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PURIFICATION OF SEMINOLIPID AND ITS CLEAVAGE TO LYSO-SEMINOLIPID. INTERACTION OF SEMINOLIPID WITH PHOSPHOLIPASE A2

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Seminolipid was purified from boar sperm and characterized by chemical, chromatographical and spectroscopical (IR, NMR) means. Phospholipase A2 from two sources were used in this experiment: from viper (*Vipera ammodytes*) venom, and bull seminal vesicle. It was proved that seminolipid cannot constitute a biochemical substrate for the two phospholipases. Two substrates were used for the two enzymes, egg yolk lecithin for viper venom phospholipase A2 and [^{14}C-linoleoyl]phosphatidyl ethanolamine for bull seminal vesicle phospholipase A2. The action of seminolipid on the latter enzyme, in quantitative terms, is presented. A small portion of seminolipid was submitted to mild alkaline hydrolysis and lyso-seminolipid recovered from the mixture was peracetylated and its ^{1}H and ^{13}C NMR spectra registered.

INTRODUCTION

Seminolipid (1-O-alkyl-2-O-acyl-3-(β-D-(3'-sulfo)galactopyranosyl-*sn*-glycerol) is an important representative of glycoglycerolipids, a sub-group of the extremely vast group, glycolipids.[1] It was discovered by a group of Japanese glycobiologists[2] in a tight scientific international competition with other laboratories and named "the lipid of semen" – seminolipid. Seminolipid is the major glycolipid of spermatozoa from the following mammals: human, swine, bovine, ovine, rabbit, guinea-pig, rat, mice.[3] Investigations concerning lipidic moiety metabolism of brain seminolipid indicated that in new-born rats, 1,2-di-O-fatty acyl molecular species is predominant, they being gradually replaced by 1-O-alkyl 2-O-acyl, till the ratio is completely reversed in the favor of the latter one.[4] More precisely, in the day 19 the concentration of diacyl species is double the concentration of 1-O-alkyl 2-O-acyl, while in the day 48 the latter one is more than four times higher.[5] At least two different enzymes are directly involved in the metabolism of seminolipid concerning its electric charge: an (aryl)sulfatase hydrolyses it to 1-O-alkyl-2-O-acyl-3-β-D-galactopyranosyl-*sn*3-glycerol,[6] while the neutral compound is converted to seminolipid by cerebroside sulfotransferase, sulfate donor being 3'-phosphoadenosyl 5'-phosphosulfate (PAPS).[3,7,8]

In this paper, ^{1}H and ^{13}C NMR spectra of seminolipid and lyso-seminolipid, both in peracetylated form, have been registered. Moreover, the interaction of seminolipid with phospholipase A2 from two biological sources, viper (*Vipera ammodytes*) venom and bull seminal vesicle was studied and measured.

RESULTS

Lipidic solution of the lower phases, after spermatozoa extraction and Folch partition, was chromatographed on a Florisil column, elution being made with a gradient of methanol in chloroform. Fractions were analyzed by TLC and for the presence of lipid bound sugar (glycolipids). A mixture of a remarcable diversity in lipids and glycolipids – inevitably connected with the complex biological functions of spermatozoa – has been found by TLC. As is evident from the

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chromatogram of Fig. 1 (the lane labeled *Am*), the mixture of all lipids contained compounds with increasing polarity from cholesterol esters and triglycerides through phospholipids to gangliosides.[9-11] The fractions containing glycolipids were mixed and applied on a column of DEAE-Sephadex A-25 (acetate form). The unbound lipids were washed with four vols of chloroform-methanol-water, 30/60/8 (v/v) and then, glycolipids eluted with chloroform-methanol-aq. sodium acetate, 30/60/8, (v/v) (Fig. 2) were mixed, dialysed against distilled water and the content of the dialysis bag concentrated to dryness by rotavapor. The residue was resumed in a small volume of chloroform-methanol and applied on a column of silicagel. Fractions containig lipid bound sugar were mixed and analyzed. A small sample was submitted to mild alkaline hydrolysis and the produced glycolipid separated by column chromatography of silica gel. As is evident from Fig. 3, both seminolipid and lyso-seminolipid produced only one spot in three different solvent systems.

