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# Hydrophilic interaction chromatography of intact, soluble proteins

Tim Tetaz[a,*], Simon Detzner[a], Arno Friedlein[b], Birgit Molitor[c], Jean-Luc Mary[c]

[a] F. Hoffmann-La Roche Ltd, Discovery Technologies, PROBMX21, Grenzacherstrasse 124, CH-4070 Basel, Switzerland
[b] F. Hoffmann-La Roche Ltd, Translational Research Sciences, PRLP, Grenzacherstrasse 124, CH-4070 Basel, Switzerland
[c] F. Hoffmann-La Roche Ltd, Metabolic and Vascular Research, PRDM, Grenzacherstrasse 124, CH-4070 Basel, Switzerland

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ABSTRACT

The separation of intact proteins by means of Hydrophilic Interaction Chromatography (HILIC) was demonstrated with human apoA-I, recombinant human apoM, and equine cytochrome C. Five different commercially available HILIC columns were compared. Using one of these columns, different glycosylated isoforms of apoM were separated from each other and from the aglyco-form.



## 1. Introduction

Hydrophilic Interaction Chromatography (HILIC) is the term coined by Alpert in 1990 [1] to describe a variant of normal-phase chromatography (NPC). HILIC differs from NPC in that while NPC uses hydrophilic stationary phases and highly nonpolar mobile phases such as heptane, HILIC uses a combination of hydrophilic stationary phases and more polar organic mobile phases. Thus, by the use of water-miscible solutes such as alcohols (e.g. isopropanol) or acetonitrile, HILIC has the possibility to be useful for the separation of biomolecules.

Fractionation by HILIC has been proposed to involve a mechanism of partitioning between the hydrophobic mobile phase and a layer of mobile phase enriched with water and partially immobilized on the stationary phase [1]. The phenomena responsible for this partitioning are not well understood.

HILIC can be used to separate peptides and other lower molecular weight compounds such as carbohydrates [2], with a selectivity that is complementary to other modes of chromatography. Typically, the order of elution is the opposite of that seen in reversed-phase HPLC (RP-HPLC). However, while retention in RP-HPLC is generally determined by the overall hydrophobicity of a protein, retention in HILIC is more dependent on the presence of hydrophilic domains within the protein [3].

In his original paper on HILIC, Alpert concentrated mainly on the chromatography of amino acids, peptides and carbohydrates, although he did mention the separation of H1 histones from chicken erythrocytes on a cation exchange column by HILIC [1]. Apart from that reference, very little has been published on the purification of proteins by HILIC. Rubinstein and colleagues [4,5] described the use of a LiChrosorb Diol column to purify bovine serum proteins and human leukocyte interferon using HILIC. Grego and colleagues [6,7] used non-endcapped reversed-phase columns to chromatograph various proteins in a technique referred to by Simpson et al. [7] as inverse gradient chromatography (IGC), but which is now known to be HILIC. IGC was widely used by Simpson et al. for the desalting of electroeluates; a later refinement of this desalting procedure used a standard HILIC column in place of the reversed-phase columns [8].

One explanation for the lack of publications describing the application of HILIC to purification of proteins is that many proteins, especially enzymes, would be denatured in the presence of the high concentrations of organic solvent which are necessary to perform HILIC. However, many proteins (for example, apolipoproteins) function in the hydrophobic environment of membranes and lipids and might thus be expected to tolerate exposure to mobile phases with a high organic solvent content [3]. Furthermore, the concern in proteomics analyses is the detection and measurement of proteins, not their biological activity.

Another factor which may have limited the application of HILIC to purification of proteins is that many proteins are not soluble in the high concentrations of organic solvents necessary for HILIC. To some extent this lack of solubility may be countered by the addition of solubilizing agents such as hexafluoroisopropanol (HFIP) or unbuffered acids to the solvents used for HILIC. Solubilizing agents such as HFIP or unbuffered acids are chaotropic and thus may act as eluting agents in HILIC by antagonizing the formation of the immo-

* Corresponding author. Tel.: +41 61 687 1877; fax: +41 61 688 7408.
*E-mail address:* tim.tetaz@roche.com (T. Tetaz).

