVYQ PKDLTPQIPL FIYHGTLDAL VPIVNSRKTF
QQWCDWGLKS GEYNEDLTNG HITESIVGAP AALTWIINRF NGQPPVDGCQ HNVRSANLEY PGTPQSIKNY FEALHAILG FDLGPDVKRD
KVTLGGLLKL ERFAFH H
```

Figure 77

SEQ ID No. 65

```
1      migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avgrllldqi
61      vaeqvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaaltaaa gtlvttgfd
121     trgvpvklhl rgkياتyngh vraiadrygc pvldlwsllrs vqdrrawdada rhlhspeght
181     rvalraggal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241     hvtakgtlsp daiktriaav a
```



Figure 78

SEQ ID No. 66

ZP 00058717

```

1 mlphpagerg evgaffallv gtpqdrirl echetrplrg rcgcgerrvp pltlpgdgvl
61 cttssstrdae tvwrkhlqpr pdggfrphlg vgcllagqgs pgvlwcgreg crfevcrdt
121 pglstrngd ssppfragws lppkceisq sarktpavpr ysllrtdrpd gprgrfvsg
181 praatrrrlf lgipalvlvt altlavlapt gretlwrwmc eatgdwclgv pvdsrgqpae
241 dgeflllspv qaatwgnyya lgdsyssgdg ardyypgtav kggcwsana ypelvaeayd
301 faghlsflac sgqrgyamld aidevgsqld wnsphtslvt igiggndlgf stvlktcmvr
361 vplldskact dgedairkrm akfettfeel isevrtrapd arilvgypr ifpeeptgay
421 yltltnqrw lnetiqefnq qlaeavavhd eeiaasggvg svefvdvyha ldgheigsde
481 pwvngvqlrd latgvtvdrs tfhpnaaghr avgervieqi etgpgprplya tfavvagatv
541 dtlagevg

```

Figure 79

SEQ ID No. 67

```

1 mgsgpraatr rrlflgipal vlvtaltlvt avptgretlw rmwceatqdw clgvpvdsrg
61 qpaedgefll lspvqaatwg nyyalgsys sgdgardyp gtavkggcw sanaypelva
121 eaydfaghls flacsgqrgy amldaidevg sqldwnspht slvtigiggn dlqfstvlkt
181 cmvrvpplds kactdgedai rkrmakfett feelisevrt rapdarilv gyprifpeep
241 tgayytltns nqrwlnetiq efnqqlaeav avhdeeiaas ggvgsvfvd vyhaldghei
301 gsdepwvngv qlrdlatgvt vdrstfhpna aghravgerv ieqietgpgr plyatfavva
361 gatvdtlage vg

```

Figure 80

SEQ ID No. 68

```

1 mrttviaasa llllagcadg areetagapp gessggiree gaeastsitd vyialgdsya
61 amggrdqplr gepfclrssg nypellhaev tdltcggavt gdllprrtlg ertlpaqvda
121 ltedttlvtl siggndlgfg evagcireri agenaddcvd llgetigeql dqlppqldrv
181 heairdragd aqvvtgylp lvsagdcpep gdvseadrw aveltgqine tvreaaerhd
241 alfvlpddad ehtscappqg rwadiqqgqt dayplhptsa gheamaaavr dalglepvqp

```

Figure 81

SEQ ID No. 69

ZP\_00094165

```

1 mgqvklfarr capvllalag lapaatvare aplaegaryv algssfaagp gvgpnagpss
61 ercgrgtlly phllaealkl dlvdatsga tthhvlgpwn evppqidsvn gdtrlvtlti
121 ggndvsfvgn ifaaacekma spdprcgkwr eiteewqad eermrsivrq iharaplarv
181 vvdvitytvl psgtcaamai spdrlaqsrs aakrlarita rvareegasl lkfshisrrh
241 hpcsakpwsn glsapaddgi pvhpnrlgha eaaaalvklv klmk //

```



## Figure 82

SEQ ID No. 70

NP\_625998.

```
1 mrrfrlvgl sslvlaagaa ltgaataqaa qpaaadgyva lgdsyssgvg agsyisssgd
61 ckrstkahpy lwaaahspst fdftacsgar tgdvlsqqlg plssgtglvs isiggn dagf
121 adtmittcvlq sessclsria taeayvdstl pgkl dgyvsa isdkapnahv vvigyp rfyk
181 lgttciglse tkrtainkas dhlntvlaqr aaahgftfgd vrttftghel csgspwlhsv
241 nwl nigesyh ptaagqsggy lpv lngaa
//
```

## Figure 83

SEQ ID No. 71

NP\_827753.

```
1 mrrsritayv tslllavgca ltgaataqas paaaatgyva lgdsyssgvg agsylsssgd
61 ckrsskaypy lwqaahspss fsfmacsgar tgdvlanqlg tlnsstglvs ltiggn dagf
121 sdvmttcvlq sdsaclsrin takayvdstl pgql dsvyta istkapsahv avlgyprfyk
181 lggsclagls etkrsainda adylnsaiak raadhgftfg dvkstftghe icssstwlhs
241 ldllnigqsy hptaagqsgg ylpvmnsva
//
```

## Figure 84

SEQ ID No. 72

```
MRLTRSLAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE
SYHPTSTGHQSGYLPVLNANSST
```

## Figure 85

SEQ ID No. 73

```
ADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRFSNGP VWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWN
PKYQVINNLDYEVTQFLQKDSFKPDDLVI LWVGANDYLAYGWNT EQDAKRVRDAISDAANRMVLNGAKEILLFNLPDLGQ
NPSARSQKVVEAASHVSAYHNQLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQRNACYGGSYVWKPFASRSA
STDSQLSAFNPQERLAIAGNPLLAQAVASPMARSASTLNCEGKMFWQVHPTTVVHAALSEPAATFIESQYEF LAH
```



Figure 86

## SEQ ID No. 74

```
1  ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTQFPGLT
61  IANEAEggAT AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVL WVGANDYLAY
121 GWNTEQDAKR VRDAISDAAN RMVLNGARQI LLFNLPDLGQ NPSARSQKVV EAVSHVSAYH
181 NKLLLNLARQ LAPTMVKLF EIDKQFAEML RDPQNFGLSD VENPCYDGGY VWKPFATRSV
241 STDRLSAFS PQERLAIAGN PLLAQAVASP MARRSASPLN CEGKMFWDQV HPTTVVHAAL
301 SERAATFIET QYEFLAHG
```

Figure 87

## SEQ ID No. 75

```
ACAGGCCGATGCACGGAACCGTACCTTTCCGCAGTGAAGCGCTCTCCCCCATCGTTCGC
CGGGACTTCATCCGCGATTTTGGCATGAACACTTCCTTCAACGCGCGTAGCTTGCTACAA
GTGCGGCAGCAGACCCGCTCGTTGGAGGCTCAGTGAGATTGACCCGATCCCTGTGGCCG
CATCCGTCATCGTCTTCGCCCTGCTGCTCGCGCTGCTGGGCATCAGCCCGGCCAGGCAG
CCGGCCCGCCTATGTGGCCCTGGGGGATTCTTCTCCTCGGGCAACGGCGCCGGAAGTT
ACATCGATTGAGCGGTGACTGTACCGCAGCAACAACGCGTACCCCGCCGCTGGGCGG
CGGCCAACGCACCGTCCTCCTTACCTTCGCGGCCTGCTCGGGAGCGGTGACCACGGATG
TGATCAACAATCAGCTGGGCGCCCTCAACGCGTCCACCGGCCTGGTGAGCATCACCATCG
GCGGCAATGACGCGGGCTTCGCGGACGCGATGACCACCTGCGTCACCAGCTCGGACAGCA
CCTGCCTCAACCGGCTGGCCACCGCCACCAACTACATCAACACCACCCTGCTCGCCCGC
TCGACGCGGTCTACAGCCAGATCAAGGCCCCGTGCCCCAACGCCCCGCGTGGTCGTCCTCG
GCTACCCGCGCATGTACCTGGCCTCGAACCCCTGGTACTGCCTGGGCCTGAGCAACACCA
AGCGCGCGGCCATCAACACCACCGCCGACACCCTCAACTCGGTGATCTCCTCCCGGGCCA
CCGCCCACGGATTCCGATTGCGCGATGTCCGCCCCGACCTTCAACAACCACGAACTGTTCT
TCGGCAACGACTGGCTGCACTCACTACCCTGCCGGTGTGGGAGTCGTACCACCCACCA
GCACGGGCCATCAGAGCGGCTATCTGCCGGTCCTCAACGCCAACAGCTCGACCTGATCAA
CGCACGGCCGTGCCCGCCCCGCGGTACGCTCGGCGGGGCGCCGAGCGCGTTGATCA
GCCACAGTGCCGGTGACGGTCCCACCGTCACGGTCGAGGGTGTACGTACGGTGGCGCC
GCTCCAGAAGTGGAACGTCAGCAGGACCGTGGAGCCGTCCCTGACCTCGTCGAAGAACTC
CGGGGTCAGCGTGATCACCCCTCCCCCGTAGCCGGGGGCGAAGGCGGCGCGAACTCCTT
GTAGGACGTCCAGTCGTGCGGCCCGGCGTTGCCACCGTCCGCGTAGACCGCTTCCATGGT
CGCCAGCCGGTCCCCGCGGAACTCGGTGGGGATGTCCGTGCCCAAGGTGGTCCCGGTGGT
GTCCGAGAGCACCGGGGGCTCGTACCGGATGATGTGCAGATCCAAAGAATT
```



Figure 88

## SEQ ID No. 76

MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN  
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT  
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNFWYC  
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE  
SYHPTSTGHQSGYLPVLNANSST

Figure 89

## SEQ ID No. 77

1 mlphpagerg evgaiffallv gtpqdrllrl echetrplrg rcgcgerrvp pltlpgdgvl  
61 cttssrdae tvwrkhlqpr pdggfrphlg vgcilagqgs pgvlwcgreg crfevcrdt  
121 pglstrngd ssppfragws lppkcgeisq sarktpavpr ysllrtdrpd gprgrfvsg  
181 praatrrrlf lgipalvlvt altlvlavpt gretlwrnwc eatqdwclgv pvdsrgqpae  
241 dgeflllspv qaatwgnyya lgdsyssgdg ardyypgtav kggcwrsana ypelvaeayd  
301 faghlsflac sgqrgyamld aidevgsqld wnsphtslvt igiggndlgf stvlktcmvr  
361 vplllaskact dgedairkrm akfettfeel isevrtrapd arilvgypr ifpeeptgay  
421 yltasnqrw lnetigefnq qlaeavavhd eeiaasggvg svefvdvyha ldgheigsde  
481 pwvngvqlrd latgvtvdrs tfhpnaaghr avgervieqi etgprplya tfavvagatv  
541 dtlagevg



Figure 90

## SEQ ID No. 78

```
1 ggtggtgaac cagaacaccc ggtcgtcggc gtgggcgtcc aggtgcaggt gcaggttctt
61 caactgctcc agcaggatgc cgccgtggcc gtgcacgatg gccttgggca ggctgtggt
121 ccccgacgag tacagcaccc atagcggatg gtcgaacggc agcggggtga actccagttc
181 cgcgccttcg cccgcggctt cgaactccgc ccaggacagg gtgtcggcga cagggccgca
241 gcccaggtag ggccaggacga cgggtgtgctg caggctgggc atgccgtcgc gcagggcttt
301 gagcacgtca cggcgggtga agtccttacc gccgtagcgg tagccgtcca cggccagcag
361 cactttcggg tcgatctgcg cgaaccggtc gaggaacgtg cgcaccocga agtcggggga
421 acaggacgac caggtcgcac cgatcgcggc gcaggcgagg aatgcggccg tcgcctcggc
481 gatgttcggc aggtaggcca cgaccggtc gccggggccc accccgaggc tgcggagggc
541 cgcagcgatc gcggcgggtg gggtcgcgag ttctccocag gtccactcgg tcaacggccg
601 gagttcggac gcgtgccgga tcgccacggc tgatgggtca cggtcgcgga agatgtgctc
661 ggcgtagttg aggggtggcg cggggaacca gacggcgccg ggcatggcgt cggaggcgag
721 cactgtggtg tacggggtgg cggcgcgcac ccggtagtag tcccagatcg cggaccagaa
781 tccttcgagg tcggttaccg accagcgcca cagtgcctcg tagtcgggtg cgtccacacc
841 gcggtgctcc cgcaccacgc ggggtgaacgc ggtgaggttg gcgcgttctt tgcgctcctc
901 gtcgggactc cacaggatcg gcggctgcgg cttgagtgtc atgaaacgcg accccttcgt
961 ggacgggtgc gatgcggtga gcgtcgggtg cctcccctaa cgctcccggg tgacggagtg
1021 ttgtgcacca catctagcac gcgggacgcg gaaaccgtat ggagaaaaca cctacaaccc
1081 cggccggacg gtgggtttcg gccacactta ggggtcgggt gcctgcttgc cgggcagggc
1141 agtcccgggg tgctgtggtg cgggcgggag ggctgtcgtc tcgaggtgtg ccggcgggac
1201 actccgggcc tcagccgtac ccgcaacggg gacagttctc ctcccttcgg ggctggatgg
1261 tcccttcccc cgaaatgcgg cgagatctcc cagtcagccc ggaaaacacc cgctgtgccc
1321 aggtactctt tgcttcgaac agacaggccg gacggtccac gggggagggt ttgtgggcagc
1381 ggaccacgtg cggcgaccag acgacgggtg ttctcggta tcccgcctct tgtacttgtg
1441 acagcgctca cgctggtctt ggctgtcccg acggggcgcg agacgctgtg gcgcatgtgg
1501 tgtgaggcca cccaggactg gtgcctgggg gtgccggtcg actcccgcg acagcctgcg
1561 gaggaacggc agtttctgct gctttctccg gtccaggcag cgacctgggg gaactattac
1621 gcgctcgggg attcgtactc ttccgggggac ggggcccggc actactatcc cggcaccgcg
1681 gtgaaggggc gttgctggcg gtccgctaac gcctatccgg agctggtcgc cgaagcctac
1741 gacttcggcg gacactgtc gttcctggcc tgacggggcc agcgcggtc cgccatgctt
1801 gacgctatcg acgaggtcgg ctgcgagctg gactggaact cccctcacac gtcgctggtg
1861 acgatcggga tcggcggcaa cgatctgggg ttctccacgg ttttgaagac ctgcatgggtg
1921 cgggtgccgc tgctggacag caaggcgtgc acggaccagg aggacgctat ccgcaagcgg
1981 atggcgaaat tcgagacgac gtttgaagag ctcatcagcg aagtgcgcac ccgcgcgccg
2041 gacgcccgga tccttgtcgt gggctacccc cggatttttc cggagggaacc gaccggcgcc
2101 tactacaogc tgaccgcgag caaccagcgg tggtcaacg aaaccattca ggagttcaac
2161 cagcagctcg ccgaggctgt cgcgggtccac gacgaggaga ttgccgcgtc gggcggggtg
2221 ggcagcgtgg agttcgtgga cgtctaccac gcgttgagcg gccacgagat cggctcggac
2281 gagccgtggg tgaacggggt gcagttgcgg gacctcgcca ccggggtgac tgtggaccgc
2341 agtaccttcc accccaacgc cgctgggcac cgggcggtcg gtgagcgggt catcgagcag
2401 atcgaaaccg gcccgggccg tccgtctctat gccactttcg cgggtggtggc gggggcgacc
2461 gtggacactc tcgcgggcga ggtgggggtga cccggcttac cgtecgggcc gcaggtctgc
2521 gagcactgcg gcgatctggt ccaactgcca gtgcagttcg tcttcgggtg tgaccagcgg
2581 cggggagagc cggatcgttg agccgtgcgt gtctttgacg agcacacccc gctgcaggag
2641 ccgttcgcac agttctcttc cgggtggccag agtcgggtcg acgtcgatcc cagcccacag
2701 gccgatgctg cgggcgcgca ccacgccgtt gccgaccagt tggtcgaggc gggcgcgcag
2761 cacggggggc agggcgcgga catgggtccag gtaagggccg tcgcggacga ggctcaccac
2821 ggcagtgcgg accgcgcagg cgaggcggtt gccgccgaag gtgctgccgt gctggccggg
2881 gcggatcacg tcgaagactt ccgcgtcgcc taccgccgcc gccacgggca ggatgcggcc
2941 gcccagcgct ttgccgaaca ggtagatatc ggcgtcgact ccgctgtggt cgcaggcccc
```



Figure 91

SEQ ID No. 79

```
1  vsgsptraatr  rrlflgipal  vlvtaittlvtl  avptgretlw  rmwceatqdw  clgvvpvdsrg
61  qpaedgefll  lspvqaatwg  nyyalgdsys  sgdgardyyp  gtavkggcwr  sanaypelva
121 eaydfaghl  flacsgqrgy  amldaidevg  sqldwnspht  slvtigiggn  dlqfstvlkt
181 cmvrvpllds  kaetdqedai  rkrmakfett  feelisevrt  rapdarilvv  gypriapeep
241 tgayytltas  nqrwlneiti  efnqqlaeav  avhdeeiaas  ggvgsefvvd  vyhaldghei
301 gsdepwngv  qlrdlatgvt  vdrstfhpna  aghravger  ieqietgpgr  plyatfavva
361 gatvdtlage  vg
```

Figure 92

SEQ ID No. 80

```
1  mrttviasa  llllagcadg  areetaggp  gessggiree  gaeastsitd  vyialgdsya
61  amggrdgplr  gepfclrssg  nypellhaev  tdltcggavt  gdllleprtlg  ertlpaqvda
121 ltedttlvtl  siggndlgfg  evagcireri  agenaddcvt  llgetigeql  dqlppqldrv
181 heairdragd  aqvvtgylp  lvsagdcpl  gdvseadrrw  aveltgqine  tvreaaerhd
241 alfvlpddad  ehtscappq  rwadiqqqt  dayplhptsa  gheamaaavr  dalglepvqp
```



Figure 93

SEQ ID No. 81

```
1  ttctggggtg ttatggggtt gttatcggct cgtcctgggt ggatcccgcc aggtggggtg
61  ttcacggggg acttttgtgt ccaacagccg agaatgagtg ccctgagcgg tgggaatgag
121 gtgggcgggg ctgtgtcgcc atgagggggc ggcgggctct gtggtgccc gcgaccccgc
181 gccccggtga gcggtgaatg aaatccggct gtaatcagca tcccgtgcc acccgtcgg
241 ggaggtcagc gcccgagtg tctacgcagt cggatcctct cggactcggc catgctgtcg
301 gcagcatcgc gctcccgggt cttggcgctc ctcggtgtgt ctgcctgtg tccctggaag
361 gcgaaatgat caccggggag tgatacaccg gtggtctcat ccggatgcc cacttcggcg
421 ccatccggca attcgggcag ctccgggtgg aagtaggtgg catccgatgc gtcggtgacg
481 ccatagtggg cgaagatctc atcctgtctg aggtgtctca ggccactctc cggatcgata
541 tcgggggctg ccttgatggc gtccttgctg aaaccgaggt gcagcttggt ggcttccaat
601 ttcgcaccac ggagcgggac gaggctggaa tgacggccga agagcccgtg gtggacctca
661 acgaaggtgg gtagtcccgt gtcattctg aggaacacgc cctccaccgc acccagcttg
721 tggccggagt tgcgtaggc gctggcatcc agaagggaaa cgtctcata tttgtcgggtg
781 tgctcagaca tgatcttctc ttgctgtcgg tgtctggtac taccacggta gggctgaatg
841 caactgttat tttctgttta ttttaggaat tgggtccatat cccacaggct ggctgtggtc
901 aaatcgtcat caagtaatcc ctgtcacaca aaatgggtgg tgggagccct ggtcgcgggtt
961 ccgtgggagg cgcctgccc cgcaggatcg tcggcatcgg cggatctggc cggtaaccgc
1021 cgggtgaataa aatcattctg taaccttcat cacggttggg tttaggtatc cgccttctc
1081 gtcctgaccc cgtcccgcgc gcgggggagc ccgcggttg cggtagacag gggagacgtg
1141 gacaccatga ggacaacggt catcgcagca agcgcattac tcttctcgc cggatgcgcg
1201 gatggggccc gggaggagac cgcgggtgca ccgcggttg agtctccgg gggcatccgg
1261 gaggaggggg cggaggcgtc gacaagcatc accgacgtct acatcgccct cggggattcc
1321 tatgcggcga tggcgggcg ggatcagccg ttacgggggt agccgttctg cctgcgtcg
1381 tccggttaatt acccggaact cctccacgca gaggtcacog atctcacctg ccagggggcg
1441 gtgaccgggg atctgtcga acccaggacg ctgggggagc gcacgctgcc ggcgcagggtg
1501 gatgcgtga cggaggacac caccctggtc accctctcca tcgggggcaa tgacctcgga
1561 ttcggggagg tggcgggatg catccgggaa cggatcgccg gggagaacgc tgatgattgc
1621 gtggacctgc tgggggaaac catcggggag cagctcgatc agcttcccc gcagctggac
1681 cgcgtgcacg aggtatccg ggaccgcgc ggggacgcgc aggttgtggt caccggttac
1741 ctgcccgtcg tgtctgccgg ggaactgccc gaactgggg atgtctccga ggcggtcgt
1801 cggtgggagg ttgagctgac cgggcagatc aacgagaccg tgcgcgaggc ggccgaacga
1861 cacgatgccc tctttgtcct gccgacgat gccgatgagc acaccagttg tgcacccca
1921 cagcagcgct gggcgatat ccagggccaa cagaccgatg cctatccgct gcacccgacc
1981 tccgcccggc atgaggcgat ggccgcccgc gtcggggacg cgtgggcctt ggaaccggtc
2041 cagccgtagc gccgggcgcg cgttgtcga cgaccaacc atgccaggct gcagtcacat
2101 ccgcacatag cgcgcgcggg cgatggagta cgcaccatag aggatgagcc cgtgcccagc
2161 gatgatgagc agcacactgc cgaagggttg ttcccagagg gtgcgcagag ccgagtccag
2221 acctgcggcc tgcctcggat catgggccc accggcgatg acgatcaaca ccccaggat
2281 cccgaaggcg ataccacggg cgacataacc ggctgttcg gtgatgatga tcgcggtccc
2341 gacctgccct gacccgcac ccgcctccag atcctcccgc aaatcccggg tggccccctt
2401 ccagagggtt tagacacccg ccccagtac caccagccc gcgaccacaa ccagcaccac
2461 accccagggt tgggatagga cgggtggcgt gacatcggtg gcggtctccc catcgagggt
2521 gctgccgcc cgggcgaagg tggaggtggt caccgccagg gagaagtaga ccatggccat
2581 gaccgcccc ttggcccttt ccttgaggtc ctgcccgc agcagctggc tcaattgcca
2641 gagtcccagg gccgccagg cgatgacggc aaccacagg aggaactgcc caccgggagc
2701 ctccgcgatg gtggccagg cacctgaatt cgaggcctca tccccgaac cgcggatcc
2761 agtggcgatg cgcaccgca tccaccgat gaggatgtgc agtatgccc ggacaatgaa
2821 accacctctg gccagggtg tcagcgcggt gtggtcctcg gcctggtcgg cagccggtc
2881 gatcgtccgt ttcgcggatc tgggtgtgcc cttatccata gctccattg aaccgccttg
2941 aggggtgggc ggccactgtc agggcggtt gtgatctgaa ctgtgatgtt ccatcaaccc
```



Figure 94

## SEQ ID No. 82