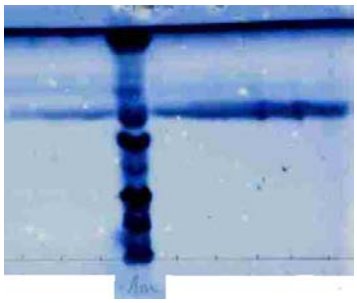

Fig. 1 – TLC Analysis of fractions obtained by purification of seminolipid by Florisil column chromatography. Migration, SS I, chloroform-methanol-water, 60/25/4 (v/v). Visualization, mostain. The lane marked by Am indicates the initial mixture.

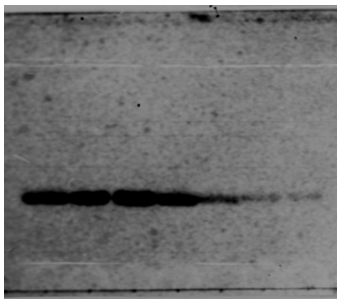

Fig. 2 – Fractions from seminolipid purification by ion-exchange chromatography on DEAE-Sephadex A-25, as monitored by TLC. Migration, SS II, chloroform-methanol-water, 50/10/1 (v/v); visualization, orcinol.

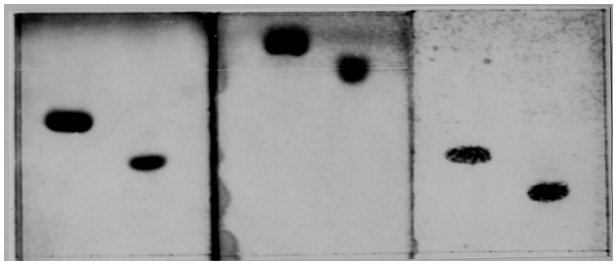

Fig. 3 – Purity of seminolipid and lyso-seminolipid, as checked by TLC. Start 1, on all three plates, seminolipid; start 2, on all three plates, lyso-seminolipid; migration: plate 1, SS III, chloroform-methanol-water-conc. ammonia, 70/30/3/1 (v/v); plate 2, SS IV, n-propanol-water, 8:2 (v/v); plate 3, SS V, chloroform-methanol-acetone-acetic acid-water, 10/2/4/2/1 (v/v); visualisation, orcinol.

NMR spectra of peracetylated seminolipid (structure in Fig. 4). The ^{1}H- and ^{13}C-NMR data support the indicated structures. The assignments presented below have been made by comparison with previous literature data for related compounds.[12-14]

^{1}H-NMR ($CDCl_3$; δ, ppm): 3.75 (H-1a, 1H), 3.54 (H-1b, 1H), 4.75 (H-2, 1H), 3.71 (H-3a, 1H), 3.52 (H-3b, 1H), 4.69 (d, 6.4, H-1', 1H), 5.07 (H-2', 1H), 6.0 (H-3', 1H), 5.2 (H-4', 1H), 5.05 (H-5', 1H), 3.72 (H-6'a, 1H), 3.43 (H-6'b, 1H), 2,27 (t, 7.2, H-2''', 2H), 1.25 (s, methylene groups of alkyl and acyl group), 1.90, 2.03, 2.10 (s, methyl groups of acetate esters, 9H), 0.88 (t, 6.8, methyl groups of alkyl and acyl chain, 3H).

^{13}C-NMR ($CDCl_3$): 69.34 (C-1), 77.33 (C-2), 71.45 (C-3), 101.23 (C-1'), 69.95 (C-2'), 69.49 (C-3'), 69.49 (C-4'), 68.41 (C-5'), 68.41 (C-6'), 14.11 (C''-16 and C'''-16), 22.69 (methylene groups of alkyl and acyl chain), 29.19-29.73 (methylene groups of alkyl and acyl chain), 169.12, 170.3, 171.5, 172.8 (C=O group of acetate ester).

NMR spectra of peracetylated lyso-seminolipid (Fig. 4): ^{1}H-NMR ($CDCl_3$; δ, ppm): 3.75 (H-1a, 1H), 3.53 (H-1b, 1H), 4.7 (H-2, 1H), 3.97 (H-3a, 1H), 3.66 (H-3b, 1H), 5.75 (d, 5.6, H-1', 1H), 5.1 (H-2', 1H), 6.0 (H-3', 1H), 5.35 (H-4', 1H), 5.37 (H-5', 1H), 3.74 (H-6'a, 1H), 3.45 (H-6'b, 1H), 2,43-2.54 (m, H-2''-H-4'', 4H), 1.26 (s, methylene groups of alkyl group), 2.07, 2.11, 2.18 (s, methyl groups of acetate esters, 12H), 0.88 (t, 6.8, methyl groups of alkyl chain, 3H).