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bilized hydration layer on either the stationary phase surface or on the proteins themselves. However, proteins have so many potential binding sites that solubilization and elution are more likely to be of concern than is retention.

Recently, Carroll et al. [9] have reported using HILIC to purify membrane proteins from the water-insoluble pellets of mitochondrial preparations. Many of the mitochondrial proteins they purified by HILIC were found to bind irreversibly in RP-HPLC, a technique which is not generally of use in the purification of very hydrophobic proteins, except under certain conditions [10,11].

Starting from the initial chromatographic conditions described by Carroll et al. [9], we have developed a modified method which allows us to chromatograph various intact, soluble proteins by HILIC and, in some cases, even to recover functionally active protein. The following paper describes the separation of two different lipophilic proteins (apoA-I and a truncated version of apoM lacking its signal peptide) plus a mitochondrial, membrane-associated protein on five different commercially available columns. In addition, we show that HILIC may be used to separate different glycosylated isoforms of human apoM.

## 2. Experimental (materials and methods)

### 2.1. Reagents

Isopropanol (2-PrOH), hexafluoroisopropanol (HFIP), trifluoroacetic acid, trichloroacetic acid (TCA), formic acid and ammonium formate were obtained from Fluka. Acetonitrile (ACN) was purchased from Merck, while L-tryptophan, potassium bromide (KBr), urea and horse heart cytochrome c were from Sigma. Human plasma was obtained from the Blood Bank of the Kantonsspital in Basel, Switzerland.

### 2.2. HILIC columns

Five different HILIC columns were used in this study:

(1) PolyHYDROXYETHYL A was item# 204HY0503 (200 mm × 4.6 mm i.d., 5 μm particle size, 300 Å pore size) or # 254HY0501 (250 mm × 4.6 mm i.d., 5 μm particle size, 100 Å pore size) from PolyLC (Columbia, MD, USA) and is silica coated with polyhydroxyethyl aspartamide.
(2) ZIC-HILIC PEEK (150 mm × 4.6 mm i.d., 5 μm, 200 Å) was purchased from SeQuant (Germany) and has a zwitterionic ligand which is covalently attached to porous silica.
(3) ProntoSIL 300-5-Si (200 mm × 4.6 mm i.d., 5 μm, 300 Å) is a bare silica column and was obtained from Bischoff Chromatography (Germany).
(4) TSKgel Amide 80 (250 mm × 4.6 mm i.d., 5 μm, 80 Å) was purchased from Tosoh Bioscience (Japan) and is silica coated with a polymer with attached carbamoyl groups.

### 2.3. HPLC methodology

HILIC separations were performed on an Agilent 1200 Series HPLC equipped with preparative pumps (G1361A), a Rheodyne sample injector (1 ml loop) and a diode array detector (G1315C). Solvent A was 50 mM ammonium formate pH 3.7 + 0.5% HFIP + 50% 2-PrOH + 25% ACN while solvent B was 50 mM formic acid (unbuffered = pH 2.5) + 0.5% HFIP + 10% 2-PrOH + L-tryptophan (4–5 mg/L). Purified proteins (typically 100 μg per run) were dissolved or diluted in solvent A and applied to the column at a flow rate of 1 ml/min. Column temperature was maintained at 24 °C. Standard chromatographic conditions were 5 min isocratic with solvent A, followed by a 30 min linear gradient from solvent A to solvent B followed by 10 min isocratic with solvent B. Protein elution was monitored at 215, 254 and 280 nm. Fractions were collected using an analytical fraction collector (G1364C).