```
1 mrrfrlvglf sslvlaagaa ltgaatagaa qpaaadgyva lgdsyssgvv agsyisssgd
61 ckrstkahpy lwaaahspst fdftacsgar tgdvlsqqlg plssgtglvs isiggnadagf
121 adtmmtcvlq sessclsria taeayvdstl pgkldgvysa isdkapnahv vvigypfrfyk
181 lgttciglse tkrtainkas dhlntvlaqr aaahqftfgd vrttftghel csgspwlhsv
241 nwl nigesyh ptaagqsggy lpvlnгаа
```

Figure 95

## SEQ ID No. 83

```
1 cccggcgggc cgtgcaggag cagcagccgg cccgcgatgt cctcggggcgt cgtcttcac
61 aggcggtcca tcgcgtcggc gaccggcgcc gtgtagttgg .cccggacctc gtcccagggtg
121 cccgcggcga tctggcgggt ggtgcggtgc gggccgcgcc gaggggagac gtaccagaag
181 cccatcgtca cgttctccgg ctgcgggttcg ggctcgtccg ccgctccgtc cgtcgcctcg
241 ccgagcacct tctcggcgag gtcggcgctg gtcgcgctca ccgtgacgtc ggcgccccgg
301 ctccagcgcg agatcagcag cgtccagccg tcgccctccg ccagcgtcgc gctgcgggtcg
361 tcgtcgcggg cgatccgcag cacgcgcgcg ccggggcgga gcagcgtggc gccggaccgt
421 acgcgggtcga tgttcgccgc gtgcgagtac ggctgctcac ccgtggcgaa acggccgagg
481 aacagcgcggt cgacgacgtc ggacggggag tcgctgtcgt ccacgttgag ccggatcggc
541 agggcttcgt gcgggttcac ggacatgtcg ccatgatcgg gcacccggcc gccgcgtgca
601 cccgctttcc cgggcacgca cgacaggggc tttctcgcgg tcttcgtcc gaacttgaac
661 gagtgtcagc catttcttgg atggacact tccagtcaac gcgcgtagct gctaccacgg
721 ttgtggcagc aatcctgcta agggagcttc catgagacgt ttccgacttg tgggttcct
781 gagttcgctc gtccctcgcg ccggcgccgc cctcaccggg gcagcgaccg cccaggcggc
841 ccaaccgcgc gccgcgacg gctatgtggc cctcggcgac tccactcct cccgggtcgg
901 agcgggcagc tacatcagct cgagcggcga ctgcaagcgc agcacgaagg cccatcccta
961 cctgtggggc gccgcccact cgcctccac gttcgacttc accgcctgtt ccggcgcccg
1021 tacgggtgat gttctctccg gacagctcgg cccgctcagc tccggcaccg gcctcgtctc
1081 gatcagcatc ggcggaacg acgcgggttt cgccgacacc atgacgacct gtgtgctcca
1141 gtccgagagc tcctgcctgt cgcggatcgc caccgcccag gcgtacgtcg actcgacgct
1201 gcccggaag ctcgacggcg tctactcggc aatcagcgac aaggcgccga acgcccacgt
1261 cgtcgtcatc ggctaccgcg gcttctacaa gctcggcacc acctgcatcg gcctgtccga
1321 gaccaagcgg acggcgatca acaaggcctc cgaccacctc aacaccgtcc tcgcccagcg
1381 cgccgcggcc cacggcttca ccttcggcga cgtacgcacc accttcaccg gccacgagct
1441 gtgctccggc agcccctggc tgcacagcgt caactggctg aacatcggcg agtcgtacca
1501 cccacccgcg gccggccagt ccggtggcta cctgcgggtc ctcaacggcg ccgcctgacc
1561 tcaggcggaag ggagaagaag aaggagcgga gggagacgag gagtgggagg ccccgcccga
1621 cggggtcccc gtcccgtct ccgtctccgt cccggtcccg caagtcaccg agaacgccac
1681 cgcgtcggac gtggcccga ccggactccg caccctccag cgcacggcac tctcgaacgc
1741 gccggtgtcg tcgtgcgtcg tcaccaccac gccgtcctgg cgcgagcgct cgcgcgccga
1801 cgggaaggac agcgtccgcc accccggatc ggagaccgac ccgtcccgcg tcacccacgg
1861 gtagccgacc tccgcgggca gccgcccga cgtgaacgtc gccgtgaacg cgggtgcccg
1921 gtcgtcggc ggcgacagg ccccgagta gtgggtgcgc gagcccacca cggtcacctc
1981 caccgactgc gctgcggggc
```



Figure 96

## SEQ ID No. 84

```
1 mrrsritayv tslllavgca ltgaataqas paaaatgyva lgdsyssgv agsylsssgd
61 ckrsskaypy lwqaahspss fsfmacsgar tgdvlanglg tlnsstglvs ltiggndagf
121 sdvmttcvlq sdsaclsrin takayvdstl pgqldsvyta istkapsahv avlgyprfyk
181 lggsclagls etkrsainda adylnsaiak raadhgftfg dvkstftghe icssstwlhs
241 ldllnigqsy hptaagqsgg ylpvmnsva
```

Figure 97

## SEQ ID No. 85

```
1 ccacgcgcgg gtcggcgggc agtctcctgg cctcggtcgc ggagaggttg gccgtgtagc
61 cggtcagcgc ggcgcggaac gtcttcttca ccgtgcgcgc gtactcgttg atcaggccct
121 tgcccttgct cgacgcggcc ttgaagccgg tgcccttctt gagcgtgacg atgtagctgc
181 ccttgatcgc ggtgggggag ccggcggcga gcaccgtgcc ctggcgcggg gtggcctggg
241 cgggcagtg cgtgaatccg cccacgaggg cgccggtgcg cacggcggtt atcgcggcga
301 tccggtatct cttgctacgc agctgtgcca tacgagggag tctcctctg ggcagcggcg
361 cgccctgggt gggcgcacgg ctgtgggggg tgcgcgcgtc atcacgcaca cggccctgga
421 gcgtcgtgtt ccgccctggg ttgagtaaag cctcgcccat ctacgggggt ggctcaaggg
481 agttgagacc ctgtcatgag tctgacatga gcacgcaatc aacggggcgg tgagcaccoc
541 ggggcgaccc cgaaagtgc cgagaagtct tggcatggac acttctctgc aacacgcgta
601 gctggtacga cggttacggc agagatcctg ctaaaggag gttccatgag acgttccga
661 attacggcat acgtgacctc actcctctc gccgtcggct ggcctctcac cggggcagcg
721 acggcgaggg cgtccccagc cgcgcgggcc acgggctatg tggccctcgg cgactcgtac
781 tcgtccgggt tcggcgccgg cagctacctc agctccagcg gcgactgcaa gcgcagttcg
841 aaggcctatc cgtacctctg gcaggccggc cattcaccct cgtcgttcag tttcatggct
901 tgctcggggc ctcgtacggg tgatgtctct gccaatcagc tcggcacccct gaactcgtcc
961 accggcctgg tctccctcac catcggaggg aacgacgcgg gcttctccga cgtcatgacg
1021 acctgtgtgc tccagtccga cagcgcctgc ctctcccgca tcaacacggc gaaggcgtac
1081 gtgcactcca ccctgcccg ccaactcgac agcgtgtaca cggcgatcag cacgaaggcc
1141 ccgtcggccc atgtggccgt gctgggctac ccccgcttct acaaactggg cggctcctgc
1201 ctcgcggggc tctcggagac caagcggtec gccatcaacg acgcggccga ctatctgaac
1261 agcgccatcg ccaagcgcg cgcggaccac ggcttcacct tcggcgacgt caagagcacc
1321 ttcacggccc atgagatctg ctccagcagc acctggctgc acagtctcga cctgctgaac
1381 atcgccaggt cctaccaccc gacgcgggcc ggccagtcgg gcggctatct gccggtcatg
1441 aacagcgtgg cctgagctcc caccggcctga atttttaagg cctgaatttt taaggcgaag
1501 gtgaaccgga agcggaggcc ccgtccgtcg gggctctccg cgcacaggtc accgagaacg
1561 gcacggagtt ggacgtcgtg cgcaccgggt cgcgcacctc gacggcgatc tcgttcgaga
1621 tcgttcggct cgtgtcgtac gtggtgacga acacctgctt ctgctgggtc tttccgcgcg
1681 tcgcccggaa ggacagcgtc ttccagcccc gatccgggac ctcgcccttc ttggtcacc
1741 agcgggtact cacctcgacc ggcacccggc ccaccgtgaa ggtcgccgtg aacgtgggcg
1801 cctggggcgt gggcgggcgg caggcaccgg agtagtcggg gtgcacggcg gtgaccgtca
1861 ccttcacgga ctgggcgggc ggggtcgtcg taccggccgc gccacggcg cctcccgag
1921 tggagcccga gctgtggtcg ccccgccgt cggcggtgtc gtcctcgggg gttttcgaac
```



Figure 98

SEQ ID No. 86

```
1 mgsqpraatr rrlflgipal vlvtaatlvl avptgretlw rmwceatqdw clgvvpdsrg
61 qpaedgefll lspvqaatwg nyyalgdsys sgdgadyyp gtavkggcwr sanaypelva
121 eaydfaghl flacsggrgy amldaidevg sqldwnspht slvtigiggn dlqfstvlkt
181 cmvrvpllds kactdgedai rkrmakfett feelisevrt rapdarilvv gyprifpeep
241 tgayytlts nqrwlnetiq efnqqlaeav avhdeeiaas ggvgsefvvd vyhaldghei
301 gsdepwvngv qlrdlatgvt vdrstfhpna aghravgerv ieqietgpgr plyatfavva
361 gatvdtlage vg
```

Figure 99

SEQ ID No. 87

```
1 ctgcagacac ccgccccgcc ttctcccgga tcgtcatgtt cggcgactcc ctcagcgaca
61 ccggcaagat gtactccaag atgcgcggct acctgccgtc ctccccgccg tactacgagg
121 gccgcttctc gaacggcccc gtctggctgg agcagctgac gaagcagttc cccggcctga
181 cgatcgccaa cgaggccgag gggggcgcgga ccgcagtcgc ctacaacaag atctcctgga
241 acccgaagta ccaggtcatt aacaacctcg actacgaggt caccagttc ttgcagaagg
301 actcgttcaa gcccgacgac ctggtcatcc tgtgggtggg cgccaacgac tacctggcct
361 acggttgga cagggagcag gacgccaagc ggggtgcgga cgccatctcg gacgcggcaa
421 accgcatggt cctgaacggc gcgaagcaga tcctgctgtt caacctgccg gacctgggcc
481 agaaccgctc cgcccgtccc cagaaggctc tcgaggccgt ctgcacgtg tccgcctacc
541 acaacaagct gctcctcaac ctgcgccggc agctcgcccc gacgggcatg gtcaagctgt
601 tcgagatcga caagcagttc gcggagatgc tgcgcgaccc ccagaacttc ggcctgagcg
661 acgtggagaa cccgtgctac gacggcggct acgtgtggaa gccgttcgcc acccggtccg
721 tctcgaccga ccggcagctg tcggccttct cgccccagga gcgcctggcg atcgctggca
781 acccgctcct ggcacaggcg gtagcttcgc cgatggcccc ccgctcggcc tcgcccctca
841 actgcgaggg caagatgttc tgggaccagg tccacccac caccgtggtc cagccgccc
901 tctcgagcgc cgccgccacc ttcacgcaga ccagtacga gttcctcgcc cactagtcta
961 gaggatcc
```



Figure 100

SEQ ID No. 88

```
1  ATGAAACAAC AAAAACGGCT TTACGCCCCG TTGCTGACGC TGTTATTTGC
   TACTTTGTTG TTTTGGCCGA AATGCGGGCT AACGACTGCG ACAATAAACG

51  GCTCATCTTC TTGCTGCCTC ATTCTGCAGC TTCAGCAGCA GATACAAGAC
   CGAGTAGAAG AACGACGGAG TAAGACGTCG AAGTCGTCGT CTATGTTCTG

101 CGGCGTTTAG CCGGATCGTC ATGTTTGGAG ATAGCCTGAG CGATACGGGC
   GCGGCAAATC GGCCTAGCAG TACAAACCTC TATCGGACTC GCTATGCCCCG

151 AAAATGTATA GCAAATGAG AGGCTATCTT CCGTCAAGCC CGCCGTATTA
   TTTTACATAT CGTTTTACTC TCCGATAGAA GGCAGTTCGG GCGGCATAAT

201 TGAAGGCCCG TTTAGCAATG GACCGGTCTG GCTGGAACAA CTGACGAAAC
   ACTTCCGGCG AAATCGTTAC CTGGCCAGAC CGACCTTGTT GACTGCTTTG

251 AATTTCCGGG ACTGACGATC GCTAATGAAG CAGAAGGAGG AGCAACAGCG
   TTAAAGGCCG TGAAGCTAG CGATTACTTC GTCTTCCTCC TCGTTGTCGC

301 GTCGCCTATA ACAAATCAG CTGGGACCCG AAATATCAGG TCATCAACAA
   CAGCGGATAT TGTTTTAGTC GACCCTGGGC TTTATAGTCC AGTAGTTGTT

351 CCTGGACTAT GAAGTCACAC AGTTTCTTCA GAAAGACAGC TTAAACCCGG
   GGACCTGATA CTTCAGTGTG TCAAAGAAGT CTTTCTGTCT AAATTTGGCC

401 ATGATCTGGT CATCCTTTGG GTCGGCGCCA ATGATTATCT GCGGTATGGC
   TACTAGACCA GTAGGAAACC CAGCCGCGGT TACTAATAGA CCGCATACCG

451 TGGAACACAG AACAAAGATG CAAAAGAGTC AGAGATGCCA TCAGCGATGC
   ACCTTGTGTC TTGTTCTACG GTTTTCTCAG TCTCTACGGT AGTCGCTACG

501 CGCTAATAGA ATGGTCTCTG ACGGCGCCAA ACAAATCCTG CTGTTTAACC
   GCGATTATCT TACCAGGACT TGCCGCGGTT TGTTTAGGAC GACAAATTGG

551 TGCCGGATCT GGGACAAAAT CCGAGCGCCA GAAGCCAAAA AGTCGTCGAA
   ACGGCCTAGA CCCTGTTTTA GGCTCGCGGT CTTGCGTTTT TCAGCAGCTT

601 GCAGTCAGCC ATGTCAGCGC CTATCATAAC AAAGTCTGTC TGAACCTGGC
   CGTCAGTCGG TACAGTCGCG GATAGTATTG TTTGACGACG ACTTGGACCG

651 AAGACAATTG GCACCGACGG GAATGGTTAA ATTGTTTGAA ATTGACAAAC
   TTCTGTTAAC CGTGGCTGCC CTTACCAATT TAACAACTT TAACTGTTTG

701 AGTTTGCCGA AATGCTGAGA GATCCGCAA ATTTTGGCCT GAGCGATGTC
   TCAAACGGCT TTACGACTCT CTAGGCGTTT TAAACCGGA CTCGCTACAG

751 GAAAACCCGT GCTATGATGG CGGATATGTC TGGAACCCGT TTGCCACAAG
   CTTTTGGGCA CGATACTACC GCCTATACAG ACCTTTGGCA AACGGTGTTT

801 AAGCGTCAGC ACGGATAGAC AACTGTCAGC GTTTAGCCCG CAAGAAAGAC
   TTCGCAGTCG TGCTTATCTG TTGACAGTCG CAAATCGGGC GTTCTTTCTG

851 TGGCAATCGC CGGAAATCCG CTTTTGGCAC AAGCAGTTGC TTCACCGATG
   ACCGTTAGCG GCCTTTAGGC GAAAACCGTG TTCGTCAACG AAGTGGCTAC

901 GCAAGAAGAT CAGCAAGCCC GCTGAATTGC GAAGGCAAAA TGTTTTGGGA
   CGTTCTTCTA GTCGTTCCGG CGACTTAACG CTTCCGTTTT ACAAACCCCT

951 TCAGGTCCAT CCGACAACAG TTGTCCATGC TGCCCTTTCA GAAAGAGCGG
   AGTCCAGGTA GGCTGTTGTC AACAGGTACG ACGGGAAAGT CTTTCTCGCC

1001 CGACGTTTAT CGAAACACAG TATGAATTC TGGCCCATGG CTGA
   GCTGCAAATA GCTTTGTGTC ATACTTAAAG ACCGGGTACC GACT
```



## Figure 101

## SEQ ID No. 89

MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN  
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT  
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC  
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE  
SYHPTSTGHQSGYLPVLNANSST

## Figure 102

## SEQ ID No. 90

1 ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTQOFFPGLT  
61 IANEAEGGAT AVAYNKISWD PKYQVINNLD YEVTQFLQKD SFKPDDLVL WVGANDYLAY  
121 GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLENLPDLGO NPSARSQKV EAVSHVSAYH  
181 NKLLLNLARQ LAPTMVKLF EIDKQFAEML RDPONFGLSD VENPCYDGGY VWKPFRSASP  
241 LNCEGKMFWD QVHPTTVVHA ALSERAATFI ETQYEFLAHG



Figure 103

1. L131
2. S. avermitilis
3. T. fusca
4. Consensus

```

      1                                     50
1  (1) -----MRLTRSLAASVIVFALLLALLGISPAQAAG-----
2  (1) -----MRRSRITAYVTSLLLAVGCALTGAATAQASPA-----
3  (1) VSGGPRAATRRLFLGIPALVLVTALTVLAVPTGRET LWRMWCEATQDW
4  (1)          MRRSRFLA  ALILLTLA  AL  GAA  ARAAP

      51                                     100
1  (32) -----P-AYVALGDSYSSGNGAGSYID
2  (33) -----AAATGYVALGDSYSSGVGAGSYLS
3  (51) CLGVPVDSRGQPAEDGEFLLLSPVQAATWGNYYALGDSYSSGDGARDYYP
4  (51)          A A  YVALGDSYSSG  GAGSY

      101                                    150
1  (53) SSGD---CHRSNNAYPARWAAANAP---SSFTFAACSGAVTTDVIN----
2  (57) SSGD---CKRSSKAYPYLWQAAHSP---SSFSEMACSGARTGDVLA----
3  (101) GTAVKGGCWRSANAYPELVAEAYDFA--GHLSFLACSGQRGYAMLDAIDE
4  (101) SSGD  C RSTKAYPALWAAHA      SSFSF  ACGARTYDVLA

      151                                    200
1  (93) --NQLGALNAST--GLVSITIGGNDAGFADAMTTCVTS-----SDSTCL
2  (97) --NQLGTLNSST--GLVSLTIGGNDAGFSDVMTTCVLQ-----SDSACL
3  (149) VGSQLDWNSPHT--SLVTIGTIGGNDLGFEFSTVLKTCMVR-----VPLLDS
4  (151)  QL  LNS T   LVSITTIGGNDAGFAD MTTCVL      SDSACL

