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Synthesis of designer lipids using papaya (*Carica papaya*) latex lipase

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Abstract

Ethyl esters (EE) of C_2 to C_{14} saturated acids were interesterified with tripalmitin using papaya (*Carica papaya*) lipase to produce structured triacylglycerols (TG) with palmitoyl moieties in the secondary (*sn*-2), and short-chain or medium-chain acyl moieties in the primary (*sn*-1,3) positions. It was found that the incorporation of the acyl moieties rose with time and chain length of the ethyl ester. Little reaction occurred with ethyl acetate. The positional analysis of the structured TG formed revealed an increase in preference of the lipase for the primary positions as compared to the secondary position with increasing chain length of the acyl donor from C_2 to C_{14}.

Keywords: Acyl donor; Ethyl ester; Interesterification; Lipase; Papaya; Structured lipids

1. Introduction

Lipases are useful enzymes for several synthetic reactions. However, their immense industrial potential has not been translated into reality, primarily due to their cost (Gandhi [1]). From this point of view, lipases obtained from plants are relatively inexpensive, readily available and regenerable, and are generally more acceptable for food applications.

Latex of papaya (*Carica papaya*) containing the proteolytic enzymes, papain and chymopapain, has been used in food and beverage industries over a large part of this century. Less well known, but not less significant, is the lipolytic activity found in crude papaya latex (Giordani et al. [2]). The ability of the papaya lipase to function at moderately high temperatures and in water-immiscible organic solvents makes this lipase attractive for employment in predominantly nonaqueous media for synthetic reactions, such as esterification and transesterification (Villenueve et al. [3–5], Mukherjee and Kiewitt [6,7], Foglia and Villeneuve [8]). The reported typoselectivity of the *Carica papaya* lipase (CPL) towards shorter-chain fatty acids (FA) (Giordani et al. [2], Villenueve et al. [4]), *sn*-3 regioselectivity (Villenueve et al. [3]), and preference for FA having *cis*-5 and *cis*-9 unsaturation, while discriminating against those with *cis*-4, *cis*-6, and *cis*-8 double bonds (Mukherjee and Kiewitt [6]) could be tapped for the synthesis of designer lipids containing a definite fatty acid composition and/or positional distribution.

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One such structured lipid that is currently of great interest is the human milk fat replacer for use in infant food formulations (Fitch Haumann [9], Kavanagh [10], Jensen [11]). For a lipid to be used for this function, it should mimic the fatty acid composition of human milk triacylglycerols (TG). The latter contain palmitic acid predominantly (about 70 %) esterified at the *sn*-2 position. This unique positioning of palmitoyl moieties at the *sn*-2 position is of considerable significance and consequence for the absorption of human milk fat in infants (Kavanagh [10], Jensen [11], Akoh [12]). TG containing short-chain and medium-chain fatty acyl moieties are an instant source of energy for infants and stressed adults (Quinlan [13]) since these are readily absorbed as free FA, which can be directly catabolized via energy-yielding pathways. It is therefore envisaged that structured TG containing palmitic acid esterified at the *sn*-2 position and short- or medium-chain FA at the *sn*-1,3 positions should be useful products for infant nutrition and dietetics. Based on earlier work from this laboratory (Mukherjee and Kiewitt [7]), synthesis of the above type of structured TG was attempted by using the regio-selective and typo-selective, thermostable and inexpensive papaya lipase in the interesterification reaction between tripalmitin and ethyl esters (EE) of short-chain and medium-chain FA.

2. Experimental

2.1. Chemicals

Crude preparation of papaya (*Carica papaya*) latex, CPL, was obtained from Sigma-Aldrich-Fluka, Deisenhofen, Germany. The granular CPL preparation was ground in a mortar with pestle to a fine powder and sieved to 0.8 mm mesh size. The Grignard reagent (ethylmagnesium bromide), methyl myristate, methyl stearate, ethyl laurate, ethyl myristate, ethyl oleate, ethyl caproate, ethyl caprylate, tripalmitin, and 2′,7′-dichlorofluorescein were also procured from Sigma-Aldrich-Fluka. Solvents, ethyl caprate, ethyl butyrate, ethyl acetate and Silica Gel 60 were purchased from E. Merck, Darmstadt, Germany. Trimethylsulfonium hydroxide (TMSH) was procured from Macherey-Nagel, Düren, Germany.