### 2.4. Purification of proteins

ApoA-I was purified from human plasma by KBr density centrifugation followed by delipidation and lyophilization of HDL, resuspension in 5 M urea and isoelectric focusing using a preparative Rotofor (BioRad). Selected fractions from the Rotofor were precipitated with 10% TCA, washed with 0.1% TCA, dissolved again in urea and dialyzed against 0.9% sodium chloride. The purified product was demonstrated to be active in promoting cholesterol efflux from THP-1 cells in an ABCA1-dependent manner.

Recombinant apoM was expressed in HEK293 cells as a truncated form lacking the signal peptide. The protein was purified by chromatography on HisPrep 16/10 FF (GE Healthcare) followed by size exclusion chromatography on a Superdex 200 column (GE Healthcare). The purified product was assayed using the method described by Ahnström et al. [12] and shown to bind retinoic acid with a Kd of 1.0 μM, which is comparable to the values quoted by these authors.

The purity of both native human apoA-I and recombinant human apoM purified as described above was shown by SDS-PAGE to be ≥99% and the expected molecular weights were confirmed by mass spectrometry.

### 2.5. Mass spectrometry

Nanoelectrospray MS: 2 μl aliquots of the collected HILIC fractions of interest were directly transferred into a nanoelectrospray needle. Mass spectra were acquired on a QSTAR Pulsar i quadrupole TOF tandem mass spectrometer (AB-Sciex) equipped with a nanoelectrospray ion source (Proxeon).

### 2.6. Assay for cholesterol efflux

Macrophage-like cells were obtained by exposing THP-1 monocytic leukemia cells to phorbol myristate acetate and subsequently loaded with lipids by further culture in the presence of acetylated LDL containing $^{3}H$ cholesterol tracer. The model foam cells thus obtained were then exposed for 8 h to cholesterol acceptor test protein. Cell culture supernatants were harvested and filtrated and cells lysed in 5% NP40. Fractional efflux was calculated as the ratio of radioactivity in the supernatant to the sum of the radioactivity in the cells and supernatant.

Parallel experiments were performed using cells exposed to 5 nM of the RXR agonist Beraxotene (Ligand Pharmaceuticals) and 50 nM of the LXR agonist TO901317 (Tularik).

## 3. Results and discussion

The initial chromatographic conditions employed were identical to those used by Carroll et al. [9], except that solvent A contained 50% 2-PrOH and 25% ACN, while solvent B contained 10% 2-PrOH, and the ammonium formate concentration in both solvents was 50 mM. Under these conditions (on the 204HY0503 column from PolyLC), human apoA-I and apoM both eluted at the very end of the 30 min gradient (Fig. 1). Since apolipoproteins are known to be very hydrophobic [10,11] this might be unexpected. However, as explained in Section 1, in HILIC proteins tend to interact with the stationary phase via hydrophilic domains, such as the amphipathic helices known to be present in apolipoproteins [13].

In order to facilitate an earlier elution of apoA-I and apoM, unbuffered formic acid (a weak chaotrope) was substituted for ammonium formate in solvent B (Fig. 2). Addition of L-tryptophan

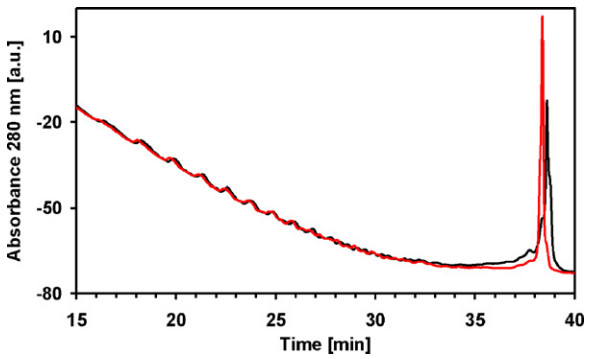


**Fig. 1.** HILIC separation of human apoM (black) and human apoA-I (red) on a PolyHYDROXYETHYL A 204HY0503 column, using ammonium formate in solvent B.