      201                                    250
1  (133) NRLATATNYINTTLA-----RLDAVYSQIKARAPNARVVVLGYPRMY
2  (137) SRINTAKAYVDSTLPG-----QLDSVYTAISTKAPSAHVAVLGYPRFY
3  (191) KACTDQEDAIRKMAKF-----ETTFEELISEVRTRAPDARILVVGYPRIE
4  (201)  RIA AK YI  TLPA      RLDSVYSAI  TRAP  ARVVVLGYPRIY

      251                                    300
1  (176) LASNPWYCLGLSNTKRAAINTTADTLNSVISSRATAH-----GF
2  (180) KLGG-SCLAGLSETKRSAINDAADYLNSAIAKRAADH-----GF
3  (237) PEEPTGAYYTLTASNQRWLNETIQEFNQQLAEAVAVHDEEIAASGGVGSV
4  (251)  SG      LGLS  TKRAAINDAAD  LNSVIAKRAADH      GF

      301                                    350
1  (215) RFGDVRPTFNNHELFFGNDWLHSLTLP-----VWESYH
2  (218) TFGDVKSTFTGHEICSSSTWLHSLDLLN-----IGQSYH
3  (287) EFVDVYHALDGHEIGSDEPWVNGVQLRDLATG-----VTVDRSTEH
4  (301) TFGDV  TF  GHLCSA  PWLHSLTLP          V  SYH