2.2. Interesterification

To a mixture of 404 mg tripalmitin (0.5 mmole) and 1 mmole of the ethyl ester taken in a magnetically stirred, teflon-lined, screw-capped reaction vial, CPL (27 mg) was added. The temperature was maintained at 63°C (66°C in case of ethyl myristate and ethyl oleate). Sample aliquots of 50 μl were withdrawn at specified intervals during the 24-h reaction period, taken up in dichloromethane, centrifuged to remove the residual CPL granules, and the supernatants analyzed further.

2.3. Lipid analysis

Aliquots of reaction products to which a known amount of methyl stearate, the internal standard, was added were fractionated by thin-layer chromatography (TLC) on 0.3 mm Silica Gel H plates to separate the various lipid components (ethyl plus methyl esters, TG, diacylglycerols (DG) and monoacylglycerols (MG), as well as unesterified FA) using isohexane: diethyl ether: acetic acid (80:20:1, v/v/v). Chromatoplates were dried and sprayed with 0.1 % solution of 2′,7′-dichlorofluorescein in ethanol and the different lipid components marked under ultraviolet light. The latter were scraped off and converted to methyl esters using a mixture of methanol and concentrated sulfuric acid (24:1, v/v) at 80°C for an hour with occasional shaking. The methyl esters were extracted using isohexane, the extract washed till neutral with distilled water, and the organic layer dried and used for gas chromatography after adding a known amount of methyl myristate or heptadecanoate as internal standard, to determine the relative proportion of individual lipid classes in the reaction products. The techniques used were essentially similar to those described by Christie [14] with a precision and coefficient of variation similar to those for GC analysis (Firestone and Horwitz [15]).

2.4. Gas chromatography (GC)

A Hewlett-Packard (Böblingen, Germany) HP-5890 Series II instrument equipped with a flame ionization detector was used. Fatty acid methyl esters of lipids were separated on a 0.25 μm CS-FFAP-CB

free fatty acid phase column (25 m × 0.25 mm i.d., J&W, ASS-Chem, Bad Homburg, Germany) using hydrogen as the carrier gas (linear velocity 20 cm s^{-1}) and linear temperature programming from 160°C to 240°C at 4°C min^{-1}, followed by 5 min at 240°C. The split ratio was 1:10 and the injector as well as flame ionization detector temperatures 270°C. Peak areas and percentages were calculated using a Hewlett-Packard PC Integration Pack (HP 3365 Series ChemStation Version A.03.21) using response factors. Total incorporation of acyl moieties from the EE into TG was calculated from the amount of ethyl palmitate formed, which was in turn determined from the peak areas of ethyl palmitate and the internal standard, methyl stearate, in TLC ester fractions consisting of methyl stearate, newly formed ethyl palmitate and unreacted EE. Methyl myristate was used as the internal standard for determining the relative proportions of the individual lipid classes in all cases except for reactions with ethyl oleate and ethyl myristate when methyl heptadecanoate was used as internal standard.

2.5. Grignard hydrolysis and positional analysis

The TG isolated by TLC of the products after 24 h of reaction were subjected to partial Grignard degradation (Christie [14]) to determine the composition of acyl moieties esterified at the secondary and primary hydroxy positions of glycerol. The resultant mixture was subjected to TLC on 0.3 mm Silica Gel H plates (impregnated with 5 % boric acid) using isohexane: diethyl ether: acetic acid (70:30:1, v/v/v). The 1(3)-MG, 2-MG and 1,3-DG were scraped off and eluted with diethyl ether saturated with water. After removal of diethyl ether by evaporation, the acylglycerol fractions were redissolved in dichloromethane and analyzed by GC after derivatization to methyl esters using TMSH (Schulte and Weber [16]). The column used and conditions of GC were similar to before, but the temperature program was modified for the reactions with different ethyl esters (EE) as follows:

2:0-EE: 5 min at 28°C, followed by heating at 1°C min^{-1} to 30°C, and after 1 min, heating at 7°C min^{-1} to 220°C, finally at 220°C for 5 min;

4:0-EE: 5 min at 30°C, followed by heating at 7°C min^{-1} to 220°C, finally at 220°C for 5 min;

6:0-EE onwards: 5 min at 50°C, followed by heating at 7°C min^{-1} to 220°C, finally at 220°C for 5 min.