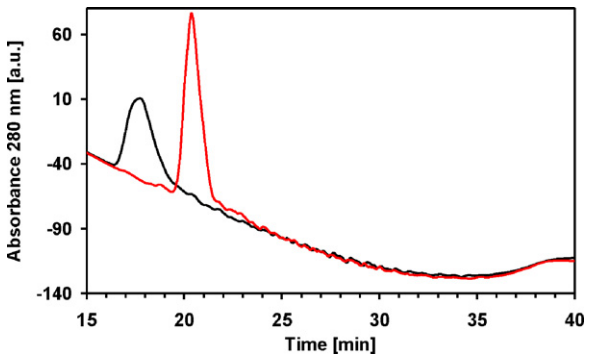


**Fig. 2.** Identical to Fig. 1, except that unbuffered formic acid was substituted for ammonium formate in solvent B (which contained no tryptophan).

to solvent B (based on an idea from Tempst and Riviere [14]) led to a significant improvement in the baseline (Fig. 3).

The separation of human apoA-I, human apoM and equine cytochrome c was compared on five different commercially available columns, as described in Section 2. The results are shown in Figs. 4–8. Comparison of Figs. 3 and 4 shows a significant difference in the separation of apoM and apoA-I, which was ascribed to the use of columns packed with PolyHYDROXYETHYL A from two different batches. In addition to there being observable differences in HILIC selectivity between PolyHYDROXYETHYL A columns with packing from different batches, it was also observed that the selectivity of these columns tended to change during the lifetime of the column. A similar shift in retention time was observed for apoM on the TSKgel Amide 80 column (Figs. 8 and 9), but this may be at least partially a result of a heavier loading in the case of Fig. 9. Since the ZIC-HILIC and ProntoSIL columns were not employed over an

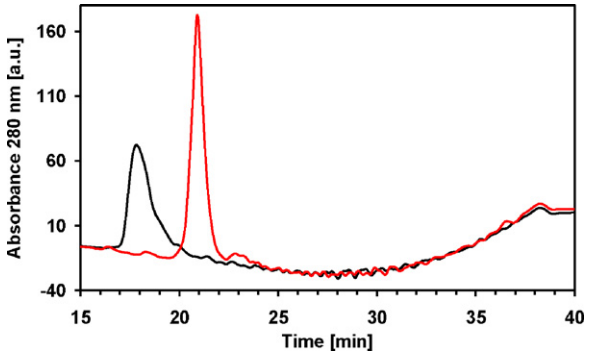


**Fig. 3.** HILIC separation of human apoM (black) and human apoA-I (red) on a PolyHYDROXYETHYL A 204HY0503 column. Conditions were identical to those described in Section 2 (where solvent B now contained tryptophan).

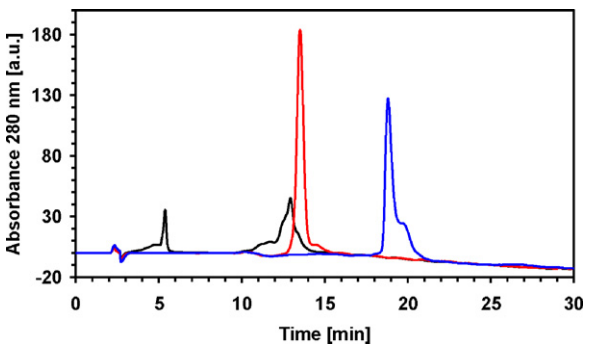


**Fig. 4.** HILIC separation of human apoM (black), human apoA-I (red) and equine cytochrome C (blue) on a PolyHYDROXYETHYL A 204HY0503 column from a different batch than in Fig. 3. Conditions were as described in Section 2.