      351                                    395
1  (248) PTSTGHQSGYLPVLNANSST-----
2  (252) PTAAGQSGGYLPVMNSVA-----
3  (328) PNAAGHRAVGERVIEQIETGPGRPLYATFAVVAGATVDTLAGEVG
4  (351) PTA  GHAAGYLPVLNSI  T

```



Figure 104

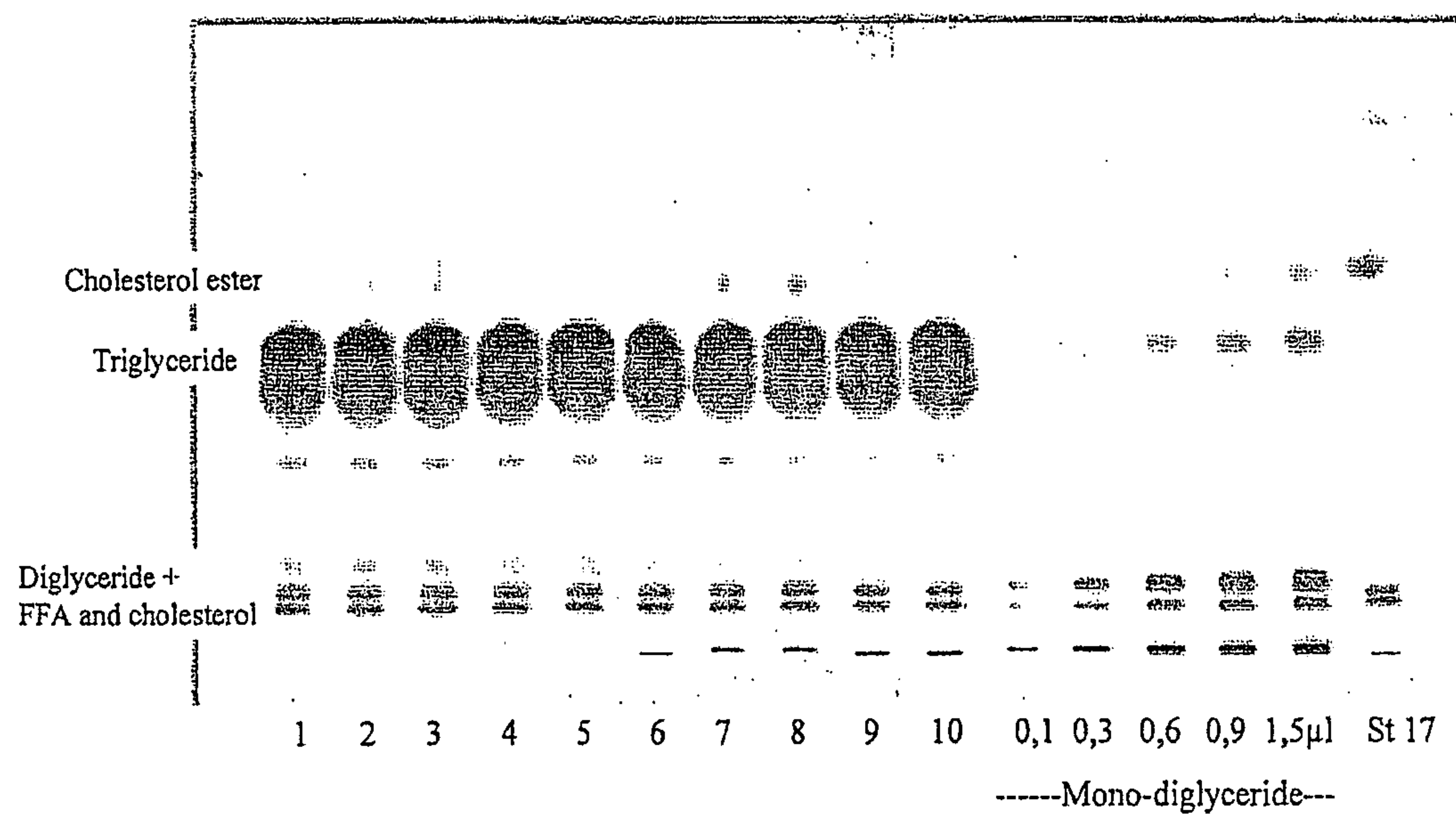


Figure 105

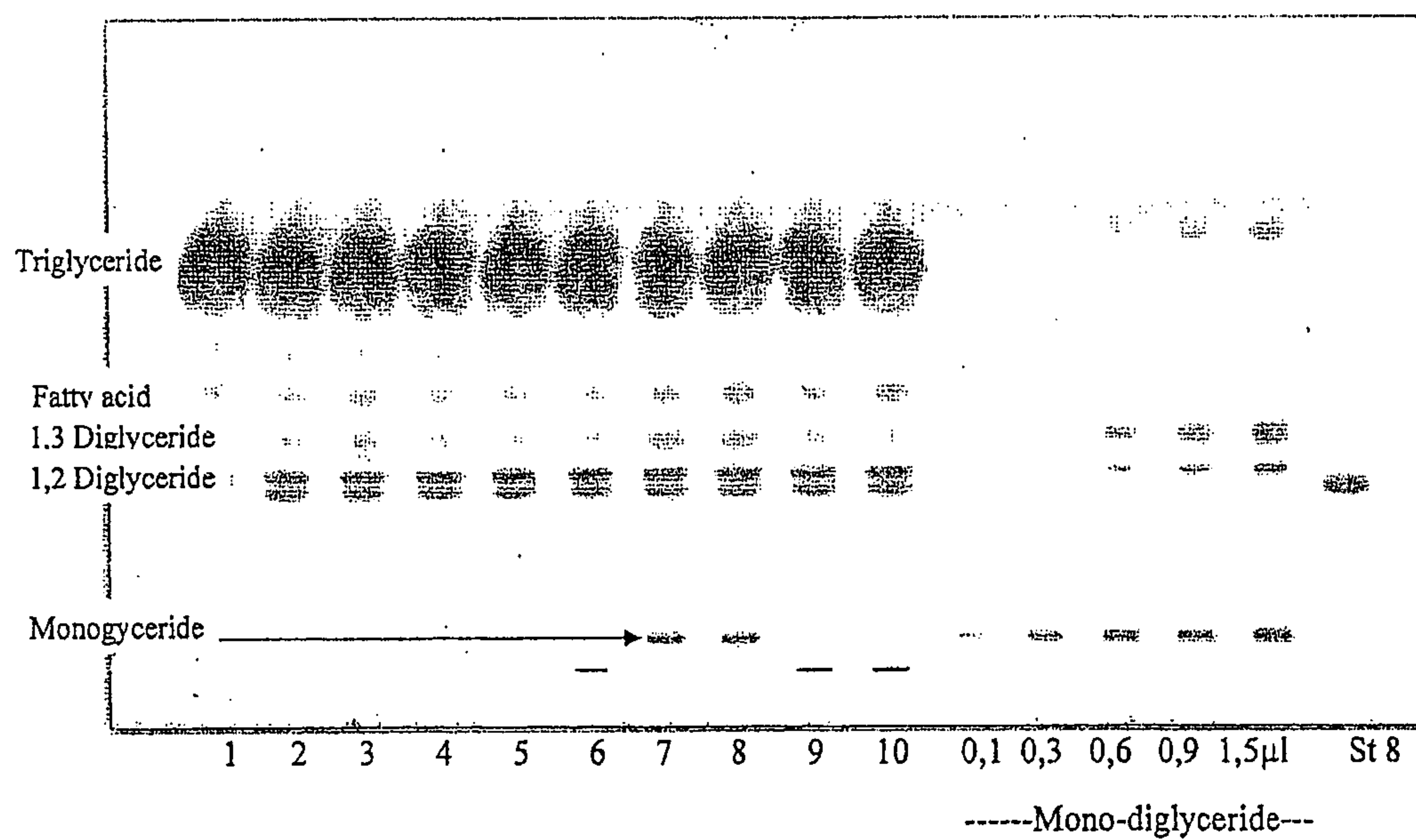




Figure 106

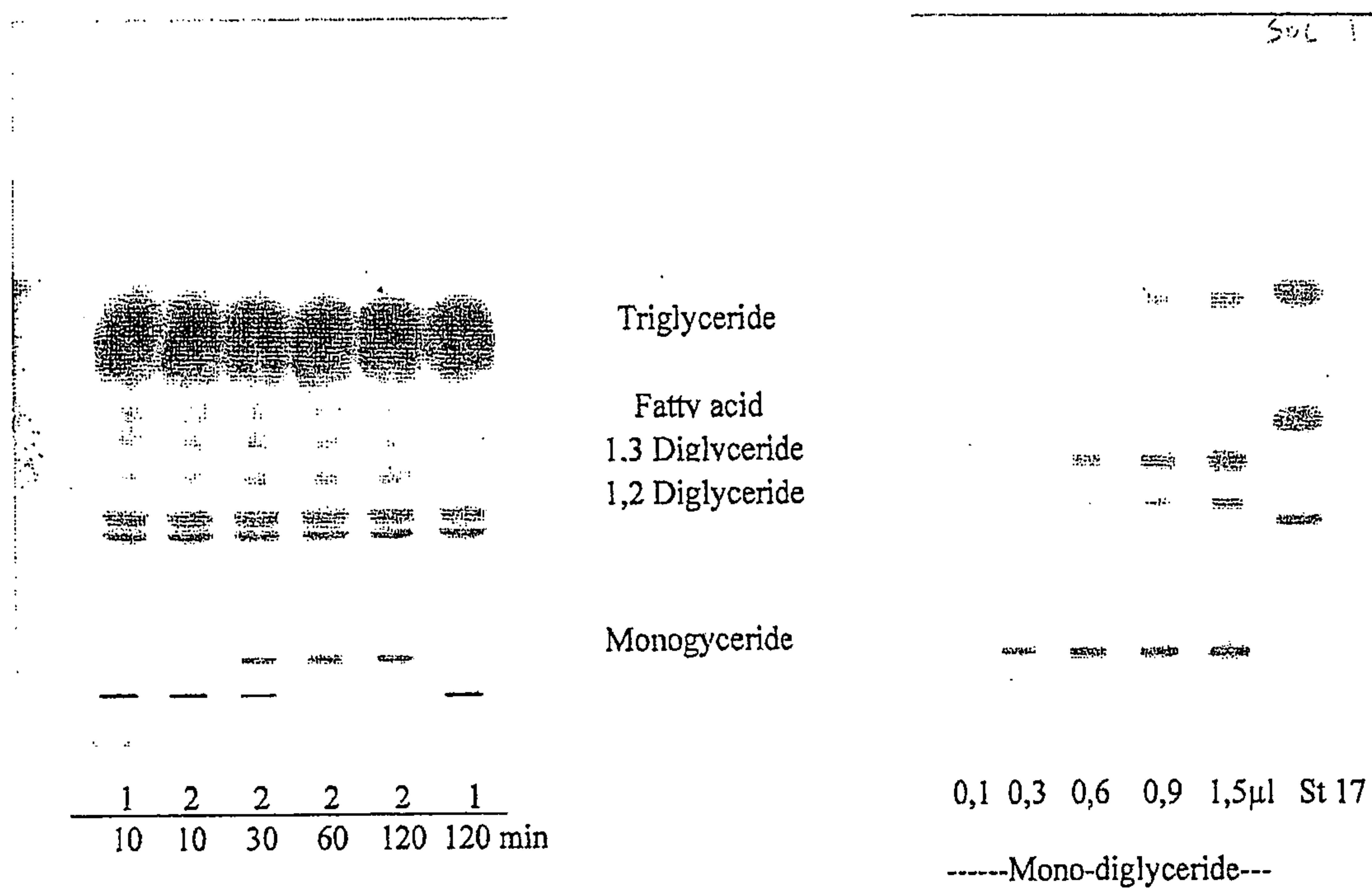


Figure 107

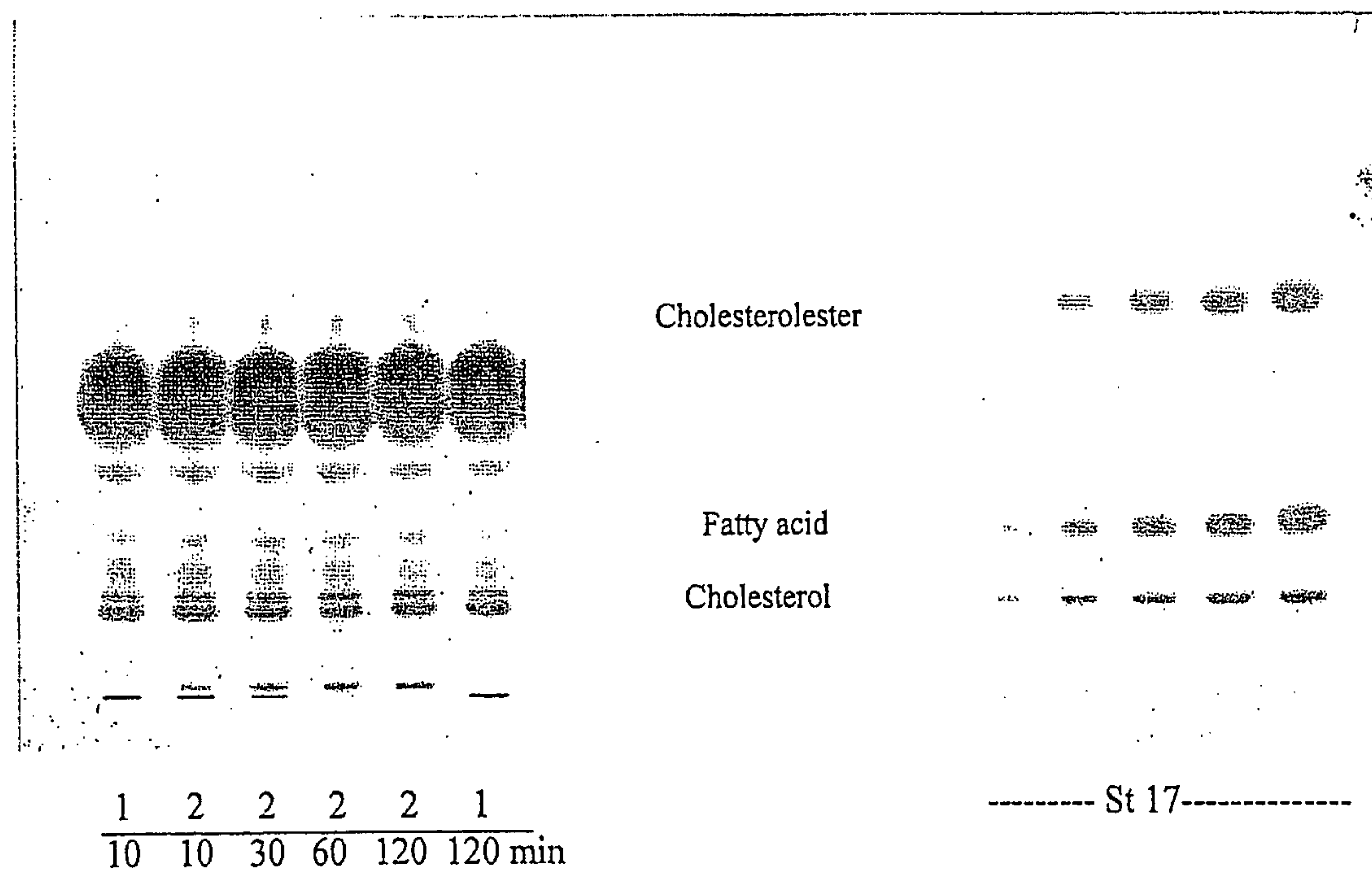




Figure 108

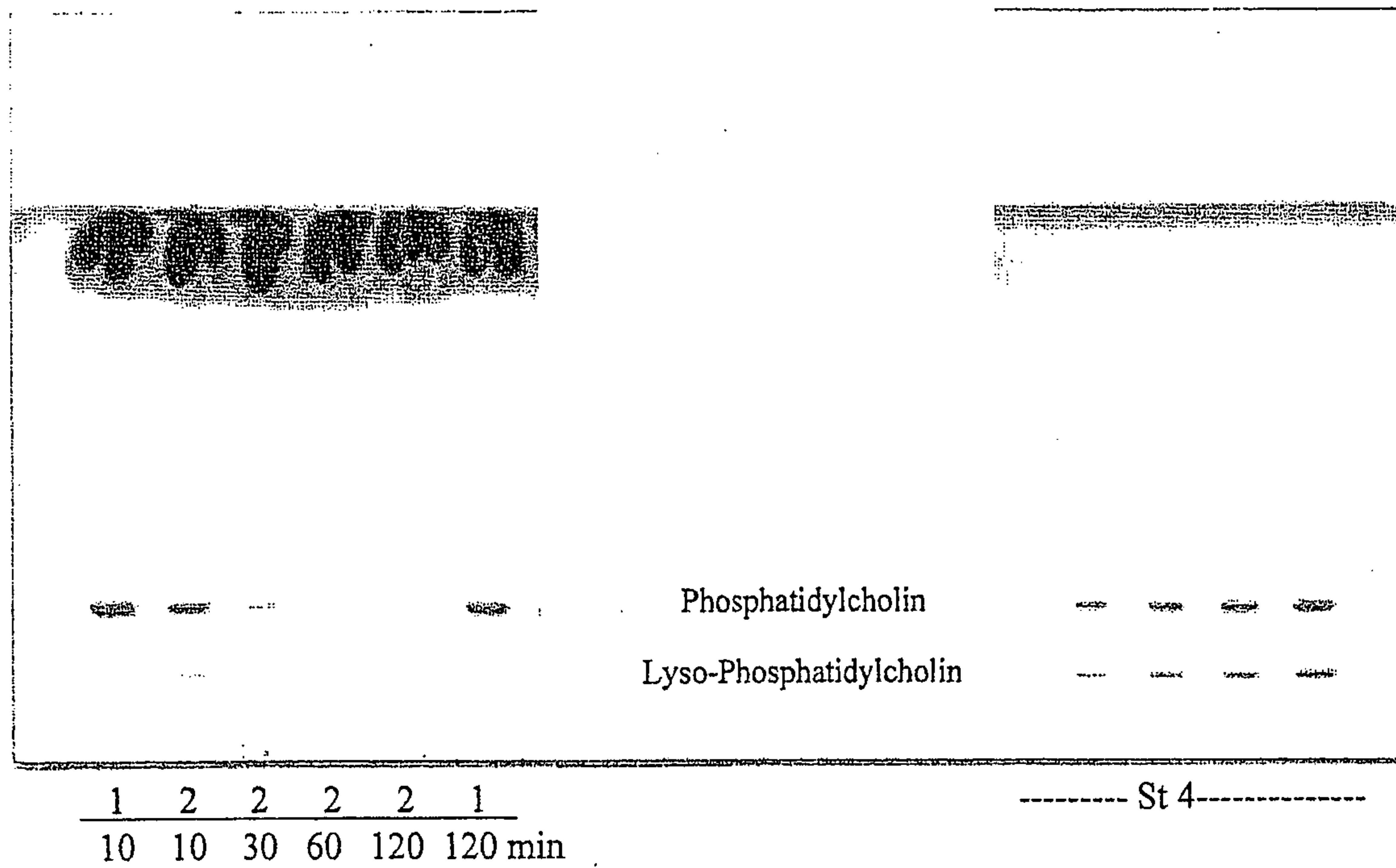




Figure 109

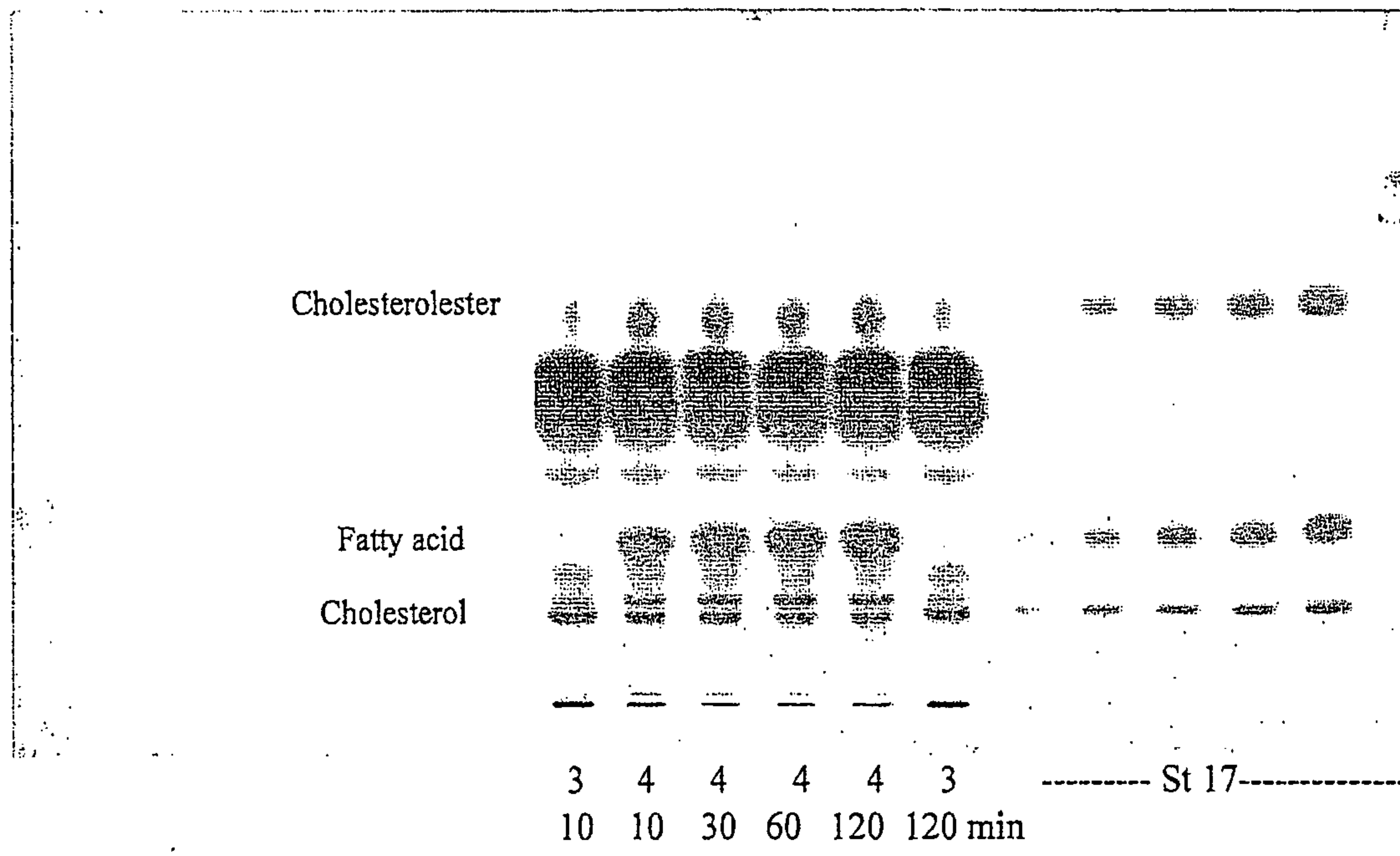




Figure 110

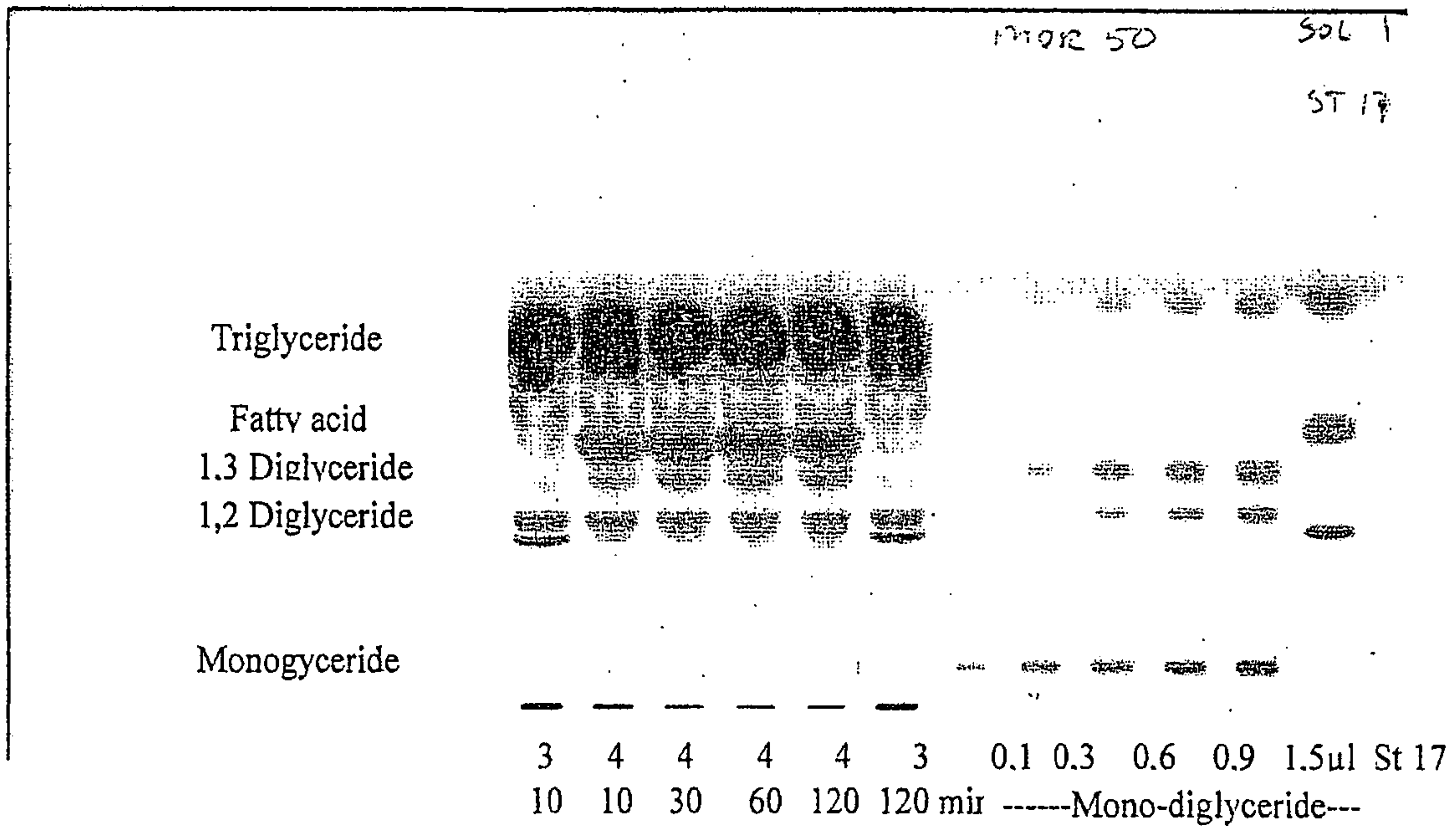


Figure 111

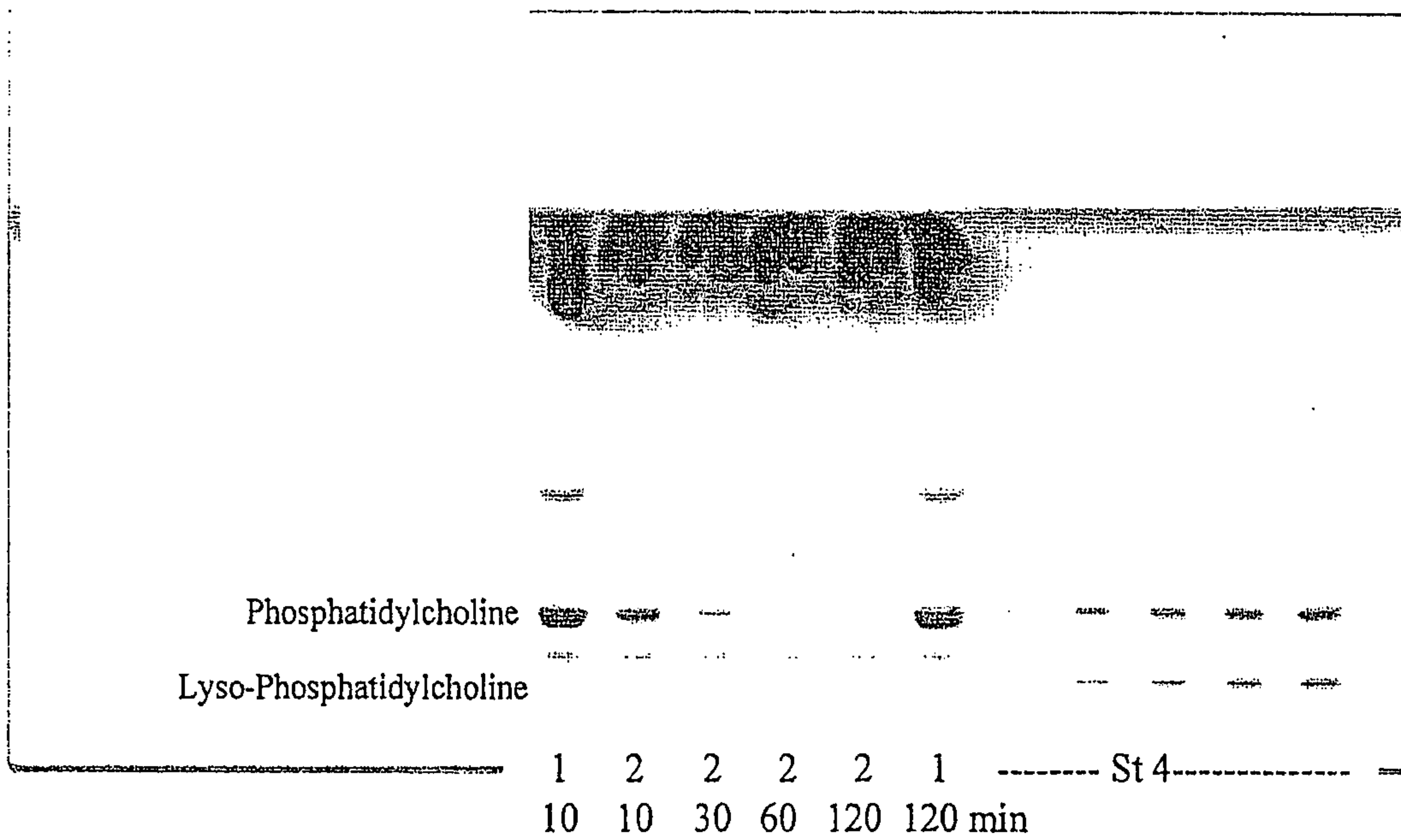




Figure 112

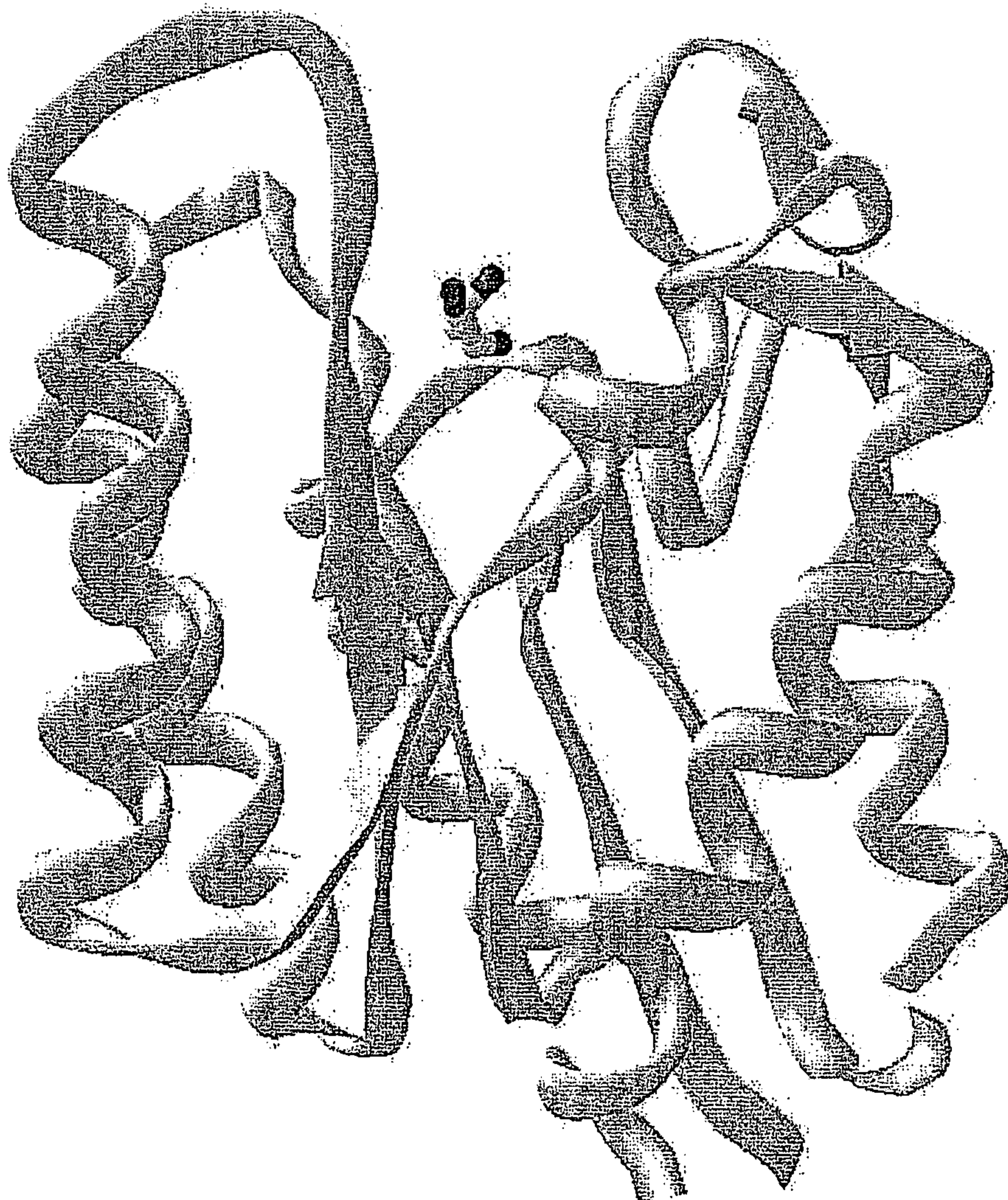




Figure 113

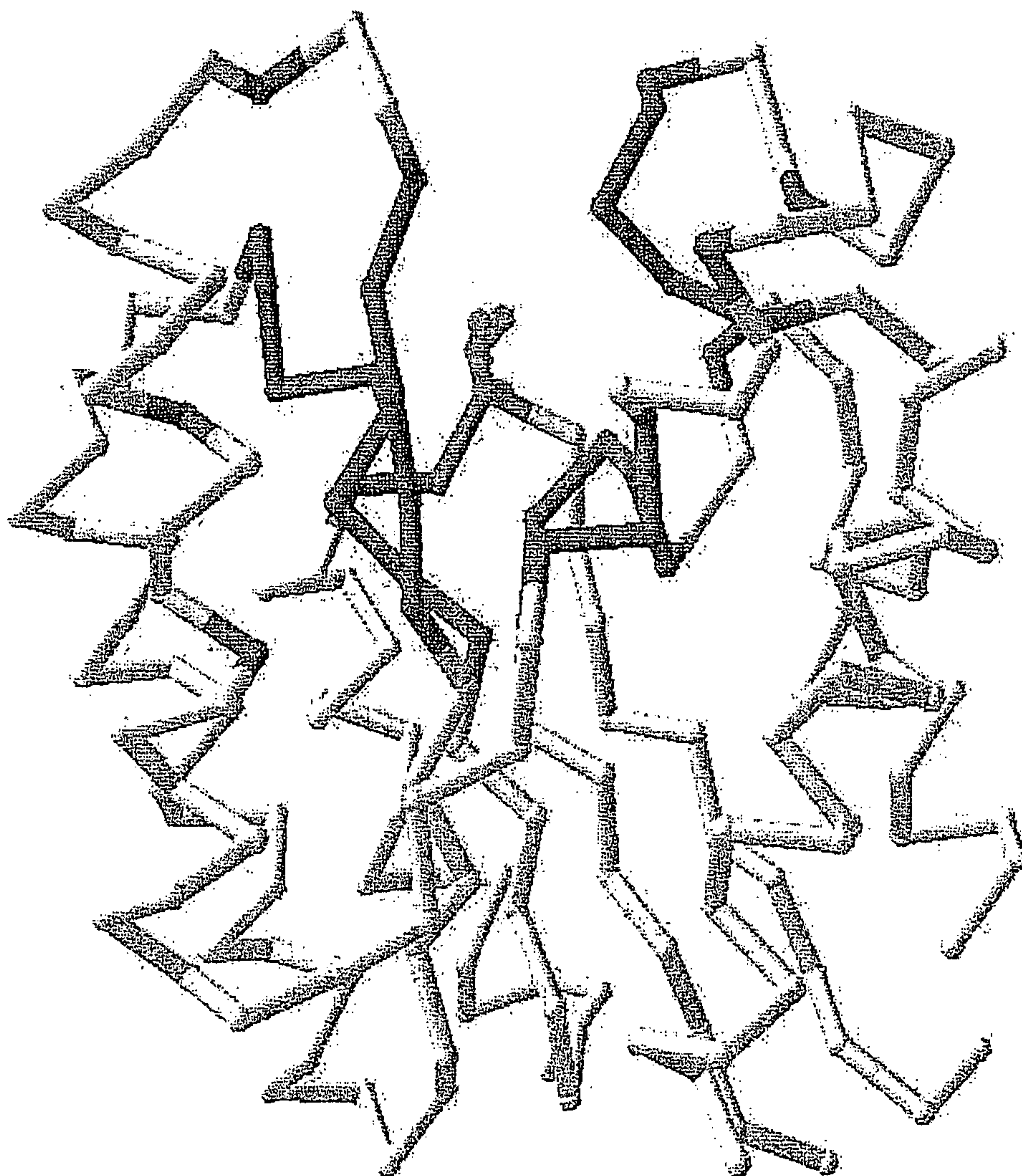
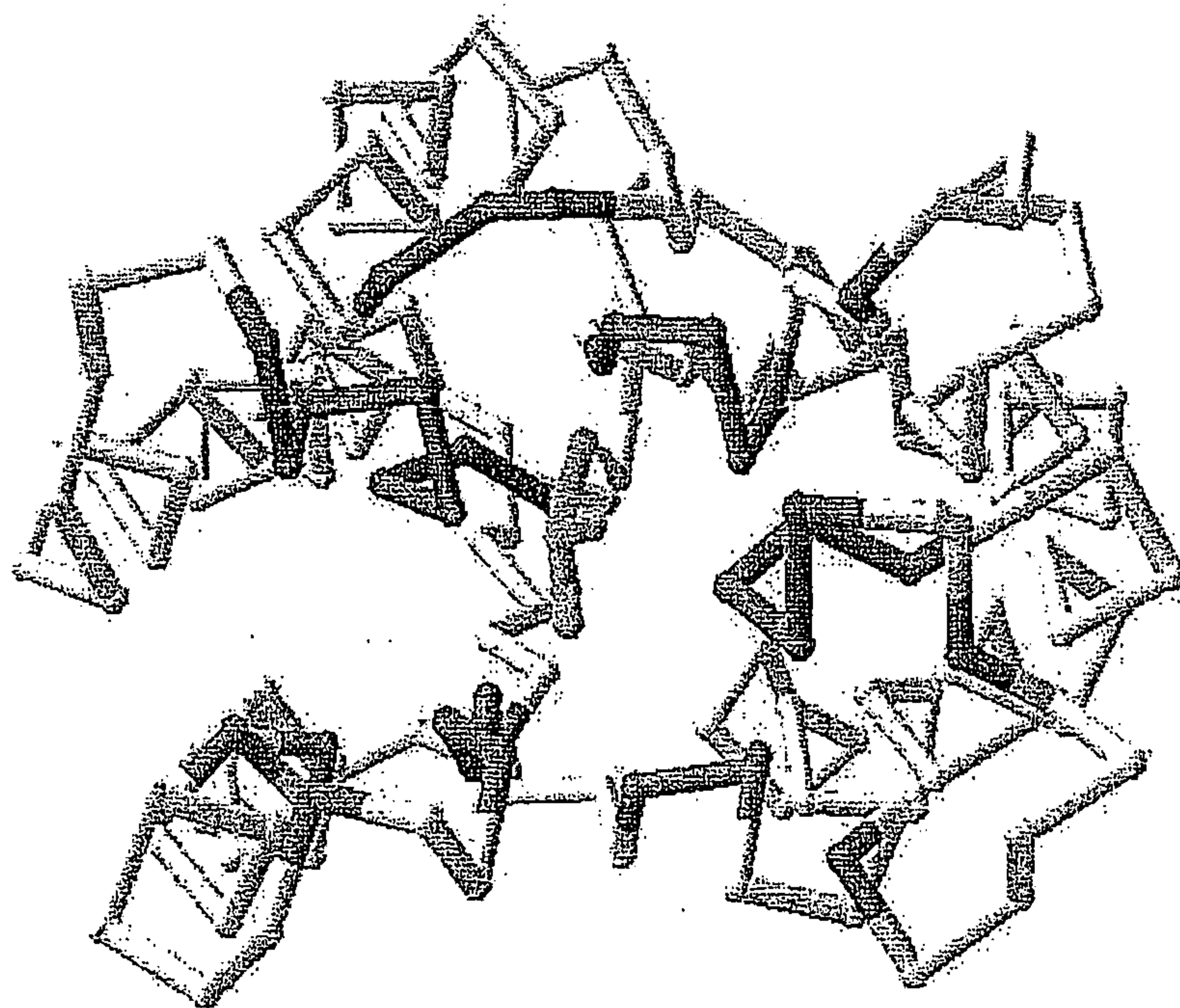




Figure 114





[illegible]



[illegible]



[illegible]



[illegible]



Figure 118