3. Results and discussion

3.1. Incorporation of acyl moieties into tripalmitin

Interesterification reactions were carried out between tripalmitin and EE of 2:0 to 14:0 and 18:1 acids using CPL as the enzyme catalyst. Incorporation of acyl moieties from the EE into tripalmitin was determined by the amount of ethyl palmitate formed with respect to the EE added:

$$\%\ \text{incorporation} = \frac{\text{moles ethyl palmitate formed}}{\text{mmoles ethyl ester added}} \times 100$$

No correction was made for hydrolysis; therefore, the data given are approximate estimates of the percentage incorporation of acyl moieties.

In Fig. 1, the acyl incorporation was plotted as a function of reaction time for different EE. Overall,

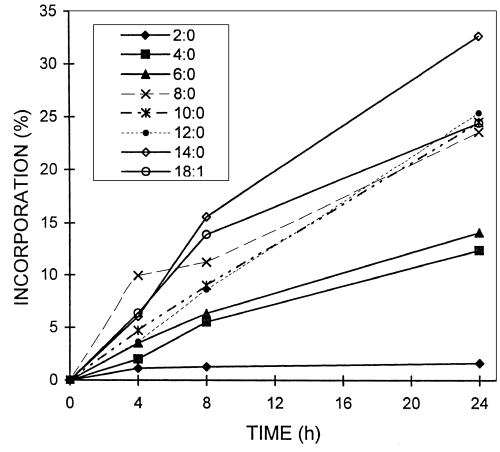

Fig. 1. Incorporation (%) of acyl moieties from ethyl esters into tripalmitin during interesterification with various ethyl esters, catalyzed by papaya lipase.

percent incorporation rose with time and chain length of the acyl donor. Ethyl oleate, however, showed some deviation from this trend (Fig. 1).

In case of reactions with EE longer than C_6 almost linear increases of acyl incorporation with time are clearly seen (Fig. 1). The acetyl moiety was poorly incorporated, indicating ethyl acetate to be a poor substrate for the papaya lipase.

Fig. 2 shows the variation of initial rate (calculated from the gradient of the tangent to each curve at time $t = 0$) with chain length of the ethyl ester.

It is clearly evident from Fig. 2 that the initial rate of incorporation increased with chain length indicating that CPL prefers long-chain (12:0, 14:0 and 18:1) and medium-chain (6:0 to 10:0) substrates to short-chain (2:0 and 4:0) ones. The highest rate was obtained in the case of reaction with ethyl myristate (Fig. 2). The rate of incorporation with ethyl oleate was slightly lower than the observed trend with C_2 to C_{14} ethyl ester series, and could be due to either the greater chain length or unsaturation, or both.

However, with increasing acyl chain length from 2:0 to 18:1, a decrease in hydrolysis was obtained by Giordani et al. [2] as opposed to increase in extent of interesterification with increasing chain length of the ethyl ester that was obtained in the present study (Figs. 1 and 2). Obviously, the parameters affecting hydrolytic reactions are different from those affecting the interesterification reactions implying that enzyme behavior may be reaction-specific (Muderwha et al. [17]).

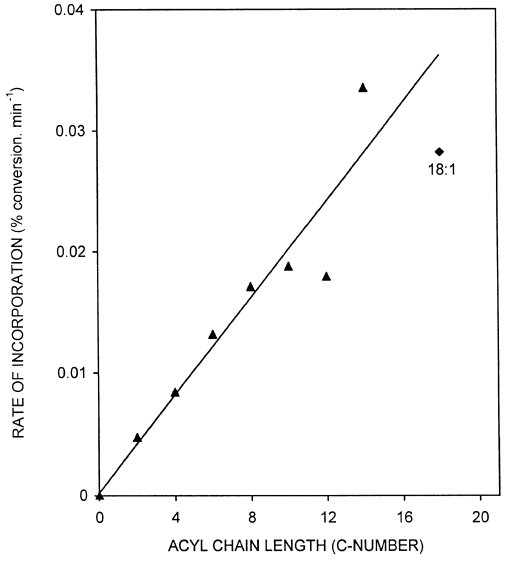

Fig. 2. Rate of acyl incorporation into tripalmitin as a function of chain length of the acyl donor during interesterification with ethyl esters catalyzed by papaya lipase.

The maximum conversion obtained was about 32 % with ethyl myristate after 24 h (Fig. 1). This modest conversion can be attributed to two factors. First, the ethyl ester is a poor acyl donor relative to other donors, such as free acids and vinyl esters (Villeneuve et al. [5], Schuch and Mukherjee [18], Millqvist Fureby et al. [19]). However, disadvantages, such as the higher tendency of acyl migration when free acids are used and acetaldehyde formation with vinyl esters, were the prime reasons for selection of EE as acyl donors in the present study.