^{13}C-NMR ($CDCl_3$): 107.31 (C-1'), 68.93-77.33 (C-1 – C-3 and C-2' – C-5'), 14.1 (C''-16), 29.34-29.69 (methylene groups of alkyl chain), 169.3, 170.38, 170.79, 171.8 (C=O group of acetate ester).

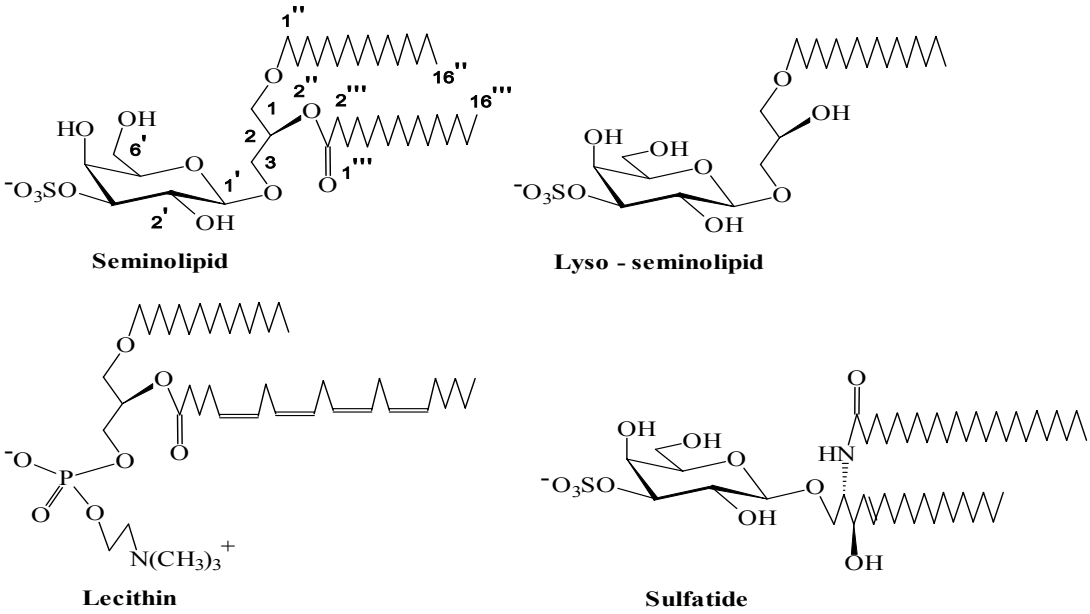

Fig. 4 – Comparative structure of some representatives of glycoglycerolipids, phospholipids, glycosphingolipids. (The same numbering system is valid for lyso-seminolipid).

IR Spectra of seminolipid: 3500-3250 cm^{-1} (hydroxy groups of D-galactose), 1750 cm^{-1} (carboxyl ester), 1250, 875 cm^{-1} (sulfate ester).
IR Spectra of lyso-seminolipid: 3500-3250 cm^{-1} (hydroxy groups of D-galactose and C-2 of glycerol), 1750 cm^{-1} (carboxyl ester), 1240, 880 cm^{-1} (sulfate ester).

The interaction of seminolipid with phospholipase A2. The interaction of seminolipid with phospholipase A2 from bull seminal vesicle, by using [^{14}C-linoleoyl]phosphatidyl ethanolamine as substrate, is indicated in Fig. 5.