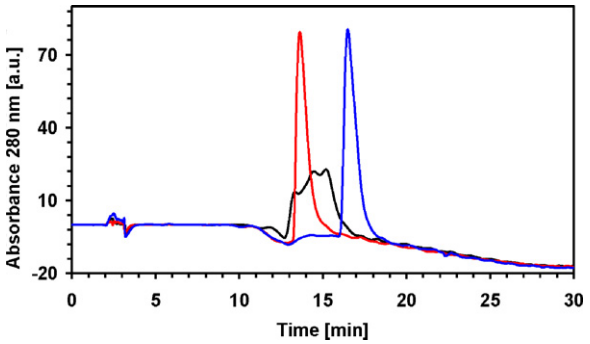


**Fig. 5.** HILIC separation of human apoM (black), human apoA-I (red) and equine cytochrome C (blue) on a PolyHYDROXYETHYL A 254HY0501 column. Conditions were as described in Section 2.

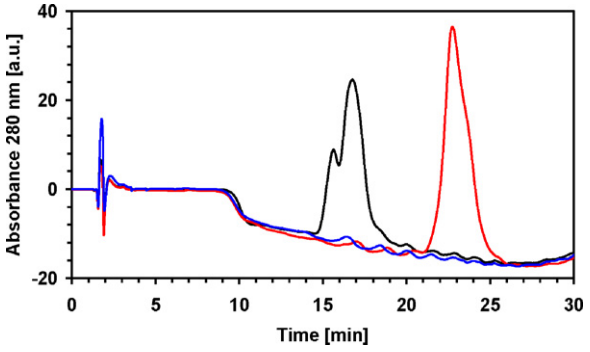


**Fig. 6.** HILIC separation of human apoM (black), human apoA-I (red) and equine cytochrome C (blue) on a ZIC-HILIC PEEK column. Conditions were as described in Section 2.

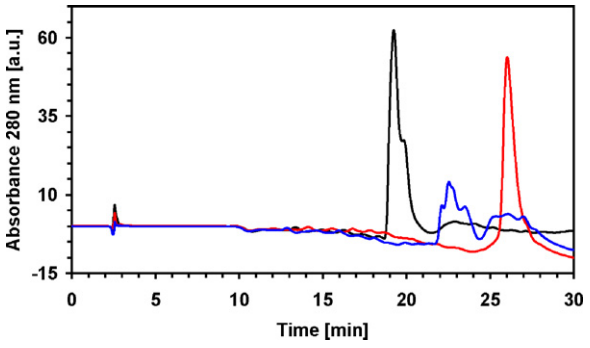


**Fig. 7.** HILIC separation of human apoM (black), human apoA-I (red) and equine cytochrome C (blue) on a ProntoSIL 300-5-Si column. Conditions were as described in Section 2.

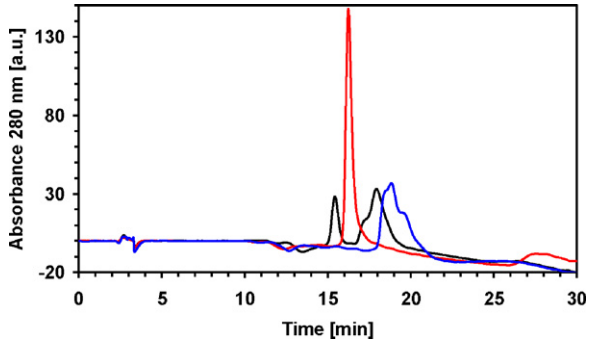


**Fig. 8.** HILIC separation of human apoM (black), human apoA-I (red) and equine cytochrome C (blue) on a TSKgel Amide80 column. Conditions were as described in Section 2.