3.2. Profile of lipid classes during reaction

The different components of the reaction products identified by TLC were the TG including tripalmitin, as well as the newly formed ones; EE fraction comprising unreacted ethyl ester, as well as newly formed ethyl palmitate; and the MG, and FA fractions, which resulted from hydrolysis taking place concomitantly (Figs. 3–5).

Of the various lipid components in the reaction products, the MG fraction was the smallest in all cases. In transesterification with the 2:0 to 8:0 EE, 0.6–1 % MGs were formed, while with the 10:0 to 14:0 and 18:1 EE, even lesser amounts of MGs were obtained (Figs. 3–5). Overall, the levels of TG containing palmitoyl moieties dropped from about 80 % after 4 h to 60 % after a 24-h reaction and correspondingly, the proportion of ethyl palmitate formed increased. It is evident from the rather high levels of DG and FA that a considerable degree of hydrolysis of TG and/or ethyl palmitate occurred in all cases (Figs. 3–5). However, it was noted that the greater the chain length of the acyl donor, the lower was the level of hydrolysis products and the higher the levels of ethyl palmitate formed. This appears to be in agreement with Giordani et al. [2] who found that degree of ester hydrolysis decreases with increasing chain length. However, it cannot be ruled out that the observed effect of chain length of the acyl donor on

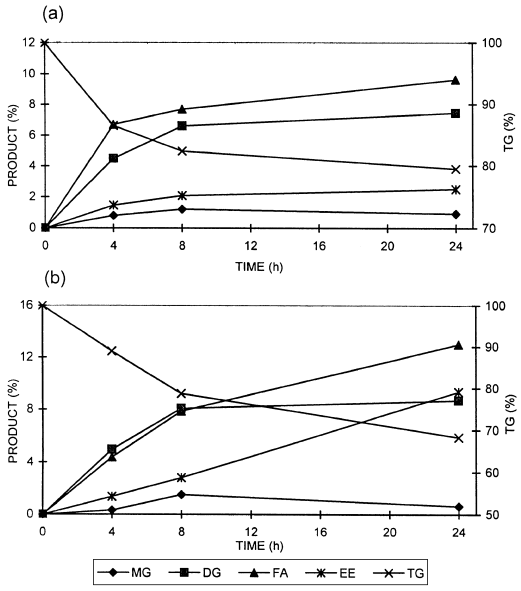

Fig. 3. Composition of lipid classes in reaction products during interesterification of tripalmitin with short-chain ethyl esters, catalyzed by papaya lipase (TG = Triacylglycerols; DG = Diacylglycerols; MG = Monoacylglycerols; FA = Fatty Acids; EE = Ethyl Esters). (a) Incorporation of acetyl moieties. (b) Incorporation of butyryl moieties.

the hydrolysis was partly due to differences in water activities of the system (Bell et al. [20]).

3.3. Positional analysis of the TG

TG fractions in the products obtained after a 24 h reaction of tripalmitin with different EE using the papaya lipase as biocatalyst were isolated, purified and subjected to partial Grignard degradation. The 1(3)-MG, 2-MG and 1,3-DG obtained were isolated by TLC and analyzed by GC as described in the Experimental section. The composition of 2-acylglycerols was taken to calculate the incorporation of acyl moieties at the *sn*-2 position of the TG. Composition of acyl moieties at the *sn*-1,3 positions of TG was calculated from the average of the composition of 1(3)-MG and that of the 1,3-DG. Results obtained for the percent incorporation of the short-chain, medium-chain and long-chain acyl moieties in the primary and secondary positions of TG are shown in Table 1.

It can be clearly seen from Table 1 that the regio-preference of the papaya lipase for the primary positions relative to the secondary one, expressed as a ratio of acyl moieties incorporated into *sn*-1,3 to those incorporated in *sn*-2 position, is a function of chain length of the acyl donor. The regiopreference increases with the increase in chain length of the ethyl ester. Ethyl esters with chain length between C_8 and C_{14} are the acyl donors on which CPL exercises its regioselectivity the best compared with other EE. It should also be noted that considerable incorporation of the acyl moieties was found in the *sn*-2 position which may be explained by acyl migration during transesterification (Millqvist Fureby et al. [19], Serdarevich [21], Xu et al. [22]).