```

      10      20      30      40      50      60
      .....|.....|.....|.....|.....|.....|
1IVN_A  4 LLILGDSLSAG-----YRMSASAAWPALLNDKWqsk----- 34
P10480 28 IVMFGDSLSDTgkmyskmgylpssppyyeGRFSNGFPVWLEQLTNEFPGLTianeaeegg 87

      70      80      90     100     110     120
      .....|.....|.....|.....|.....|.....|
1IVN_A 35 -tsvVNASISGDT-----SQQGLARLPALLKQHQP RW 65
P10480 88 tavaYNKISWNPkyq-----vINNLDYEVtQFLQKDSFKPDDL 125

      130     140     150     160     170     180
      .....|.....|.....|.....|.....|.....|
1IVN_A 66 VLVELGGNDG-----LRGFQPPQTEQT 87
P10480 126 VILWVGANDY-----LA--YGWNT EQDAKRVRDA 152

      190     200     210     220     230     240
      .....|.....|.....|.....|.....|.....|
1IVN_A 88 LRQILQDVkaANAEPllmqiRLPANYGR----- 115
P10480 153 ISDAANRMV-LNGAK-----EILLFNLPdlg-----qnP 180

      250     260     270     280     290     300
      .....|.....|.....|.....|.....|.....|
1IVN_A 116 -----RYNEAFSAIYPKLake-----fDVPLLPFFME 142
P10480 181 SARSQKVVEAASHVSAYHNQLLLNLArqlaptg-----mvklfeiDKQFAEMLRD 230

      310     320     330     340     350     360
      .....|.....|.....|.....|.....|.....|
1IVN_A 143 EVYLPQW----- 150
P10480 231 PQNFGLSDQRNacyggsyvwkpfasrsastdsqf safnpqerlaiagnpllaqavaspma 290