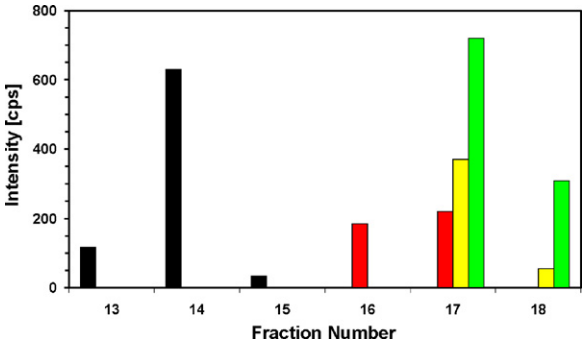


**Fig. 10.** MS analysis of fractions collected from the HILIC separation of 1 mg human apoM. Non-glycosylated apoM (19,015 Da; black bars) was found in fractions 13–15. The different glycosylated isoforms of apoM are represented as follows: (1) complex biantennary, no galactose (20,517 Da; red bars), (2) complex non-biantennary, 2-galactose, 1-sialic acid (20,930 Da; yellow bars) and (3) complex non-biantennary, 2-galactose, 2-sialic acid (21,221 Da; green bars).

extensive period of time as were the PolyHYDROXYETHYL A and TSK columns, it is not known at this stage whether this change in selectivity is a feature which is common to all columns used for HILIC of proteins.

Compared to PolyHYDROXYETHYL A, apoA-I and apoM elute later on the ZIC-HILIC PEEK column and with much broader peaks (Fig. 6). The broad peaks might be explained by the smaller pore size of the ZIC-HILIC stationary phase, although surprisingly the TSKgel Amide80 column (with 80 Å pores) gives a very sharp peak for apoA-I (Fig. 8). The later elution of apoA-I and apoM on the ZIC-HILIC PEEK column could be due to electrostatic interactions with the coating ligand. This column is supposed to be zwitterionic but also exhibits significant cation-exchange properties under some circumstances [15]. This might also explain why cytochrome c (which is highly basic) did not elute from the ZIC column.

All of the columns tested seemed to indicate that apoM could be separated into multiple isoforms. We speculated that these represented different glycosylated isoforms of apoM and chose the TSKgel Amide 80 column for further studies. After application of 1 mg human apoM to this column (Fig. 9), fractions were collected and analyzed by MS. As shown in Fig. 10, the earliest eluting peak at 13 to 15 min was shown to be non-glycosylated apoM, while the fractions across the peak from 16 to 18 min represented different glycosylated isoforms of apoM. To the authors' knowledge, this is the first description of a technique which has the potential to facilitate the separation of different glycosylated isoforms of an intact glycoprotein other than affinity chromatography with an immobilized lectin, antibody, or boronic acid group.

As mentioned in Section 1, a common criticism of HILIC is that it may yield purified but biologically inactive proteins due to the use of high concentrations of organic solvents. In this study, samples of apoA-I and apoM eluted from the PolyHYDROXYETHYL A and TSKgel Amide 80 columns, respectively, were tested to see whether they had retained any measurable biological activity. Although no binding of retinoic acid to HILIC-purified apoM could be detected, HILIC-purified apoA-I promoted higher cholesterol efflux from model foam cells than did an albumin negative control. The effect was most evident when ABC transporter expression was enhanced by combined liver X receptor (LXR) and retinoid receptor (RXR) agonists priming of the cells, suggesting an ABCA-1 transporter mediated effect (Fig. 11).

The successful chromatography of apoA-I and apoM is perhaps not too surprising, considering the good results previously afforded with HILIC of other proteins not normally found in aqueous solution [4–9]. The results presented in this study extend the utility of HILIC to an additional class of lipophilic proteins. The successful results obtained with the separation of glycosylation variants of apoM are more novel, and point to the potential of HILIC for separations not tried before. For HILIC of water-soluble proteins, the main concern is probably going to be maintaining them in solution in the starting mobile phase. The difficulty of doing this requires further investigation. Another factor which needs further exploration is the optimization of pore diameter. One might anticipate that chromatography of intact proteins would best be performed on a material with pores wide enough to preclude steric interference; 300–1000 Å, for example. However, in this study selectivity was greatest in some cases using material with pores in the range 80–100 Å. This is inconsistent with results obtained in other chromatographic modes such as ion-exchange chromatography. HILIC may be anomalous in this regard. Such anomalies are potentially the key to important advances in chromatography and, as such, are worthy of systematic investigation.