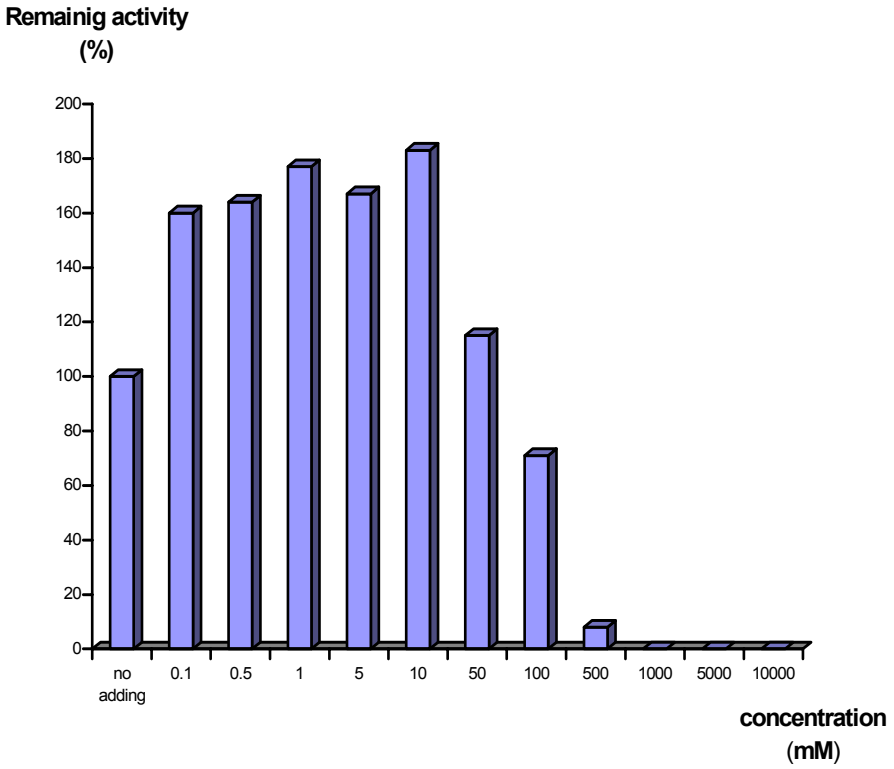

Fig. 5 – Influence of seminolipid on phospholipase A_2 purified from bovine seminal vesicle fluid.

DISCUSSION

Purification of seminolipid by a combination of adsorption and ion-exchange chromatography produced seminolipid and lyso-seminolipid of a remarcable purity (Fig. 3). Seminolipid was isolated and characterized for the first time by Ishizuka et al.,[2] from boar spermatozoa and testis. The same authors accidentally obtained small amounts of lyso-seminolipid as a secondary product of seminolipid de-sulfation.[2] ^{1}H NMR Spectra were used to confirm that fatty acyl group is attached to the *sn*-2 carbon atom of glycerol as well as for structure confirmation.[2]

A detailed ^{1}H NMR investigation of native seminolipid has been undertaken.[12] In our paper, peracetylated form of seminolipid and lyso-seminolipid have been used for ^{1}H and ^{13}C NMR spectra registration. There have been a good agreement between our NMR results concerning seminolipid and the results of others,[13,14] both about peracetylated 1,2-di-O-acyl-3-β-D-(2',3',4',6'-tetra-O-acetyl)galactopyranosyl-*sn*-glycerol. In case of peracetylated lyso-seminolipid, a unexpected high value have been obtained for C-1' (107.31).

An entirely chemical synthesis of seminolipid has been accomplished[15] by using alkenyl ethers (2-butenyl, allyl) as protecting groups. This synthesis has had at least two important implications: on one hand confirmation of structure and chirality of seminolipid and, on the other hand, the increasing of potential of choice structural modifications or labelling of seminolipid.

Surprisingly, seminolipid and sulfatide (galactocerebroside 3-sulfate) (Fig. 4) are produced in living organisms by the same enzymes: *sn* 1,2-di-O-acylglycerol (or *sn* 1-O-alkyl 2-O-acylglycerol) and ceramide are glycosylated by an UDP-galactose:ceramide galactosyltransferase to the corresponding glycolipids.[7] A cerebroside sulfotransferase converts *sn* 1,2-di-O-acylglycerol 3-β-D-galactopyranoside (or *sn* 1-O-alkyl 2-O-acylglycerol 3-β-D-galactopyranoside) to seminolipid and galactocerebroside to sulfatide.[8,16] On the other hand, hydrophobic moiety of seminolipid is similar to phospholipids *e. g.*, lecithin (Fig. 4). However, in conditions where lecithin was completely cleaved by venom phospholipase A2, seminolipid remained intact, and the same results were obtained when phospholipase A2 from bull seminal vesicle was alternatively incubated with seminolipid and phosphatidyl ethanolamine. A conclusion was drawn, that seminolipid cannot be cleaved by the two phospholipases A2 used in this paper. As is evident from Fig. 5, relatively small amounts of seminolipid play an activation role while higher concentrations are inhibitory.