      370     380     390     400
      .....|.....|.....|.....|
1IVN_A 151 -----MQDDGI-----HPNRDAQPFADWM 170
P10480 291 arsastlncegkMFWDQV-----HPTTVVHAALSEPA 322
```



Figure 119

|           |       |                                                    |     |
|-----------|-------|----------------------------------------------------|-----|
|           |       | 1                                                  | 50  |
| P10480    | (1)   | MKKWFVCLLGLVALTVQAADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLP |     |
| A. sal    | (1)   | -----ADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLP              |     |
| A. hyd    | (1)   | -----ADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLP              |     |
| Consensus | (1)   | AD*RPAFSRIVMFGDSLSDTGKMYSKMRGYLP                   |     |
|           |       | 51                                                 | 100 |
| P10480    | (51)  | SSPPYYEGRFSNGPVWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWNPK |     |
| A. sal    | (33)  | SSPPYYEGRFSNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPK |     |
| A. hyd    | (33)  | SSPPYYEGRFSNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPK |     |
| Consensus | (51)  | SSPPYYEGRFSNGPVWLEQLT**FPGLTIANEAEGG*TAVAYNKISWNPK |     |
|           |       | 101                                                | 150 |
| P10480    | (101) | YQVINNLDYEVTQFLQKDSFKPDDLVLWVGANDYLAYGWNTEQDAKRVR  |     |
| A. sal    | (83)  | YQVINNLDYEVTQFLQKDSFKPDDLVLWVGANDYLAYGWNTEQDAKRVR  |     |
| A. hyd    | (83)  | YQVINNLDYEVTQFLQKDSFKPDDLVLWVGANDYLAYGWNTEQDAKRVR  |     |
| Consensus | (101) | YQVINNLDYEVTQFLQKDSFKPDDLVLWVGANDYLAYGWNTEQDAKRVR  |     |
|           |       | 151                                                | 200 |
| P10480    | (151) | DAISDAANRMVLNGAKEILLFNLPDLGQNPSARSQKVVEAASHVSAYHNQ |     |
| A. sal    | (133) | DAISDAANRMVLNGAKQILLFNLPDLGQNPSARSQKVVEAVSHVSAYHNK |     |
| A. hyd    | (133) | DAISDAANRMVLNGAKQILLFNLPDLGQNPSARSQKVVEAVSHVSAYHNQ |     |
| Consensus | (151) | DAISDAANRMVLNGAK*ILLFNLPDLGQNPSARSQKVVEA*SHVSAYHN* |     |
|           |       | 201                                                | 250 |
| P10480    | (201) | LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQRNACYGGSYVW |     |