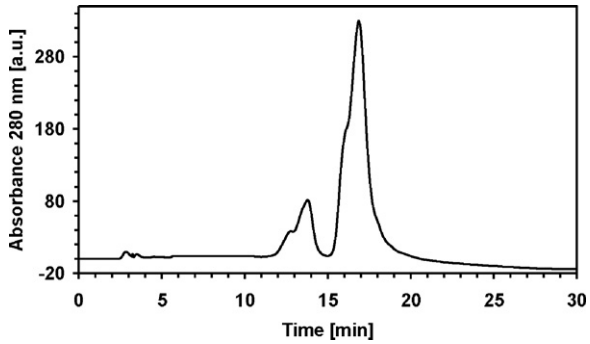


**Fig. 9.** HILIC separation of human apoM (1 mg) on a TSKgel Amide80 column. Conditions were as described in Section 2.

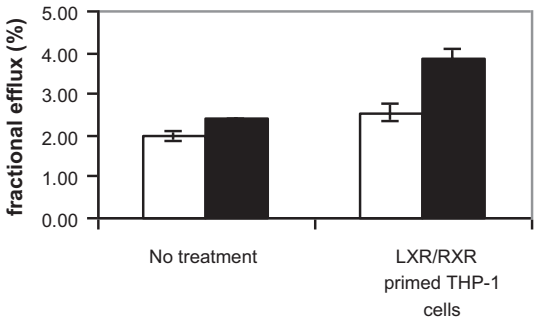


**Fig. 11.** Cholesterol efflux from THP-1 derived foam cells: Fractional efflux was measured as described in Section 2 in the presence of 0.2% albumin (□), average from 3 determinations; or: 10 μg/ml purified ApoA-1 (■), average from 2 determinations. Error bars represents the standard deviation.

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## References

[1] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[2] A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, R. Orlando, J. Chromatogr. A 676 (1994) 191.
[3] A.J. Alpert, Adv. Chromatogr. 44 (2006) 317.
[4] M. Rubinstein, Anal. Biochem. 98 (1979) 1.
[5] M. Rubinstein, S. Rubinstein, P.C. Familletti, R.S. Miller, A.A. Waldman, S. Pestka, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 640.
[6] M.T.W. Hearn, B. Grego, J. Chromatogr. 255 (1983) 125.
[7] R.J. Simpson, R.L. Moritz, E.E. Nice, B. Grego, Eur. J. Biochem. 165 (1987) 21.
[8] P. Jenö, P.E. Scherer, U. Manning-Krieg, M. Horst, Anal. Biochem. 215 (1993) 292.
[9] J. Carroll, I.M. Fearnley, J.E. Walker, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 16170.
[10] T. Tetaz, E. Kecorius, B. Grego, N. Fidge, J. Chromatogr. 511 (1990) 147.
[11] B. Meyer, E. Kecorius, P. Barter, N. Fidge, T. Tetaz, J. Chromatogr. 540 (1991) 386.
[12] J. Ahnström, K. Faber, O. Axler, B. Dahlbäck, J. Lipid Res. 48 (2007) 1754.
[13] J.P. Segrest, M.K. Jones, H. De Loof, C.G. Brouillette, Y.V. Venkatachalapathi, G.M. Anantharamaiah, J. Lipid Res. 33 (1992) 141.
[14] P. Tempst, L. Riviere, Anal. Biochem. 183 (1989) 290.
[15] Y. Takegawa, K. Deguchi, H. Ito, T. Keira, H. Nakagawa, S.I. Nishimura, J. Sep. Sci. 29 (2006) 2533.