EXPERIMENTAL

Materials

[^{14}C-Linoleoyl]phosphatidyl ethanolamine, $CDCl_3$ containing TMS, egg yolk lecithin, Florisil, DEAE-Sephadex A-25 and dialysis bag were from Sigma. Acetic anhydride, pyridine, ready-to-use glass plates covered with silica gel 60 for thin layer chromatography (TLC), silica gel for column chromatography, were from Merck. Venom from viper (*Vipera ammodytes*), a male and a female, both adults, was collected by slight electrical discharges in aseptic conditions. Phospholipase A2 from bull seminal vesicle was purified by classical means. Whole boar semen was obtained from Peris Institute, Romania, and spermatozoa were separated by repeated cycles of centrifugation, decantation, resuspension in a buffered isotonic saline.

Methods

NMR Spectra Registration

^{1}H and ^{13}C NMR spectra of seminolipid and lyso-seminolipid were registered in peracetylated form in $CDCl_3$ containing TMS.

A.One-Dimensional NMR Studies

NMR experiments were performed on a Bruker Avance DRX 400 spectrometer using 400 and 100 MHz for ^{1}H and ^{13}C frequencies, respectively.

B.Two-Dimensional NMR Experiments

The ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe.

IR spectra were recorded as KBr pellets on a Bruker Equinox 55 FT-IR spectrometer.

Extraction and Folch partition

The sediment of cells from the last centrifugation was repeatedly extracted with chloroform-methanol mixtures and then submitted to Folch Partition.[17,18] Finally, all lower phases were mixed and they constituted the main source of seminolipid, while all upper phases were used for purification of spermatozoa gangliosides (D. P. Iga, to be published). Sulfatide from bovine brain[18] was used to monitor migration of seminolipid by thin layer chromatography.

Thin-Layer and Column Chromatography

The following solvent systems (SS) were used for TLC: chloroform-methanol-water, 60/25/4 (v/v, SS I); chloroform-methanol-water, 50:10:1 (v/v, SS II); chloroform-methanol-water-conc. ammonia, 70/30/3/1 (v/v, SS III); n-propanol-water, 8:2 (v/v, SS IV); chloroform-methanol-acetone-acetic acid-water, 10/2/4/2/1 (v/v, SS V). Visualization was made by dipping the plates in a solution of amonium molybdate, sulfuric acid and cerium(IV) sulfate, or in an orcinol/$FeCl_3$ reagent, followed by heating in both cases.

Column chromatography on Florisil or silica gel was made in a gradient of methanol in chloroform (0-40%) and ion-exchange chromatography on DEAE-Sephadex A-25 (acetate form) in a gradient of chloroform-methanol-water, 30/60/8 (v/v)-chloroform-methanol-1.2 M aq. sodium acetate, 30/60/8,

(v/v). Chromatographical materials were used in the following order: Florisil, DEAE-Sephadex A-25, silica gel.

Acidic Hydrolysis and Chemical Constituents

Acidic hydrolysis was accomplished by heating the glycolipid for 24 h at 80 ^{0}C in an ampule in dry 0.8 M HCl in methanol.[2] Methanolic mixture was extracted with n-hexane and in hexane phase fatty acids as methyl esters were determined by GLC.[19] Methanolic phase was evaporated to dryness, the residue solved in pyridine and peracetylated. Acetylated products, 1-O-alkyl 2,3-di-O-acetyl *sn*-glycerol and αβ-1-O-methyl-2,3,4,6-tetra-O-acetyl-D-galactopyranose, were separated by a small column of silica gel in a gradient of ethanol in toluene and then submitted to mild alkaline hydrolysis. 1-O-Methyl-D-galactopyranose was determined by anthrone reaction[17,18] and 1-O-alkyl *sn*-glycerol by periodate oxidation.[18,20] A small portion of methanolic phase was repeatedly evaporated with water in order to remove HCl and inorganic sulfate was determined in the residue by rhodizonate method.[21]

Phospholipase A2 determination

Enzymatic activity determination of phospholipase A2 was made as indicated by.[22] Initially, it was proved that seminolipid did not constitute a substrate for this enzyme. In case of [^{14}C-linoleoyl]phosphatidyl ethanolamine, the released [^{14}C]-linoleic acid was separated before being counted.

CONCLUSIONS

A remarcable purity of seminolipid was accomplished by a combination of adsorption and ion-exchange chromatography; mild alkaline hydrolysis of seminolipid prod uced lyso-seminolipid.