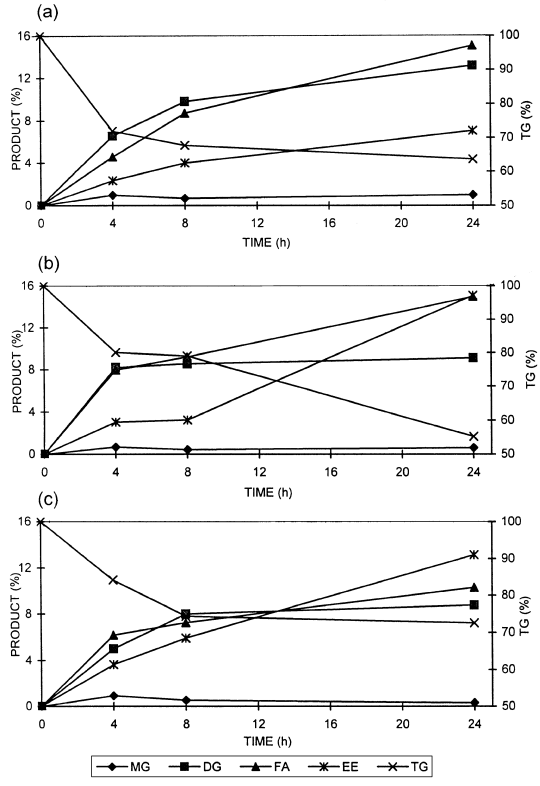

Fig. 4. Composition of lipid classes in reaction products during interesterification of tripalmitin with medium-chain ethyl esters, catalyzed by papaya lipase (TG = Triacylglycerols; DG = Diacylglycerols; MG = Monoacylglycerols; FA = Fatty Acids; EE = Ethyl Esters). (a) Incorporation of caproyl moieties. (b) Incorporation of caprylyl moieties. (c) Incorporation of capryl moieties.

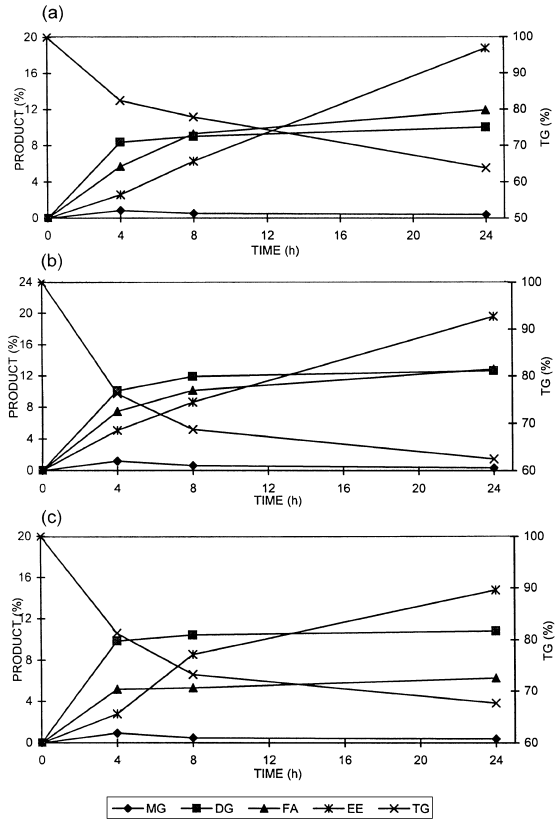

Fig. 5. Composition of lipid classes in reaction products during interesterification of tripalmitin with long-chain ethyl esters, catalyzed by papaya lipase (TG = Triacylglycerols; DG = Diacylglycerols; MG = Monoacylglycerols; FA = Fatty Acids; EE = Ethyl Esters). (a) Incorporation of lauroyl moieties. (b) Incorporation of myristoyl moieties. (c) Incorporation of oleoyl moieties.

At first sight, the present results appear to be in direct contrast to the *sn*-3 and short-chain fatty acid specificity reported earlier (Giordani et al. [2], Villeneuve et al. [3]) and selective incorporation of unsaturated FA into the primary position of tripalmitin observed recently (Mukherjee and Kiewitt [7]). This could possibly reflect reactant-dependent positional specificity of lipases (Seriburi and Akoh [23,24]).