| A. sal    | (183) | LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVW |     |
| A. hyd    | (183) | LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVW |     |
| Consensus | (201) | LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSD**N*CY*G*YVW |     |
|           |       | 251                                                | 300 |
| P10480    | (251) | KPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMARSASTLNCE  |     |
| A. sal    | (233) | KPFATRSVSTDRLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE  |     |
| A. hyd    | (233) | KPFATRSVSTDRLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE  |     |
| Consensus | (251) | KPFA*RS*STD*QLSAF*PQERLAIAGNPLLAQAVASPMARSAS*LNCE  |     |
|           |       | 301                                                | 336 |
| P10480    | (301) | GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH-               |     |
| A. sal    | (283) | GKMFWDQVHPTTVVHAALSERAAATFIETQYEFLAHG              |     |
| A. hyd    | (283) | GKMFWDQVHPTTVVHAALSERAAATFIANQYEFLAH-              |     |
| Consensus | (301) | GKMFWDQVHPTTVVHAALSE*AATFI**QYEFLAH*               |     |



Page 95 of 106

Figure 120

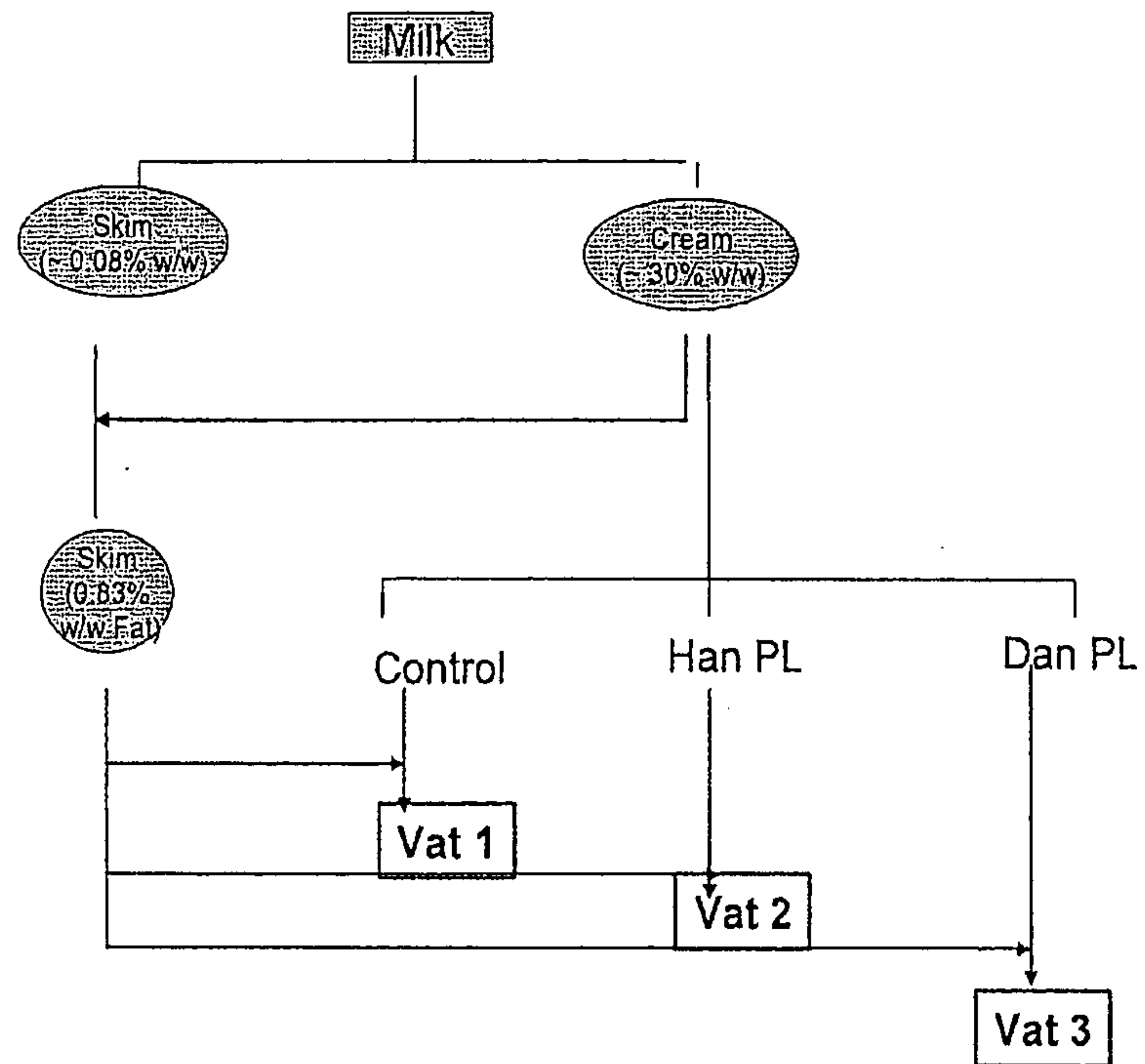




Figure 121

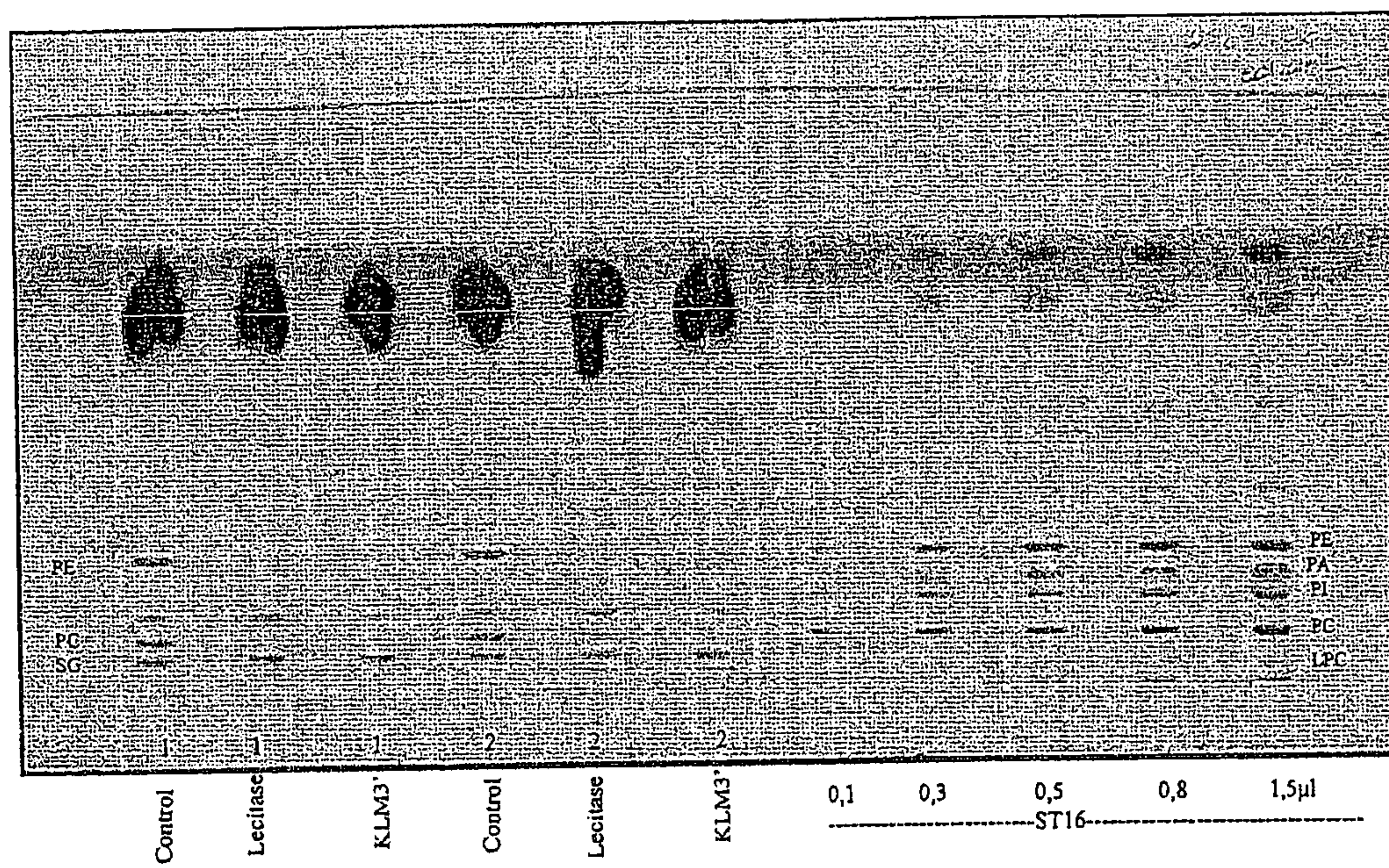


Figure 122

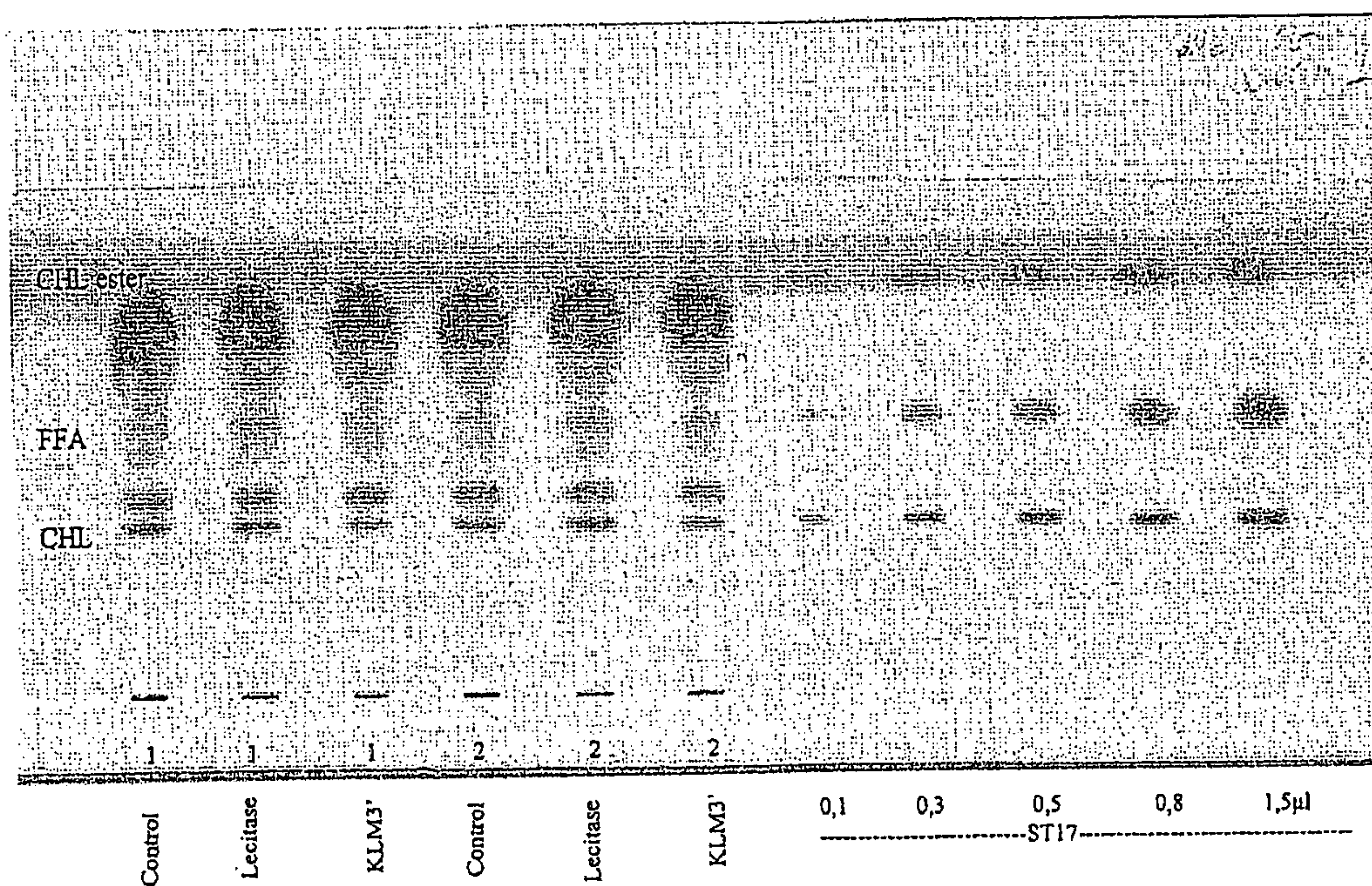




Figure 123

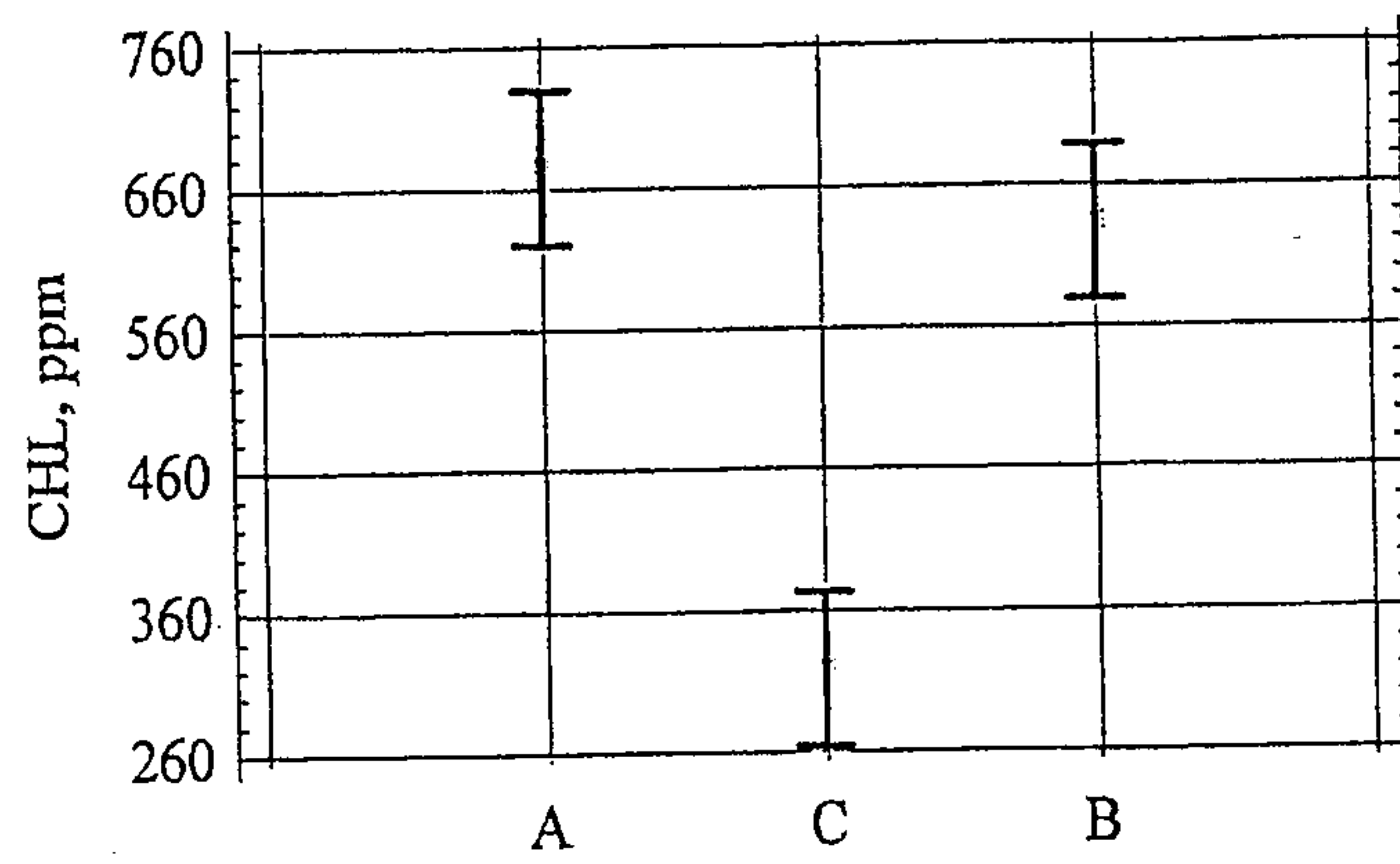


Figure 124

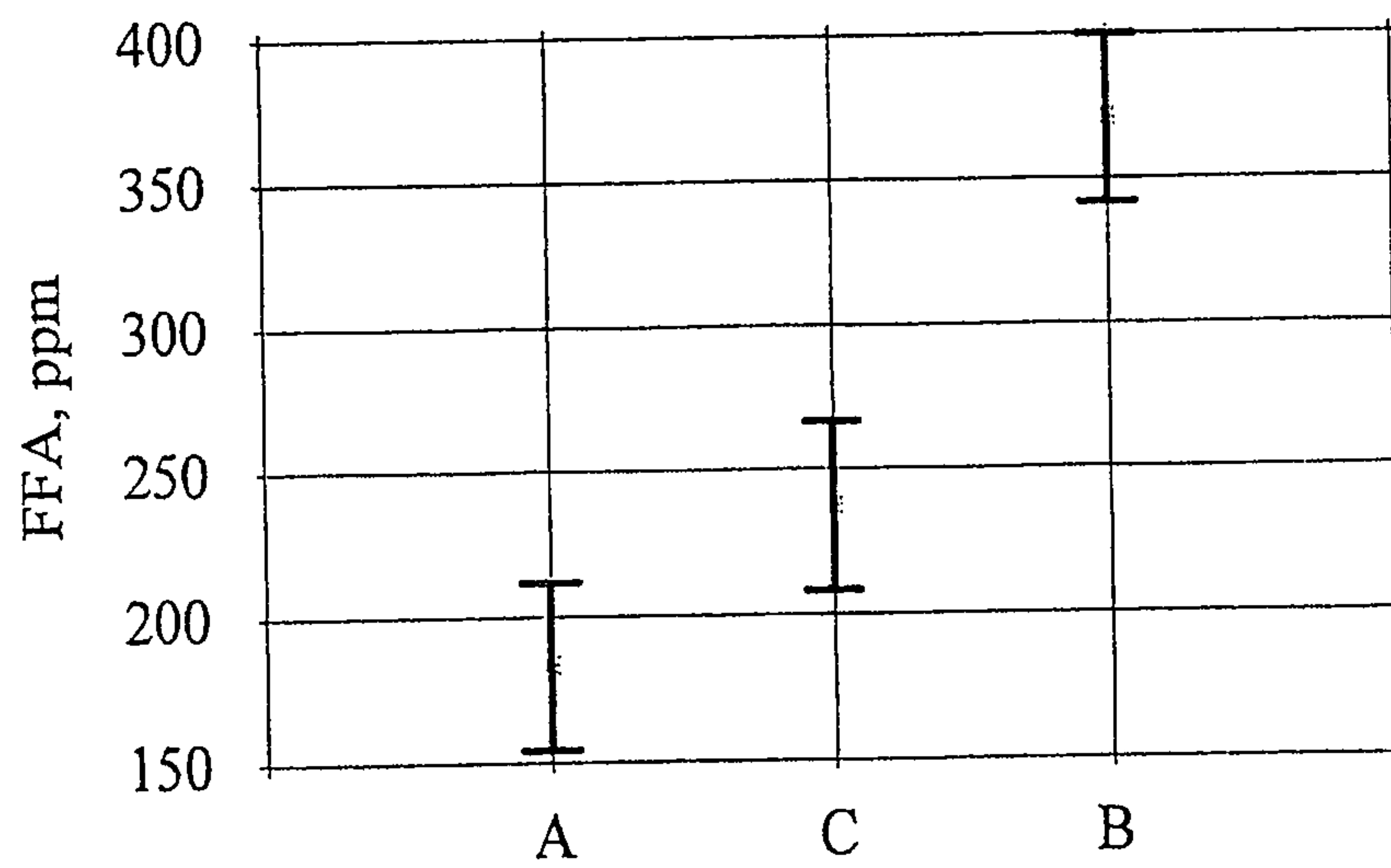




Figure 125

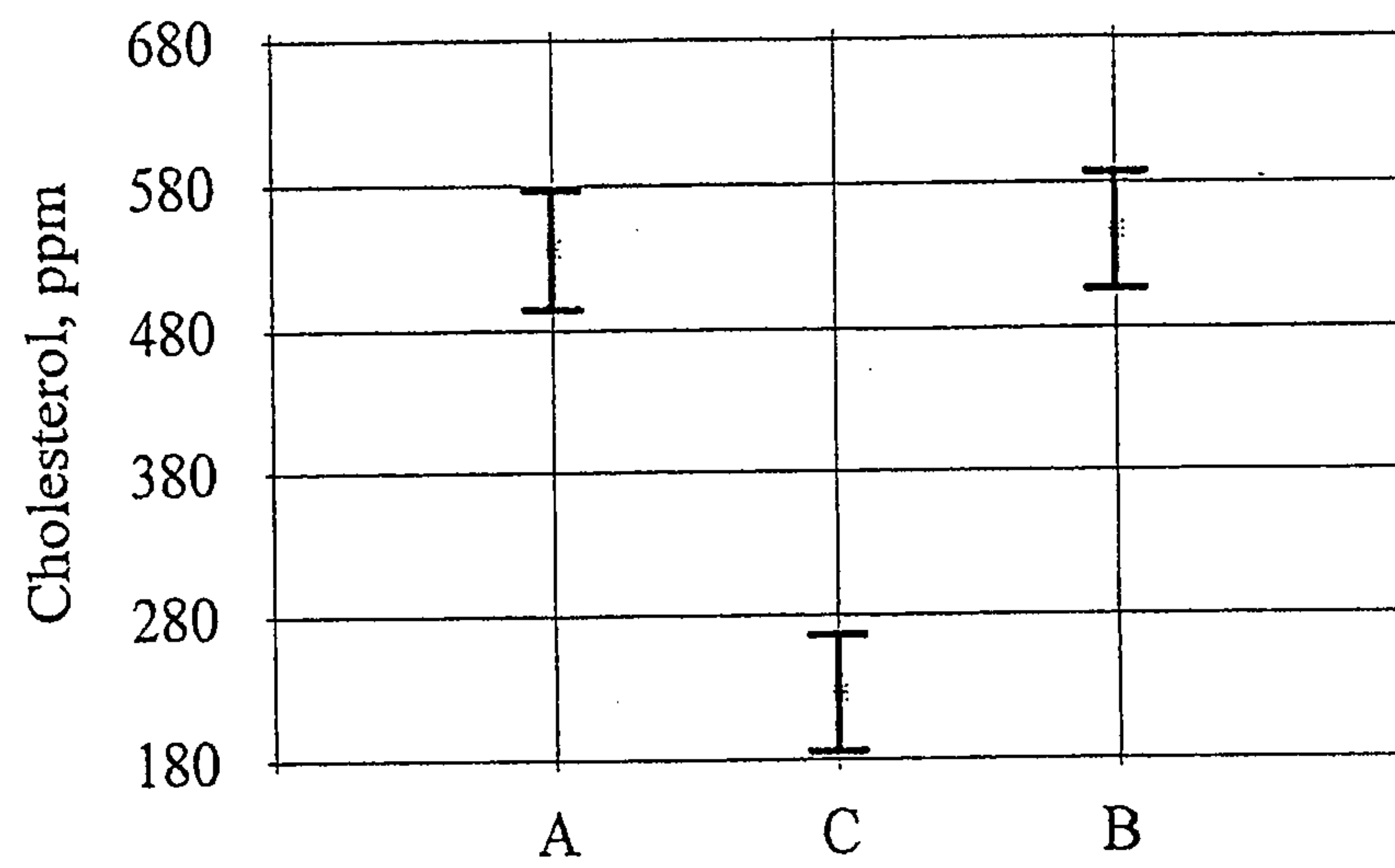


Figure 126

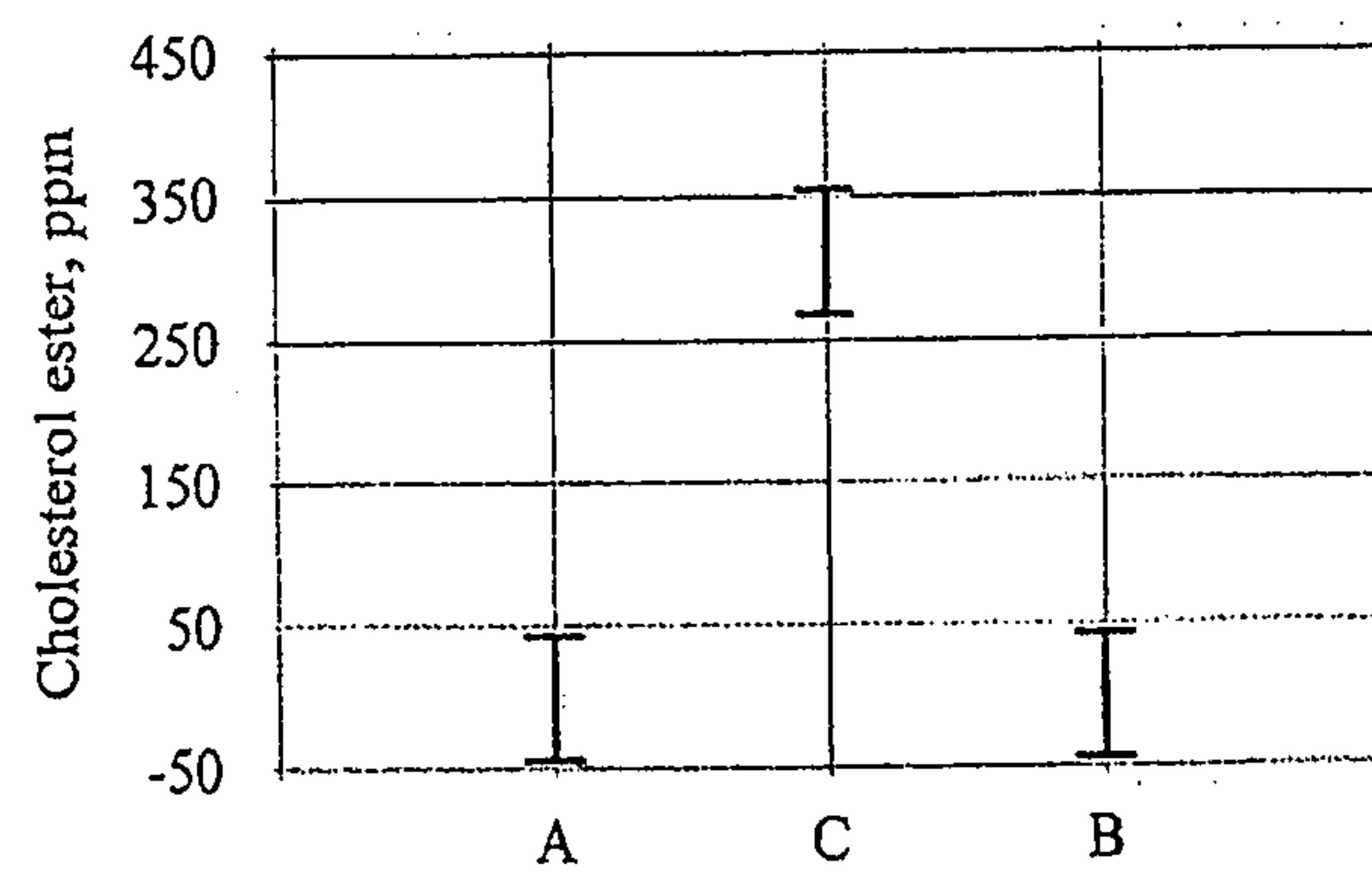




Figure 127

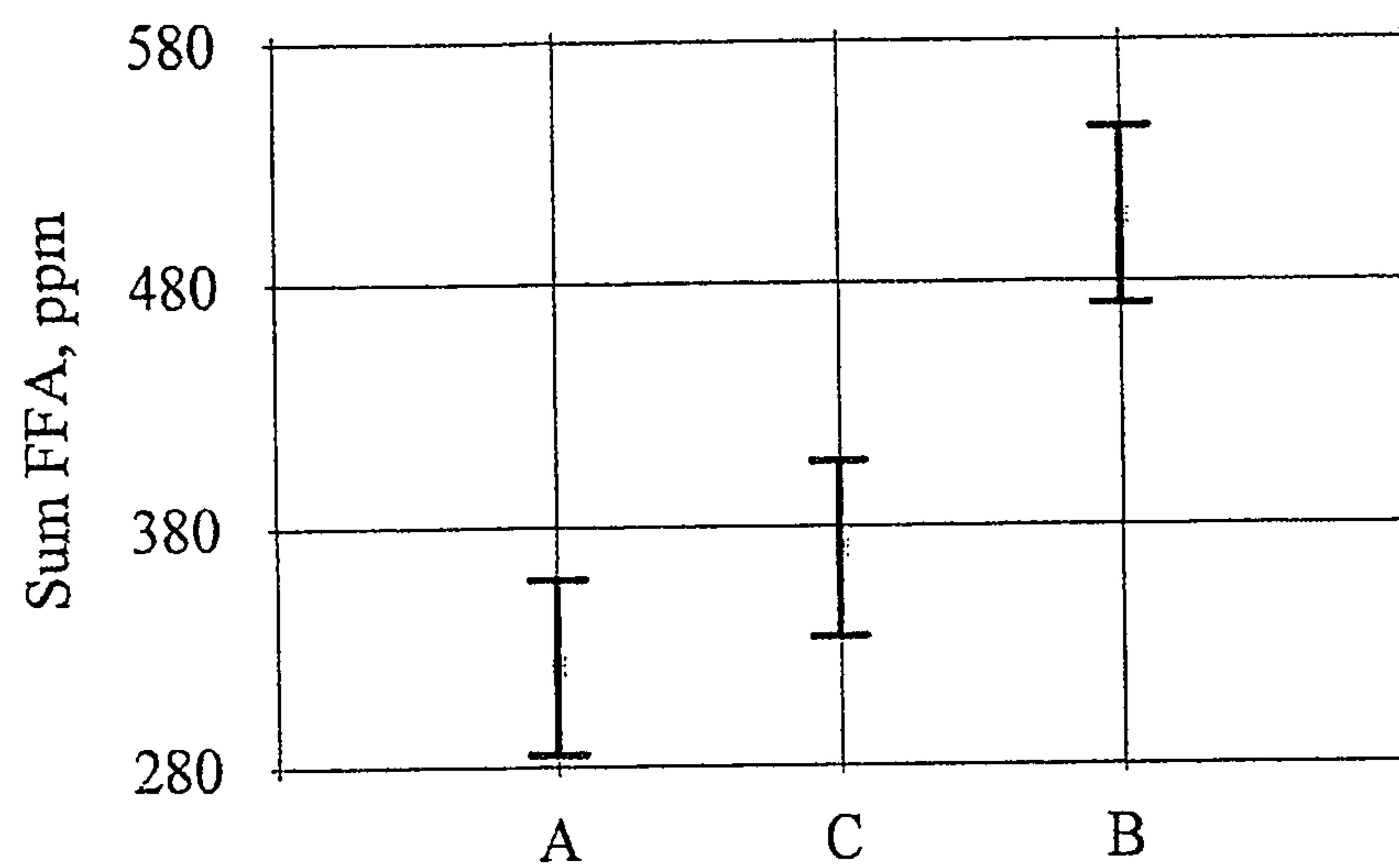


Figure 128

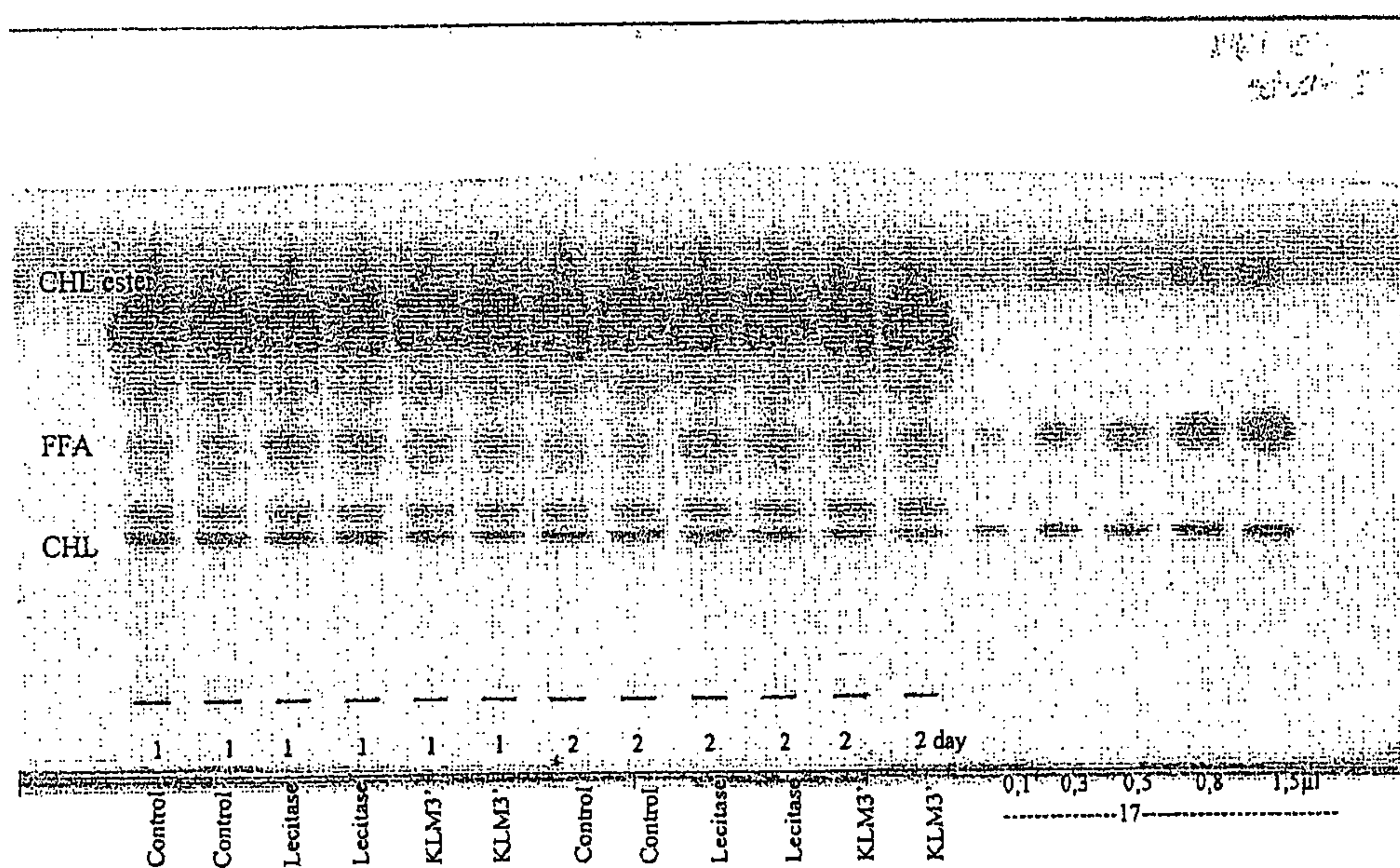




Figure 129

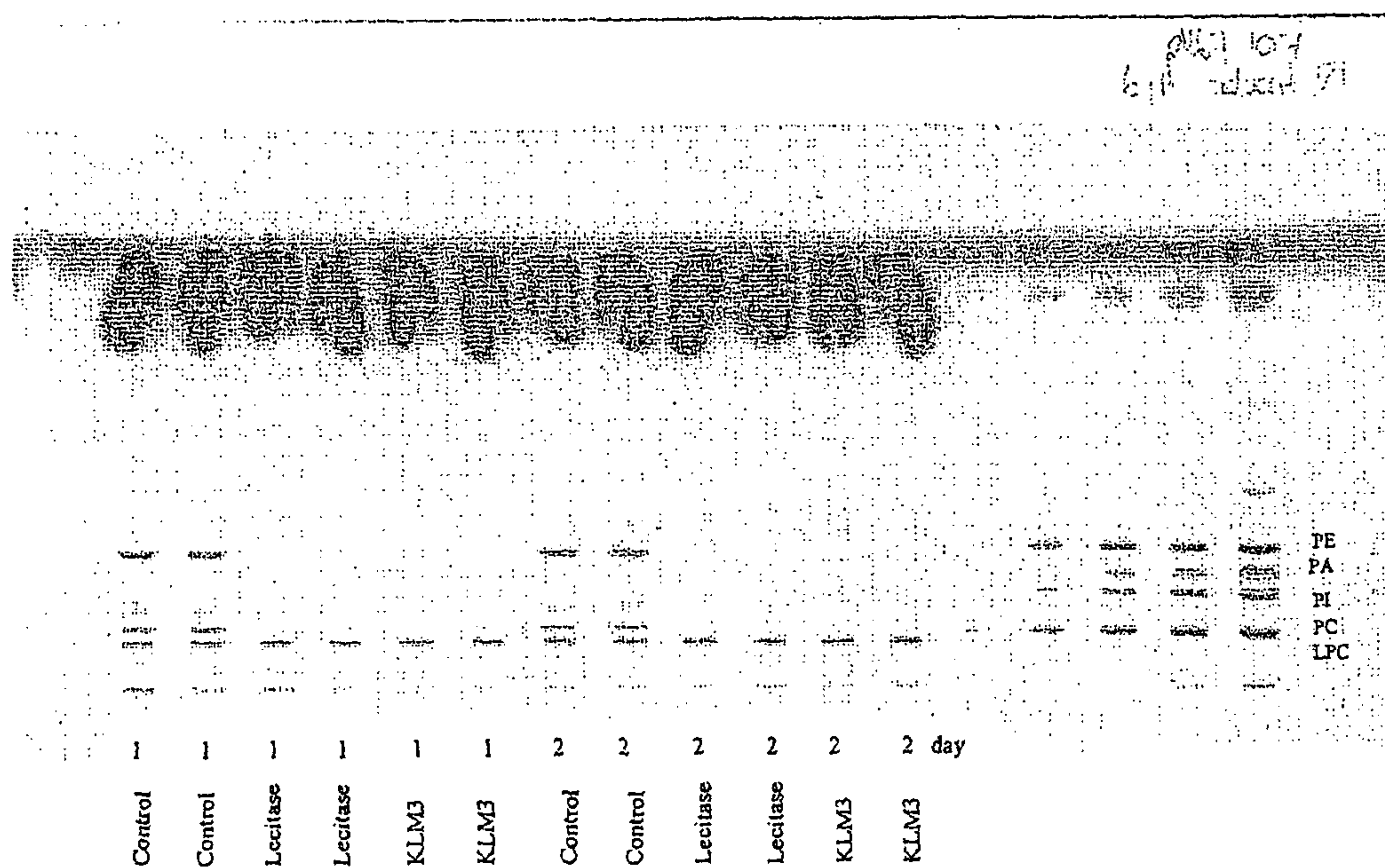


Figure 130

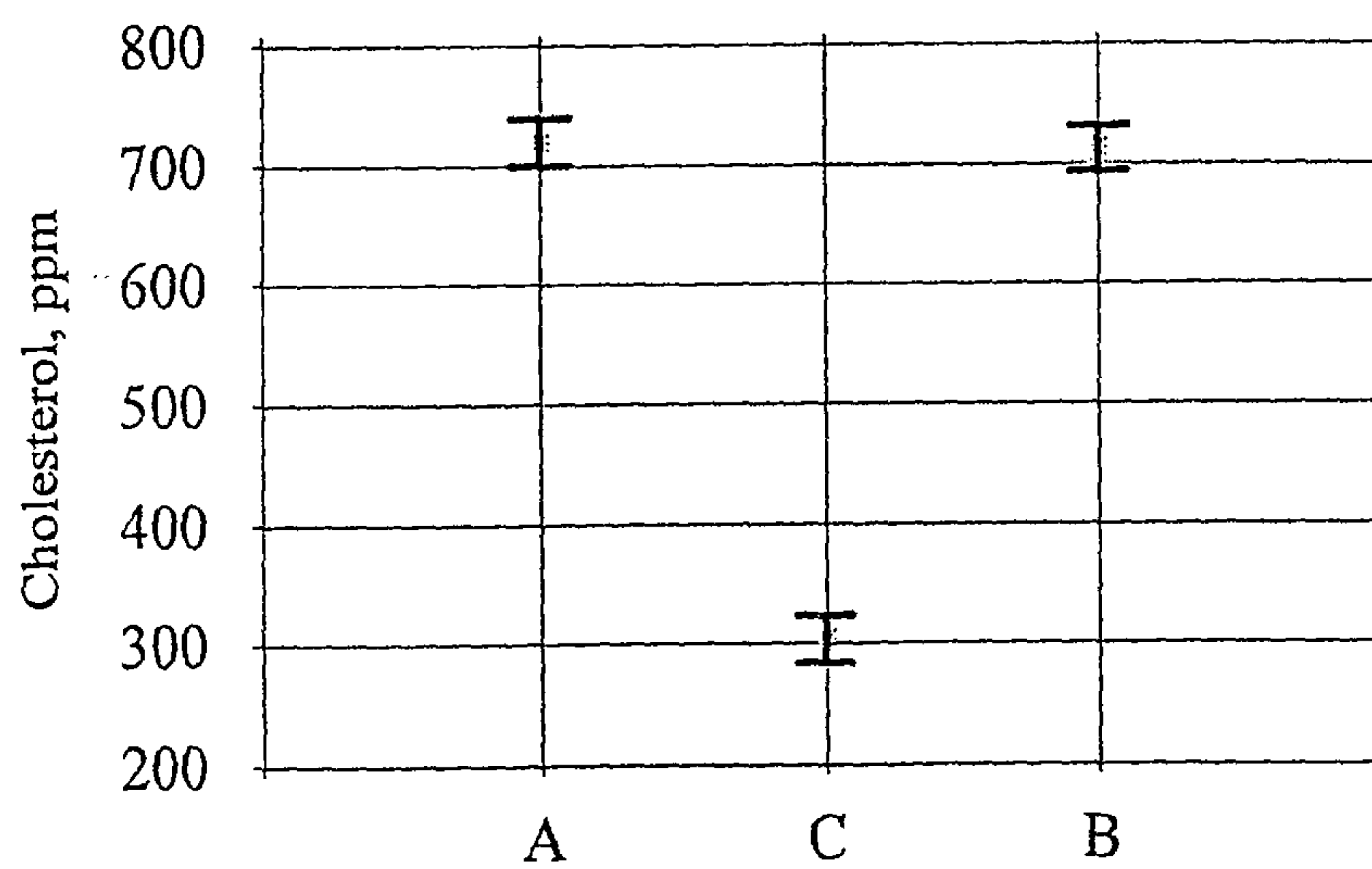




Figure 131

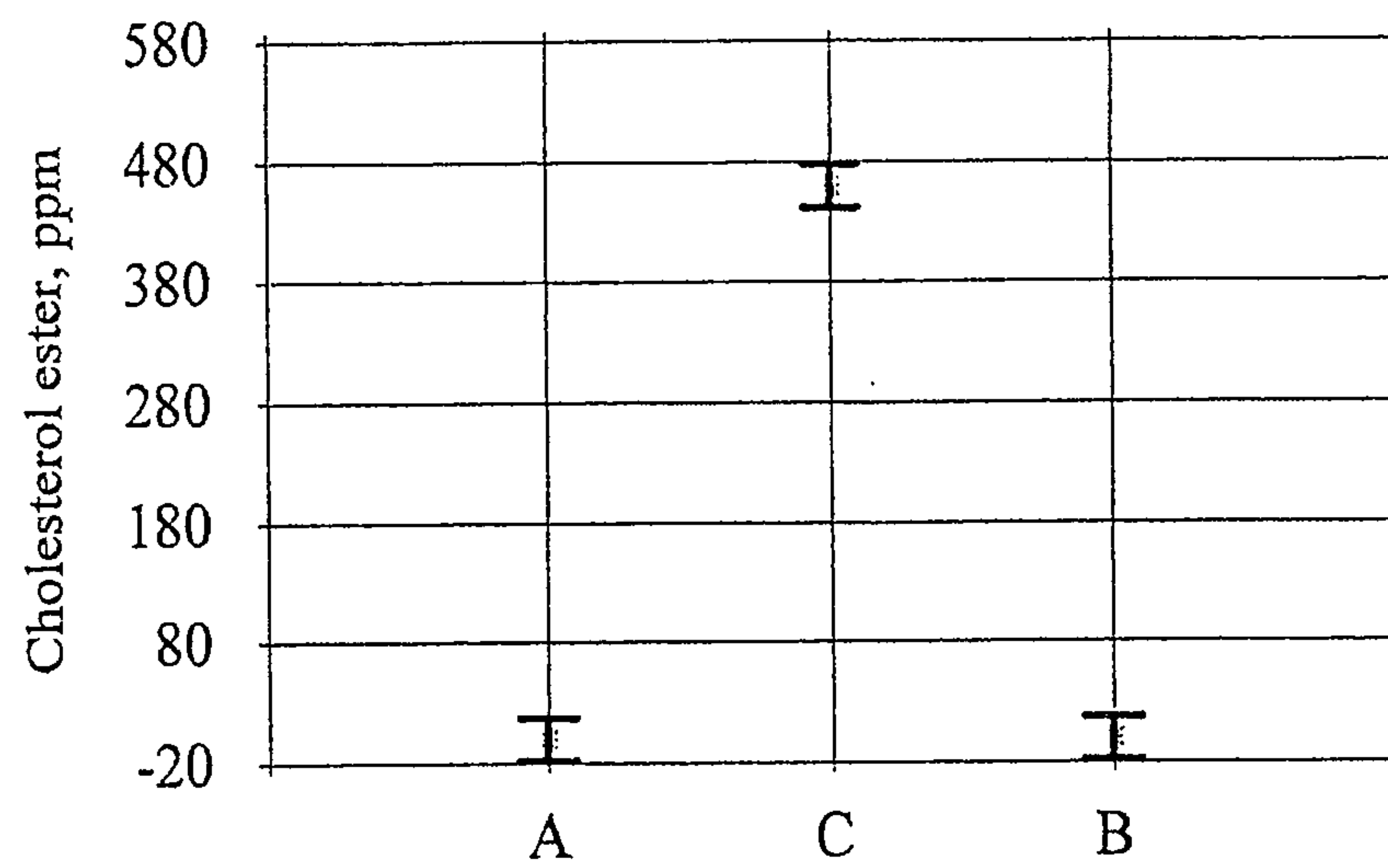


Figure 132

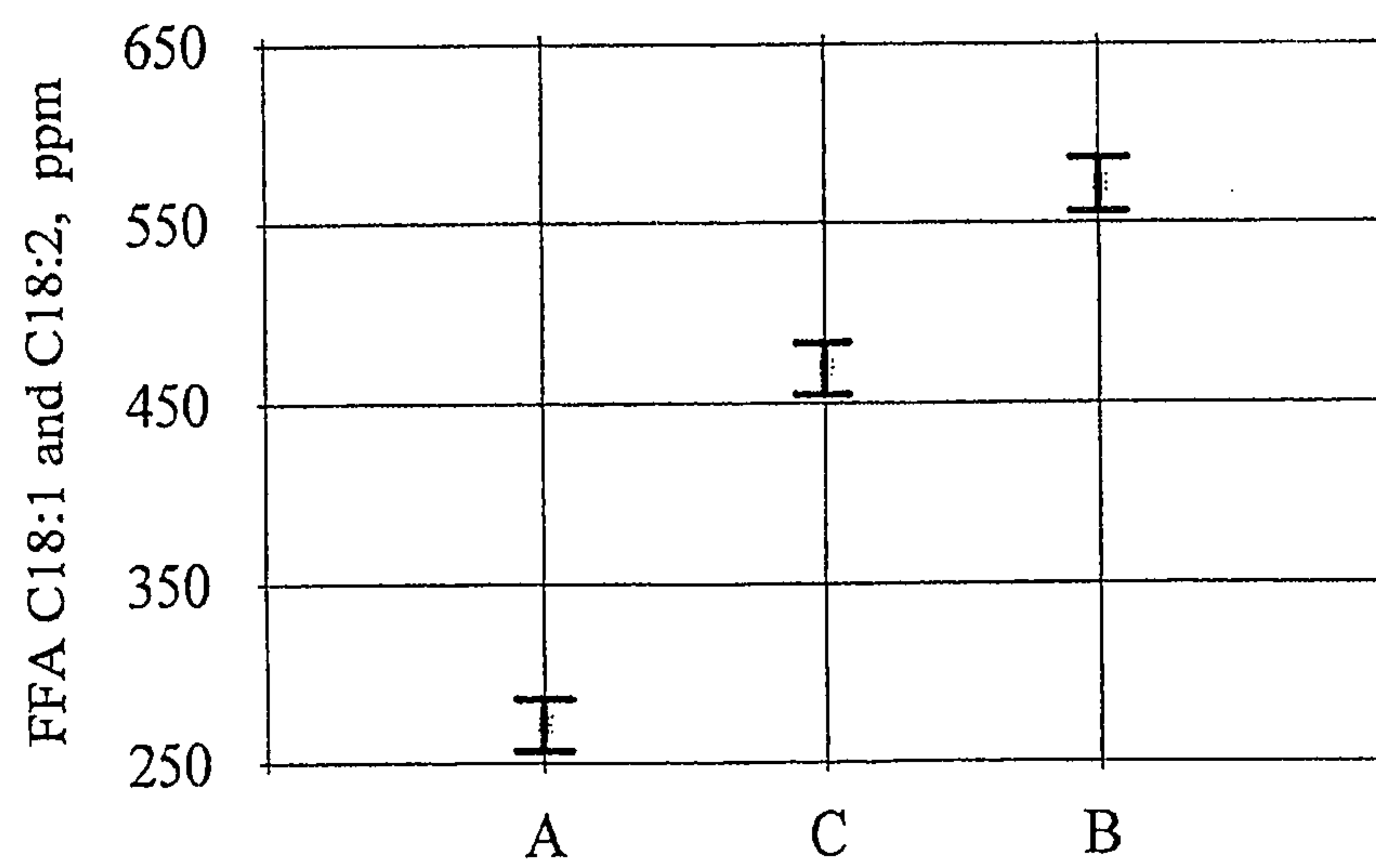




Figure 133

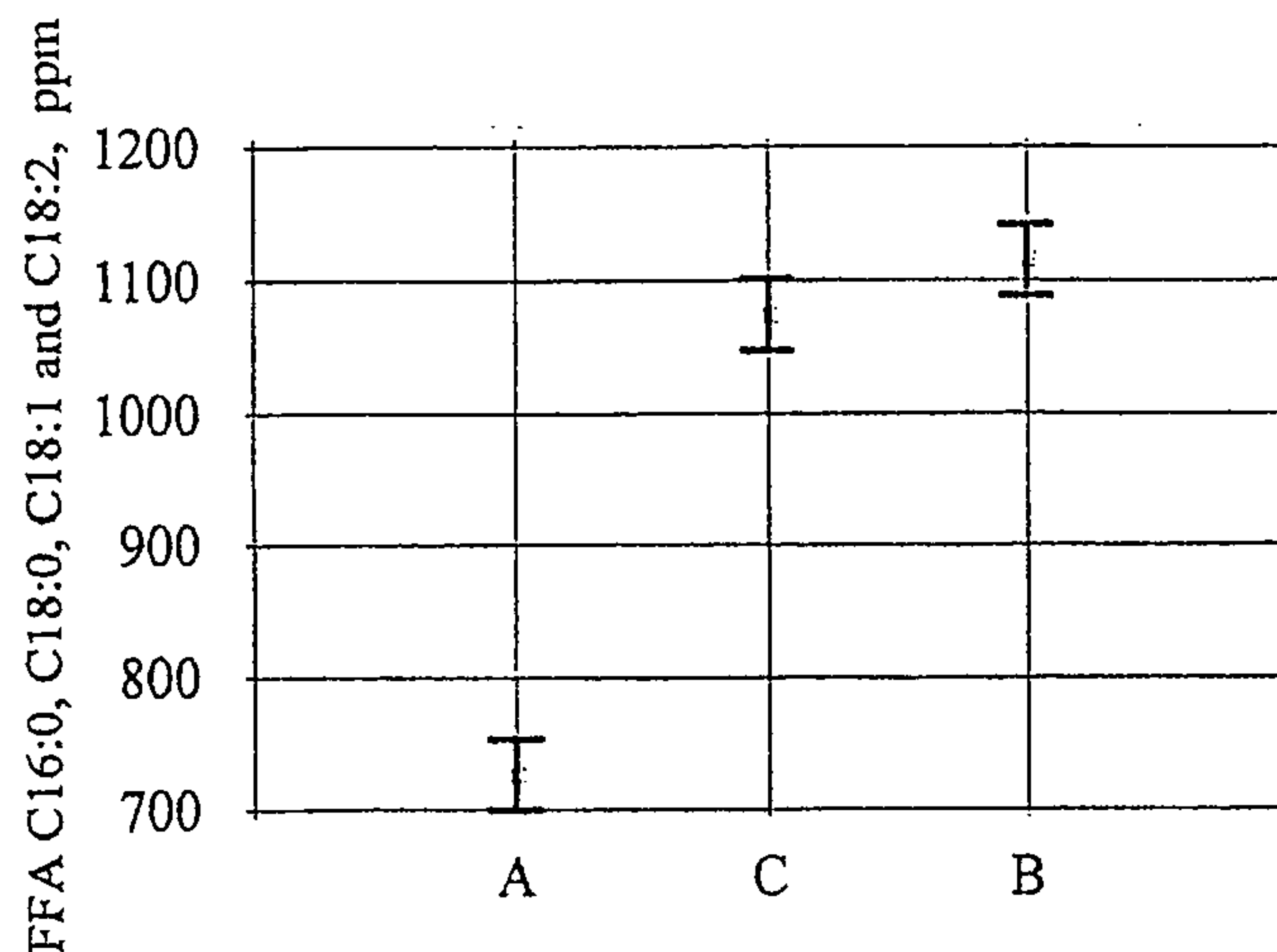


Figure 134

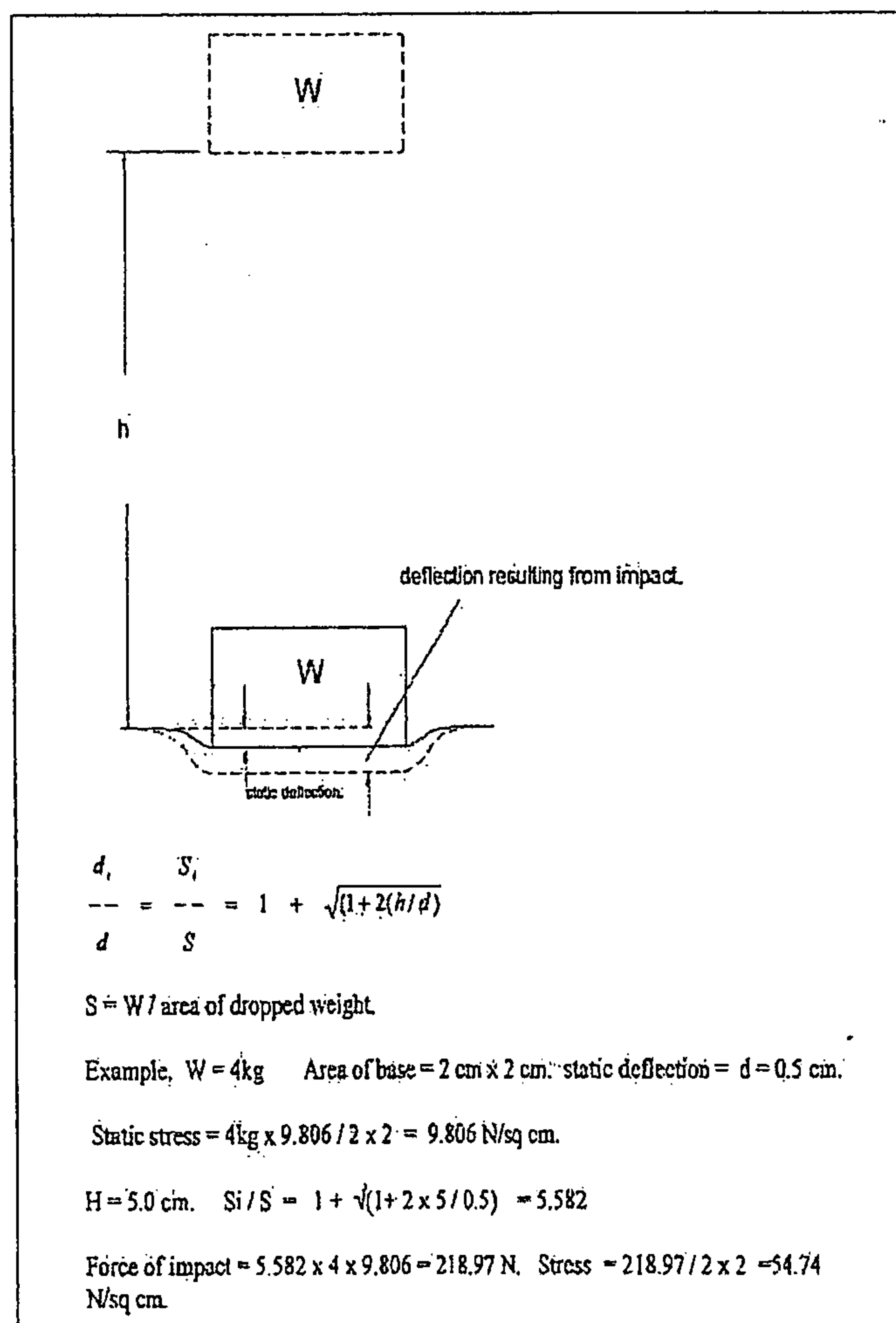




FIG. 135

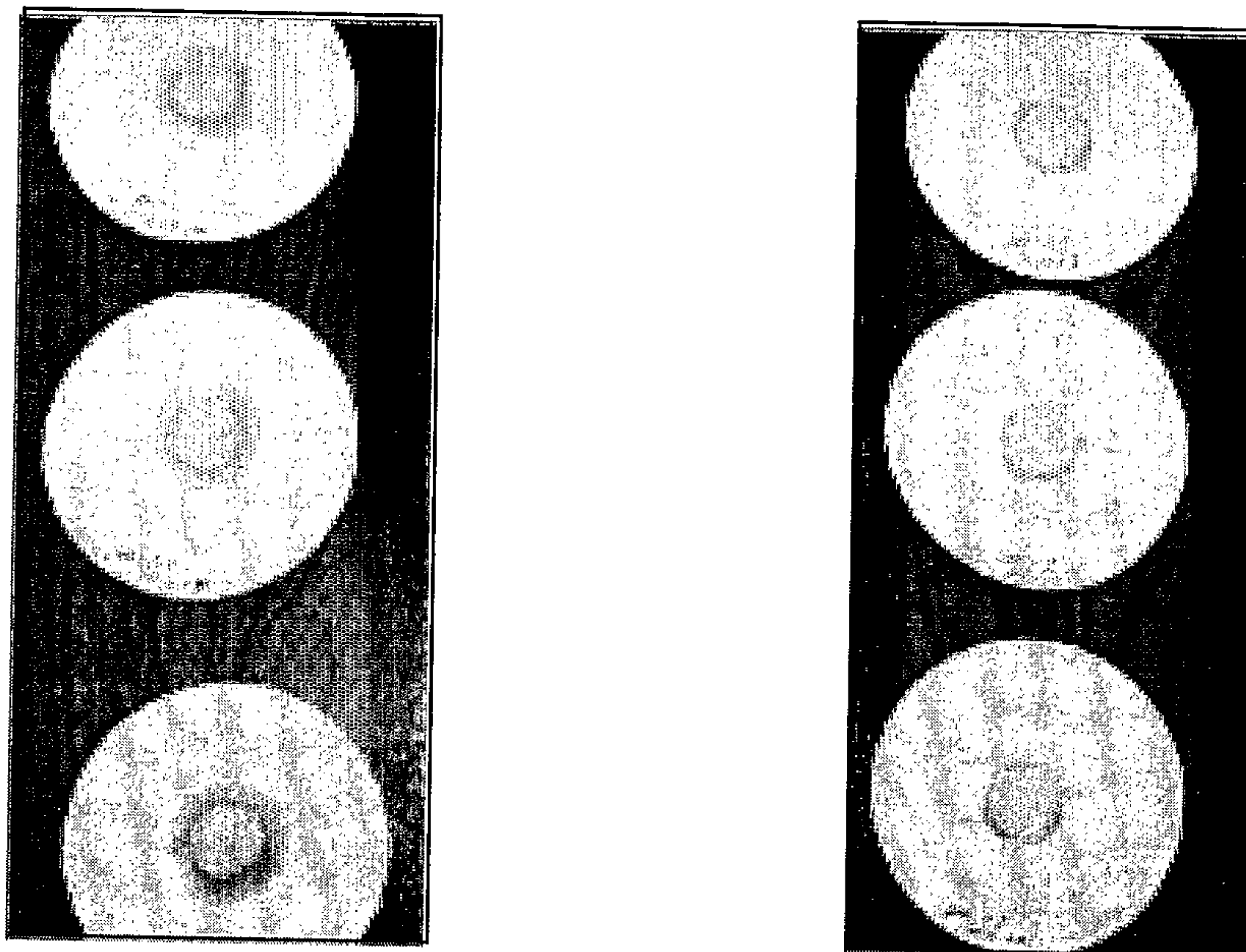


FIG. 136

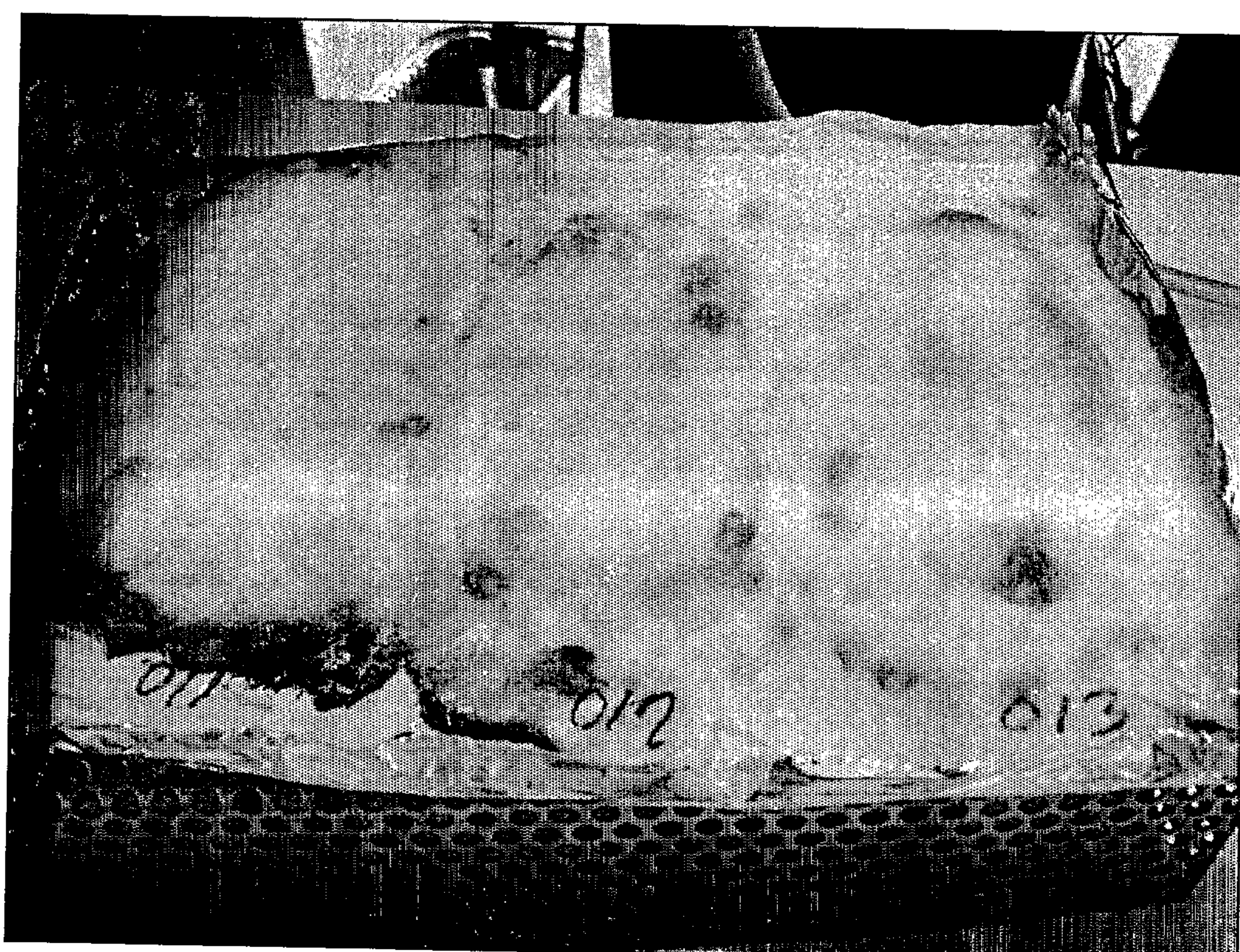




FIG. 137

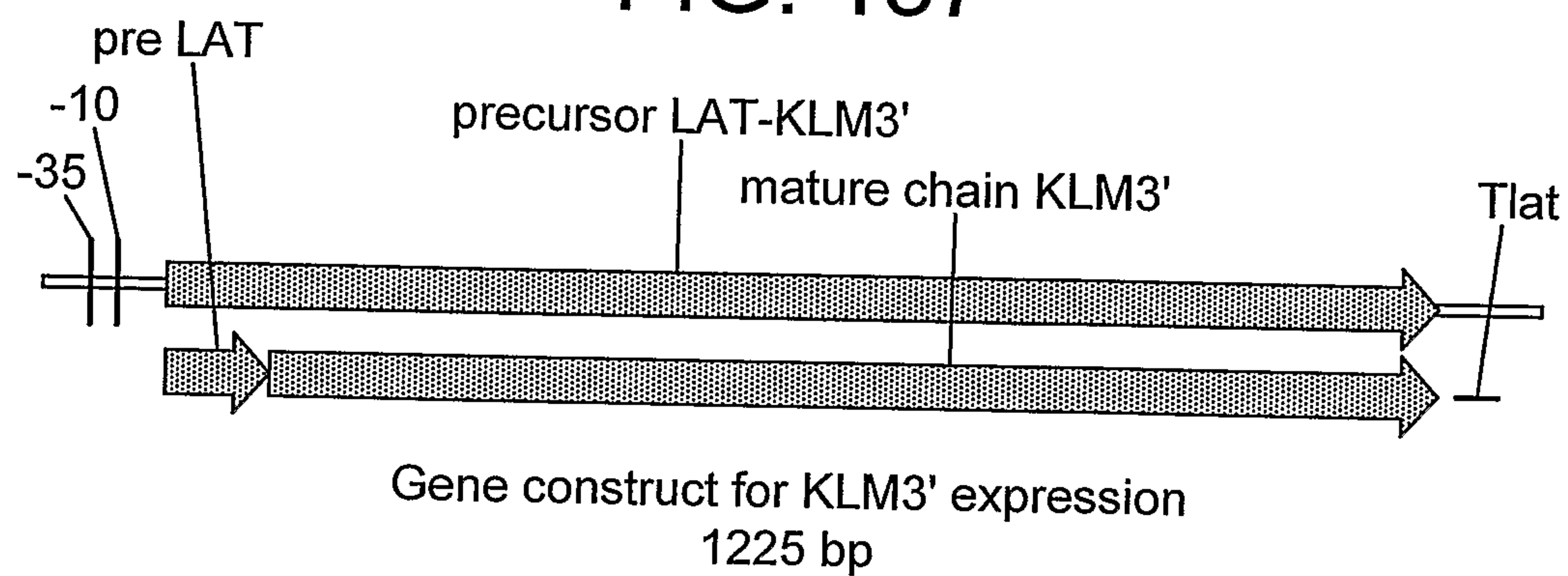


FIG. 138

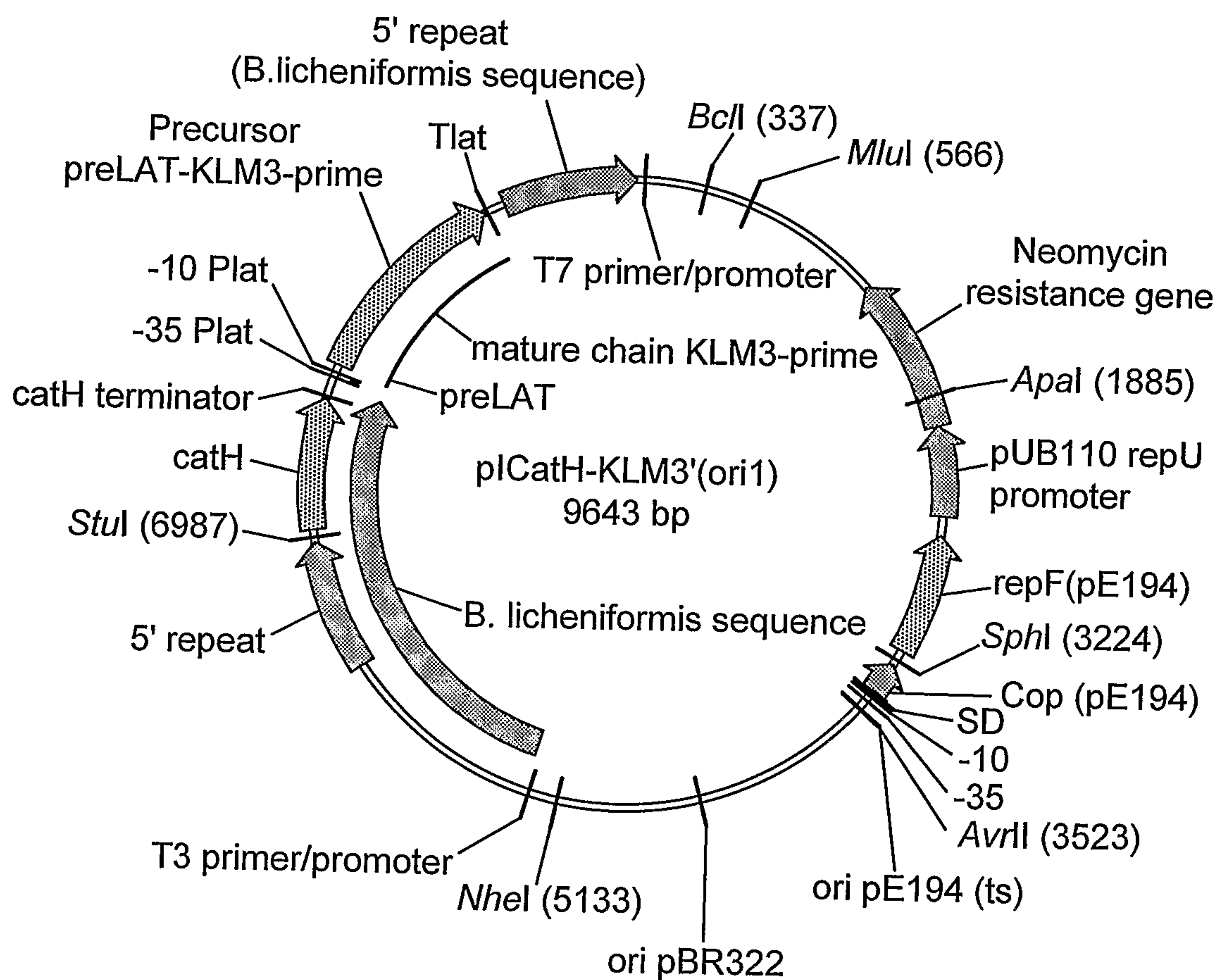




Figure 139

-35