The structure of seminolipid and lyso-seminolipid was confirmed by chemical, chromatographical and spectroscopical methods.

The action of seminolipid on phospholipase A2 from bull seminal vesicle was dependent on concentration: relatively small amounts played an activation role, while higher ones were inhibitory.

REFERENCES

1. R. K. Murray and R. Narasimhan, "Handbook of Lipid Research: Glycolipids, Phosphoglycolipids and Sulfoglycolipids", vol. 6., M. Kates, (Ed.), Plenum Press, New York, 1990, p. 321-365.
2. I. Ishizuka, M. Suzuki and T. Yamakawa, *J. Biochem. (Tokyo)*, **1973**, *73*, 77- 87.
3. A. Knapp, M. J. Kornblatt, H. Schachter and R. K. Murray, *Biochem. Biophys. Res. Com*., **1973**, *55*, 179-186.
4. I. Ishizuka, M. Inomata, K. Ueno and T. Yamakawa, *J. Biol. Chem*., **1978**, *253*, 898-907.
5. I. Ishizuka and M. Inomata, *J. Neurochem*., **1979**, *33*, 387-388.
6. A. L. Fluharty, R. L. Stevens, R. T. Miller and H. Kihara, *Biochem. Biophys. Res. Commun*., **1974**, *61*, 348-354.
7. H. Fujimoto, K. Tadano-Aritomi, A. Tokumasu, K. Ito, T. Hikita, K. Suzuki and I. Ishizuka, *J. Biol. Chem*., **2000**, *275*, 22623–22626.
8. K. Honke, Y. Hirahara, J. Dupree, K. Suzuki, B. Popko, K. Fukushima, J. Fukushima, T. Nagasawa, N. Yoshida, Y. Wada and N. Taniguchi, *Proc. Natl. Acad. Sci. U. S. A*., **2002**, *99*, 4227–4232.
9. D. P. Iga and S. Iga, *Analele Univ Bucuresti*, **1996**, XLV, 9-11.
10. D. P. Iga, S. Iga and V. Mihaila, *Analele Univ. Bucuresti*, **1995**, XLIV, 13-17.
11. S. Iga and D. P. Iga, *Analele Univ Bucuresti*, **1994**, XLIII, p. 26-27.
12. J. G. Alvarez, B. T. Storey, M. L. Hemling and R. L. Grob, *J. Lipid Res*., **1990**, *31*, 1073-1081.
13. P. Jorasch, D. C. Warnecke, B. Lindner, U. Zähringer and E. Heinz *Eur. J. Biochem*., **2000**, *267*, 3770-3783.
14. G. Ben-Menachem, J. Kubler-Kielb, B. Coxon, A. Yergey and R. Schneerson, *Proc. Natl. Acad. Sci. U. S. A*., **2003**, *100*, 7913-7918.
15. R. Gigg, *J. Chem. Soc. Perkin Trans. I*, **1978**, 712-718.
16. K. Honke, M. Tsuda, Y. Hirahara, A. Ishii, A. Makita and Y. Wada, *J. Biol. Chem*., **1997**, *272*, 4864-4868.
17. D. P. Iga, S. Iga, T. Larsson, J. Angstrom, B. Soussi, O. Rakotonirainy and H. Miller-Podraza, *Glycoconjugate J*., **1998**, *15*, 1111-1114.
18. D. P. Iga and S. Iga, *Open Org. Chem. J*., **2008**, *2*, 46-51.
19. A. A. AbuGhazaleh, D. J. Schingoethe, A. R. Hippen, K. F. Kalscheur and L. A. Whitlock, *J. Dairy Sci*., **2002**, *85*, 2266-2276.
20. L. Voris, G. Ellis and L. A. Maynard, *J. Biol. Chem*., **1940**, *133*, 491-498.
21. A. M. Khorrami, A. Choudhury, M. Andrianifahanana, G. C. Varshney, S. N. Bhattacharyya, M. A. Hollingsworth, B. Kaufman and S. K. Batra, *J. Biochem*., **2002**, *131*, 21-29.
22. M. Kates "Techniques in lipidology. Isolation, analysis and identification of lipids", vol. 3, T. S. Work and E. Work (Eds.), North-Holland Publishing Co. Amsterdam, 1972, p. 269-610.