4. Conclusions

The present study shows that the papaya lipase has a greater preference for the medium-chain and long-chain EE as compared to short-chain ones as acyl donors in the interesterification with tripalmitin. The medium-chain and long-chain acyl moieties are esterified preferentially to the primary hydroxy positions. Also, the papaya lipase is active at high temperatures, and functional in low-water environments. These properties make it an attractive lipase for the synthesis of structured TG for infant food formulations wherein medium-chain acyl moieties are desired in the primary positions. Besides, the lipase preparation used for the synthesis of structured lipids was the crude papaya latex, with the enzyme associated naturally with the latex. This naturally immobilized lipase has the added advantages of immobilized enzymes, such as facile recovery and reuse, making it even more attractive for industrial employment.

Table 1
Positional analysis of acyl moieties incorporated into *sn*-1,3 and *sn*-2 positions of triacylglycerols after 24 h interesterification of tripalmitin with various ethyl esters, catalyzed by papaya lipase

Acyl moiety	Distribution (%) of acyl moieties			*sn*-1,3/*sn*-2 ratio
	Triacylglycerols	*sn*-2 position	*sn*-1,3[a] positions	
2:0	4.2	8.9	1.6	0.2
4:0	8.6	19.8	7.6	0.4
6:0	16.5	19.1	20.7	1.1
8:0	17.5	23.3	30.6	1.3
10:0	17.1	16.7	25.5	1.5
12:0	22.5	16.0	27.2	1.7
14:0	24.0	14.9	27.9	1.9
18:1	12.8	12.2	20.0	1.6

[a]This value was obtained from the average experimental values of 1(3)-MG and 1,3-DG.

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References

[1] N.N. Gandhi, J. Am. Oil Chem. Soc. 74 (1997) 621.
[2] R. Giordani, A. Moulin, R. Verger, Psytochemistry 30 (1991) 1069.
[3] P. Villeneuve, M. Pina, D. Montet, J. Graille, J. Am. Oil Chem. Soc. 72 (1995) 753.
[4] P. Villeneuve, M. Pina, A. Skarbek, J. Graille, T.A. Foglia, Biotechnol. Tech. 11 (1997) 91.
[5] P. Villeneuve, A. Skarbek, M. Pina, J. Graille, T.A. Foglia, Biotechnol. Lett. 11 (1997) 637.
[6] K.D. Mukherjee, I. Kiewitt, J. Agric. Food Chem. 44 (1996) 1948.
[7] K.D. Mukherjee, I. Kiewitt, Biotechnol. Lett. 20 (1998) 613.
[8] T.A. Foglia, P. Villeneuve, J. Am. Oil Chem. Soc. 74 (1997) 1447.
[9] B. Fitch Haumann, Int. News Fats, Oils Relat. Mater. 8 (1997) 1004.
[10] A.R. Kavanagh, J. Franc, Oleagineux, Corps Gras, Lipides 4 (1997) 165.
[11] R.G. Jensen, Lipid Technol. 12 (1998) 34.
[12] C.C. Akoh, Lipid Technol. 11 (1997) 61.
[13] P. Quinlan, Proc. 21st World Congr. Int. Soc. Fat Res. (ISF), The Hague, Vol. 3, P.J. Barnes & Assoc., UK, 1996, p. 587.
[14] W.W. Christie, Lipid Analysis, 2nd edn., Pergamon, London, 1982.
[15] D. Firestone, W. Horwitz, J. Assoc. Off. Anal. Chem. 62 (1979) 709.
[16] E. Schulte, K. Weber, Fat Sci. Technol. 91 (1989) 181.
[17] J. Muderwha, M. Pina, J. Graille, Oleagineux 43 (1988) 385.
[18] R. Schuch, K.D. Mukherjee, J. Agric. Food Chem. 35 (1987) 1005.
[19] A.M. Millqvist Fureby, P. Adlercreutz, B. Mattiasson, J. Am. Oil Chem. Soc. 73 (1996) 1489.
[20] G. Bell, P.J. Halling, B.D. Moore, J. Partridge, D.G. Rees, Trends Biotechnol. 13 (1995) 468.
[21] B. Serdarevich, J. Am. Oil Chem. Soc. 44 (1967) 381.
[22] X. Xu, A.R.H. Skands, C.-E. Hoy, H. Mu, S. Balchen, J. Adler-Nissen, J. Am. Oil Chem. Soc. 75 (1998) 1179.
[23] V. Seriburi, C.C. Akoh, J. Am. Oil Chem. Soc. 75 (1998) 511.
[24] V. Seriburi, C.C. Akoh, J. Am. Oil Chem. Soc. 75 (1998) 711.