```
1  GCTTTTCTTT TGGAAGAAAA TATAGGGAAA ATGGTACTTG TTAAAAATTC GGAATATTTA
   CGAAAAGAAA ACCTTCTTTT ATATCCCTTT TACCATGAAC AATTTTAAAG CCTTATAAAT
   -10                                     M K Q Q K R L .
61  TACAATATCA TATGTTTCAC ATTGAAAGGG GAGGAGAATC ATGAAACAAC AAAAACGGCT
   ATGTTATAGT ATACAAAGTG TAACTTTCCC CTCCTCTTAG TACTTTGTTG TTTTGGCCGA
   . Y A R L L T L L F A L I F L L P H S A A .
121 TTACGCCCGA TTGCTGACGC TGTATTTTGC GTCATCTTC TTGCTGCCTC ATTCTGCAGC
   AATGCGGGCT AACGACTGCG ACAATAACG CGAGTAGAAG AACGACGGAG TAAGACGTCG
   . S A A D T R P A F S R I V M F G D S L S .
181 TTCAGCAGCA GATACAAGAC CGCGGTTTAG CCGGATCGTC ATGTTTGGAG ATAGCCTGAG
   AAGTCGTCGT CTATGTTCTG GCCGCAAATC GGCCTAGCAG TACAAACCTC TATCGGACTC
   . D T G K M Y S K M R G Y L P S S P P Y Y .
241 CGATACGGGC AAAATGTATA GCAAAATGAG AGGCTATCTT CCGTCAAGCC CGCCGTATTA
   GCTATGCCCG TTTTACATAT CGTTTACTC TCCGATAGAA GGCAGTTCGG GCGGCATAAT
   . E G R F S N G P V W L E Q L T K Q F P G .
301 TGAAGGCCGC TTTAGCAATG GACCGGTCTG GCTGGAACAA CTGACGAAAC AATTTCCGGG
   ACTTCCGGCG AAATCGTTAC CTGGCCAGAC CGACCTTGTT GACTGCTTTG TTAAAGGCCC
   . L T I A N E A E G G A T A V A Y N K I S .
361 ACTGACGATC GCTAATGAAG CAGAAGGAGG AGCAACAGCG GTCGCTATA ACAAATCAG
   TGACTGCTAG CGATTACTTC GTCTTCTCC TCGTTGTCGC CAGCGGATAT TGTTTTAGTC
   . W D P K Y Q V I N N L D Y E V T Q F L Q .
421 CTGGGACCCG AAATATCAGG TCATCAACAA CCTGGACTAT GAAGTCACAC AGTTTCTTCA
   GACCTTGGGC TTTATAGTCC AGTAGTTGTT GGACCTGATA CTTCACTGTG TCAAAGAAGT
   . K D S F K P D D L V I L W V G A N D Y L .
481 GAAAGACAGC TTAAACCGG ATGATCTGGT CATCCTTTGG GTCGGCGCCA ATGATTATCT
   CTTTCTGTCG AAATTTGGCC TACTAGACCA GTAGGAAACC CAGCCGCGGT TACTAATAGA
   . A Y G W N T E Q D A K R V R D A I S D A .
541 GCGGTATGGC TGGAACACAG AACAGATGC CAAAAGAGTC AGAGATGCCA TCAGCGATGC
   CCGCATACCG ACCTTGTGTC TTGTTCTACG GTTTTCTCAG TCTCTACGGT AGTCGCTACG
   . A N R M V L N G A K Q I L L F N L P D L .
601 CGCTAATAGA ATGGTCTGA ACGGCGCCAA ACAAATCCTG CTGTTTAAAC TGCCGGATCT
   GCGATTATCT TACCAGGACT TGCCGCGGTT TGTTTAGGAC GACAAATTGG ACGGCCTAGA
   . G Q N P S A R S Q K V V E A V S H V S A .
661 GGGACAAAAT CCGAGCGCCA GAAGCCAAAA AGTCGTCGAA GCAGTCAGCC ATGTCAGCGC
   CCCTGTTTGA GGCTCGCGGT CTTGCGTTTT TCAGCAGCTT CGTCAGTCGG TACAGTCGCG
   . Y H N K L L L N L A R Q L A P T G M V K .
721 CTATCATAAC AAAGTCTGC TGAACCTGGC AAGACAATTG GCACCGACGG GAATGGTTAA
   GATAGTATTG TTTGACGACG ACTTGACCG TTCTGTTAAC CGTGGCTGCC CTTACCAATT
   . L F E I D K Q F A E M L R D P Q N F G L .
781 ATGTTTGAA ATTGACAAAC AGTTTGCCGA AATGCTGAGA GATCCGAAA ATTTTGGCCT
   TAACAACTT TAACTGTTT TCAAACGGCT TTACGACTCT CTAGGCGTTT TAAACCGGA
   . S D V E N P C Y D G G Y V W K P F A T R .
841 GAGCGATGTC GAAAACCCGT GCTATGATGG CGGATATGTC TGGAACCCGT TTGCCACAAG
   CTCGCTACAG CTTTGGGCA CGATACTACC GCCTATACAG ACCTTGGCA AACGGTGTTC
   . S V S T D R Q L S A F S P Q E R L A I A .
901 AAGCGTCAGC ACGGATAGAC AACTGTCAGC GTTTAGCCCG CAAGAAAGAC TGGCAATCGC
   TTGCGAGTCG TGCCTATCTG TTGACAGTCG CAAATCGGGC GTTCTTTCTG ACCGTTAGCG
   . G N P L L A Q A V A S P M A R R S A S P .
961 CGGAATCCG CTTTGGCAC AAGCAGTTGC TTCACCGATG GCAAGAAGAT CAGCAAGCCC
   GCCTTTAGGC GAAAACCGTG TTCGTCAACG AAGTGGCTAC CGTTCTTCTA GTCGTTCCGG
   . L N C E G K M F W D Q V H P T T V V H A .
1021 GCTGAATTGC GAAGGCAAAA TGTTTTGGGA TCAGGTCCAT CCGACAACAG TTGTCCATGC
   CGACTTAACG CTTCCGTTTT ACAAACCCCT AGTCCAGGTA GGCTGTTGTC AACAGGTACG
   . A L S E R A A T F I E T Q Y E F L A H G .
1081 TGCCCTTTCA GAAAGAGCGG CGACGTTTAT CGAAACACAG TATGAATTC TGGCCCATGG
   ACGGGAAAGT CTTTCTCGCC GCTGCAAATA GCTTTGTGTC ATACTTAAAG ACCGGGTACC
   .stop
1141 CTGAGTTAAC AGAGGACGGA TTTCTGAAG GAAATCCGTT TTTTATTTT AAGCTTGGAG
   GACTCAATTG TCTCCTGCCT AAAGGACTTC CTTTAGGCAA AAAAATAAAA TTCGAACCTC
1201 ACAAGGTAAA GGATAAAACC TCGAG
   TGTTCATT TCTATTTTGG AGCTC
```



FIG. 140

